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Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30329-4027, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

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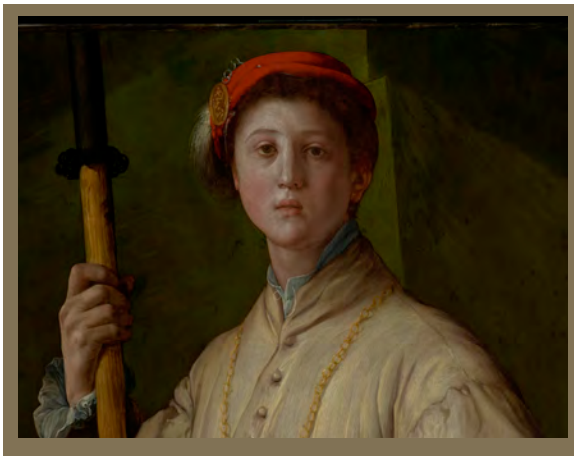

On the Cover

Pontormo (Jacopo Carucci) (1494–1557), Portrait of a Halberdier (ca. 1528–1530) (detail).

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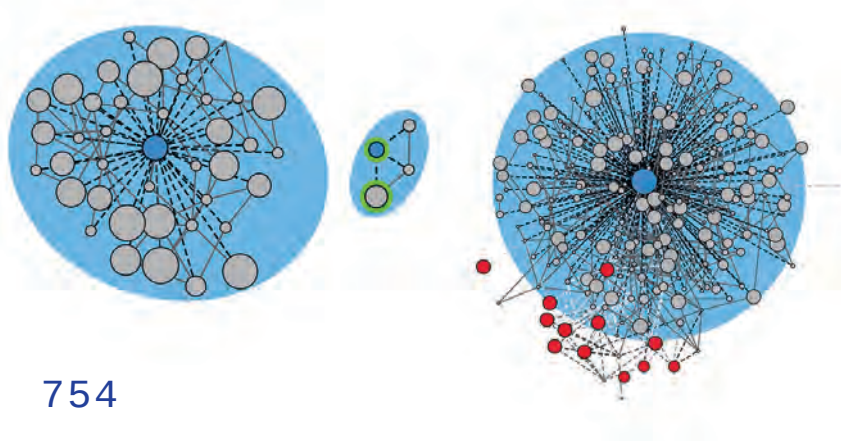

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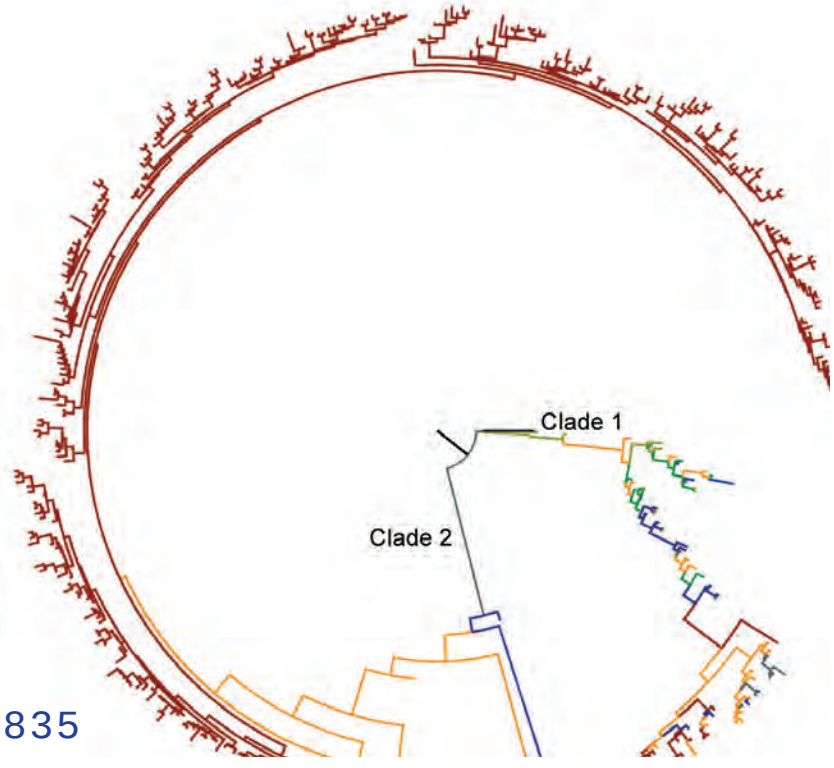

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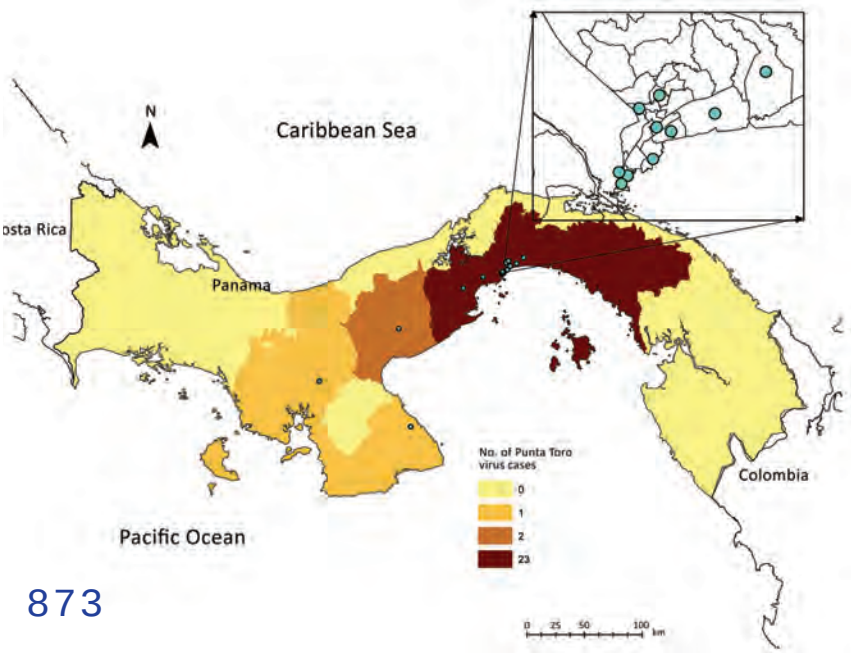

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Exposure Characteristics of Hantavirus Pulmonary Syndrome Patients, United States, 1993–2015

Annabelle de St. Maurice, Elizabeth Ervin, Mare Schumacher, Hayley Yaglom, Elizabeth VinHatton, Sandra Melman, Ken Komatsu, Jennifer House, Dallin Peterson, Danielle Buttke, Alison Ryan, Del Yazzie, Craig Manning, Paul Ettestad, Pierre Rollin, Barbara Knust

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Release date: April 12, 2017; Expiration date: April 12, 2018

Learning Objectives

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

- Assess the geographic distribution, seasonal pattern, and clinical presentation of hantavirus infection
- Distinguish the most common subtype of hantavirus associated with infection in the United States
- Evaluate the epidemiology of hantavirus pulmonary syndrome
- Identify the most common setting for exposure to hantavirus among cases of hantavirus pulmonary syndrome in the current study.

CME Editor

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DOI: http://dx.doi.org/10.3201/eid2305.161770

Rodents can transmit hantaviruses to humans. In the Americas, human infection causes severe respiratory illness known as hantavirus pulmonary syndrome. Using national surveillance system data, we assessed demographics and rodent exposure settings for 662 case-patients during 1993–2015. American Indians accounted for 18% of case-patients, and case-fatality rates for this population (46%) were higher than those for whites (33%). Case-patients reported rodent exposures in the home (71%), at work (32%), or in a recreational setting (24%). Cars, trailers, or mobile homes accounted for 7% of rodent exposures; 17% of case-patients reported having cleaned rodent-infested areas. Of those whose exposure was work related, 53% had jobs with potential risk for rodent exposure. The proportion of recreational exposures was significantly higher among case-patients residing in the eastern (47%) than in the western (23%) United States. Regionally and culturally appropriate educational materials can be used to direct prevention messages to persons in these risk groups.

Hantaviruses are negative-sense, single-stranded RNA viruses in the family *Bunyaviridae* (*1*). Hantavirus infections in humans are associated with several disease syndromes, including hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS; also known as hantavirus cardiopulmonary syndrome) (*2*,*3*). Although hantavirus infections had long been recognized in Asia and Europe, a 1993 outbreak of severe pulmonary disease in the Four Corners area of the United States (i.e., Utah, New Mexico, Arizona, Colorado) led to the discovery of Sin Nombre virus, the leading cause of HPS in the United States (*1*,*4*). In 1995, HPS became a nationally notifiable disease; the Viral Special Pathogens Branch (Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases) at the Centers for Disease Control and Prevention (CDC) maintains an HPS surveillance system and registry of reported HPS cases in the United States (*5*,*6*).

In the United States, most HPS cases are caused by Sin Nombre virus, for which the North American deer mouse (*Peromyscus maniculatus)* serves as reservoir (*7*). Other New World hantaviruses that cause human disease in the United States include New York and Monongahela viruses, transmitted by the North American deer mouse and white-footed deer mouse (*Peromyscus leucopus*); Black Creek Canal virus, transmitted by the hispid cotton rat (*Sigmodon hispidus*); and Bayou virus, transmitted by the marsh rice rat (*Oryzomys palustris*). Infected rodents excrete virus in their saliva, urine, and feces; inhalation of virus in rodent-infested areas is thought to be the primary mode of transmission to humans, although direct inoculation through a rodent bite is possible (*8*–*10*). Previous case–control studies have identified risk factors for HPS, such as having high rodent densities in the home; handling rodents; and performing cleaning activities, such as sweeping, in rodent-infested areas (*9*,*10*). Other factors that may precipitate exposure to hantaviruses include occupational and recreational activities, such as working outdoors or camping (*9*,*11*).

We sought to further describe demographics of HPS case-patients and possible occupational and environmental exposures associated with HPS. We examined surveillance data collected by the national HPS surveillance system.

Methods

Since 1993, as part of national surveillance activities, state and local health departments have provided CDC with standardized clinical and exposure information for all laboratory-confirmed HPS cases (*12*). To be included as an HPS case-patient, patients were required to have no other cause of illness and to have an acute febrile illness with unexplained acute respiratory distress syndrome or evidence of interstitial pulmonary infiltrates on chest radiograph or to have an unexplained respiratory illness that resulted in death and an autopsy finding compatible with noncardiogenic pulmonary edema (*13*). In addition, all case-patients had laboratory confirmation of infection by either hantavirus-specific serologic testing (IgM and IgG) or reverse transcription PCR.

State and local health departments recorded all case-patient data on standardized surveillance case report forms, which asked closed-ended questions about case-patient demographics and open-ended questions about exposure (location and activities) and occupation (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/5/16-1770-Techapp1.pdf). For 158 cases from 1993–1999, investigators used a structured questionnaire to interview case-patients or family member proxies as part of routine surveillance; the methods used to collect these data and preliminary summary data have been published (*9*). The data gathered from the questionnaires were used to supplement the data included in the surveillance case report forms for the early case-patients. These data contained more detailed, systematically collected information about exposures, including specific questions about rodent exposure at home, in a recreational setting, and in the workplace.

On the basis of the free-text descriptions of reported rodent exposures and their locations, we classified rodent exposures as occurring at the case-patient's home, at work, or in a recreational setting. Case-patients could be classified by >1 potential exposure setting. We also noted whether reported rodent exposure occurred in cars, trailers, or mobile homes and whether reported exposure included cleaning a rodent-infested area, regardless of the setting in which the exposure probably occurred. Among case-patients with a reported occupation, we created 2

categories: occupations for which direct or indirect contact with rodents was likely (i.e., outdoor activities or cleaning) and occupations for which such contact was unlikely (i.e., primarily indoors and office based). We subclassified occupations with opportunities for rodent contact as forestry/outdoor recreation, agriculture/ranching, construction/landscaping, professional cleaning, animal handling (e.g., wildlife biologist or exterminator), or oil field work. We defined the eastern United States as states east of the Mississippi River. We compared differences in frequency distribution between groups by using the Pearson χ^2 for categorical variables and differences in means for all continuous variables by using the Student *t* test with unequal variance. We considered results statistically significant if the p value was <0.05.

Results

During 1993–2015, a total of 662 laboratory-confirmed HPS case-patients were reported to CDC and included in the analysis. Of 651 case-patients for whom outcome information was recorded, 230 (35%) died; case-fatality rates did not vary by geographic region (p>0.05).

Race information was recorded for 648 case-patients. Most (78%) were white, although American Indians accounted for 18% of case-patients (Table 1). Most (89%) American Indian case-patients resided in the Four Corners area. American Indian case-patients were significantly younger than white case-patients (mean 34 vs. 39 years of age, respectively; $t = 2.71$, df = 164, p = 0.01), and the case-fatality rate among American Indian case-patients was significantly higher than that among white case-patients (46% vs. 33%, respectively; $\chi^2 = 6.4$, df = 1, p = 0.01). After stratification by age group, case-fatality rates were significantly higher among American Indian women 40–64 years of age than among white women of the same age group (Table 2).

Rodent exposure was reported for 319 persons. We classified rodent exposure settings as being in the home, in a recreational setting, or at work (Table 3). Home exposure was most frequent in the eastern and western United States; however, home exposure was significantly more common among case-patients residing in the western United States (Table 3). Rodent exposure in a recreational setting was more common among case-patients residing in the eastern United States. Rodent exposures in cars, trailers, or mobile homes were reported for 49 (7%) case-patients. A history of cleaning a probable rodent-infested area (e.g., crawl spaces or outbuildings) was reported for 114 (17%) case-patients. The proportion of home exposures was greater for American Indian than for white case-patients (Table 4).

Occupation status was reported for 450 (68%) case-patients, and a specific occupation was reported for 354. Those with occupations for which contact with rodents was deemed unlikely (e.g., teaching or clerical work) accounted for 54% of case-patients with a reported occupation (Table 1). Further analysis of the frequency of occupational exposure among 187 persons (28% of total case-patients) with both reported occupation and exposure (Table 5) indicated that those who worked in an occupation for which frequent rodent contact was possible were more likely to be occupationally exposed than those who worked in an occupation without the potential for frequent rodent exposure.

Discussion

Using exposure data for >600 case-patients reported by the national HPS surveillance system, we were able to define occupations and exposures that may contribute to increased risk of acquiring HPS; in this regard, our findings are consistent with those of previous studies. Early surveillance data identified possible risk factors for acquiring hantavirus infection as cleaning or entering structures that had been previously closed or uninhabited for long periods (*8*). Our

Table 1. Demographics of laboratory-confirmed hantavirus pulmonary syndrome case-patients, United States, 1993–2015*

Case-patients, n = 662	No. (%)
Age, y	
<18	53 (8)
18–39	303 (46)
40–64	250 (38)
≥65	48 (7)
Race, n = 648	
White	488 (78)
American Indian	113 (18)
Black	8 (1)
Asian/Pacific Islander	10 (2)
Other	1 (<1)
Male, n = 655	414 (63)
US Region, n = 662	
Eastern	27 (4)
Western	635 (96)
Not Hispanic, n = 525	404 (77)
Employment status	
Not reported	212 (32)
Unemployed	29 (4)
Retired	28 (4)
Student	39 (6)
Employed with reported occupation	354 (54)
Reported occupations, n = 354	
No frequent rodent exposure	190 (54)
Potential frequent rodent exposure	164 (46)
Agriculture/ranching†	80 (49)
Construction/landscaping‡	43(26)
Forestry/parks/outdoor recreation§	14 (9)
Cleaning¶	12 (7)
Oil field#	9 (5)
Animal work**	6 (4)

*Median patient age (interquartile range) 37 (26–50) years.
†Farmer, rancher, rodeo worker, feedlot rider, dairy manager, bovine hoof trimming specialist, hay transporter.
‡Masonry, roofer, horticulturalist, electrician, building inspector, appliance repair, field laborer, and surveyor.
§Conservation worker, rafting outfitter, fisheries technician, outdoor guide, outdoor researcher with no direct animal contact.
¶Janitor and carpet cleaner.
#Well digger, oil field worker.
**Small mammal researcher, exterminator.

Table 2. Hantavirus pulmonary syndrome deaths, stratified by patient race, sex, and age group, United States, 1993–2015

Patient age, y	American Indian male, no. (%)	White male, no. (%)	χ^2	p value*	American Indian female, no. (%)	White female, no. (%)	χ^2	p value*
<18	3 (38)	6 (26)	0.38	0.54	3 (30)	5 (56)	1.3	0.26
18–39	13 (42)	54 (38)	0.14	0.71	12 (50)	28 (37)	1.2	0.27
40–64	5 (31)	35 (29)	0.037	0.85	11 (69)	21 (30)	8.1	0.004
>64	1 (33)	6 (29)	0.029	0.87	1 (100)	3 (23)	2.7	0.10

*df = 1.

study demonstrated that a possible source of hantavirus exposure may be cleaning rodent-infested areas because 17% of case-patients had a recorded history of cleaning areas that may have been rodent infested. Zietz et al. demonstrated that HPS was more likely to develop in herders but that risk was not increased for ranchers, farmers, and construction workers; however, their study was limited by small numbers of case-patients and was restricted to the Four Corners region, where herders are relatively overrepresented among occupations with rodent-exposure risk (*9*). In addition, given the dry, dusty environment in the Four Corners region and the likelihood of inhaling infected matter, persons in this region m ay be increasingly exposed to infected dust. Previous serologic studies of persons with occupational risk for rodent exposure did not reveal many with serologic evidence of past infection (*14–17*). However, because HPS is rare (i.e., typically 20–40 cases are reported in the United States annually), serologic surveys may not accurately portray risk for exposure to hantavirus when incidence is very low. We identified 2 cases, in addition to 3 previously published case reports, of HPS in persons who were not wearing adequate personal protective equipment while trapping wild mice for field research studies (*18*,*19*) and for whom direct contact with rodents in an occupational setting may have contributed to their risk. Therefore, the use of staff training along with appropriate personal protective equipment in field research settings (*20*) should be emphasized.

We identified that persons with occupations with potential for frequent rodent exposure should be aware of the risks for hantavirus infection; these persons include those working in agriculture (e.g., farmers, ranchers, and temporary laborers), construction (e.g., electricians, carpenters and roofers), forestry/outdoor recreation, oil drilling, and the cleaning industry (e.g., janitors and house cleaners). Employers should continue to educate employees about hantavirus transmission, steps to take to reduce the risk of contracting hantavirus infection in the workplace, and signs and symptoms of hantavirus infection. Current examples of employee education programs include informational sessions for river rafters and power industry workers in Arizona and industrial hygiene workers in Colorado and prevention education for National Park Service, Bureau of Land Management, and mining industry employees in New Mexico. Online educational materials for employees with frequent rodent exposure can be found at the websites of California Department of Public Health and the National Park Service (online Technical Appendix Table).

Educational efforts to reduce exposure risk in the home should be continued because 71% of case-patients with a specified exposure reported rodent exposure at home. During the 1993 Four Corners outbreak, a case–control study found a significant association between higher rodent densities in the home and HPS (*10*). Our study echoed earlier surveillance data that identified risk factors to be cleaning or inhabiting structures that had been previously closed or uninhabited, because many of these structures may be rodent infested (*8*). Typical domestic cleaning activities, such as sweeping and vacuuming, are presumed to increase risk by aerosolizing infectious excreta. When performed in a confined area with limited ventilation, these activities may expose persons to a sufficient inoculum of virus to lead to infection. Public education programs for prevention of HPS in the residential setting, such as the Seal Up, Trap Up, Clean Up campaign launched by the New Mexico Department of Health in 1994 and adopted nationally, emphasize safe cleaning methods (e.g., wet mopping) and exclusion and removal of rodents from the peridomestic environment (online Technical Appendix Table) (*21*). Simple and relatively inexpensive rodent exclusion methods, including the application of expanding foam and wire mesh to eliminate points of entry into living spaces, effectively reduce rodent infestations in homes. Our study demonstrated that home exposure was more common among American

Table 3. Frequency of recorded rodent exposure types by US region of hantavirus pulmonary syndrome case-patients, United States, 1993–2015

Exposure	All case-patients, no. (%), n = 319	Western region, no. (%), n = 302	Eastern region, no. (%), n = 17	χ^2	p value*
Home	228 (71)	220 (73)	8 (47)	5.2	0.022
Occupational	102 (32)	96 (32)	6 (35)	0.091	0.76
Recreational	78 (24)	70 (23)	8 (47)	5.0	0.026

*df = 1.

Table 4. Frequency of recorded rodent exposures by race for hantavirus pulmonary syndrome case-patients, United States, 1993–2015

Exposure	White, no. (%), n = 255	American Indian, no. (%), n = 43	χ^2	p value*
Home	181 (71)	37 (86)	4.3	0.039
Occupational	90 (35)	7 (16)	6.1	0.014
Recreational	63 (25)	6 (14)	2.4	0.12

*df = 1.

Indian case-patients than among those of other racial/ethnic groups. Targeted rodent exclusion projects in American Indian communities have successfully decreased rodent intrusion (*22*). Support for environmental health efforts aimed at rodent exclusion should be continued in American Indian communities. More recently, Navajo Nation has worked closely with CDC on a variety of educational projects, including presentations to Navajo Department of Health and Indian Health Service clinicians, an interactive radio forum on hantavirus, development of radio public service announcements in the Navajo language, and workshops with students at Dine College (Tsaile, AZ) to develop health communication videos for the general public.

A recreational exposure was recorded for 78 (24%) case-patients, 10 of whom were exposed during the 2012 outbreak in Yosemite National Park (*11*). The National Park Service has increased its efforts to educate visitors through its website, park brochures, and posters (online Technical Appendix Table). In some settings, the National Park Service encourages overnight visitors to read a brief statement about hantavirus and prevention methods. The National Park Service is in the process of developing a comprehensive smartphone application for visitors. This application will not only serve as a resource for general details about the parks but will also contain information about safety precautions and animalborne diseases in the park. Because recreational exposures were proportionally more frequent among case-patients residing in the eastern United States, clinicians (even those caring for patients in low-incidence states) should assess recent travel history in addition to rodent exposures in the home and at work and consider hantavirus as a possible cause of disease.

Over the past few decades, educational materials on HPS and hantavirus for general audiences have been developed by health departments and distributed through local jurisdictions. A variety of local efforts to increase hantavirus awareness exist, through traditional and nontraditional news sources. These interventions are relevant, particularly in the spring when hantavirus infection prevalence may be higher among North American deer mice (*23*,*24*) and when persons may be more likely to participate in cleaning or recreational activities that could increase risk for rodent exposure. In 2016, spring electric bills in a Colorado county were accompanied by letters containing hantavirus information. Arizona works collaboratively with local public health and environmental health agencies to share prevention messages with the public to minimize the risk for rodent exposure in recreation, occupation, and peridomestic settings. The Coconino County (AZ) Public Health Department also posts preventive messages on Facebook and Twitter.

States in which risk for HPS is high send seasonal Health Alert Network messages to public health staff and clinicians. The New Mexico Department of Health answers hantavirus-related questions through an all-hours phone line and informs the public of new cases and prevention techniques through statewide press releases. On a national level, CDC manages a Hantavirus Hotline, which the general public and providers can call with hantavirus-related questions (online Technical Appendix Table). CDC, New Mexico Department of Health, and clinicians from the University of New Mexico (Albuquerque, NM) have given educational seminars to healthcare providers through the University of New Mexico Project ECHO, which targets Indian Health Service clinicians, and through Clinician Outreach and Communication Activity calls, which target a wide range of clinical professionals (online Technical Appendix Table).

It is useful not only to define settings where HPS risk is increased because of rodent exposure but also to define demographic risk factors for HPS and subsequent death. HPS disproportionately affects American Indians, who represent ≈2% of the US population (*25*) yet account for 18% of reported US HPS cases. Because 89% of American Indian HPS case-patients reside in the Four Corners region, where most HPS cases occur, the disproportionate number

Table 5. Occupation risk and frequency of reported rodent exposure type for hantavirus pulmonary syndrome case-patients with specified occupation and exposure, United States, 1993–2015

Exposure	Occupation without frequent rodent exposure, n = 91	Occupation with potential frequent rodent exposure, n = 96	χ^2	p value*
Home	67 (74)	60 (63)	2.7	0.10
Occupational	34 (37)	51 (53)	4.7	0.030
Recreational	22 (24)	15 (16)	2.2	0.14

*df = 1.

of American Indian case-patients may in part result from environmental factors that increase the risk of inhaling infected dust particles. Biological factors that may increase HPS risk among American Indians have not been identified. We found that American Indians with HPS were younger and that mortality rates were significantly higher than those among whites of the same age group, particularly among American Indian women 40–64 years of age. According to the 2010 US Census, the median age for American Indians and Alaskan Natives is 28.8 years, compared with the median age for white Americans of 38.4 years (*25*); therefore, the age difference in our study may be a result of overall differences in age distribution between American Indians and white Americans. Sex disparities in death from HPS, by age, have been noted both within and outside the United States but are poorly understood (*26*–*29*). Different mortality rates could result from hormonal effects on the immune response, concurrent medical conditions, or exposure type. Among Norway rats infected with Seoul virus, immune responses vary by sex; Th1 response is greater for males than females (*30*). Of note, male and female humans with acute hantavirus infection have similar Th1 and Th2 responses but different levels of other cytokines, including interleukin-9, fibroblast growth factor 2, granulocyte macrophage colony–stimulating factor, and interleukin-8 (*31*). To prevent more cases and improve outcomes, investigations of the health disparities observed for American Indians and the increased mortality rates observed for American Indian women should continue.

Although we did not systematically collect information on physical location of rodent exposure, 49 case-patients were exposed in a vehicle, trailer, or mobile home. More information is needed to better understand if manufactured housing and vehicles increase the risk for rodent infestation and hantavirus exposure because of their construction. A recent HPS outbreak among overnight visitors to Yosemite National Park led to an association between staying in a particular type of housing (i.e., tents with drywall interiors) and risk for HPS (*32*). These tents were noted to have evidence of active rodent infestation, holes in the canvas, and gaps between the tent and insulated wall, enabling rodent entry. National Park Service employees and migrant workers (*33*) may also reside in temporary on-site housing or use vehicles provided by their employers; therefore, employers should also be prudent about excluding rodents from these items.

Our findings have several limitations. Because of underreporting or misdiagnosis, we may not have captured all cases of HPS in the United States. Ethnicity and race data were missing from 7% and 26% of case report forms, respectively, because some states have only recently begun collecting that information. Because occupation and exposure history were collected by use of free-text responses, data for these variables were not collected systematically for all reported cases. Persons completing case report forms may have overreported occupations for those persons who are more likely to have been exposed to rodents at work, and the HPS-associated exposure could have occurred at another site not reported on the case report form. In addition, for case-patients who lived at their workplace (e.g., forestry, agriculture), it was difficult to distinguish where rodent exposure occurred. As a result of this analysis, we have modified our case report form to systematically capture more detailed information regarding type of exposure and setting.

Although HPS is rare in the United States, surveillance data suggest that persons in certain occupations and certain populations may be at increased risk for HPS because of potential for rodent exposure. Physicians should recognize HPS risk factors and consider HPS for patients with documented rodent exposure or who are at high risk for rodent exposure. Educational efforts and awareness focused on high-risk populations should continue so that persons can decrease their risk of acquiring HPS.

Acknowledgment

We are grateful to public health staff who completed the case report forms and provided assistance with hantavirus surveillance. In particular, we would like to thank Curtis Fritz for his contributions.

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Increased Neurotropic Threat from *Burkholderia pseudomallei* Strains with a *B. mallei*–Like Variation in the *bimA* Motility Gene, Australia

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Release date: April 14, 2017; Expiration date: April 14, 2018

Learning Objectives

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

- Distinguish differences in virulence among *Burkholderia pseudomallei* bim_{Bm} sequence variants, based on a mouse model of neurologic melioidosis
- Distinguish differences in progression among *B. pseudomallei* bim_{Bm} sequence variants
- Determine the implications for clinical disease of these differences among *B. pseudomallei* bim_{Bm} sequence variants in progression and severity of experimental melioidosis.

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Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. *Disclosure: Laurie Barclay, MD, has disclosed the following relevant financial relationships: owns stock, stock options, or bonds from Alnylam; Biogen; Pfizer.*

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DOI: http://dx.doi.org/10.3201/eid2305.151417

Neurologic melioidosis is a serious, potentially fatal form of *Burkholderia pseudomallei* infection. Recently, we reported that a subset of clinical isolates of *B. pseudomallei* from Australia have heightened virulence and potential for dissemination to the central nervous system. In this study, we demonstrate that this subset has a *B. mallei*–like sequence variation of the actin-based motility gene, *bimA*. Compared with *B. pseudomallei* isolates having typical *bimA* alleles, isolates that contain the *B. mallei*–like variation demonstrate increased persistence in phagocytic cells and increased virulence with rapid systemic dissemination and replication within multiple tissues, including the brain and spinal cord, in an experimental model. These findings highlight the implications of *bimA* variation on disease progression of *B. pseudomallei* infection and have considerable clinical and public health implications with respect to the degree of neurotropic threat posed to human health.

Burkholderia mallei, the etiologic agent of glanders, is thought to have evolved from a single strain of *B. pseudomallei*, becoming highly specialized for intracellular persistence (*1*). *B. mallei* and *B. pseudomallei* share sequence similarity and are highly pathogenic through the respiratory route, often initiating rapid disease progression resulting in high mortality (*2*). Unlike *B. pseudomallei*, *B. mallei* has a narrower host range and is less capable of extended persistence in the environment.

Knowledge of the virulence factors responsible for inducing the diverse spectrum of clinical manifestations of *B. pseudomallei* infection remains limited (*3*). Similar to bacteria of other genera, such as *Listeria*, *Rickettsia*, *Mycobacterium*, and *Shigella*, intercellular and intracellular movement of *Burkholderia* are facilitated by actin polymerization at 1 pole of the bacterium (*4*). The putative autotransporter protein *Burkholderia* intracellular motility A (BimA) has been shown to mediate actin-based motility in *B. pseudomallei* and *B. mallei*, promoting bacterial dissemination while shielding the pathogen from immune surveillance and autophagy (*5*). Differences in the structure of the *bimA* gene in *B. mallei* and *B. pseudomallei* (*6*–*8*) suggest that actin assembly might occur through distinct mechanisms in these 2 *Burkholderia* species. *B. mallei*–like *bimA* variants (bim_{Bm}) have been identified in a subset of *B. pseudomallei* isolates from Australia and 2 *B. pseudomallei* isolates from India (*9*,*10*). This allele has not yet been identified in isolates from Southeast Asia.

Neurologic melioidosis is a serious, potentially fatal form of *B. pseudomallei* infection. Recently, we reported that although *B. pseudomallei* isolates from patients with neurologic melioidosis do not demonstrate selective neurotropism in an experimental model, a distinct subset of *B. pseudomallei* isolates appeared equipped for rapid dissemination to multiple tissues, including the central nervous system (CNS), after infection (*11*). Correlation of virulence genes of *B. pseudomallei* with clinical presentations of melioidosis identified the bim_{Bm} allele as a risk factor for neurologic melioidosis (*12*). Given the importance of BimA in intercellular and intracellular spread of *Burkholderia* spp. and the recognition of bim_{Bm} variants of *B. pseudomallei* in northern Australia, we hypothesized that bim_{Bm} variants of *B. pseudomallei* would have an increased advantage for establishment of infection and dissemination compared with typical bim_{Bp} strains. Therefore, we used a well-characterized animal model of melioidosis to compare virulence and disease progression after infection with clinical isolates of *B. pseudomallei* collected in the Northern Territory of Australia during October 1989–October 2012 and identified as having either the bim_{Bm} or bim_{Bp} allele (*13*).

Methods

B. pseudomallei Isolates

B. pseudomallei strains were isolated from patients with melioidosis. Clinical details and the sequence type determined from multilocus sequence typing of the *B. pseudomallei* strains investigated are noted (Table). Additional details are described elsewhere (*11*,*12*,*14*,*15*). These isolates were chosen to represent *B. pseudomallei* strains previously identified as having bim_{Bm} (n = 7) and bim_{Bp} (n = 8) alleles within the *bimA* gene (*10*,*12*).

Animal Infection

We used 8- to 12-week-old C57BL/6 and BALB/c mice purchased from the Small Animal Breeding Facility at James Cook University. Experiments were approved by the Institutional Animal Ethics committee (A1500). To mimic natural routes of infection, intranasal or subcutaneous routes were used for inoculation by using methods described previously (*16*). *B. pseudomallei* isolates were cultured to logarithmic phase and prepared for inoculations as previously described (*11*).

Virulence Determination

Virulence of bim_{Bm} (n = 7) and bim_{Bp} (n = 6) isolates were compared in mice as described previously (*11*). The 50% infectious dose (ID_{50}) was determined by using a modified version of the Reed and Meunch method (*17*). Virulence, as defined by the ID_{50} values for *B. pseudomallei* strains, were compared in BALB/c and C57BL/6 mice after intranasal and subcutaneous infection. Data for bim_{Bm} and bim_{Bp} strains are expressed as mean $\log_{10}$ ID_{50} $\pm$SD

Bacterial Dissemination and Disease Progression

We selected bim_{Bm} (MSHR543) and bim_{Bp} (MSHR305) strains of comparable virulence (determined by intranasal ID_{50} values as 2.6×10^2 CFU and 2.9×10^2 CFU,

Table. Clinical and patient characteristics and sequence type diversity of *bim*$_{Bm}$ and *bim*$_{Bp}$ *Burkholderia pseudomallei* isolates, Australia

Isolate no.	Age, y/sex	Risk factors	Clinical presentation	Outcome	MLST genotype
bim$_{Bm}$					
MSHR62	23/M	None	Brainstem encephalitis	Survived	148
MSHR435	37/M	None	Brainstem encephalitis	Survived	126
MSHR543	22/F	None	Skin ulcer	Survived	294
MSHR668	53/M	None	Diffuse encephalitis	Survived	129
MSHR1153	59/M	DBT	Brainstem encephalitis	Died	117
MSHR2138	49/F	DBT	Bacteremia	Survived	456
NCTC13178	6/M	None	Brainstem encephalitis	Died	286
bim$_{Bp}$					
MSHR305	64/M	ALC	Encephalitis, myelitis	Died	36
MSHR346	49/M	ALC, COPD	Pneumonia	Survived	243
MSHR465	67/M	DBT, COPD	Pneumonia, septic shock	Died	132
MSHR1655	61/F	COPD	Pneumonia	Survived	131
MSHR3709	14/M	None	Brainstem encephalitis	Survived	132
MSHR974*	16/F	None	Skin ulcer	Survived	554
MSHR4237*	45/F	None	Pneumonia	Survived	868
NCTC13179	54/M	DBT	Skin ulcer	Survived	613

*Additional isolates included for internalization and persistence assays. ALC, hazardous alcohol use; COPD, chronic obstructive pulmonary disease; DBT, diabetes; MLST, multilocus sequence typing.

respectively) for comparison of bacterial dissemination after intranasal infection of C57BL/6 mice. C57BL/6 mice provide a more accurate model for neurologic melioidosis because this form of the disease tends to occur in otherwise healthy persons without known risk factors (*13*). MSHR543 (*bim*$_{Bm}$) was isolated from a localized skin infection in a healthy 22-year-old with a cut on her hand that was exposed to muddy water. Blood cultures were negative, and she remained systemically well with no evidence of dissemination of *B. pseudomallei.* The *bim*$_{Bp}$ (MSHR305) strain was isolated from a patient with a fatal case of neurologic melioidosis. The 64-year-old patient had a history of excessive alcohol consumption and had had onset of flaccid paralysis after a period of influenza-like illness (*14*). An equivalent dose of MSHR543 (1.4×10^4 CFU) or MSHR305 (1.1×10^4 CFU) was used to inoculate mice. Survival rates and signs of disease were monitored daily for a period of 21 days (n = 10 mice per isolate). Mice that became moribund during the experimental period were euthanized, and bacterial loads were determined in organs and pathology of CNS investigated. Parallel groups of mice were inoculated with MSHR543 (*bim*$_{Bm}$) (n = 15) and MSHR305 (*bim*$_{Bp}$) (n = 15) for assessment of bacterial loads within blood, liver, spleen, lung, cervical lymph node , brain, and nasal-associated lymphoid tissue (NALT) at 2 hours, 1 day, and 3 days postinfection (n = 5 mice per time point) by using methods described previously (*11*). The detection limit of bacteria in blood and organs was 2 CFU. Data are expressed as the mean $\log_{10}$ CFU ±SD.

Bacterial Growth Rate

The growth of *B. pseudomallei* isolates in trypticase soy broth (TSB) was compared. Overnight broth cultures of *B. pseudomallei* isolates were diluted 1:10 in fresh TSB and incubated in triplicate at 37°C with shaking at 120 rpm. Absorbance (600 nm) was measured hourly for 10 hours with a microplate reader (Fluostar Omega; BMG Labtech, Mornington, VIC, Australia) and the exponential growth rate for each isolate determined. Data are presented as the mean gradient (μhr^{-1}) ±SD for *bim*$_{Bm}$ and *bim*$_{Bp}$ strains.

Internalization and Persistence of Bacteria in Phagocytic Cells

We determined internalization and intracellular persistence of *B. pseudomallei* isolates (n = 7 *bim*$_{Bm}$; n = 8 *bim*$_{Bp}$) in mononuclear phagocytes after co-culture with murine leukocytes. Leukocytes were isolated from spleen and peripheral lymph nodes (cervical, mediastinal, axillary, inguinal, and popliteal) of uninfected female C57BL/6 mice (*18*). *B. pseudomallei* isolates were grown to logarithmic phase, washed then added to leukocyte cultures at a multiplicity of infection of 1 (mononuclear cell): 5 (bacteria) (*19*). After 2 hours of co-culture, kanamycin (250 μg/mL) was added to wells to limit extracellular bacterial growth (*18*). Internalization (2 h) and persistence (8 and 24 h) of *B. pseudomallei* isolates in leukocytes was determined by flow cytometry. Uninfected and *B. pseudomallei*–infected leukocytes were fluorescently stained with a combination of anti-mouse fluorescein isothiocyanate–conjugated CD45 and F4/80 (BD Biosciences, North Ryde, NSW, Australia) and peridinin chlorophyll-cyanine 5.5 (PerCP-Cy5.5)–conjugated CD11c (eBioscience, San Diego, CA, USA) by using methods described previously (*18*). After fixation and permeabilization, leukocytes were stained with polyclonal rabbit anti–*B. pseudomallei* outer membrane protein antibody (BpOMP). A secondary biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA, USA) monoclonal antibody and streptavidin–phycoerythrin conjugate (eBioscience) was used for

detection of the primary antibody. Acquisition (2×10^5 leukocytes) was performed by using a FACSCalibur with Cell Quest software (BD Biosciences) and FlowJo software (Tree Star, Inc., San Carlos, CA, USA) was used for postacquisition analysis. The fluorescence of extracellular bacteria was quenched with Trypan blue (0.2%). Data are expressed as the percentage or total number of leukocytes ($CD45^+$), macrophages ($F4/80^+$), or dendritic cells ($CD11c^+$) positive for intracellular BpOMP staining. Two independent experiments were conducted, and the mean ±SD of data from both experiments is shown. Microbiologic culture was used to confirm intracellular *B. pseudomallei* numbers estimated by BpOMP staining (*20*).

Statistical Analysis

We performed statistical analysis by using Graphpad Prism Version 6 (Graphpad Software, La Jolla, CA, USA) and used Kaplan–Meier survival curves to compare susceptibility to infection with *B. pseudomallei* isolates. Virulence parameters (ID_{50} values, time for development of neurologic symptoms, and intracellular bacterial loads within leukocytes) for bim_{Bm} and bim_{Bp} strains were compared by using the Mann-Whitney U test. Bacterial load kinetics in organs after infection with MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) were tested for significance using 2-way analysis of variance with Sidak's post hoc analysis. We considered comparisons significant at $p \leq 0.05$.

Results

High Virulence of Bim_{Bm} Variants in Murine Models of Melioidosis

We compared virulence, as defined by ID_{50}, for bim_{Bm} and bim_{Bp} strains in *B. pseudomallei*–susceptible (BALB/c) and *B. pseudomallei*–partially resistant (C57BL/6) mice after intranasal and subcutaneous infection (*16*,*21*). *B. pseudomallei* bim_{Bm} strains were significantly more virulent for BALB/c and C57BL/6 (Figure 1, panels A and B) mice than bim_{Bp} strains, regardless of route of infection. These

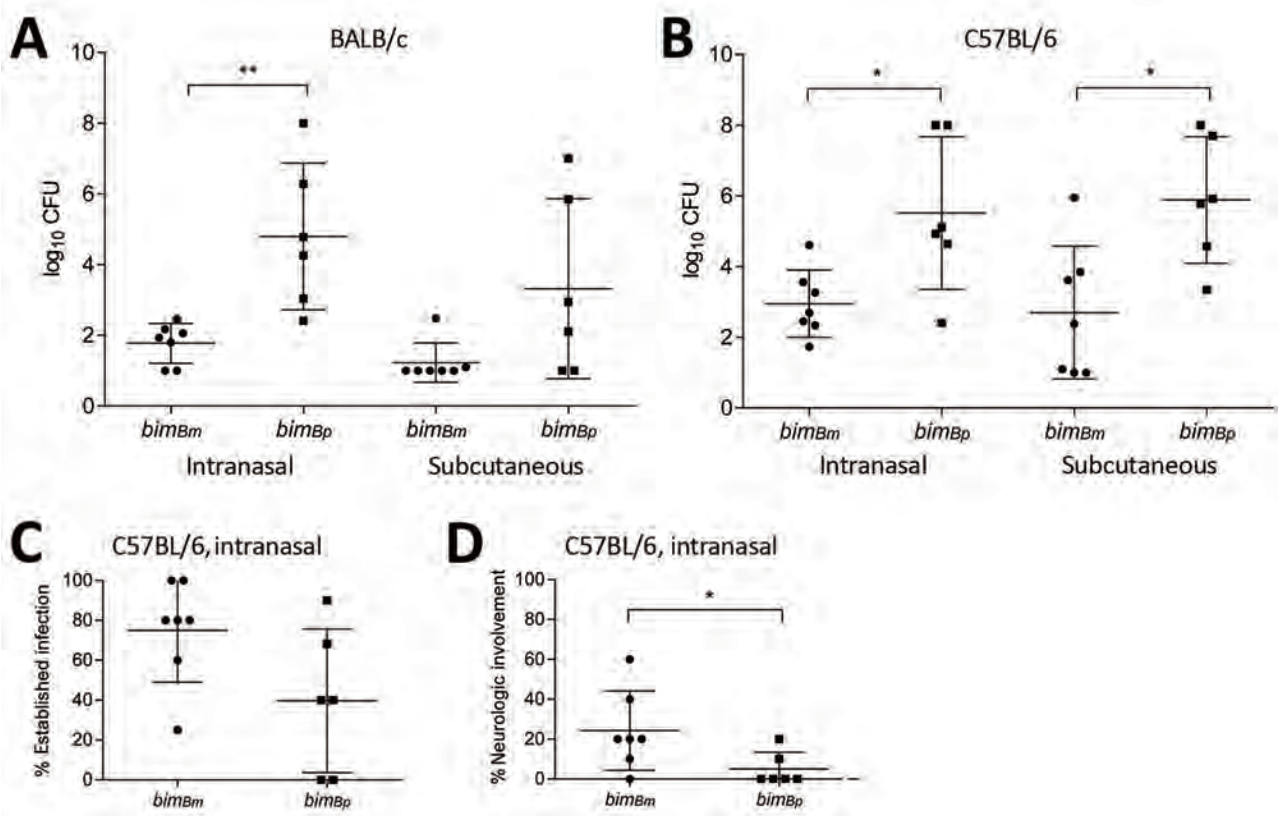

Figure 1. Virulence of bim_{Bm} and bim_{Bp} *Burkholderia pseudomallei* isolates. Day 21 50% infectious dose values after intranasal and subcutaneous infection of BALB/c (A) and C57BL/6 (B) mice with bim_{Bm} (n = 7) and bim_{Bp} (n = 6) *B. pseudomallei* isolates. Groups of 5 mice were inoculated via intranasal and subcutaneous routes at 10-fold increasing doses of *B. pseudomallei*, ranging from 10^0 CFU to 10^7 CFU. Virulence of bim_{Bm} isolates was significantly greater for both mouse strains, regardless of the infection route. Data are expressed as mean $\log_{10}$ CFU ±SD. C57BL/6 mice (n = 10) were infected with bim_{Bm} (n = 7) and bim_{Bp} (n = 6) *B. pseudomallei* isolates at equivalent doses (10^4 CFU) and monitored for 21 days postinfection. The percentage of mice for a given bacterial strain for which evidence indicated establishment of *B. pseudomallei* infection (culture-positive growth from tissues) (C) and signs of neurologic involvement (e.g., head tilt, spinning behavior, and hind leg paresis) (D) was increased for animals exposed to bim_{Bm} compared with bim_{Bp} isolates. *p<0.05; **p<0.01.

findings are consistent with the BALB/c–C57BL/6 model of contrasting resistance to *B. pseudomallei* (*21*).

When equivalent inoculating doses of *B. pseudomallei* strains were compared (10^4 CFU), bim_{Bm} strains were more likely to establish persistent infection with bacteria recoverable from multiple organs at 21 days postinfection after intranasal infection of C57BL/6 mice (p = 0.077) (Figure 1, panel C). Additionally, neurologic involvement occurred with more frequency in animals infected through the intranasal route with bim_{Bm} compared with those infected with bim_{Bp} strains when an equivalent inoculating dose (10^4 CFU; n = 10 mice/*B. pseudomallei* strain) was used (p = 0.046) (Figure 1, panel D). Most *B. pseudomallei* strains tested were capable of CNS infection; however, neurologic involvement tended to occur at comparatively lower inoculating doses for bim_{Bm} than bim_{Bp} strains. The mean number of bacteria required to infect C57BL/6 mice through the respiratory tract and result in the development of neurologic signs in ≥20% of mice was 9×10^3 CFU (range 5.3×10^1 to 2×10^4 CFU) for bim_{Bm} and 3.7×10^5 CFU (range 2.6×10^4 to 6.6×10^5) for bim_{Bp} (p = 0.048). Despite infection of C57BL/6 mice with doses as high as 10^8 CFU, neurologic symptoms were never observed after infection with 2 strains (MSHR3709 and MSHR1655), both of which are type bim_{Bp}.

The mean number of bacteria required to infect susceptible BALB/c mice via the respiratory route and manifest neurologic signs in ≥20% of mice was 8.6×10^3 CFU (range 4×10^1 to 3×10^4 CFU) for bim_{Bm} and 1.5×10^5 CFU (range 2.6×10^4 to 4.2×10^5 CFU) for bim_{Bp} (p = 0.03). For C57BL/6 mice, the mean number of days postinfection for onset of neurologic symptoms was 9 (range 5–16) days; for BALB/c mice, it was 11 (range 4–18) days. These findings indicate that bim_{Bm} variants are significantly more virulent than bim_{Bp} strains in murine models of melioidosis and suggest that fewer inoculating bacteria are required to establish CNS infection.

Differing Disease Progression for bim_{Bm} and bim_{Bp} Strains after Intranasal Infection

We selected a bim_{Bm} (MSHR543) and bim_{Bp} (MSHR305) strain of comparable virulence to compare organ tropism after intranasal infection (intranasal ID_{50} values of 2.6×10^2 and 2.9×10^2 CFU, respectively). Twenty-one day mortality rates were comparable after intranasal infection with either MSHR543 (bim_{Bm}) or MSHR305 (40% and 50%, respectively). However, of the animals monitored for survival, 2 of the 5 mice that succumbed to infection with MSHR305 (bim_{Bp}) had neurologic symptoms (1 with head tilt on day 7, another with hind limb paresis on day 14). In contrast, all of the 4 mice that succumbed to infection with MSHR543 (bim_{Bm}) had symptoms of neurologic melioidosis (3 with head tilt on day 5 and day 7, the other with hind leg paresis on day 7). Moribund mice were euthanized and tissues processed for bacterial load determination. Bacterial loads were high in brains of moribund mice (Figure 2). *B. pseudomallei* was typically recovered from all tissues investigated, although levels tended to be low or undetectable in the blood of moribund mice that had signs of neurologic infection in the first week postinfection. Compared with moribund animals infected with MSHR543 (bim_{Bm}), bacterial loads were significantly higher in NALT of moribund mice infected with MSHR305 (bim_{Bp}, p = 0.025), with a similar trend observed in lung. Abscessation was observed in the nasal epithelium, with extensive suppurative inflammation in the olfactory submucosa extending to the olfactory bulb and moderate infiltration in the trigeminal nerve branches (Figure 3, panels A and B) in mice that had signs of neurologic involvement at day 5 postinfection with MSHR543 (bim_{Bm}). Leptomeningitis and encephalomyelitis were cardinal features in these animals (Figure 3, panels C and D). We also observed cranial microabscesses were in animals that succumbed to infection, although the area affected varied and included the cerebellum, brainstem, and cerebral cortex (Figure 3, panel E).

Systemic dissemination occurred rapidly for MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}); bacteria were recovered from multiple sites by day 1 postinfection (Figure 4). At 2 hours postinfection, NALT was the only tissue that bacteria were cultured from, with levels comparable for mice infected with MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) ($\log_{10}$ CFU of 0.9 ±1.1 and 0.3 ±1.1, respectively). Compared with

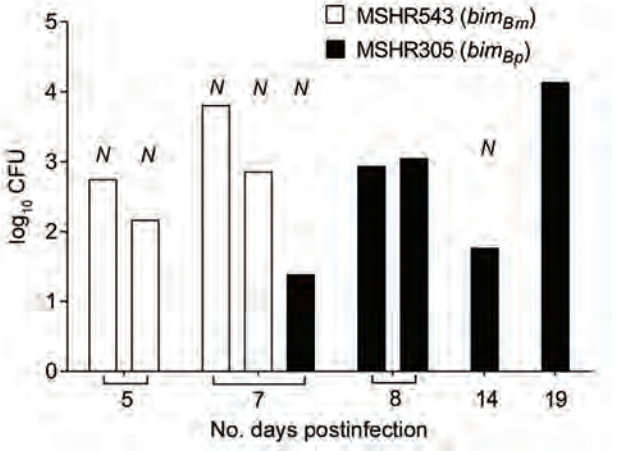

Figure 2. Brain bacterial loads in mice that had signs of neurologic involvement and succumbed to infection with MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) *Burkholderia pseudomallei* isolates. Bacterial loads in brains of C57BL/6 mice (MSHR543, n = 4; MSHR305, n = 5) that had become moribund and required euthanasia within the 21-day experimental period after intranasal infection with MSHR543 (1.4×10^4 CFU; white bars) and MSHR305 (1.1×10^4 CFU; black bars). *N* indicates mice that displayed symptoms of neurologic involvement. Data are expressed as $\log_{10}$ CFU. Mice exposed to MSHR543 (bim_{Bm}) had signs of neurologic involvement and became moribund within 7 days of exposure.

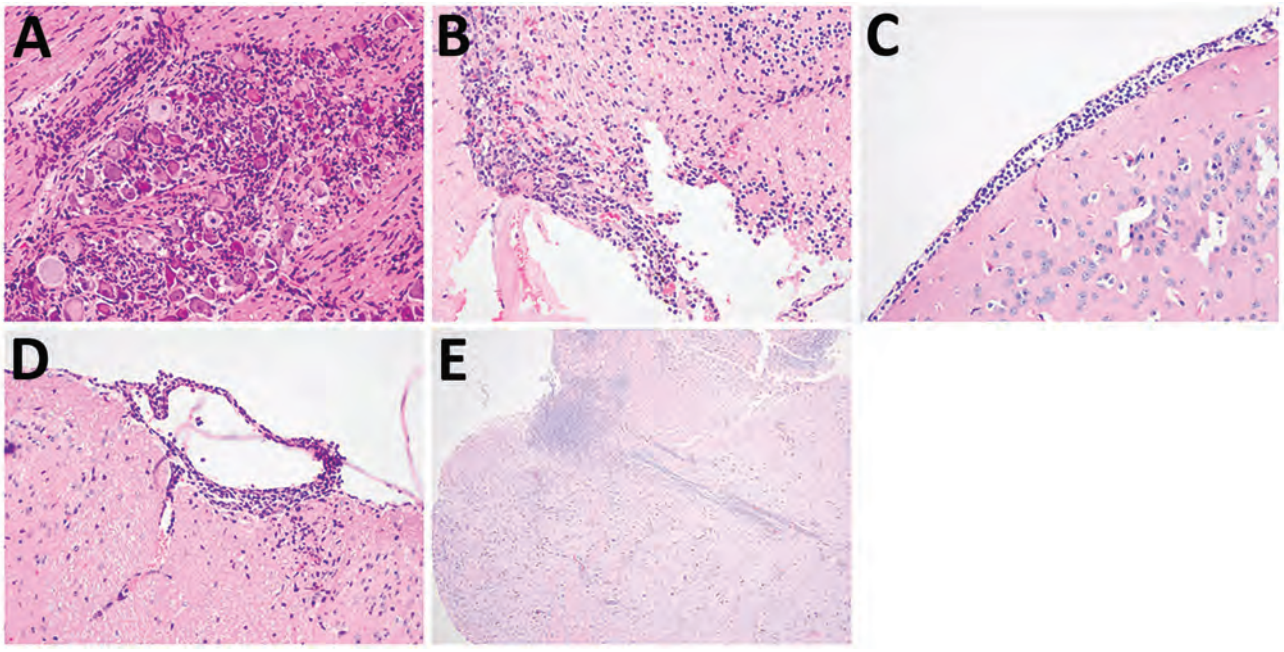

Figure 3. Central nervous system pathology in mice that had signs of neurologic involvement and succumbed to infection with bim_{Bm} and bim_{Bp} *Burkholderia pseudomallei* isolates. Evidence of central nervous system pathology was demonstrated in these mice. Inflammatory infiltrates were prominent in trigeminal nerve branches and ganglion (original magnification ×400) (A) and in the olfactory bulb (original magnification ×200) (B). Cranial meningitis (C) and spinal (D) meningitis were observed, often with involvement of underlying parenchyma (original magnification ×400). Microabscesses were frequently observed in cerebral cortex (original magnification ×100) (E), brainstem (not shown) and cerebellum (not shown) of mice that had neurologic symptoms and succumbed to infection.

MSHR305 (bim_{Bp}), replication of MSHR543 (bim_{Bm}) was significantly higher in cervical lymph nodes and spleen (Figure 4). Bacterial loads were low in brains of mice infected with MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) within 3 days of infection despite signs of neurologic involvement by day 5 postinfection in 4 mice infected with MSHR543 (bim_{Bm}), corresponding to bacterial loads in the brain in excess of 10^2 CFU (Figure 2). In comparison, only 1 animal infected with MSHR305 (bim_{Bp}) had symptoms of neurologic melioidosis and required euthanasia within 7 days.

Five mice (50%) survived to 21 days after intranasal infection with MSHR305 (bim_{Bp}), of which 4 had evidence of persistent *B. pseudomallei* infection, with bacteria recovered from the brain of 1 mouse (Figure 4, panel C). Six mice (60%) survived after intranasal infection with MSHR543 (bim_{Bm}), and all had evidence of persistent infection, with bacteria recovered from the brains of 5 mice (Figure 4, panel C).

These findings demonstrate that despite equivalent inoculating doses and similar 21-day mortality rates, the pattern and kinetics of dissemination differ for MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) after intranasal infection, with neurologic involvement occurring with more frequency after infection with MSHR543 (bim_{Bm}).

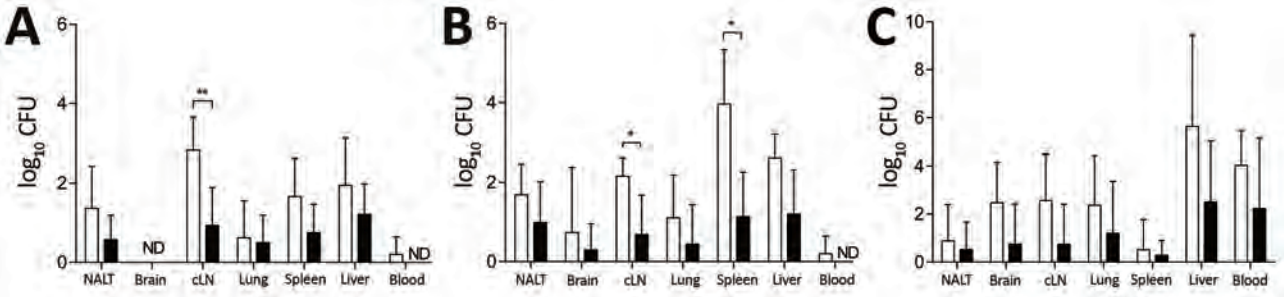

Figure 4. Comparison of early bacterial dissemination and persistence after intranasal infection of C57BL/6 mice with MSHR543 (bim_{Bm}) and MSHR305 (bim_{Bp}) *Burkholderia pseudomallei* isolates. A, B) Bacterial load at day 1 (A) and day 3 (B) postinfection in nasal-associated lymphoid tissue, brain, cervical lymph nodes, lung, spleen, liver, and blood after intranasal infection of C57BL/6 mice (n = 5/time point) with MSHR543 (1.4×10^4 CFU; white bars) and MSHR305 (1.1×10^4 CFU; black bars). C) Bacterial organ loads in mice that survived the 21-day experimental period (MSHR543, n = 6; MSHR305, n = 5). Data are expressed as mean $\log_{10}$ CFU ±SD (upper bars only). cLN, cervical lymph nodes; NALT, nasal-associated lymphoid tissue; ND, not detected. *p<0.05; **p<0.01.

Increased Persistence of bim_{Bm} Strains in Mononuclear Phagocytic Cells

To investigate whether differences observe in systemic dissemination in vivo might be attributable to inherent differences in multiplication of bim_{Bm} and bim_{Bp} strains, we compared the in vitro growth rate of isolates in broth culture. No significant differences were observed for the exponential growth of bim_{Bm} and bim_{Bp} variants in TSB (slope, μhr^{-1}, 0.105 $\pm$0.02 and 0.092 $\pm$0.02, respectively). Having demonstrated that bim_{Bm} and bim_{Bp} strains multiply at the same rate in cell-free media, we next investigated whether intracellular growth rates were comparable for the 2 groups of isolates. Because macrophages and dendritic cells play a pivotal role in protection against *B. pseudomallei* infection (*3*), we compared the uptake and persistence of bim_{Bm} (n = 7) and bim_{Bp} (n = 8) isolates in ex vivo cultures of murine spleen and lymph node–derived macrophages and DC. Absolute numbers of leukocytes were comparable for bim_{Bm}- and bim_{Bp}-infected cultures at 2, 8, and 24 hours postinfection (Figure 5, panel A). The percentage of leukocytes positive for BpOMP staining was also comparable in cultures infected with bim_{Bm} and bim_{Bp} strains at 2 and 8 hours postinfection (Figure 5, panel B). However, by 24 hours, the proportion of $BpOMP^+$ leukocytes was significantly higher in cultures infected with bim_{Bm} than bim_{Bp} strains (p = 0.002), and persistence of bim_{Bm} isolates was greater in $CD11c^+$ dendritic cells (p = 0.012) and $F4/80^+$ macrophages (p = 0.006) than bim_{Bp} strains (Figure 5, panel C). Overall, these data suggest that bim_{Bm} strains of *B. pseudomallei* might possess mechanisms to facilitate their internalization and intracellular persistence within professional phagocytes.

Discussion

Although uncommon, neurologic melioidosis is a severe and debilitating form of *B. pseudomallei* infection, primarily affecting healthy persons with no recognizable risk factors and occurring with increased frequency in Australia (*13*,*14*,*22*). Diagnosis and management of neurologic melioidosis is challenging because of nonspecific clinical presentation, poor diagnostics, and intrinsic resistance to antibiotics. Similar to other intracellular bacteria, *B. pseudomallei* and *B. mallei* are able to spread to adjacent host cells and evade immune surveillance through the formation of actin tails in a process that involves polymerization of host actin monomers (*5*,*10*,*23*,*24*). Polymorphisms in machinery used for actin assembly in other obligate intracellular bacteria have been reported to influence virulence and tissue tropism (*25*–*27*). Recently, isolates possessing a *B. mallei*–like *bimA* allele (bim_{Bm}) were shown to be associated with neurologic involvement in human melioidosis (*12*). Our study provides in vivo evidence of the implications of the bim_{Bm} sequence variation on disease progression and severity of experimental melioidosis. Compared with *B. pseudomallei* isolates with typical BimA motifs, bim_{Bm} variants were more virulent in an animal model of melioidosis when delivered intranasally or subcutaneously. This subset of strains was associated with increased persistence within phagocytic cells and increased likelihood of establishing CNS infection compared with bim_{Bp} strains of *B. pseudomallei*.

Although no evidence from our study indicates preferential seeding of the CNS compared with other tissues, CNS infection did occur with increased frequency and at lower inoculating doses after infection of mice with bim_{Bm} than bim_{Bp} strains of *B. pseudomallei*. Neurologic involvement was observed after intranasal and subcutaneous inoculation with *B. pseudomallei* isolates, although the frequency of CNS infection increased after intranasal infection. Neurologic involvement, as evidenced by bacterial colonization of the brain

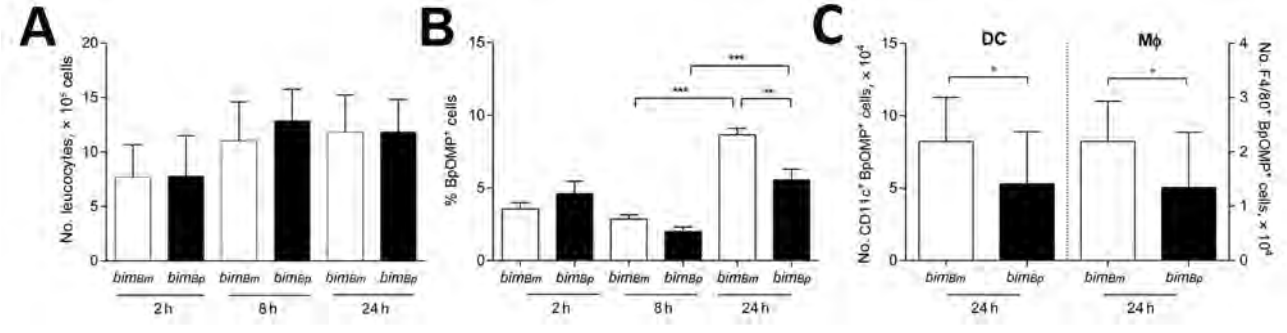

Figure 5. Internalization and persistence of bim_{Bm} and bim_{Bp} *Burkholderia pseudomallei* isolates within murine leukocytes. Spleen and lymph node–derived leukocytes were co-cultured with *B. pseudomallei* isolates at multiplicity of infection 1:5. A) At 2, 8, and 24 hours postinfection, absolute numbers of $CD45^+$ leukocytes were comparable in cultures infected with bim_{Bm} and bim_{Bp} strains. B) Bacterial uptake (2 h) and persistence (8 h and 24 h) was compared by assessing the percentage of $CD45^+$ leukocytes that were positive for intracellular *B. pseudomallei* outer membrane protein antibody (BpOMP) staining using flow cytometry. BpOMP staining increased within leukocytes between 8 hours and 24 hours of cultures. Compared with bim_{Bp}, the percentage of leukocytes positive for intracellular BpOMP was significantly higher in cultures stimulated with bim_{Bm} isolates at 24 hours postinfection. Internalization of bim_{Bm} or bim_{Bp} isolates by $CD11c^+$ dendritic cells and $F4/80^+$ macrophages was comparable (not shown). C) However, persistence of bim_{Bm} strains was significantly higher in dendritic cells and macrophages after 24 hours of culture. Data reflect the mean $\pm$SD of 2 independent experiments. BpOMP, *B. pseudomallei* outer membrane protein antibody; DC, dendritic cells; Mϕ, macrophages. *p<0.05; **p<0.01; ***p<0.001.

and neutrophil infiltration to the cranial and spinal meninges, occurred with more frequency in animals infected with MSHR543 (bim_{Bm}) than those exposed to MSHR305 (bim_{Bp}). Although we observed considerable variability in the sites of abscessation in the CNS, leptomeningitis, meningoencephalitis, and encephalomyelitis were common features in animals that succumbed to infection. Similar neuropathology has been reported in experimental models using intravenous (rather than intranasal) challenge of mice with *B. pseudomallei* (*28*). Furthermore, the neuropathology observed in our study is consistent with the only published histopathologic study of human CNS from patients with melioidosis encephalomyelitis (*14*).

Clinical and experimental data suggest *B. pseudomallei* is capable of using ≥1 mechanism for entry into the brain and spinal cord (*28*–*37*). *B. pseudomallei* has been shown to take advantage of olfactory and trigeminal nerve branches to gain direct access to the brain after respiratory infection of mice (*29*–*32*), and St. John et al. (*32*) recently demonstrated a role for *bimA* in direct CNS invasion by *B. pseudomallei*. Clinical reports also support progression of sinusitis or upper respiratory tract infection with *B. pseudomallei* to neurologic melioidosis (*33*–*35*). Additionally, cortical brain abscesses, a clinical presentation commonly reported for neurologic melioidosis in Southeast Asia (*33*), were observed and are consistent with bacteremic spread of *B. pseudomallei*, directly or through transmigration of infected leukocytes, to the CNS (*28*). In addition to direct infection through the upper respiratory tract, cases of neurologic melioidosis from the Darwin Prospective Melioidosis Study have recently provided strong support for direct brainstem or spinal cord infection occurring through nerve root translocation of bacteria secondary to skin inoculation with *B. pseudomallei* on the face/scalp or limbs (*36*,*37*). The observation of hind leg paraparesis in some animals after *B. pseudomallei* infection in our study provides additional support for this postulated mechanism of CNS entry.

In our study, rapid systemic dissemination to secondary lymphoid tissues was observed for *B. pseudomallei* bim_{Bm} and bim_{Bp} variants, with significantly higher bacterial loads observed earlier in these tissues after infection with the bim_{Bm} variant. Moreover, despite significant reduction in intracellular bacterial loads, persistence of *B. pseudomallei* was evident in vitro in dendritic cells and macrophages, tissue phagocytic cells that *B. pseudomallei* would be exposed to in the early stages of subcutaneous and intranasal infection. We acknowledge that other leukocyte subsets might support intracellular infection with B. pseudomallei and therefore potentially contribute to rapid dissemination of this bacterium in vivo. We limited our assessment to dendritic cells and macrophages because these cells are among the earliest responders to infection and are critical for controlling *B. pseudomallei* infection (*3*,*20*,*21*). Skin dendritic cells also migrate to secondary lymphoid tissues, facilitating the trafficking and systemic dissemination of live intracellular *B. pseudomallei* (*18*). Our data support a potential role for professional phagocytic cells in rapid systemic dissemination of *B. pseudomallei* to distant sites such as the CNS.

As an increasing number of clinically derived strains are genotyped, it is becoming apparent that the manifestations of melioidosis are likely to be influenced by the infecting strain, as well as the route of infection, infecting dose, and host risk factors for melioidosis. Our findings from this current study provide strong support to our clinical observations (*12*) that bim_{Bm} variation is a predictor for severe forms of melioidosis, including neurologic involvement. Despite comparative interrogation of genomes between *B. pseudomallei* strains of contrasting virulence (*38*,*39*), to date *bimA* has been identified as the only gene with a strong association with neurologic melioidosis. However, our observation that bim_{Bp} strains have the potential to invade the CNS, albeit typically at higher inoculating doses than bim_{Bm} strains, suggest that genes other than *bimA* also contribute to *B. pseudomallei* invasion and dissemination in vivo. Under favorable circumstances, avirulent *B. pseudomallei* strains and even the closely related but avirulent bacterium, *B. thailandensis*, can initiate systemic and lethal infection (*40*,*41*). Identifying and characterizing bacterial effector proteins involved in the intracellular and intercellular spread and persistence of *B. pseudomallei* and *B. mallei* will be critical for identification of novel agents to manipulate these processes with therapeutic application.

Acknowledgment

We thank Christopher Davis and Ifor Beacham of Griffith University for their helpful discussions and contribution to the work described in this article.

Dr. Morris is a postdoctoral researcher in the Australian Institute of Tropical Health and Medicine at James Cook University. Her research interests include the immunopathogenesis of *Burkholderia pseudomallei* infection.

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Population Genomics of *Legionella longbeachae* and Hidden Complexities of Infection Source Attribution

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Legionella longbeachae is the primary cause of legionellosis in Australasia and Southeast Asia and an emerging pathogen in Europe and the United States; however, our understanding of the population diversity of *L. longbeachae* from patient and environmental sources is limited. We analyzed the genomes of 64 *L. longbeachae* isolates, of which 29 were from a cluster of legionellosis cases linked to commercial growing media in Scotland in 2013 and 35 were non–outbreak-associated isolates from Scotland and other countries. We identified extensive genetic diversity across the *L. longbeachae* species, associated with intraspecies and interspecies gene flow, and a wide geographic distribution of closely related genotypes. Of note, we observed a highly diverse pool of *L. longbeachae* genotypes within compost samples that precluded the genetic establishment of an infection source. These data represent a view of the genomic diversity of *L. longbeachae* that will inform strategies for investigating future outbreaks.

Legionellosis presents as 2 clinically distinct forms: an influenza-like illness called Pontiac fever and a severe pneumonia known as Legionnaires' disease (*1*). In Europe and the United States, most legionellosis cases are caused by *Legionella pneumophila* serogroup 1 (*1*,*2*); <5% of cases are caused by nonpneumophila *Legionella* spp. (*3*,*4*). In Australasia, New Zealand, and some countries in Asia, infections caused by *L. longbeachae* occur at comparable levels to infections caused by *L. pneumophila* (*5*–*7*). Unlike *L. pneumophila* infections, which are typically linked to artificial water systems, *L. longbeachae* infections are associated with exposure to soil, compost, and potting mixes (*8*).

The number of legionellosis cases caused by *L. longbeachae* is increasing worldwide (*7*), with a notable rise reported across Europe (*9*–*11*). Within the United Kingdom, most *L. longbeachae* infections have been identified in Scotland, where 6 cases were diagnosed during 2008–2012 (*12*) and another 6 were diagnosed in the summer of 2013 and represented a singular increased incidence or cluster with all patients requiring intensive care hospitalization (*11*). Epidemiologic investigation revealed that most patients from the 2013 cluster were avid gardeners, and *L. longbeachae* was isolated from respiratory secretions and from samples of the growing media they had used for gardening before becoming ill (*11*,*12*). However, an investigation into the provenance of the growing media did not reveal a single commercial or manufacturing source that would suggest a common origin for the *L. longbeachae* associated with the outbreak (*11*).

Molecular typing methods used to discriminate between *L. longbeachae* and other *Legionella* spp. and between the 2 *L. longbeachae* serogroups have limited efficacy, and although considerable evidence supports growing media as a source for *L. longbeachae* infections (*13*,*14*), there is still a lack of genetic evidence for an epidemiologic link. Furthermore, a population genomic study involving large numbers of *L. pneumophila* isolates has been conducted (*15*,*16*), but the same has not been done for *L. longbeachae*, so the diversity of environmental and pathogenic genotypes and the relationship between them remains unknown for *L. longbeachae*. To examine the etiology of the 2013 cluster of legionellosis cases in Scotland in the context of *L. longbeachae* species diversity, we analyzed the genomes of 70 *Legionella* spp. isolates from 4 countries over 18 years.

Materials and Methods

Bacterial Isolates

We sequenced 65 isolates that had previously been identified as *L. longbeachae*. These isolates were obtained during 1996–2014 from several patients, growing media samples (including compost and soil), and a hot water supply. Of these isolates, 55 were from Scotland (29 from the 2013 cluster of infections and 26 from other clinical and environmental samples) and 10 were from patients and

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DOI: http://dx.doi.org/10.3201/eid2305.161165

environmental compost samples in New Zealand (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/5/16-1165-Techapp1.pdf).

In our analysis, we also included all publicly available genome sequences for *L. longbeachae*: *L. longbeachae* NSW150 (serogroup 1) and *L. longbeachae* C-4E7 (serogroup 2) isolated from patients in Australia; and *L. longbeachae* D-4968 (serogroup 1), *L. longbeachae* ATCC39642 (serogroup 1), and *L. longbeachae* 98072 (serogroup 2) isolated from patients in the United States (*17*–*19*). We sequenced multiple isolates (n = 2 to 5) for each of 3 patients and their linked growing media samples from the 2013 outbreak in Scotland and for 2 additional compost samples. The species of all isolates had been determined by serotyping or macrophage infectivity potentiator (mip) gene sequencing (*20*,*21*).

Bacterial Culture, Genomic DNA Isolation, and Whole-Genome Sequencing

We cultured *Legionella* spp. isolates in a microaerophilic and humid environment at 37°C on BCYE (buffered charcoal yeast extract) agar plates for 48 h. We then picked individual colonies from the plates and grew them in ACES-buffered yeast extract broth containing *Legionella* BCYE Growth Supplement (Oxoid Ltd., Basingstoke, UK) with shaking at 37°C for 24–48 h. We extracted genomic DNA from fresh cultures by using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Benelux B.V., Venlo, the Netherlands).

We prepared sequencing libraries by using the Nextera XT kit for MiSeq or HiSeq (all from Illumina, San Diego, CA, USA) sequencing at Edinburgh Genomics, University of Edinburgh (Edinburgh, Scotland, UK). For each isolate, one 2 × 250–bp or two 2 × 200–bp paired-end sequencing runs were carried out using the MiSeq and HiSeq technologies, respectively. Raw reads were quality checked using FastQC v0.10.1 (*22*), and primers were trimmed by using Cutadapt (*23*). We used wgsim software (*24*) to simulate sequence reads for publicly available, complete whole-genome sequences.

Bioinformatic Analysis and Data Deposition

A detailed description of the bioinformatic analyses is available in the online Technical Appendix. The sequence data for the 65 genomes of *Legionella* spp. sequenced in this study were deposited in the SRA database (accession no. PRJEB14754).

Results

Limitations of Current Typing Approaches for *Legionella* spp. Identification

We sequenced 65 isolates obtained from several patients and environmental samples over 18 years in different countries and previously identified as *L. longbeachae*. To confirm the species identity of the *Legionella* isolates, we constructed a phylogenetic tree that included all *Legionella* type strains for which cultures are available, based on the 16S rRNA gene sequence (*25*). We also built phylogenetic trees based on the whole-genome content and core-genome diversity. For each approach, 64 of the 70 isolates examined co-segregated within the *L. longbeachae*–specific clade, 4 isolates clustered with *Legionella anisa*, and 2 belonged to a separate clade that was distinct from all known *Legionella* spp. (Figure 1; online Technical Appendix Figures 1, 2). The species identities were further supported by determination of the average nucleotide identity values (online Technical Appendix Figure 3), a widely used method for bacterial species delineation based on genomic relatedness (*26*). Of note, *L. anisa* is the most common nonpneumophila *Legionella* spp. in Europe (*27*–*29*). In addition, *L. longbeachae* isolates 13.8642 (from a compost sample from Scotland) and 13.8295 (from a patient in New Zealand) belong to a putative novel *Legionella* spp. Overall, the data indicate that current serotyping methods and *mip* gene sequencing are limited in their capacity to identify *L. longbeachae* to the species level.

To investigate the genetic relatedness of *L. longbeachae* strains associated with the 2013 outbreak to temporally and geographically distinct isolates, we constructed a core genome–based neighbor-joining tree of the 64 confirmed *L. longbeachae* isolates obtained from 4 countries over 18 years (online Technical Appendix Figure 4). This phylogenetic tree presents a comet-like pattern, with 2 distinct clades separated by 9,911 single-nucleotide polymorphisms, representing the major serogroups (serogroups 1 and 2) previously identified for *L. longbeachae* (*20*), each containing isolates from patient and environmental samples from different years. In contrast with findings from a previous analysis of 2 isolates of *L. longbeachae* serogroup 1 (*20*), we observed a higher diversity among the 56 isolates within serogroup 1 (online Technical Appendix Figures 1, 4); this finding is not unexpected, given the difference in the number of genomes examined. Nevertheless, compared with isolates from the same serogroup in other *Legionella* spp., such as *L. pneumophila* serogroup 1 (2% polymorphism) (*20*), *L. longbeachae* serogroup 1 exhibits a lower diversity (<0.1% polymorphism). Although serogroup 1 and 2 clades contained isolates from Scotland, Australasia, and the United States, 96% of the isolates from Scotland (including all of the 2013 outbreak isolates) belonged to serogroup 1, suggesting that serogroup 1 may be more clinically relevant in Scotland than in some other countries where *L. longbeachae* is a more established cause of legionellosis. However, analysis of more isolates from different countries would be required to investigate this observation further.

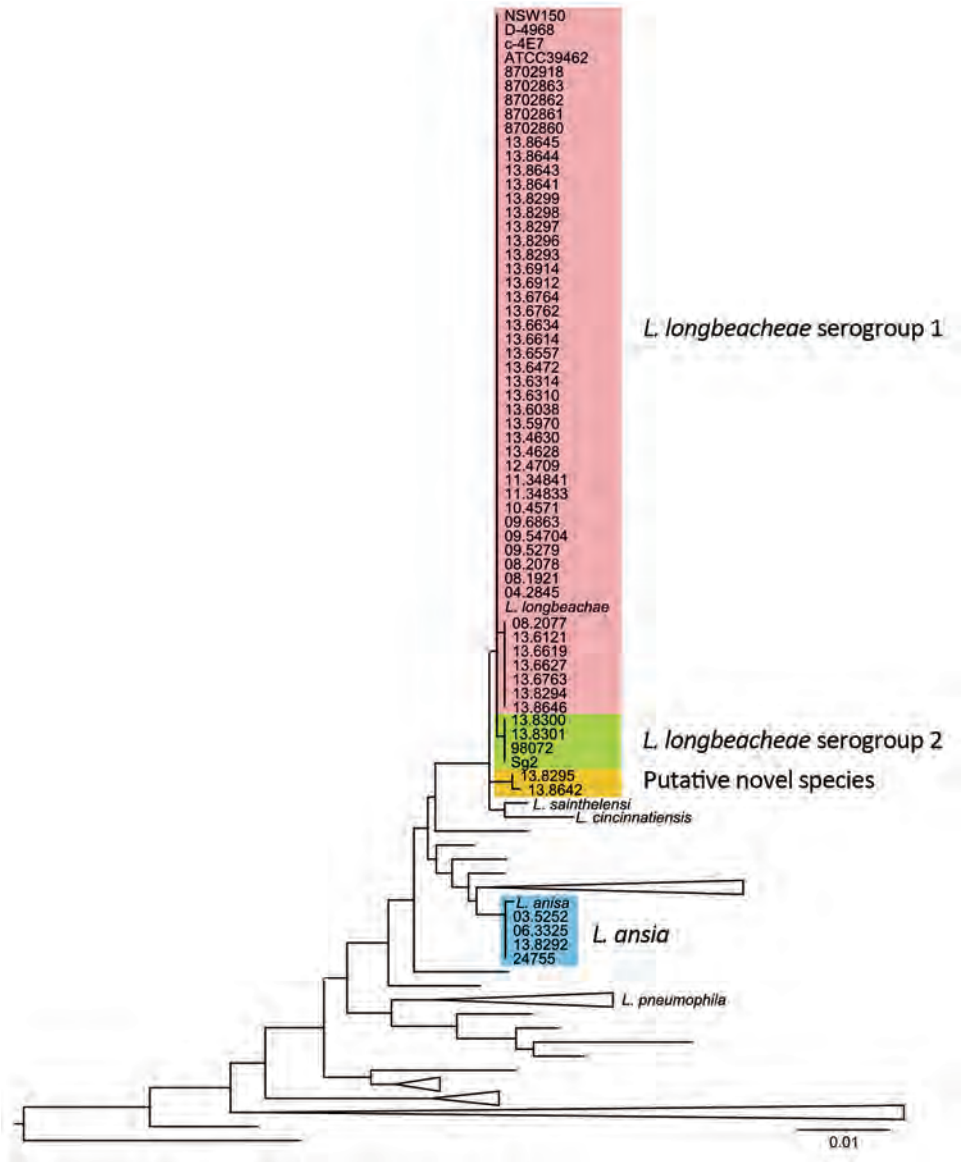

Figure 1. 16S rRNA gene–based phylogenetic tree of the sequenced genomes and all the cultured and type *Legionella* spp. strains available in the ribosomal database project (http://rdp.cme.msu.edu/), as accessed in May 2015. Scale bar indicates the mean number of nucleotide substitutions per site.

Effect of Recombination on *L. longbeachae* Serogroup 1 Population Structure

It is established that recombination has played a key role in shaping the evolutionary history of *L. pneumophila*, but its effect on *L. longbeachae* population structure is unknown (*22*,*30*). This knowledge is critical because for highly recombinant bacteria, recombination networks may represent evolutionary relationships more explicitly than traditional phylogenetic trees. Therefore, we constructed a recombination network of all serogroup 1 isolates by using the neighbor-net algorithm of SplitsTree4 (*31*). The resultant network displayed a reticulate topology with an extensive reticulated background from which clusters of isolates emerge, supporting an evolutionary history involving recombination ($p< 0.01$ by ϕ test) (*32*), followed by clonal expansion and subsequent additional recombination events among some lineages (online Technical Appendix Figure 5). Using BratNextGen (*33*), we identified a total of 94 predicted recombination events affecting more than half of the core genome (1.74 Mb of 3.36 Mb) and representing recent and ancient recombination events of different sizes (range 1,350 bp–350 Kbp) distributed across the phylogeny (online Technical Appendix Figure 6). Given the reported limitation in sensitivity of BratNextGen for the identification of all recombination events (*34*), we also used ClonalFrameML (*35*), an algorithm that uses maximum likelihood inference to simultaneously detect recombination in bacterial genomes and account for it in phylogenetic reconstruction. The estimated average length of the recombined fragments was 8,047 bp, and the ratio of recombination to mutation was 1.42, indicating a greater role for recombination over mutation in the diversification

of *L. longbeachae*. This estimate is in accordance with early estimates for *L. pneumophila* based on multiple gene sequence data (*36*), but it is low compared with recent estimates based on whole-genome sequence data [recombination to mutation ratios of 16.8 (*30*) or 47.93 (*37*)]. Differences in the clonal diversity of *Legionella* spp. sequence datasets used to determine recombination rates could affect the estimates. Reconstruction of the phylogeny after removal of all predicted recombinant sequences resulted in a tree with largely similar clusters of isolates but with reduced branch lengths and variation in the position of nodes deep in the phylogeny (Figure 2).

Accessory Genome Analysis Indicates Extensive Interspecies and Intraspecies Gene Flow

The extent to which horizontal gene transfer occurs among *L. longbeachae* isolates and between *L. longbeachae* and other *Legionella* spp. is unknown. In our study, the pangenome of *L. longbeachae* represented by the 56 serogroup 1 isolates was 6,890 genes, including a core genome of 2,574 genes; the average gene content was 3,558 genes per strain. The accessory genome, which included only strain-dependent genes varied from 809 to 1,155 genes, depending on the strain. A parsimony clustering analysis based on the presence or absence of all genes classified the isolates in a manner distinct from that in a core genome–based maximum-likelihood tree, suggesting extensive horizontal gene transfer among *L. longbeachae* isolates (online Technical Appendix Figures 1, 2). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of all assembled contigs was used to filter for plasmid-related homologous sequences, revealed 2 major plasmids: pLLO, described previously in *L. longbeachae* NSW150 (*20*), and pLELO, originally identified

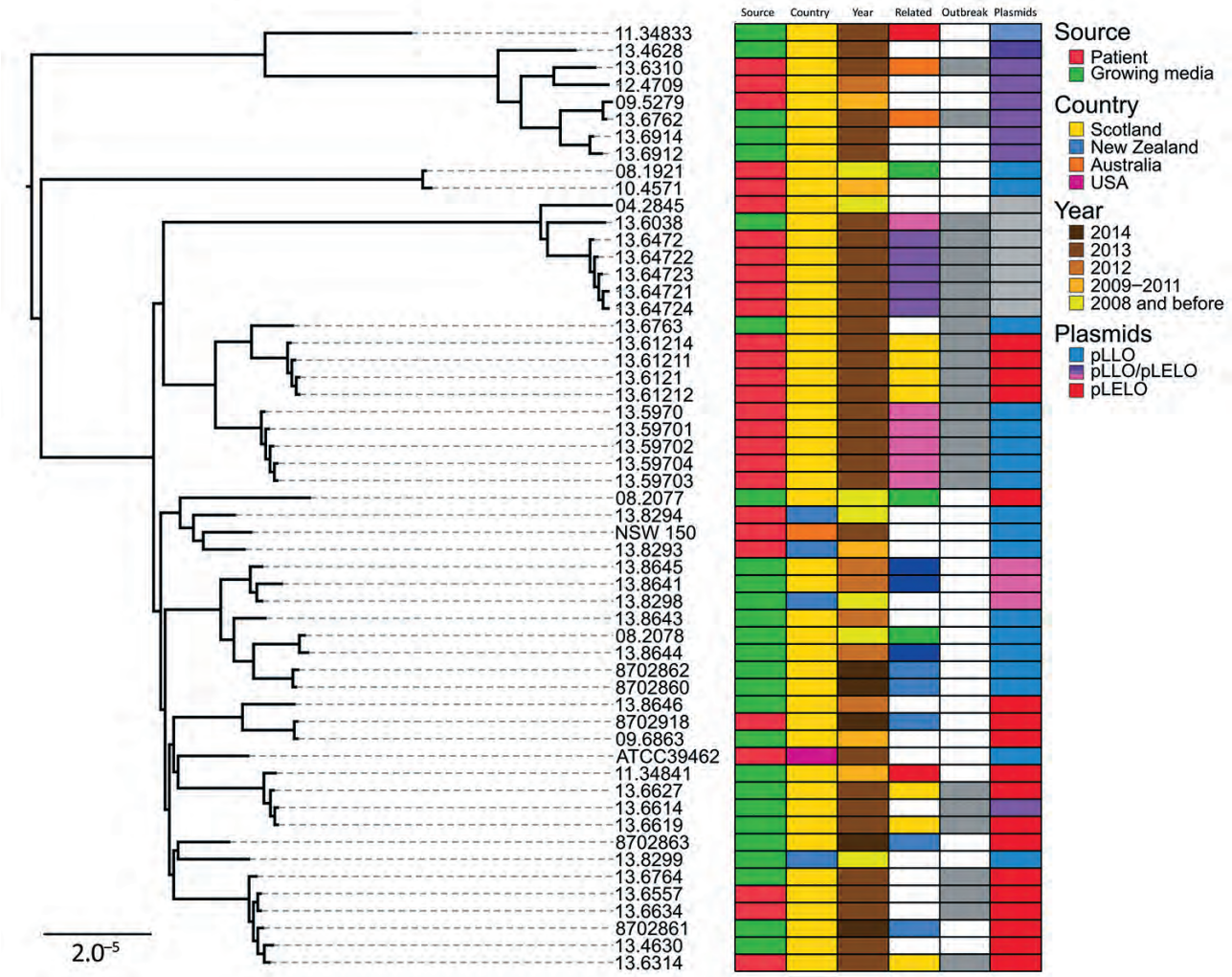

Figure 2. Core genome–based maximum-likelihood phylogeny of *Legionella longbeachae* serogroup 1 isolates corrected for recombination; source, country, year of isolation, relatedness and plasmid carriage are indicated. Related isolates are shown in the same color; those from the 2013 outbreak are indicated by gray. Isolates from the same patient are clustered together but do not co-segregate with cognate compost samples. Scale bar indicates the mean number of nucleotide substitutions per site.

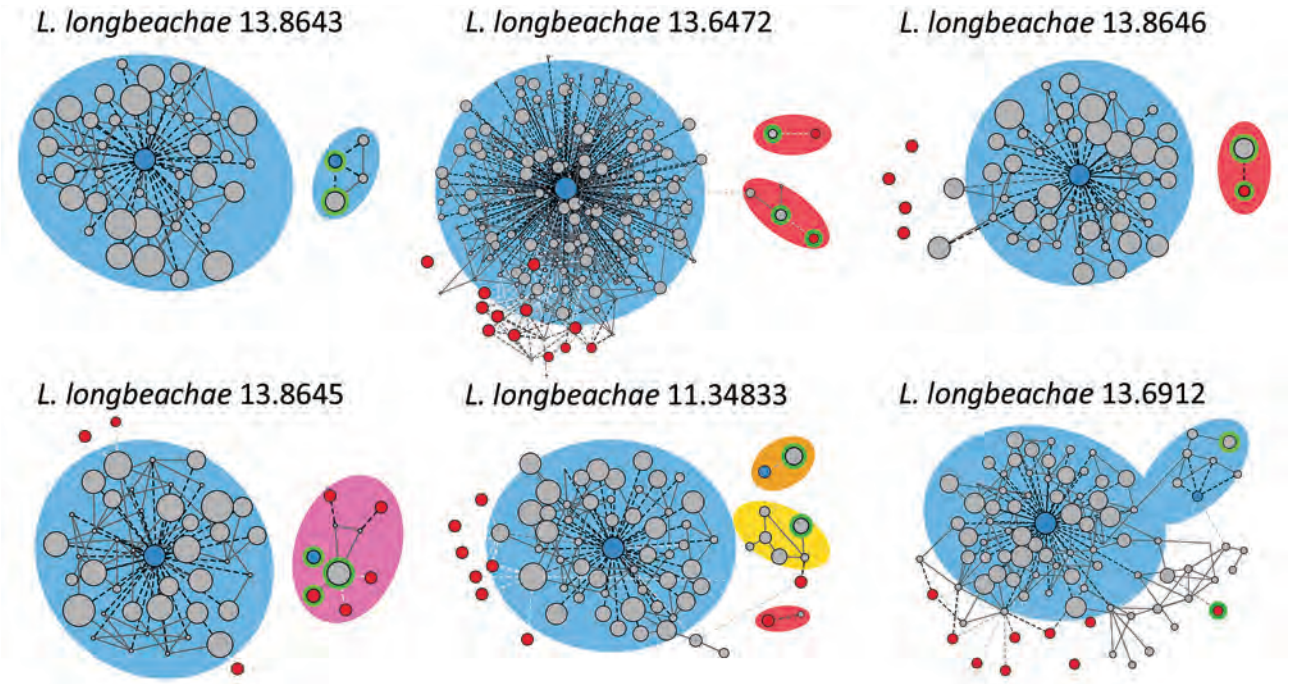

Figure 3. *Legionella longbeachae* plasmid analysis: contigs networks reconstructions for 6 representative *L. longbeachae* types of plasmid content. The networks of the contigs representing the main chromosome and plasmids comprising the genome obtained by using PLACNET (*38*), a program enabling reconstruction of plasmids from whole-genome sequence datasets. The sizes of the contig nodes (in gray) are proportional to their lengths; continuous lines correspond to scaffold links. Dashed lines represent BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) hits to the *L. longbeachae* (blue) or *L. pneumophila* (red) strains; intensity of the line is proportional to the hit (white indicates low, black indicates high). Green lines correspond to plasmid contigs. Background colors indicate species relatedness for the main chromosome and plasmids (blue for *L. longbeachae*, red for *L. pneumophila*, pink for a combination of both, and yellow for previously unidentified genomic content).

in *L. pneumophila subsp. pneumophila* (*22*). Of the 55 serogroup 1 isolates, 36 contained sequences for the pLLO and pLELO plasmids. Of note, the distribution of these plasmids among the *L. longbeachae* isolates correlated with the gene content–based clustering, whereas the distribution of plasmids in the core genome–based tree was independent of the phylogeny (Figure 2). In addition, 11 isolates appeared to contain plasmids with sequences homologous to those for pLLO and pLELO, which is indicative of recombinant forms of the plasmid. Further examination of plasmid diversity using a modified version of PLACNET (*38*), a program enabling reconstruction of plasmids from whole-genome sequence datasets, confirmed that some plasmids consisted of a mosaic of recombinant fragments homologous to pLELO, pLLO, or other unknown plasmids (Figure 3). Overall, these data indicate the high prevalence of specific plasmids among *L. longbeachae* isolates and reveal extensive recombination and horizontal gene transfer among different *Legionella* spp (*39*). The high prevalence of plasmids in *L. longbeachae* is notable, considering these elements may be less common in *L. pneumophila* (*30*).

To examine the possibility that clinical and environmental isolates of *L. longbeachae* contained genomic differences reflecting their distinct origins, we compared their accessory genome content. For isolates obtained from a single patient sample, the accessory genome was highly conserved compared with those for environmental isolates from a single compost sample or closely related environmental isolates from distinct compost samples (Figure 4, panel A). In addition, considering the average gene content of all sequenced isolates (28 clinical and 27 environmental), the gene content for *L. longbeachae* from growing media samples (3,586 genes) was significantly higher than that for isolates from patients (3,533 genes; 2-sample *t*-test, $t = 2.5213$; d.f. = 53; $p = 0.01474$) (Figure 4, panel B). The data imply that gene loss occurs during human infection or that *L. longbeachae* strains with reduced gene content have enhanced human infectivity. However, we did not identify a specific enriched gene or functional category in clinical or environmental samples (data not shown).

Source Attribution Confounded by Complex Serogroup 1 Populations within Environmental Samples

Having accounted for the influence of recombination on the phylogeny of *L. longbeachae*, we investigated the diversity of isolates associated with 5 patients and their linked compost samples obtained during 2008–2014, including

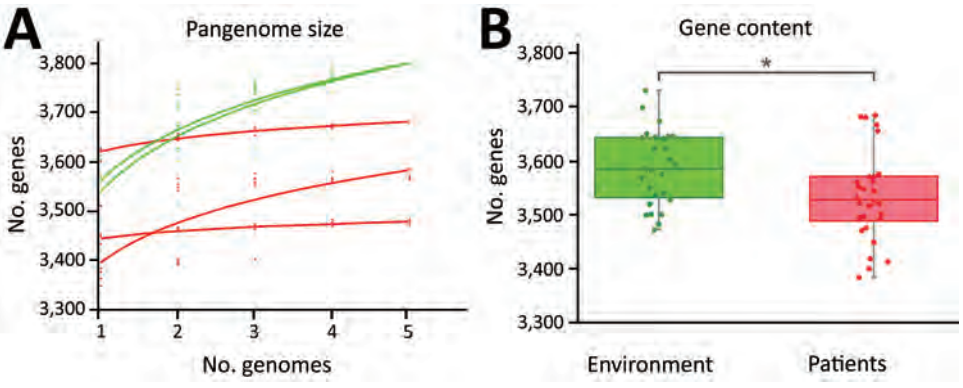

Figure 4. Variation in gene content between environmental and patient *Legionella longbeachae* samples. A) Increase in pangenome size with every addition of a *L. longbeachae* genome. Environmental isolates pangenomes (green) are larger and continue increasing after the addition of 5 genomes, consistent with an open pangenome, but the within-patient pangenome plateaus quickly, consistent with a more closed pangenome. B) Average gene content of environmental isolates is significantly higher than that of clinical isolates (p = 0.01474).

3 patients from the 2013 outbreak in Scotland. Of note, isolates from the 2013 outbreak were distributed across several subclades of the tree, indicating that the infections were caused by different strains (Figure 2). However, all isolates from a single patient clustered together, consistent with a monoclonal etiology of each infection. Of note, for all 5 patients, clinical isolates were not closely allied to the environmental isolates obtained from linked compost samples, and therefore a genetic link between patient and compost samples could not be established. Most subclades included isolates of diverse geographic origin, consistent with a wide distribution for *L. longbeachae* strains; however, 3 *L. longbeachae* isolates originating from Australasia (strains 13.8294, 13.8293, and NSW150) belonged to their own region-specific cluster (Figure 2).

We hypothesized that the lack of genetic relatedness between *L. longbeachae* isolates from patients and linked compost samples could be explained by a highly diverse population of *L. longbeachae* in growing media samples compounded by a sampling strategy consisting of a single sequenced isolate. All 5 compost samples for which we had >1 isolate contained isolates distributed across multiple clades in the phylogenetic tree. In particular, 5 isolates from the same growing media sample linked to a patient infected in Edinburgh in 2014 were distributed across 4 distinct clades, demonstrating that within a single environmental sample, considerable species diversity may be represented (Figure 2). Taken together, these data suggest that for future outbreak investigations, extensive sampling of environmental samples may be required to identify genotypes responsible for episodes of legionellosis infection, if indeed they are present.

Discussion

Our findings reveal the population genomic structure for *L. longbeachae*, an emerging pathogen in Europe and the United States, and includes a genome-scale investigation into an outbreak of *L. longbeachae* legionellosis. We provide evidence for extensive recombination and lateral gene transfer among *L. longbeachae*, including the presence of widely distributed mosaic plasmids that have likely recombined with plasmids from other *Legionella* spp., suggesting an ecologic overlap or shared habitat. Our analysis highlights the need to account for recombination events when determining the genetic relatedness of *L. longbeachae* isolates.

Our application of whole-genome sequencing for diagnostic purposes revealed the misidentification, using current serotyping methods, of several *L. anisa* isolates as *L. longbeachae* and led to the identification of a putative novel *Legionella* sp. linked to legionellosis. These findings highlight the limitations of current typing methods for differentiation of *Legionella* spp. and accurate identification of legionellosis etiology.

We used whole-genome sequencing to attempt to establish a genetic link between legionellosis infections and associated compost samples. Our inability to establish a link probably reflects the traditional strategy of single isolate sampling, which when applied to a highly diverse pool of *L. longbeachae* genotypes fails to detect the infecting genotype. We suggest that the approach to investigating the source of future legionellosis cases linked to growing media will require a radical revision of sampling protocols to maximize the chances of isolating the infecting strain, if present. Taken together, our findings provide a view of the population structure of *L. longbeachae* and highlight the complexities of tracing the origin of legionellosis associated with growing media. Overall, our findings demonstrate the resolution afforded by whole-genome sequencing for understanding the biology underpinning legionellosis and provide information that should be considered for future epidemiologic investigations.

Acknowledgment

We are grateful to Carmen Buchrieser for providing the original sequence reads for *L. longbeachae* strains ATCC39642, 98072, and C-4E7. We thank David Harte for supplying the New Zealand strains.

Funding was provided by the Chief Scientist's Office Scotland (grant ETM/421 to J.R.F.) and by the Biotechnology and Biological Sciences Research Council (ISP3 grant BB/J004227/1 to J.R.F.).

Mr. Bacigalupe is a PhD candidate at the Roslin Institute, University of Edinburgh. His primary research focuses on the evolution, adaptation, and outbreak dynamics of bacterial pathogens.

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Insecticide-Treated Nets and Protection against Insecticide-Resistant Malaria Vectors in Western Kenya

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Insecticide resistance might reduce the efficacy of malaria vector control. In 2013 and 2014, malaria vectors from 50 villages, of varying pyrethroid resistance, in western Kenya were assayed for resistance to deltamethrin. Long-lasting insecticide-treated nets (LLIN) were distributed to households at universal coverage. Children were recruited into 2 cohorts, cleared of malaria-causing parasites, and tested every 2 weeks for reinfection. Infection incidence rates for the 2 cohorts were 2.2 (95% CI 1.9–2.5) infections/person-year and 2.8 (95% CI 2.5–3.0) infections/person-year. LLIN users had lower infection rates than non-LLIN users in both low-resistance (rate ratio 0.61, 95% CI 0.42–0.88) and high-resistance (rate ratio 0.55, 95% CI 0.35–0.87) villages (p = 0.63). The association between insecticide resistance and infection incidence was not significant (p = 0.99). Although the incidence of infection was high among net users, LLINs provided significant protection (p = 0.01) against infection with malaria parasite regardless of vector insecticide resistance.

The launch of the Roll Back Malaria (RBM) program in 1998 by the World Health Organization (WHO), United Nations Children's Fund, United Nations Development Partnership, and the World Bank was a catalyst for renewed global commitment to the fight against malaria, leading to massive investment (*1*). There followed a tremendous decline in disease and death caused by malaria, with a 40% reduction in the incidence of malaria cases between 2000 and 2015 and a reduction in malaria-attributable death from 839,000 in 2000 to 438,000 in 2014 (*1*,*2*). This decline has been brought about principally by the use of insecticide-based vector control tools, such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying. It is estimated that LLINs have been a key malaria prevention tool in sub-Saharan Africa, accounting for ≈68% of the decline of clinical cases (*3*).

Following the massive scale-up of insecticide-based vector control, resistance was observed in almost all countries in sub-Saharan Africa (http://www.irmapper.com) (*4*). Twelve insecticide products (containing pyrethroids, organochlorines, organophosphates, or carbamates) are available for vector control. Only pyrethroids are used for LLINs because they are safe, efficacious against malaria vectors, and relatively low cost (*5*–*7*).

On a programmatic scale, a 10-fold increase in malaria cases was observed in KwaZulu-Natal, South Africa, subsequent to the re-emergence of pyrethroid-resistant *Anopheles funestus* mosquitoes and emergence of malaria parasite drug resistance to sulfadoxine/pyrimethamine (*8*). Upon switching to DDT for indoor residual spraying and artemether lumefantrine for malaria case management, malaria parasite control was restored with a rapid decline in malaria case incidence (*8*–*10*). Similar observations were made in Uganda, where DDT and pyrethroids were used for indoor residual spraying in the presence of resistance; as soon as carbamates were deployed, the malaria parasite slide positivity rate declined substantially (*11*).

Malaria interventions including universal LLIN coverage, targeted deployment of indoor residual spraying, and prompt diagnosis and treatment have been scaled up in

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DOI: http://dx.doi.org/10.3201/eid2305.161315

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western Kenya since the early 2000s. Control tools targeting endophagic and endophilic malaria vector mosquitoes have been remarkably effective in reducing *An. gambiae* and *An. funestus* mosquito populations that were known to be anthropophilic; these tools have led to lowered malaria inoculation rates and consequently >50% declines in malaria disease and death (*12–14*). In western Kenya, malaria prevalence in children <5 years of age declined to ≈30% in 2006, after which it stabilized or slightly increased (*15*,*16*). A possible cause of this persistent infection in children is insecticide resistance in the local vector population. Concerns that resistance could be compromising malaria vector control and, therefore, hampering efforts to lower malaria parasite transmission have led to calls for more effective insecticide resistance management (*17*,*18*).

Population-based active surveillance can complement routine passive sentinel surveillance systems by providing public health data and insights into the complex epidemiology of disease. Active infection-detection cohorts are studies that involve clearing participants of infections, following them up, and testing them at regular intervals, regardless of whether they are symptomatic, until the first infection appears, at which point the follow-up is discontinued. These types of studies provide estimates of time to infection in participants and are useful because they enable estimations of various parameters associated with disease (*19*,*20*). The main advantage of using population-based malaria parasite surveillance is that it provides the data needed to determine the infection rate and the populations at risk for infection (*19*).

This study was implemented as part of a large, multicountry program set up to quantify the impact of insecticide resistance on the effectiveness of insecticide-based vector controls (*21*). To determine if insecticide resistance altered the effectiveness of LLINs in malaria endemic subcounties of western Kenya, we conducted population-based malaria parasite active infection-detection cohort studies.

Methods

Study Sites

We conducted this study in 4 malaria-endemic subcounties in western Kenya described previously (*22*). In brief, in 2014, the National Malaria Control Programme conducted a massive campaign to distribute nets; a mix of PermaNet 2.0 (treated with deltamethrin) and Olyset nets (treated with permethrin) were distributed in the 4 subcounties Bondo, Teso, Rachuonyo, and Nyando to meet the universal coverage threshold of 1 net per 2 persons. Subsequent routine distribution was conducted through health facilities to pregnant women and children <5 years of age. Twenty sublocations (hereafter referred to as clusters) were randomly selected from each of the 4 subcounties where the initial insecticide resistance assessment was conducted (*21*,*22*). After the assessment, the clusters were categorized into 3 groups: those with ≥80% mosquito mortality to deltamethrin or permethrin (categorized as low-resistance clusters), those with mosquito mortality <80% but ≥60% (categorized as medium-resistance clusters), and those with mosquito mortality <60% (categorized as high-resistance clusters). Finally, 13 low- and high-resistance clusters were selected in Rachuonyo, 11 in Teso, 16 in Bondo, and 10 in Nyando, giving a total of 50 clusters for subsequent studies. Each cluster had 10–30 villages, each with ≈100 households. We recruited children 6 59 months of age from households immediately around larval habitats that were sampled by the entomology teams for assessing insecticide resistance; moving out in concentric circles from the larva habitats, we recruited study participants until 20 eligible and consenting households were enrolled.

Study Design and Sample Collection

Community health workers were trained to use rapid diagnostic test kits SD Bioline Malaria Ag P.f/Pan (Standard Diagnostics, Gyeonggi-do, South Korea) and CareStart Malaria HRP2 (Pf) (Access Bio, Inc., Somerset, NJ, USA) and to appropriately administer artemisinin combination therapy (Coartem Dispersible [20 mg artemether/120 mg lumefantrine], Novartis, Basel, Switzerland) for the treatment of malaria. The study ran September 2013–May 2014 for cohort 1 and July–December 2014 for cohort 2. Twenty children 6–59 months of age were recruited for each cluster within each cohort. Subject to written informed consent from the parent or caregiver, 1 eligible child was enrolled from each selected household.

At recruitment, all children were treated with a standard therapeutic dose of artemether/lumefantrine. To verify clearance of malaria parasites, 14 days later, thick and thin blood smears were taken from children and assessed for infection by microscopic examination. Any children whose smear results were positive were excluded from follow-up analysis. Community health workers visited each child at home every 2 weeks to test for infection with malaria parasites using rapid diagnostic tests. Children who tested positive for malaria parasite were treated and excluded from further follow-up. LLIN use on the previous night was recorded at each visit. Data in the field was collected using paper forms and then entered into electronic forms made with Microsoft Excel and Access software (Microsoft, Redmond, WA, USA).

From July 2013 through October 2013 and August 2014 through November 2014, we conducted insecticide resistance monitoring in each of the clusters. We collected and reared *An. gambiae sensu lato* (*s.l.*) mosquito larvae and adults and tested them for susceptibility to deltamethrin insecticide using the WHO standard test

(*22*). We performed these bioassays with both permethrin and deltamethrin at baseline (*22*), but because mortality upon exposure to these 2 insecticides were positively correlated (online Technical Appendix Figures 1, 2, https://wwwnc.cdc.gov/EID/article/23/5/16-1315-Techapp1.pdf) and mosquito population size was small, only deltamethrin was used for bioassays in subsequent years. Ethical approval for this study was obtained from the Kenya Medical Research Institute Ethical Review Committee (no. SSC 1677).

Data Analysis

We used individual visit data for each child to conduct time-to-event analysis to determine incidence rates and incidence rate ratios (RRs) using survival analysis and Poisson regression models. Children who had >5 weeks between visits were censored. Incidence rates and 95% CIs were calculated per person-year for each district and year.

We used insecticide resistance data (percentage mosquito mortality upon exposure to deltamethrin) to dichotomize clusters into high- and low-resistance clusters by using the median mortality for that year, namely, 88% for 2013 (clusters with mortality rates ≥88% were categorized as low resistance and those with mortality rates <88% as high resistance) and 67% for 2014 (clusters with mortality rates ≥67% were categorized as low resistance and those with mortality rates <67% as high resistance). In combined analysis of both years, we used the overall median mortality (82%) to dichotomize clusters into high or low resistance for net users and non–net users. Recommended methods (*23*) were used to compute SEs, allowing for the correlation of responses within clusters. We used incidence RRs and corresponding 95% CIs to compare incidence rates between users and nonusers of LLINs and between high- and low-resistance clusters. Modification of the effect of net use on infection incidence depending on insecticide resistance level (mortality to deltamethrin in bioassays) was assessed through the inclusion of an appropriate interaction term in the regression model. Net use was included in models as a time-varying covariate.

We plotted cluster-specific incidence rates for each year and cluster-specific RRs for non–net users and net users against mosquito mortality with deltamethrin exposure. The slope of best-fitting straight lines were determined by using linear regression of cluster-specific incidence on cluster-specific mosquito mortality.

Table 1. Characteristics of cohorts used to detect active malaria parasite infections, Kenya, 2013 and 2014

Characteristic	Cohort 1, n = 989	Cohort 2, n = 969
Female sex, % (no.)	49 (481)	49 (478)
Median age, y (range, mo–y)	2.5 (4–5)	2.2 (1–6)
Average follow-up per child, d	80	95
No. infections	279	483

Results

Active Infection Cohorts 1 and 2

Approximately 1,000 children were recruited into each active infection cohort. The median age of children at recruitment was 2.5 years for cohort 1 and 2.2 years for cohort 2. For cohort 1, each child was followed for 80 days, and a total of 279 infections were detected; for cohort 2, each child was followed for 95 days, and a total of 483 infections were detected (Table 1). LLIN use was 81.3% for cohort 1 and 85.7% for cohort 2. The overall incidence rate of infection with the malaria parasite was 2.2 (95% CI 1.9–2.5) infections/person-year for cohort 1 and 2.8 (95% CI 2.5–3.0) infections/person-year for cohort 2. The subcounty-specific infection incidences were 1.2–3.0 infections/person-year in cohort 1 and 1.8–4.1 infections/person-year in cohort 2 (Table 2).

In low-resistance clusters, the malaria parasite infection incidence rate was 4.0 (95% CI 3.2–5.2) infections/person-year among non–net users and 2.3 (95% CI 2.1–2.5) infections/person-year among net users (RR 0.61, 95% CI 0.42–0.88; p = 0.01). In high-resistance clusters, incidence was 5.3 (95% CI 4.0–7.1) infections/person-year among non–net users and 2.9 (95% CI 1.7–3.2) infections/person-year among net users, a 45% reduction (RR 0.55, 95% CI 0.35–0.87; p = 0.01) in malaria parasite incidence among net users (Table 3).

Association between Malaria Parasite Infection Incidence and Insecticide Resistance

We found no association between malaria parasite infection incidence and insecticide resistance when comparing

Table 2. Incidence of malaria parasite infection by subcounty and cohort, Kenya, 2013 and 2014

Subcounty	Cohort	No. clusters	No. children	No. malaria episodes	Total follow-up time, person-years	Incidence, infections/person-year (95% CI)
Bondo	1	16	184	76	35.0	2.2 (1.7–2.7)
	2	16	255	154	58.5	2.6 (2.2–3.1)
Nyando	1	10	147	33	28.3	1.2 (0.8–1.6)
	2	10	180	83	47.3	1.8 (1.4–2.2)
Rachuonyo	1	13	192	97	32.2	3.0 (2.5–3.7)
	2	13	208	136	42.9	3.2 (2.7–3.8)
Teso	1	11	157	73	29.4	2.5 (2.0–3.1)
	2	11	156	110	27.0	4.1 (3.4–4.9)

Table 3. Incidence of malaria parasite infection in net users and non–net users in low– and high–insecticide resistance clusters, Kenya, 2013 and 2014

Parameter	No. children	Follow-up time, person-years	No. infections detected	Incidence, infections/person-year (95% CI)	Adjusted RR* (95% CI)	p value
Low resistance (mortality ≥82%)						
Non–net users	175	15.6	63	4.0 (3.2–5.2)	1.00	
Net users	760	182.9	415	2.3 (2.1–2.5)	0.61 (0.42–0.88)	0.01
High resistance (mortality <82%)						
Non–net users	129	9.0	48	5.3 (4.0–7.1)	1.00	
Net users	772	167.7	494	2.9 (1.7–3.2)	0.55 (0.35–0.87)	0.01
Interaction parameter					0.86 (0.48–1.55)	0.63
Change in incidence per 10% increase in mosquito mortality					0.96 (0.87–1.06)	0.45

*Adjusted for district, year, and visit month.

high- and low-resistance clusters. For cohort 1, incidence was 2.2 (95% CI 1.8–2.7) infections/person-year among children living in low-resistance clusters and 2.0 (95% CI 1.6–2.4) infections/person-year among children living in high-resistance clusters (adjusted RR 0.9, 95% CI 0.5–1.6; p = 0.68) (Table 4). For cohort 2, infection incidence was 2.8 (95% CI 2.4–3.2) infections/person-years among children residing in low-resistance clusters and 2.7 (95% CI 2.4–3.1) infections/person-years among children residing in high-resistance clusters (adjusted RR 0.8, 95% CI 0.5–1.2; p = 0.33). After plotting data from 93/100 clusters (data from all subcounties and both years), we found no association between deltamethrin insecticide resistance and malaria parasite infection incidence (Figure 1).

Insecticide Resistance

Mosquito mortality ranged 55%–100% in 2013 and 30%–98.5% in 2014. The median (25%–75% interquartile range) mortality rates were 88% (81%–97%) for 2013 and 67% (51%–80%) for 2014 (Figure 2).

Effect of Insecticide Resistance and Net Use on Malaria Parasite Infection Incidence

The interaction between resistance (high and low) and net use was not significant for either cohort (p = 0.63) (Table 3). The insecticide resistance stratum did not modify the effect of LLIN use on infection incidence.

Discussion

Because of the widespread use of insecticide-based malaria vector control tools, such as LLINs and indoor residual spraying (*3*,*17*,*24*), insecticide resistance is a rising concern in sub-Saharan Africa (*4*). Our study was designed to estimate the effect that pyrethroid resistance in local malaria vectors had on malaria parasite infection incidence in areas of varying levels of insecticide resistance in western Kenya. Net use was 81.3% in cohort 1 and 85.7% in cohort 2, a small change in net use despite the timing of the LLIN distribution campaign (just before the beginning of cohort 2). Nets were found to be effective at preventing infection in low- and high-resistance clusters. Even with rises in pyrethroid resistance among malaria vectors, nets were shown to be 39% protective in low-resistance clusters and 45% protective in high-resistance clusters. LLINs are still effective in reducing malaria parasite transmission because, aside from the insecticide's repellent and toxic properties, nets also act as natural barriers that prevent human–vector contact (*25*). Given the positive news that LLINs are still useful in environments with high levels of insecticide resistance, malaria parasite control programs should continue to provide and distribute LLINs and encourage their use in parallel with efforts to develop and evaluate new tools (*18*,*26*).

We did not find a significant association between insecticide resistance and incidence of malaria parasite

Table 4. Incidence of malaria parasite infection in low– and high–insecticide resistance clusters by year, Kenya, 2013 and 2014*

Insecticide resistance	No. children	No. malaria episodes	Total follow-up time, person-years	Incidence, infections/person-year (95% CI)	Unadjusted RR (95% CI)	Unadjusted p value	Adjusted RR (95% CI)	Adjusted p value
2013								
Low resistance	290	114	51.6	2.2 (1.8–2.7)	1.0		1.0	
High resistance	311	116	59.2	2.0 (1.6–2.4)	0.9 (0.5–1.6)	0.70	0.9 (0.5–1.6)	0.68
Per 10% increase in mosquito mortality					1.0 (0.7–1.5)	0.99	1.0 (0.7–1.5)	0.98
2014								
Low resistance	433	224	80.7	2.8 (2.4–3.2)	1.0		1.0	
High resistance	460	222	80.9	2.7 (2.4–3.1)	1.0 (0.7–1.4)	0.96	0.8 (0.5–1.2)	0.33
Per 10% increase in mosquito mortality					1.0 (0.9–1.1)	0.90	1.1 (0.9–1.2)	0.24

*In 2013, low resistance was defined as mortality ≥88% and high resistance as mortality <88%. In 2014, low resistance was defined as mortality ≥67% and high resistance as mortality <67%. RR, rate ratio.

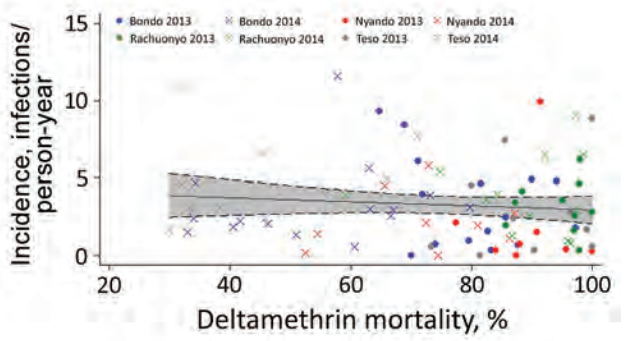

Figure 1. Relationship between deltamethrin insecticide resistance and incidence of malaria parasite infection, 4 subcounties, western Kenya, 2013 and 2014. The incidence of infection in the clusters from subcounties Bondo (blue), Ranchuonya (green), Nyando (red), and Teso (gray) in years 2013 (circles) and 2014 (Xs) were plotted against the corresponding values of mosquito mortality to deltamethrin for that year and that cluster. The best-fit line (with the 95% CI shaded in gray) for the scatterplot is nearly straight, suggesting no relationship between the incidence of infection and *Anopheles gambiae sensu lato* mosquito mortality upon exposure to deltamethrin measured by the World Health Organization bioassay.

infection in either year. Concern that insecticide resistance could compromise malaria parasite control has been expressed (*18*,*24*,*27*,*28*), and, with this, the expectation that the incidence of infection would be higher in high-resistance areas. The results of our study, therefore, are surprising, considering the failure some countries have had in malaria vector control after the development of resistance to the insecticides used in indoor residual spraying (*8*,*29*). More specifically, studies have reported resistant mosquitoes surviving exposure to potent nets (nets able to knockdown >80% of susceptible mosquitoes) (*30*,*31*); it was expected that areas with such mosquitoes would have higher malaria parasite infection incidences because the mosquitoes live longer and thus are able to spread malaria parasite for longer.

Several factors might explain why we did not observe a correlation between insecticide resistance and malaria parasite infection incidence. First, as previously mentioned, LLINs serve as a barrier to prevent human–vector contact. If the nets are in good condition and are used consistently and properly, they reduce the chances of mosquito bites and hence malaria parasite transmission (*32*). Second, the WHO tube bioassay does not indicate what level of insecticide resistance is expected to lead to vector control failure, which is a major weakness of the assay (*33*). Therefore, even though we observe insecticide resistance, the mosquito populations might still be susceptible to the toxic effects of the chemical doses used on the nets. This highlights the need for more quantitative methods for monitoring insecticide resistance (*33*,*34*). In addition, a recent study in deltamethrin-resistant mosquitoes showed that sublethal doses of pyrethroids can interfere with parasite development (*35*). Even though these mosquitoes do not succumb to exposure with insecticides, their ability to transmit the malaria parasite is reduced, and therefore, increasing insecticide resistance does not necessarily directly and immediately lead to a major increase in incidence of malaria parasite infection.

However, our results should be interpreted with caution. We have already observed instances of mosquitoes failing to succumb to control tools, such as in a report conducted in the Bungoma district, where resting but still bioactive pyrethroid-resistant *An. gambiae sensu stricto* (*s.s.*) mosquitoes were found inside of LLINs without getting killed or repelled (*30*). Also, in Benin, as many as 5 mosquitoes were found to enter damaged LLINs at night (*31*). Similarly, pyrethroid-resistant *An. funestus* mosquitoes have foiled indoor residual spraying efforts to control malaria parasite transmission in South Africa (*8*,*36*).

An. arabiensis mosquitoes were the predominant vector in Bondo, Rachuonyo, and Nyando (>90% of the *An. gambiae s.l.* population), the other vector being *An. gambiae s.s* mosquitoes. In Teso, *An. gambiae s.s.* mosquitoes were predominant (>70% of the *An. gambiae s.l.* population). It is therefore necessary that, even as programs continue to implement insecticide-based vector control, they follow the guidelines provided by global programs for insecticide resistance management (*28*). Regular insecticide resistance surveillance should continue to be conducted on a wide scale to ensure accurate reporting of the otherwise largely heterogeneous insecticide resistance trends.

Our study had weaknesses that might have affected results, the first being the highly variable nature of the

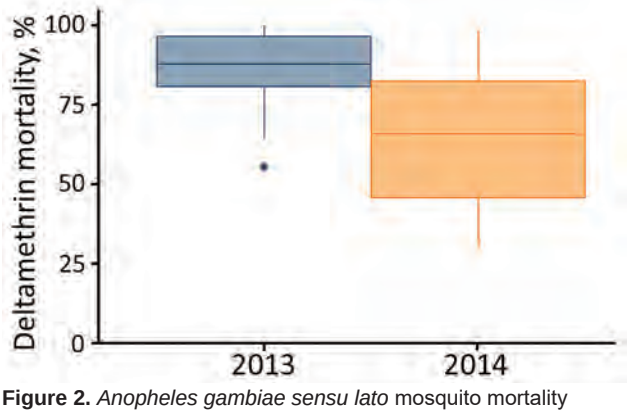

Figure 2. *Anopheles gambiae sensu lato* mosquito mortality to deltamethrin, western Kenya, 2013 and 2014. Mortality was measured using the World Health Organization tube bioassay. Whiskers indicate full range of data; top and bottom lines of boxes indicate 25%–75% interquartile ranges; horizontal lines within boxes indicate medians.

susceptibility data from 1 year to the next and from 1 cluster to the next. As mentioned previously, the WHO tube bioassay is not very informative of the intensity of insecticide resistance. The categorization of net users and non–net users might have substantially confounded results given that net use was not randomly assigned and non–net users were a relatively small number of children who did not prefer to use nets. Last, our study did not consider insecticide resistance in the population *of An. funestus* mosquitoes, a reemerging vector in the region (*37*), mostly because of the difficulty of rearing them in the lab and finding them in larval habitats.

In conclusion, insecticide resistance, especially to pyrethroids, continues to increase in countries in sub-Saharan Africa where LLINs and indoor residual spraying are the mainstays of vector control. The results of this study indicate a utility for continuing LLIN use despite the increasing levels of insecticide resistance in the malaria vector population. However, in our study, even among users of nets, malaria parasite incidence remained alarmingly high. Taken together with other reports suggesting an increase in malaria prevalence in parts of western Kenya with high LLIN coverage (*15*,*16*), the malaria parasite transmission taking place in this region urgently needs to be addressed. Because of their reduced susceptibility, LLINs might not be killing mosquitoes as effectively as they used to. More emphasis needs to be placed on maximizing the coverage and use of LLINs, fully implementing the guidelines on resistance monitoring, and developing more vector control tools to complement existing ones.

Acknowledgments

We are greatly indebted to Judith Wandera; Brigid Kemei; Mercy Nduta; Belinda Ohas; Dorcas Akach; the Kenya Medical Research Institute (KEMRI) Malaria branch staff for their technical support; KEMRI staff; CDC Kenya staff; KEMRI Centre for Global Health Research staff; KEMRI Centre for Biotechnology, Research, and Development staff; and the National Malaria Control Programme staff. We thank the community health workers and the district health management teams in the subcounties where the study was conducted. We are grateful to the director of KEMRI for the permission to publish this data.

This research was funded by the Bill and Melinda Gates Foundation through the World Health Organization (#54497 awarded to C.M.).

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Prevention of Chronic Hepatitis B after 3 Decades of Escalating Vaccination Policy, China

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China's hepatitis B virus (HBV) prevention policy has been evaluated through nationally representative serologic surveys conducted in 1992 and 2006. We report results of a 2014 serologic survey and reanalysis of the 1992 and 2006 surveys in the context of program policy. The 2014 survey used a 2-stage sample strategy in which townships were selected from 160 longstanding, nationally representative, county-level disease surveillance points, and persons 1–29 years of age were invited to participate. The 2014 sample size was 31,713; the response rate was 83.3%. Compared with the 1992 pre–recombinant vaccine survey, HBV surface antigen prevalence declined 46% by 2006 and by 52% by 2014. Among children <5 years of age, the decline was 97%. China's HBV prevention program, targeted toward interrupting perinatal transmission, has been highly successful and increasingly effective. However, this progress must be sustained for decades to come, and elimination of HBV transmission will require augmented strategies.

Hepatitis B virus (HBV) causes ≈240 million chronic infections and ≈780,000 deaths from cirrhosis and liver cancer annually (*1*). Recognizing the large global disease burden, the United Nations Sustainable Development Goals for 2030 include combating hepatitis. HBV has been highly endemic in China, where serosurveys in 1979 and 1992 indicated a 10% prevalence of HBV surface antigen (HBsAg) (*2*,*3*). High rates of chronic HBV infection among infants indicated that the infection occurred in early childhood (*4*–*6*). Historical HBV transmission built a reservoir of ≈90 million chronically infected persons in China (*7*,*8*), accounting for 30% of the global burden of chronic HBV infection (*9*).

The government of China adopted increasingly comprehensive strategies to prevent HBV transmission, including immunization, promotion of safe injection practices, blood donation screening, and surveillance (*10*). Implementation of China's immunization strategy began in 1985 with licensure of plasma-derived hepatitis B vaccine (HepB). A recombinant vaccine was licensed in 1992 and managed nationally. The strategy was designed to interrupt perinatal HBV transmission and provide newborns lifelong protection from HBV with a birth dose of HepB followed by 2 additional doses during infancy. Before 2002, HepB was managed as a type 2 vaccine for which parents or adult vaccinees had to pay out-of-pocket. In 2002, China integrated HepB into its Expanded Program on Immunization (EPI), making the vaccine available at no cost to children through 14 years of age (*11*,*12*). During 2009–2011, China conducted a HepB catch-up campaign for children <15 years of age who were born during 1994–2001; this campaign vaccinated ≈68 million children with HepB. In 2011, China launched a program integrating prevention of mother-to-child transmission of HIV, syphilis, and HBV in 1,156 counties (representing 44% of pregnant women in China) and then expanded the program nationwide in 2015, covering all pregnancies (*13*–*15*).

The effectiveness of China's HBV prevention measures is evaluated by using national serologic surveys; the fourth such survey was conducted in November 2014. We report results of this survey in the context of China's HBV prevention and control measures, along with reanalysis of the 1992 and 2006 surveys (*3*,*7*).

Methods

Survey Conduct

Target populations for all 3 surveys were local residents residing in national disease surveillance points (DSPs) for ≥6 months. DSPs were selected by the Chinese Academy of Preventive Medicine (now the Chinese Center for Disease Control and Prevention [China CDC]) to be representative of the population of China. Population demographic and socioeconomic conditions and morbidity and mortality continue to be representative (*16*–*18*).

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DOI: http://dx.doi.org/10.3201/eid2305.161477

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The surveys' targeted age ranges were 1–59 years in 1992 and 2006 and 1–29 years in 2014. The 2014 serosurvey used age groups of 1–4 years, 5–14 years, and 15–29 years; we reanalyzed the 1992 and 2006 surveys by using these groupings.

The 1992 serosurvey used a multistage cluster sampling strategy. Three villages were identified at random from each of 145 DSPs; families were randomly selected from each village from lists of residents; and all age-appropriate family members were selected (*3*,*19*). The total sample size was 67,017.

The 2006 serosurvey used a 3-stage cluster sampling strategy to identify 369 townships at random for the first stage and 369 villages at random for the second stage. Finally, 81,775 persons, stratified into age groups of 1–4 years, 5–14 years, and 15–59 years, were selected at random from a list of village residents (*7*,*20*). Children 1–14 years of age were oversampled to increase the precision of estimates for young children.

The 2014 serosurvey used a 2-stage cluster random sample from the same 160 DSPs that were used in the 2006 serosurvey. First, we allocated the same proportion of the sample to each region (eastern, middle, and western) and location type (urban and rural); second, in each region, we allocated samples to each DSP by using a probability-proportional-to-size method; third, in each DSP, we randomly selected 3 villages or communities, and from these, samples in grouping of 1–4 years, 5–14 years, and 15–29 years from each village or community were selected based on simple random sample. In all, 324 villages/communities were selected from the 38,527 villages/communities in the DSPs, with a sampling probability proportional to their size. Then persons were selected by simple random sample from local government lists of residents of sampled villages/communities into the age-group strata. The sample size calculation was based on expected HBsAg prevalence extrapolated from the 2006 survey by age group (0.7% for 1–4 years, 1.5% for 5–14 years, and 5.0% for 15–29 years) and was powered to detect differences of $\pm$50% the expected point prevalence. The final target sample size was 31,024. Lists of eligible persons were sampled systematically until the target sample size was reached.

The field investigation methods were identical in the 3 serosurveys (*3*,*7*). The interviews were carried out by trained professionals through house-to-house visits in the order of the sample listings. Communities had been notified in advance of the survey. Working persons and school children were interviewed during weekends or after school hours. For persons who were not at home, the interview staff made up to 3 additional home visits within 1 week. If, after 3 unsuccessful visits, the person could not be found, he or she was considered missing. Face-to-face interviews with the respondent or the respondent's parent were completed by trained staff by using standard questionnaires to obtain basic information, including sex, birthdate, ethnicity, birthplace, and HepB vaccination history of the children <15 years of age (validated by parent-held certificate or village vaccination record).

Laboratory Technique

All specimens were tested in the National Hepatitis Laboratory of the Institute for Viral Disease Control and Prevention, China CDC. For the 2006 (*7*,*20*) and the 2014 serosurveys, ELISA reagents were used to detect levels of HBsAg, anti-HBV surface antigens (anti-HBs), and anti-HBV core antigens (anti-HBc). HBsAg $\geq$2.1 IU was considered positive for consistency across serosurveys. Specimens yielding inconsistent or indeterminate results were retested by using microparticle enzyme immunoassay reagents (Abbott Laboratories, Chicago, IL, USA). HBsAg-positive specimens were tested for HBV e antigen and anti–HBV e antigen also by using Abbott microparticle enzyme immunoassay reagents. For the 1992 serosurvey, HBsAg, anti-HBs, and anti-HBc were tested by solid-phase radioimmunoassay (SPRIA) (*3*,*19*).

Statistical Analysis

In the 2006 and 2014 surveys, data were double-entered into EpiData version 3.02 (EpiData Association, Odense, Denmark) and verified for consistency and then were analyzed by using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Statistical methods of the 2014 serosurvey were identical to those of the 2006 serosurvey. To ensure representativeness of poststratification adjustments, sample weighting components were village selection probability and age-specific and person-selection probabilities within the village. The weight per person i was $w_{ji} = w_j \times w_{i,j} \times w_{adj}$, where w_j was the reciprocal of the probability of including village j, $w_{i,j}$ was the reciprocal of the conditional inclusion probability of person i from village j, and w_{adj} was an adjustment factor for person i so the sum of weights equaled China's population. We used the SAS procedure surveyfreq to calculate point estimates and 95% CIs of serologic markers by using weighting adjustments; Taylor series linearization was used for variance estimations. The 1992 survey had no design weighting, so we determined unweighted point prevalence and 95% CIs.

The HBsAg, anti-HBs, and anti-HBc prevalence of persons 1–29 years of age covered in the 1992 and 2006 serosurveys were reanalyzed to be consistent with the format of the 2014 serosurvey by 3 age groups (1–4 years, 5–14 years, and 15–19 years), as well as sex, ethnicity, location type (urban or rural), region, and year of birth.

Vaccination Coverage and HBsAg Prevalence by Birth Year

HepB vaccination history for children <15 years of age was coded as vaccinated (i.e., birth dose plus 2 more doses or incomplete series), unvaccinated, or unknown, based on

children's immunization certificates. Coverage levels of children born during 1985–1991, 1992–2005, and 2006–2013 were determined from the 1992, 2006, and 2014 surveys, respectively. Weighted HBsAg prevalences for the 1962–1991, 1976–2005, and 1985–2013 birth cohorts were determined by using the 1992, 2006, and 2014 surveys for each birth cohort included in the respective surveys.

Analyses of Cases Averted

We estimated the number of chronic HBV infections prevented in the 1992–2013 birth cohorts by using Goldstein's model, which was used in the 2006 survey to estimate baseline disease prevalence and cases prevented (*21*). This model provides estimates of total numbers of cases and deaths caused by acute HBV infection and numbers of cases and sequelae from chronic HBV infection, including cirrhosis and primary hepatocellular carcinoma that would develop during the lifetime of a birth cohort. The key inputs to the model are baseline HBsAg seroprevalence in the entire population and among women of childbearing age and HepB coverage. Figures on the effect of vaccination by birth cohort were summed to estimate the overall effect by using birth cohort sizes of 16.97 million persons per year. We assumed a baseline HBsAg prevalence of 8.58% among women of childbearing age, uniformly distributed, with 30% also being HBeAg positive. Among 5-year-old children, 32% were assumed to become chronically HBV-infected (anti-HBc positive) by 5 years of age and 55% to be chronically infected by 30 years of age; these percentages represented force of infection without vaccination (*6*,*20*).

Quality Control

China CDC convened expert groups to guide design, fieldwork, laboratory testing, and analyses for the 3 surveys. Pilots were conducted before each survey. County CDC staff administered questionnaires and collected and managed blood specimens.

Ethical Reviews

The 1992 survey was approved by Chinese Academy of Preventive Medicine's Ethical Review Committee; the 2006 and 2014 surveys were approved by China CDC's Ethical Review Committee. In 2006 and 2014, participants were informed of the study purpose and their right to keep information confidential. Consent was obtained before interview and blood drawing.

Results

Response Rate

In the 2014 survey, investigators visited selected houses up to 3 times and invited 38,142 persons to participate. Of those invited, 31,772 gave consent, yielding an 83.3% response rate. Among those consenting, 59 (0.2%) were excluded because of insufficient serum for laboratory analysis. The final sample was 31,713 persons (Figure 1). Demographic characteristics of the subjects in the 3 surveys were similar except that the percentage of the sample residing in urban areas increased from 25.7% to 49.6% during 1992–2006, and 49.8% in 2014, reflecting China's urbanization (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1477-Techapp1.pdf). Among respondents <15 years of age in the 1992, 2006, and 2014 surveys, 96.5% (21,638 of 22,419), 81.6% (32,732 of 40,129), and 18.0% (2,923 of 16,239) had vaccination records, respectively.

HBV Serologic Markers

HBsAg prevalence among persons 1–4 years, 5–14 years, and 15–29 years of age in 2014 was 0.3%, 0.9%, and 4.4%, respectively. We compared HBsAg, anti-HBsAg, and anti-HBc results from the 3 surveys (online Technical Appendix Table 2). HBsAg prevalence among 1–29-year-olds declined from 10.1% to 2.6% during 1992–2014. Declines were observed in all age, sex, ethnicity, location type (urban/rural), and regional groups. Among children <15 years of age, HBsAg prevalence declined from 10.5% to 0.8%. Prevalence of anti-HBs among 1–29-year-olds increased from 25.4% to 57.8% during 1992–2014. Prevalence of anti-HBc declined from 45.8% to 13.0% during 1992–2014, declining in all subpopulations.

In 1992, HBsAg prevalence was 10% across all age groups (Figure 2), consistent with HBsAg prevalence in 1979 (*22*). In 2006, HBsAg prevalence was high among 20–29-year-olds (8.3%) and low among 1–4-year-olds (1.0%). Similar trends in HBsAg prevalence were observed seen in the 2014 survey.

The relative decline in HBsAg prevalence was uneven by region and age group (Figure 3, panel A). Among 1–4-year-olds, eastern, central, and western region prevalences decreased by >95%, but among 15–29-year-olds, declines were 62.0%, 62.1%, and 37.0%, respectively. The decline in HBsAg prevalence was >95% among 1–4 year-olds regardless of rural/urban status (Figure 3, panel B), but among 15–29-year-olds, the decline was greater among urban residents than rural residents (68.4% and 44.3%). HBsAg prevalence by birth cohort and HepB coverage, when mapped against the timeline of important immunization program events, were noticeably affected as incremental interventions were added (Figure 4).

Cases Averted

During 2010–2014, China prevented an additional estimated 4 million chronic HBV infections on top of the 24 million chronic infections prevented during 1992–2009 (*21*). In total, 28 million chronic HBV infections were averted, and 5 million deaths from HBV infection complications were prevented

Discussion

Compared with the prevaccine era, chronic HBV infection in China has been reduced by 90% (from 10.5% to 0.8%) among children <15 years of age and by 97% (from 9.9% to 0.3%) among children <5 years of age. Disparities by region and urban/rural status that existed among young children in 1992 and 2006 were largely eliminated by 2014. Lower HBsAg prevalence among young children in 2014 (1.0%) compared with 2006 (0.3%) shows increasing effectiveness of the program.

HBsAg prevalence among 1–29-year-olds declined 46% during 1992–2006 (from 10.1% to 5.5%) and 52% during 2006–2014 (from 5.5% to 2.6%). As a result of China's program, an estimated 120 million HBV infections and 28 million chronic infections were averted.

Without postexposure prophylaxis provided by the HepB birth dose, 30% of infants born to HBsAg-positive mothers will become infected, and 90% of the infections will become chronic (*1*). Because administering a birth dose is challenging when childbirth happens in the home, a key element of China's success was promoting facility-based childbirth. Implementation of the timely birth dose was accelerated by the GAVI hepatitis B project, which promoted the birth-dose policy in rural and western areas of China (*12*). Additional strategies, such as promoting safe injection practices and screening donated blood, have also been important for chronic HBV prevention. In 2000, China passed a regulation banning the reuse of medical devices labeled for single use. In 2005, the Chinese Medical Association published clinical guidelines for injections and other skin-piercing procedures. In 2007, autodisable syringes became available for vaccine injections, and by 2010, reusable injection equipment was eliminated in China and disposable and autodisable syringes became universally used (*23*). Since 1988, donated blood has been screened for HBV serologic markers, and since 2015, HBsAg-negative donated blood has been tested for HBV DNA (*24*). Although HBV infection caused by unsafe injections and blood transfusion has been reduced, modeling shows that the newborn and infant vaccination strategy has been independently responsible for preventing ≈95% of chronic HBV infections in China (*25*).

The government of China regards health equity as important for social justice and fairness (*26*). In 2000, the ministries of health and finance and the State Council implemented a program to reduce maternal mortality rates and eliminate maternal/neonatal tetanus. The government established insurance plans to ensure access to healthcare and birth facilities, especially in impoverished, remote, or ethnic minority areas. The in-hospital delivery rate

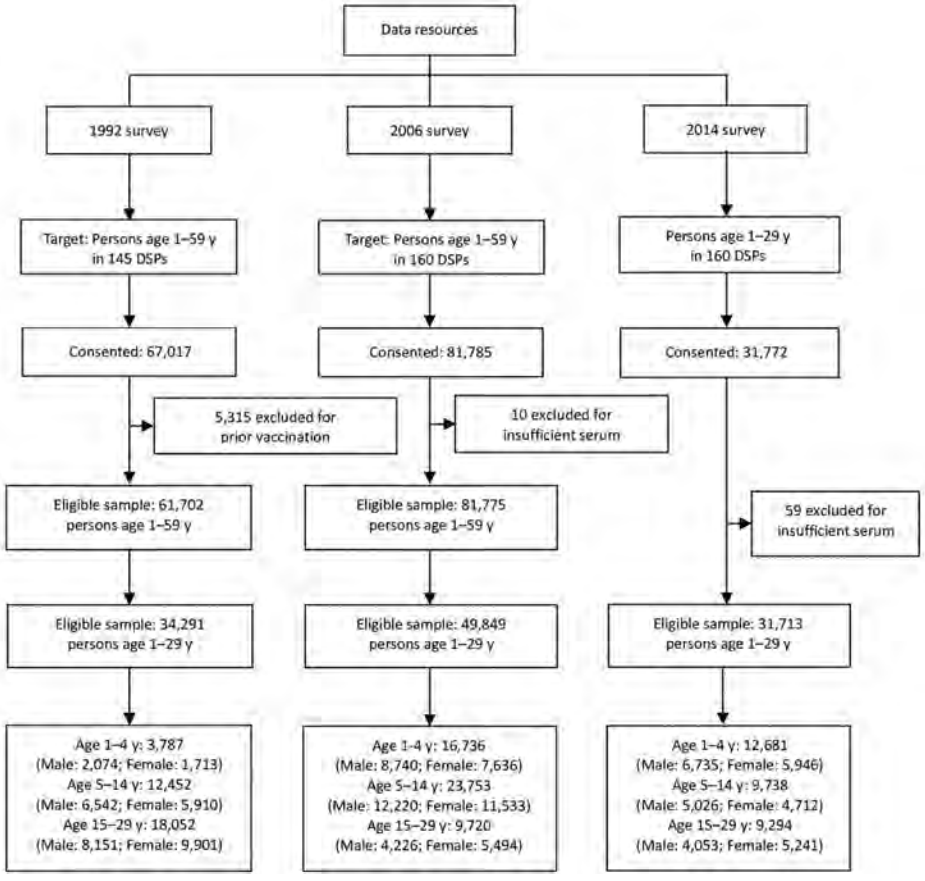

Figure 1. Study eligibility and select characteristics of persons participating in 1992, 2006, and 2014 national serosurveys for hepatitis B virus, China. DSPs, disease surveillance points.

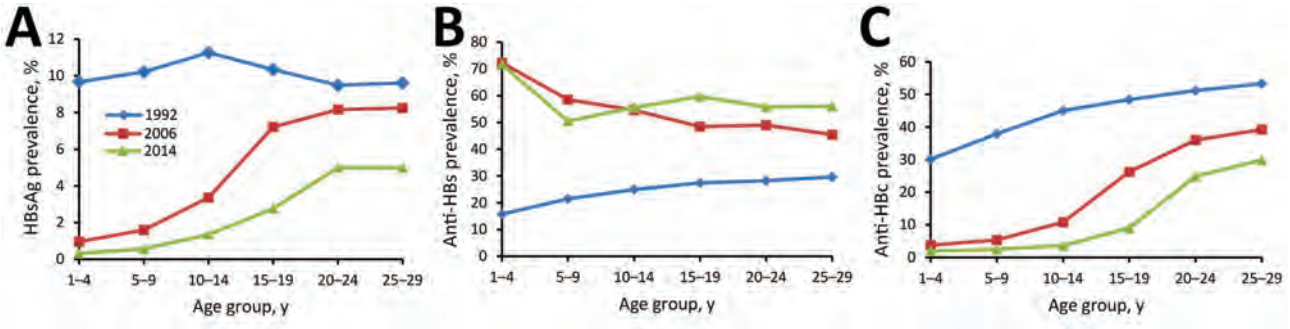

Figure 2. Longitudinal changes in prevalence of HBsAg (A), anti-HBs (B), and anti-HBc (C) among persons participating in 1992, 2006, and 2014 national serosurveys for hepatitis B virus, by age group, China. HBsAg, hepatitis B virus surface antigen; anti-HBs, antibody to hepatitis B virus surface antigen; anti-HBc, antibody to hepatitis B virus core antigen.

increased from 44% in 1985 to 99% in 2013 (*27*). By using the principle "whoever delivers the baby vaccinates the baby," virtually all infants born in birthing facilities receive a birth dose of HepB (*10*).

Timely vaccination is used as an evaluation measure of public health effectiveness (*5*,*28*–*31*). High 3-dose vaccination coverage has been maintained continuously from 2009 through 2015, having increased from 70% in 2002 to >95% in 2009 and afterward. HepB birth-dose coverage increased from 22% in 1992 to 71% in 2002 and 94% in 2013 (*32*). The national program integrating prevention of mother-to-child transmission of HIV, syphilis, and HBV has been providing HBsAg screening for pregnant women and hepatitis B immunoglobulin for all infants born to HBsAg-positive women since 2012 (*3*,*13*,*33*).

Vaccines used in the program have been evaluated periodically. When HepB became government-supported in 2002, the dose provided was 5 μg/0.5 mL, which was known to prevent infection in 85%–90% of children born to HBsAg-positive women (*34*–*37*). To improve effectiveness, the dose was increased to 10 μg/0.5 mL in 2011. China's model supports the United Nations Sustainable Development Goals and the World Health Organization's new Global Hepatitis Framework by greatly reducing HBV transmission with strategies that integrate HBV prevention into the healthcare sector.

Strengths of this study include a sound sampling strategy with comparable methods across 3 surveys separated in time, identical laboratory procedures in the 2006 and 2014 surveys, and use of sufficiently large sample sizes to support precise estimates. Our study provides previously unpublished coverage levels of plasma-derived HepB before licensure of the recombinant vaccine.

Weaknesses of the study include the use of different laboratory methods for the 1992 survey than those used for

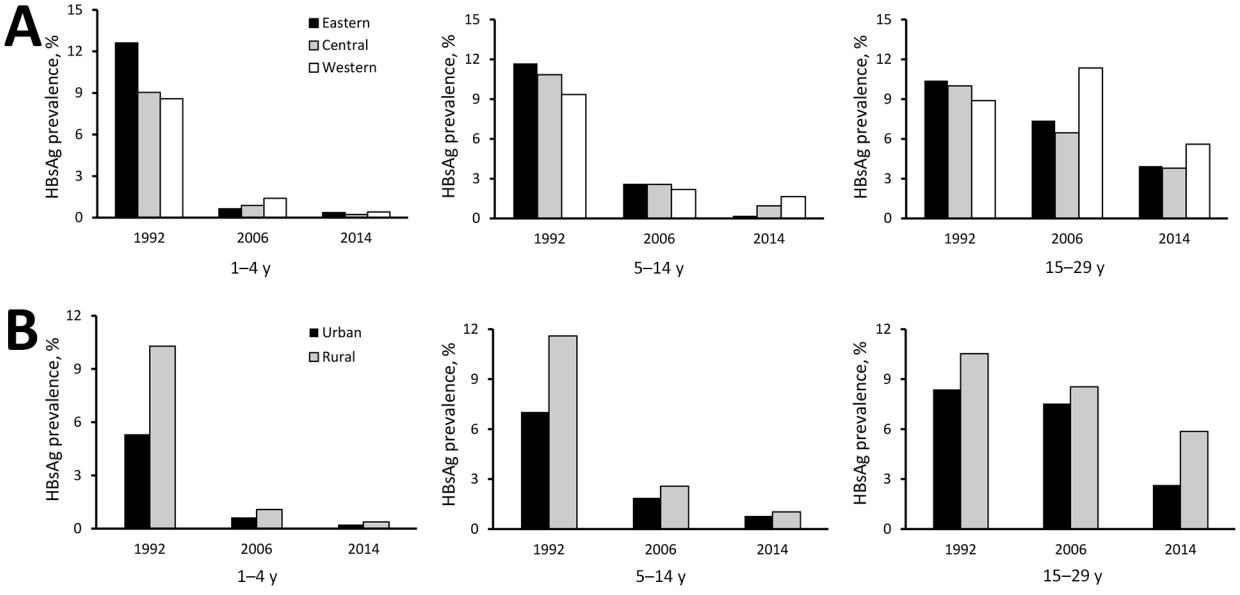

Figure 3. Prevalence of HBsAg by region, age group, and survey year (A) and by location type (urban or rural), age group, and survey year (B) among persons participating in 1992, 2006, and 2014 national serosurveys for hepatitis B virus, China. HBsAg, hepatitis B virus surface antigen.

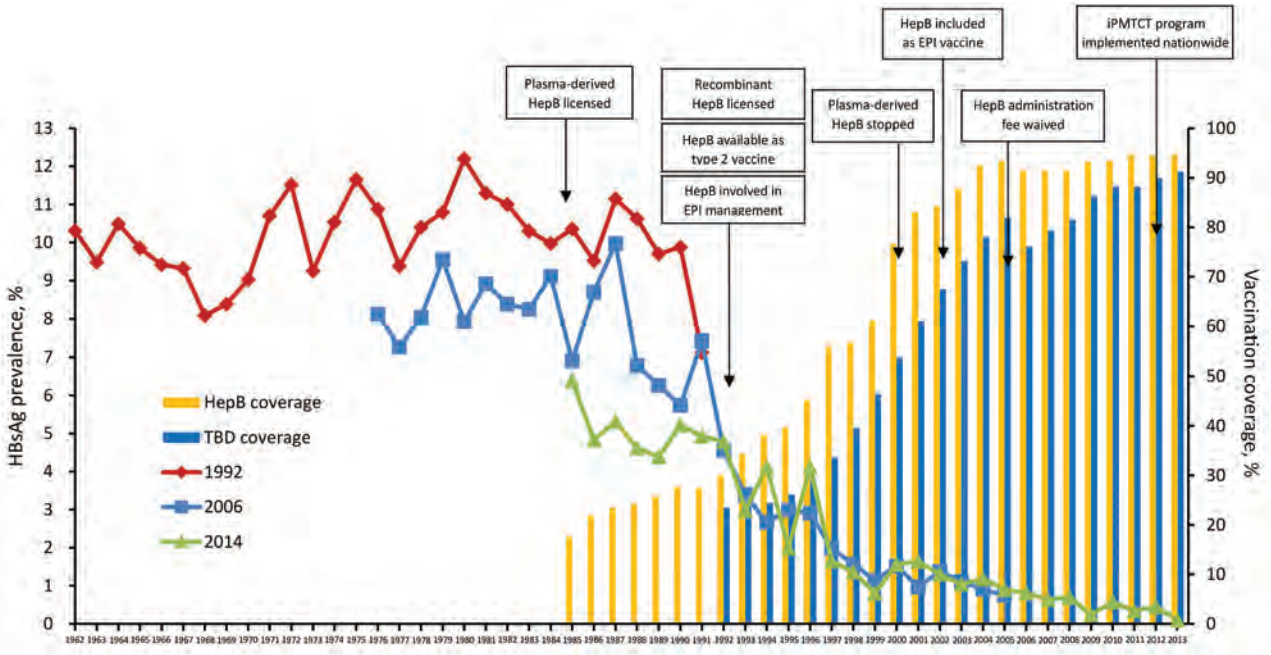

Figure 4. Prevalence of HBsAg and 3-dose HepB coverage for each birth cohort and major vaccination program milestones for hepatitis B virus, China, 1962–2014. HBsAg prevalence is shown in 3 curves, 1 for each national serologic survey (1992, 2006, and 2014). HepB coverage is shown in bars. Type 2 vaccines are private sector vaccines that are not included in the free national EPI system but must be paid for out-of-pocket. HepB coverage was defined as the percentage of children <15 years of age who received 3 doses of HepB before reaching 12 months of age. Coverage levels of children born during 1985–1991, 1992–2005, and 2006–2013 were determined from the 1992, 2006, and 2014 surveys, respectively. TBD coverage was defined as the percentage of newborn infants who received a dose of HepB within 24 hours of birth. The iPMTCT program provides free HBsAg screening of pregnant women and free hepatitis B immunoglobulin for hepatitis B virus–exposed infants. EPI, Expanded Program on Immunization; HBsAg, hepatitis B virus surface antigen; HepB, hepatitis B vaccine; iPMTCT, integrated Prevention of Mother-to-Child Transmission, TBD, timely birth dose.

the 2006 and 2014 surveys, different nonresponse rates of the 2006 and 2014 surveys, underestimation of immunity indicated by anti-HB levels (because when antibody levels wane, memory B cell mediated anamnestic response to HBV exposure can maintain protection from infection), undersampling of the migrant population (because only those residing ≥6 months in a given survey area were included), and the fact that HepB coverage levels among teens and adults are not measured in China. The 1992 survey used SPRIA for HBV infection serologic markers, and the 2006 and 2014 surveys used ELISA. According to previous studies (*38*,*39*), SPRIA is less specific than ELISA for detecting HBsAg; this difference could have led to overestimation of the relative decrease in HBsAg prevalence because ELISA testing was used in 2006 and 2014. However, we believe that the effect on our results is modest, especially for the current estimates of HBsAg prevalence, because the ELISA tests used are considered acceptable at international standards. We used the same HBsAg cutoff values for ELISA testing in the 2006 and 2014 surveys for the sake of consistency; however, higher cutoff values are used more frequently now.

HBsAg prevalence among several birth cohorts was measured by ≥1 survey (Figure 4). Of interest is that results are more consistent for younger, double-measured birth cohorts than for older, double- and triple-measured cohorts. These differences might be attributable to several reasons. Persons in the double-measured birth cohorts were 22 years older in the 2014 survey than the 1992 survey and were 8 years older in the 2014 survey than the 2006 survey. HBsAg prevalence has a small, natural decline with age (*40*). This natural decline will increase the HBsAg prevalence differences from the 1992 survey, as will the age-based accumulation of deaths caused by complications of chronic HBV. Finally, lower specificity of the 1992 survey can lead to an upward bias of the differences from the 1992 survey.

Our study has 3 main programmatic implications. First, the annual need for perinatal postexposure prophylaxis remains substantial. The prevalence of HBsAg in women of childbearing age and the size of the birth cohort in China (16.97 million), implies that 750,000 to 1 million infants are born to HBsAg-positive women annually (*13*).

Second, prevention measures must continue for decades. The age group with the highest HBsAg prevalence corresponds to the age groups with the highest fertility rate in China (69.5/1,000 for those 20–24 years and 94.0/1,000

for those age 24–29 years of age) (*27*). Even when children of today become adults, nearly 200,000 infants will be born to HBsAg carriers each year and will need postexposure prophylaxis to prevent HBV infection.

Third, many newborns still become chronically infected. Although China has reduced perinatal transmission by 97%, an HBsAg prevalence of 0.3% in a birth cohort of 16 million implies that 50,000 perinatal infections still occur annually. Additional strategies will be needed to eliminate vertical transmission.

We believe that the success HBV prevention should be communicated to stakeholders to help sustain confidence in the immunization effort. Confidence in vaccines can be fragile, as was made evident by a temporary loss of confidence in HepB in 2013 and 2014 (*41*), and showing the strongly positive impact of vaccination may help maintain or restore confidence (*42*).

Our results raise several questions. Can the current strategy eliminate perinatal transmission? HepB is not 100% effective, and additional strategies may need to be used. Antiviral prophylaxis during the third trimester for HBsAg-positive pregnant women with high HBV DNA is being shown to decrease perinatal transmission of HBV (*43*,*44*) and may need to become a standard of care in the future.

Should postvaccination serologic testing (PVST) become a recommended standard in China? PVST can help confirm whether an HBV-exposed infant is protected, is susceptible and needs to be revaccinated, or is infected and needs referral for follow-up care. The cost-effectiveness, feasibility, and acceptability of PVST for HBV-exposed infants in China should be evaluated.

Can adults at risk of HBV infection be vaccinated? Identifying cost-effective means to protect at-risk adults from HBV has potential to avert infections (*45*).

Finally, treating the estimated 90 million persons with chronic HBV infection is critically important (*9*,*46*). Prevention works, but not always perfectly, and many adults were born before prevention of HBV was possible.

Acknowledgments

Special thanks to Lance Rodewald, for his support in polishing the English manuscript and providing constructive comments.

The study was funded by the Major Science and Technology Special Project of China's 12th 5-Year Plan (grant no. 2012ZX10002001) and the National Nature Science Foundation in China (grant no. 11571272).

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Lack of Durable Cross-Neutralizing Antibodies against Zika Virus from Dengue Virus Infection

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Cross-reactive antibodies elicited by dengue virus (DENV) infection might affect Zika virus infection and confound serologic tests. Recent data demonstrate neutralization of Zika virus by monoclonal antibodies or human serum collected early after DENV infection. Whether this finding is true in late DENV convalescence (≥6 months after infection) is unknown. We studied late convalescent serum samples from persons with prior DENV or Zika virus exposure. Despite extensive cross-reactivity in IgG binding, Zika virus neutralization was not observed among primary DENV infections. We observed low-frequency (23%) Zika virus cross-neutralization in repeat DENV infections. DENV-immune persons who had Zika virus as a secondary infection had distinct populations of antibodies that neutralized DENVs and Zika virus, as shown by DENV-reactive antibody depletion experiments. These data suggest that most DENV infections do not induce durable, high-level Zika virus cross-neutralizing antibodies. Zika virus–specific antibody populations develop after Zika virus infection irrespective of prior DENV immunity.

Zika virus is a member of the flavivirus family of arthropodborne viruses, which includes West Nile virus, yellow fever virus, tick-borne encephalitis virus, and dengue virus (DENV) (*1*). The recent emergence of Zika virus in the Western Hemisphere caused widespread international concern. Although Zika virus infection is most commonly asymptomatic or causes only a mild, self-limited illness, recent epidemics have revealed new manifestations of Zika virus disease, including Guillain-Barré syndrome (*2*,*3*) and sexual transmission (*4*). Most alarmingly, and distinct from other flaviviruses, Zika virus infection during pregnancy can result in a spectrum of developmental abnormalities (congenital Zika syndrome) (*5*), which can include ocular damage, microcephaly, and fetal death (*6*). These manifestations raise public health challenges unique from those of other vectorborne diseases, particularly preventing sexual transmission and protecting pregnant women.

Given shared ecology and mosquito vectors, Zika virus is emerging in areas with endemic DENV transmission. In many areas of Latin America, most persons has been exposed to ≥1 natural DENV infections (*7*); in some regions, DENV vaccination has been implemented or is in clinical trials (*8*). Because there is known serologic cross-reactivity between Zika virus and DENV (*9*), determining how DENV immunity affects subsequent Zika virus infection is important.

The ability of flavivirus infection to induce antibodies that are virus-specific and that cross-react with different flaviviruses is well known (*10*–*13*), and flaviviruses have been grouped as serocomplexes on the basis of degree of antigenic overlap (*11*). Zika virus has not been studied extensively in this context; although it shares 54%–58% of its nucleotide sequence with the 4 serotypes of DENV in the envelope protein coding sequence (*14*), the extent to which Zika virus will group antigenically with the DENV serocomplex is unclear. Several groups recently reported that antibodies isolated from persons with prior DENV infections cross-neutralize Zika virus and cross-protect in animal models of Zika virus infection (*15*–*19*). These results raise the possibility of DENV infections or vaccines cross-protecting against Zika virus. Moreover, researchers have been concerned that the plaque/focus reduction neutralization test, the standard serologic assay for distinguishing different flavivirus serocomplexes, will be unable to differentiate Zika virus from DENV. During 2016 in North Carolina, USA, we studied whether persons exposed to DENV maintain cross-neutralizing antibodies to Zika virus.

Methods

Human Subjects and Immune Serum

Serum was collected from North Carolina residents who had probable or confirmed DENV or Zika virus infection on the basis of self-reported symptoms and travel to areas in which these viruses are endemic. Serum samples from this study were assigned consecutive and arbitrary identification numbers such as DT165. A subset of DENV immune serum was obtained from a reference panel distributed by the Pediatric Dengue Vaccine Initiative. Samples were tested by virus-capture ELISA, and DENV- or Zika virus–reactive serum was further characterized by neutralization assays on Vero cells. All donations were

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DOI: http://dx.doi.org/10.3201/eid2305.161630

collected in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill (protocol 08–0895).

Serum that had neutralizing antibodies to 1 DENV serotype or to Zika virus with minimal cross-neutralizing antibodies were defined as primary flavivirus infections (meaning that the 50% inhibitory concentration [IC_{50}] for a single DENV serotype or Zika virus was ≥4-fold higher than for any other virus tested). In most cases, the person's travel history corroborated the primary immune status. Serum that had high levels of neutralizing antibody to ≥2 flaviviruses were defined as secondary (repeat) flavivirus infections. Most secondary infection samples were from persons who had resided in DENV- or Zika virus–endemic countries for ≥5 years.

Viruses and Cells

Zika Virus Stocks

The MR766 and Dakar 41519 strains of Zika virus were obtained from R. Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX, USA) (*20*,*21*). The Centers for Disease Control and Prevention (Atlanta, GA, USA) provided Zika virus strains H/PF/2013 and PRVABC59 (*22*,*23*).

DENV Stocks

All in vitro assays were conducted with the DENV World Health Organization reference strains: DENV-1 West Pac 74, DENV-2 S-16803, DENV-3 CH54389, and DENV-4 TVP-360 (initially obtained from R. Putnak, Walter Reed Army Institute of Research, Silver Spring, MD, USA). Virus stocks were prepared in C6/36 *Aedes albopictus* mosquito cells (ATCC no. CRL-1660) or Vero *Cercopithecus aethiops* monkey cells (ATCC no. CCL-81). C6/36 cells were grown at 32°C with 5% CO_2 in minimum essential medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, nonessential amino acids, and HEPES (2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer. Vero cells were grown at 37°C with 5% CO_2 in Dulbecco modified Eagle medium supplemented with 5% FBS and L-glutamine. Virus stocks were titrated on Vero cells by plaque assay or focus-forming assay (*24*). Infected cell foci were detected at 40–48 h after infection, after fixation with 1%–2% paraformaldehyde and incubation with 500 ng/mL of flavivirus cross-reactive mouse monoclonal antibody (mAb) E60 (*25*), 2H2 (*26*), and/or 4G2 (*26*). After incubation with a 1:2,000 dilution of horseradish peroxidase–conjugated goat antimouse IgG (Sigma, St. Louis, MO, USA), foci were detected by addition of TrueBlue substrate (KPL). We analyzed foci with a CTL Immunospot instrument (CTL, Cleveland, OH, USA). All studies were conducted under biosafety level 2 containment.

ELISA

We measured binding of human serum IgG to DENV or Zika virus by ELISA as previously described (*27*). In brief, DENV virions were captured by the anti-E protein mouse mAb 4G2, blocked with 3% normal goat serum (GIBCO Life Technologies, Carlsbad, CA, USA) or 3% nonfat dairy milk (LabScientific, Inc., Highlands, NJ, USA), and incubated with human serum at indicated dilutions at 37°C for 1 h, and binding was detected with an alkaline phosphatase–conjugated antihuman secondary antibody (Sigma) and *p*-nitrophenyl phosphate substrate (Sigma). Absorbance at 405 nm was measured on an Epoch plate reader (BioTek, Suwanee, GA, USA). ELISAs used to confirm depletion were performed as given earlier, with the exception that 50 ng purified DENV was coated directly to the plate at 37°C for 1 h at 1:50 dilution before serum was tested. ELISA data are reported as optical density values that are the average of technical replicates, unless otherwise indicated. The average optical density for technical replicates using naive human serum at the same dilution factor as test samples serves as the negative control in ELISAs. In depletion experiments, the OD of depleted sample is expressed as percentage of control from same serum for some graphs as indicated.

Neutralization Assays

We adapted the previously described focus-reduction neutralization test (FRNT) (*28*) to a 96-well format (*18*). Neutralization titers were determined by FRNT by serial 3-fold dilution of human serum and mixing with ≈50–100 focus-forming units of virus in Dulbecco modified Eagle medium with 2% FBS. The virus–antibody mixtures were incubated for 1 h at 37°C and then transferred to a monolayer of Vero cells for titration by focus assay as described earlier. For neutralization assays, we calculated IC_{50} values by using the sigmoidal dose response (variable slope) equation of Prism 6 (GraphPad Software, San Diego, CA, USA). One set of DENV-1–4 neutralization values (DT003) was determined previously by U937 flow-based assay (*29*), but Zika virus neutralization was determined by Vero FRNT. IC_{50} values shown for certain Zika virus-immune serum samples were determined on 24-well plaque assay (*30*) (Table 1). Reported values were required to have an R^2 >0.75, a hill slope >0.5, and an IC_{50} within the range of the assay.

Depletions

As previously described (*31*), purified viral antigen for depletions was obtained by infecting Vero cell cultures in 850 cm^2 roller bottles (Greiner Bio-One, Monroe, NC, USA) with DENV and then concentrating DENV-containing supernatants at 4°C by tangential flow ultrafiltration using the Pellicon mini system with a 100-kD cutoff membrane (Pellicon-2 mini Holder and Pellicon-2 Mini Filters; Millipore, Darmstadt, Germany). The flow rate was 400 mL/min, and

Table 1. DENV or Zika virus neutralization profiles for persons with travel history to Zika virus–endemic areas*

Serostatus, serum sample ID	Place of Infection	IC_{50}†				
		DENV-1	DENV-2	DENV-3	DENV-4	Zika virus
Primary DENV-1						
147	Latin America	3,552	287	557	75	<20
153	Latin America	757	<20	<20	<20	<20
05/262	Asia	274	<20	<20	<20	<20
06/125	Asia	3,823	222	125	80	<20
99/1230	Asia	1,219	63	30	24	<20
Primary DENV-2						
001	Asia	49	2,188	48	89	<20
08/90	Asia	<20	2,966	<20	<20	<20
08/91	Asia	<20	838	<20	<20	<20
09/165	Asia	<20	2,093	<20	<20	<20
09/251	Asia	<20	417	<20	<20	<20
Primary DENV-3						
116	Asia	200	979	5,342	290	<20
118	Latin America	173	374	3,041	56	<20
125	Latin America	99	97	1,648	35	<20
133	Latin America	89	171	3,348	83	<20
06/297	Asia	27	<20	573	<20	<20
Primary DENV-4						
112	Latin America	908	1367	591	18,408	<20
06/105	Asia	<20	<20	<20	941	<20
06/302	Asia	<20	<20	<20	4,130	<20
09/159	Asia	115	226	478	5,694	<20
Primary Zika virus						
168	Latin America	36‡	<20	78‡	<20	1,382
172	Latin America	<20	<20	<20	<20	8,468
Secondary DENV						
000	Asia	3,306	2,087	1,162	782	<20
003	Asia	556	178	299	<20	146
115	Asia	100	355	830	245	<20
141	Latin America	1,902	1,953	4,530	664	<20
144	Asia	155	191	5,782	1,612	699
145	Asia	601	1,262	240	60	<20
146	Asia	403	1,052	1,480	451	28
155	Asia	215	299	71	27	<20
160	Latin America	947	3,564	131	1,600	<20
06/123	Asia	1,776	827	82	157	<20
06/124	Asia	1,454	1,208	1,673	1,011	<20
09/157	Asia	282	1,104	73	134	<20
09/250	Asia	375	1475	151	94	<20
Secondary Zika virus						
165	Latin America	60‡	79‡	70‡	508	1655
166	Latin America	929	393	4344	240	1814

*DENV, dengue virus; IC_{50}, 50% inhibitory concentration; ID, identification.
†All neutralization testing performed on Vero cells with the exception of DT003, which was limiting and historic values performed on a U937 Flow-based assay are shown for DENV-1–4. Zika virus IC_{50} values for DT003 are from Vero assays.
‡Curves that did not pass quality metrics are marked. Such curves might signify very low titer IC_{50} values or variable background at the lower limit of detection in serum with no specific neutralization activity.

filtration rate was ≈100 mL/min; pressure was 20–30 psi. Concentrated virus was then purified on a 15%–65% sucrose gradient by ultracentrifugation (SW 40 Ti, Beckman Coulter, Brea, CA, USA) at 21,583 relative centrifugal force for 18 h at 4°C. The fractions with maximal content of virus was determined by resolving fractions by SDS-PAGE and protein concentration was measured by Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Purified viral antigen was conjugated to Polybeads polystyrene 4.5-μ microspheres (PolyScience, Niles, IL, USA) in accordance with the manufacturer's instructions (100 μg/250 μL beads) by incubating overnight at room temperature. Control beads were incubated with equal amount of bovine serum albumin. Beads were blocked with 10 mg/mL bovine serum albumin, washed 3 times with 0.1 M borate buffer (pH 8.5), followed by 3 times with phosphate buffered saline. For depletion, serum was diluted 1:10 in phosphate buffered saline and incubated with 100 μg DENV-1 + 100 μg DENV-2 divided over 3 rounds at 37°C for 1 h each. After incubation, tubes were centrifuged at 20,800 relative centrifugal force to pellet beads with bound antibodies, and serum was pipetted off the undisturbed pellet and transferred to new vials. We confirmed depletion efficacy with direct binding ELISA. Serum with higher titers of binding antibodies was subjected to additional rounds of depletion until IgG binding was reduced to background levels.

Results

To study human antibody interactions between DENV and Zika virus, we assembled 36 late convalescent serum samples from persons exposed to DENV, Zika virus, or both (Table 1). The panel comprised serum from 21 persons exposed to primary flavivirus infections (with each DENV serotype represented and 2 cases of Zika virus) and serum from 15 persons exposed to ≥2 flavivirus infections, including 2 persons exposed to both DENV and Zika virus.

We measured total IgG binding to DENV and Zika virus using a virus capture ELISA. We observed extensive cross-reactivity between DENV serotypes and between DENV and Zika virus, confirming that cross-reactive binding antibodies are maintained for many years after infection (Figure 1). Although cross-reactive binding antibodies are commonly detected in flavivirus-immune serum, neutralization assays are more specific and can distinguish between previous exposure to various flaviviruses or even between different DENV serotypes (*32*). We therefore tested whether convalescent serum antibodies in persons exposed to DENV cross-neutralize Zika virus by a Vero cell–based neutralization assay. Serum from persons exposed to primary DENV infection of any serotype did not cross-neutralize Zika virus (Table 1; online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1630-Techapp1.pdf). In contrast, Zika virus was readily neutralized by serum from persons who had traveled to Brazil (DT168) and Colombia (DT172) in 2015 and experienced acute illnesses consistent with an arbovirus infection. Both serum samples strongly neutralized Zika virus, showing low or no cross-neutralization of the 4 DENV serotypes (Table 1; online Technical Appendix Figure 1, panels C, D), consistent with primary Zika virus infection.

Persons exposed to secondary DENV infections develop broadly neutralizing antibodies that neutralize even DENV serotypes not encountered by that person (*33*). We determined the neutralization profile for 13 serum samples from persons with secondary DENV. Although the potency of neutralization varied, all but 1 (DT155) of the 13 samples had high levels of neutralizing activity (IC_{50} >100) against at least 3 DENV serotypes, and 7 were highly neutralizing against all 4 serotypes (Table 1). Despite this extensive cross-reactivity, most (10 [77%]) secondary DENV-immune serum samples failed to neutralize Zika virus (Table 1; online Technical Appendix Figure 1). Serum from 1 person showed low levels of cross-neutralizing activity (DT146), and 2 serum samples (DT003 and DT144) had high levels of Zika virus neutralizing antibodies. Two donors (DT165 and 166) reported suspected Zika virus infections acquired while in Brazil in 2015 during a known Zika virus outbreak. Serum from these 2 persons also neutralized Zika virus and ≥1 DENV serotypes, indicating that these most likely represent secondary Zika virus infections (online Technical Appendix, Figure 1, panels E, F). These results demonstrate that the broadly cross-neutralizing antibody response that is a hallmark of repeat DENV infections is mainly confined to the DENV serocomplex, although in some cases these antibodies also might cross-neutralize Zika virus.

Zika virus strains are divided into 2 genotypes, Asian and African; contemporary Zika virus isolates from Latin America are uniformly of the Asian genotype, consistent with the model that Zika virus spread from Southeast Asia

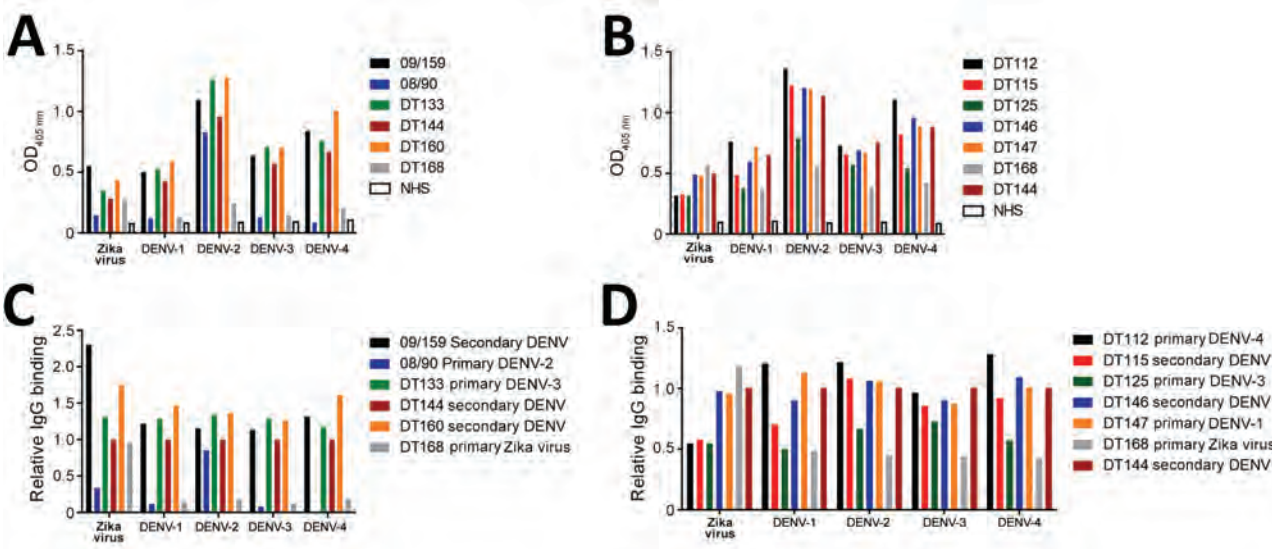

Figure 1. Binding of DENV immune serum to Zika virus virions. Zika virus and 4 DENV serotypes were captured by using plate-bound mouse monoclonal antibody 4G2 and incubated with serum from donors who had had a primary DENV, secondary DENV, or primary Zika virus infection. In 2 separate experiments (A, B), serum binding was detected by using a horseradish peroxidase–conjugated human IgG. C, D) Differential global binding of each virus was accounted for by subtracting background from native human serum and normalizing to a high binding serum common to both plates (DT144). DENV, dengue virus; NHS, naive human serum; OD, optical density.

Table 2. Zika virus stains and neutralization in selected serum*

Strain	Genotype	Origin	Year	Reference	Serum sample ID, IC_{50}			
					DT168	DT172	DT165	DT166
MR766	African	Uganda	1947	(*21*)	2,546	2,898	1,918	3,890
DAK41519	African	Senegal	1982	(*22*)	700	1,186	547	1,203
H/PF/2013	Asian	Tahiti	2013	(*23*)	609	531	516	469
PRVABC59	Asian	Puerto Rico	2015	(*24*)	436	1,606	Not tested	Not tested

*IC_{50}, 50% inhibitory concentration; ID, identification.

to Oceania and from there to Brazil (*34*). To evaluate the effect of Zika virus strain variation on cross-neutralization, we tested selected DENV and Zika virus–immune serum against 4 Zika virus strains representing diverse temporal and geographic origins (Table 2). All 4 strains exhibited similar neutralization patterns, specifically that they were neutralized by serum from primary (DT168 and DT172) or secondary (DT165 and DT166) Zika virus cases, but not by serum from a secondary DENV infection (DT145) (Table 2; online Technical Appendix Figures 2, 3), supporting the idea that Zika virus exists as a single serotype. Zika virus strain MR766 was relatively more susceptible than other strains to neutralization, and it was the only strain to exhibit even low-level neutralization by secondary DENV serum (IC_{50} 67). In general, IC_{50} titers were similar for primary Zika virus serum and for secondary Zika virus serum, consistent with the idea that cross-reactive antibodies from prior DENV infection do not contribute to Zika virus neutralization.

To test the hypothesis that Zika virus infection elicits type-specific antibody, even in the presence of DENV immunity, we incubated serum with polystyrene beads coated with purified DENV antigen to remove DENV-specific and flavivirus cross-reactive antibody (Figure 2, Table 3). We then assessed binding and neutralization of Zika virus by the depleted serum. IgG binding to DENV-1 and DENV-2 antigen was lower in depleted serum than in control serum, confirming success of this method (Figure 3, panel A). IgG binding to captured Zika virus was lower when DENV-immune serum was depleted, but depletion only partially reduced binding from secondary Zika virus serum and had little effect on serum from primary Zika virus infections (Figure 3, panels B–E). Depletion successfully removed DENV neutralizing antibodies because depleted serum from primary DENV-2 (DT001), secondary DENV (DT000), and secondary Zika virus (DT165 and 166) cases all exhibited marked reductions in ability to neutralize DENV-2 (Figure 4, Table 3). Also, broadly neutralizing serum lost the ability to neutralize a heterologous DENV serotype (DENV-4) after depletion, establishing that cross-neutralizing antibodies were effectively removed from these sera (Figure 4, Table 3). Zika virus neutralization activity was entirely maintained after DENV depletion of serum from persons with primary Zika virus (DT168 and 172) and mostly preserved in depleted serum from persons with secondary Zika virus (DT165 and 166), even when neutralization activity was lost to all DENV serotypes tested (Figure 4, Table 3).

Table 3. 50% Inhibitory concentrations for serum in depletion experiments of DENV and Zika virus*

Serum sample ID	DENV-2	DENV-4	Zika virus
DT000			
Control	744	612	29
Depleted	<20	<20	<20
DT001			
Control	1,376	60	<20
Depleted	331	24	<20
DT168			
Control	83	48	1,978
Depleted	<20	<20	2,351
DT172			
Control	22	32	2,341
Depleted	<20	<20	2,046
DT165			
Control	198	162	3,067
Depleted	31	78	2,060
DT166			
Control	1,556	1,138	1,090
Depleted	<20	126	873

*DENV, dengue virus; ID, identification. Specific antibodies were depleted using beads coated with DENV1 and DENV2 antigens. Beads coated with BSA were used as a control.

Discussion

Because Zika virus is emerging in areas with high rates of dengue prevalence, the extent of antibody cross-reactivity

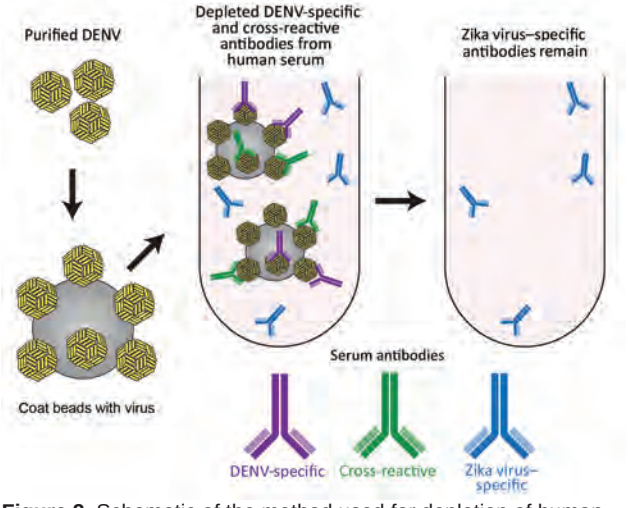

Figure 2. Schematic of the method used for depletion of human serum with DENV antigen to distinguish Zika virus–specific from cross-reactive flavivirus antibodies. Serum was incubated with DENV-1 and DENV-2 coated on polystyrene beads, enabling removal of DENV-specific and cross-reactive antibodies. DENV, dengue virus.

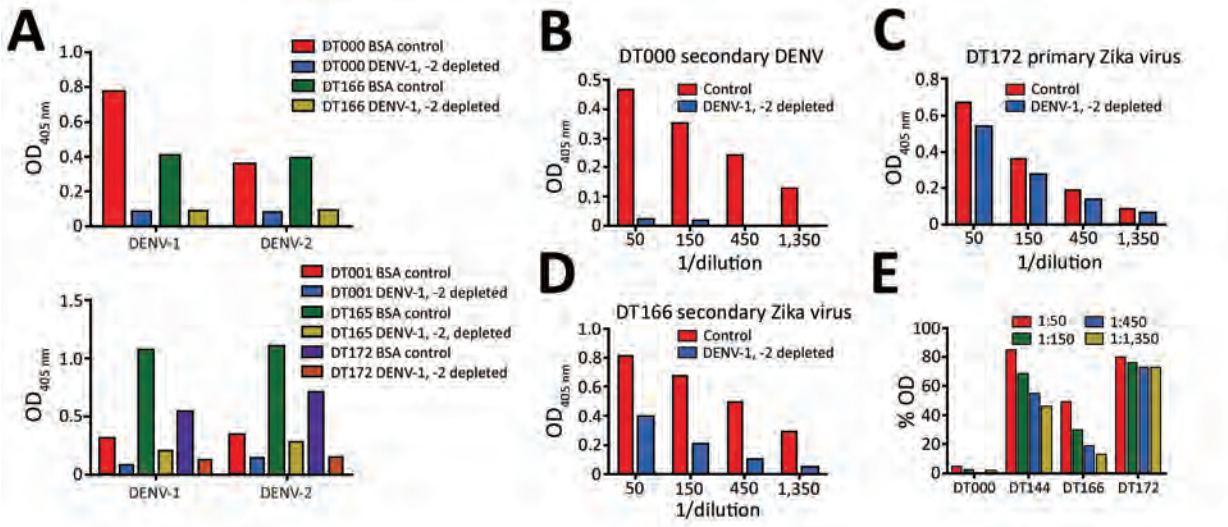

Figure 3. Use of depletion of human serum with DENV antigen to distinguish Zika virus–specific from cross-reactive flavivirus antibodies. A) Depletion efficiency was determined by direct ELISA. Plates were coated with depleting antigens (DENV-1 and DENV-2) and binding of control and depleted serum was measured. B–E) Binding of depleted serum to Zika virus H/PF/2013 was measured by capture ELISA. BSA, bovine serum albumin; DENV, dengue virus; OD, optical density.

between these 2 viruses needs to be thoroughly evaluated. Recent studies have reported that DENV infection results in antibodies that cross-neutralize Zika virus (*15*,*16*,*18*). Plasmablasts isolated from patients during or immediately after recovery from acute DENV infection produced antibodies that cross-neutralized Zika virus in cell culture (*35*) and were protective in a mouse model of Zika virus infection (*18*). Priyamvada et al. (*16*) demonstrated moderate to high-titer Zika virus neutralization in serum from 9 DENV-infected persons; Zika virus neutralization activity was maintained out to 100 days of convalescence in 1 of the 5 persons with paired samples. They further reported that 7 of 47 mAbs derived from plasmablasts from 4 patients with acute DENV infection cross-neutralized Zika virus.

Our data do not demonstrate frequent and high-level cross-neutralization of Zika virus after exposure to DENV. Of 19 persons who had recovered from primary DENV infections, none showed cross-neutralization of Zika virus. Among persons exposed to repeat DENV infections, 3 (23%) of 13 showed Zika virus neutralizing antibodies. The remaining 10 persons had no detectable Zika virus neutralizing antibodies despite having high levels of neutralizing antibodies to multiple DENV serotypes. DT165 and 166 are classified as secondary Zika virus strains on the basis of neutralization profile, epidemiologic context (these donors had fever and rash illness while residing in northeastern Brazil in 2015 during peak Zika virus transmission), and the presence of Zika virus type–specific antibodies in their serum. Accordingly, these persons have high titers of neutralizing antibodies to Zika virus in addition to neutralization activity to ≥1 DENV serotypes. These results are consistent with those reported by Swanstrom et al., who found that only 1 of 16 persons exposed to repeat DENV infections had Zika virus IC_{50} values >1:100 (*18*). Low-level cross-neutralization to heterologous DENV serotypes is also observed into late convalescence after primary DENV infection; however, this phenomenon typically does not preclude accurate diagnosis of the originally infecting DENV serotype, nor does it confer immunity to secondary DENV infection by heterologous serotypes (*30*,*36*).

A key feature of acute DENV infection is the transient induction of high levels of flavivirus cross-neutralizing and cross-protective antibody (*33*). Over a few months, the neutralizing antibody response becomes more specific to ≥1 DENV serotypes, with little to no cross-neutralization of viruses belonging to other flavivirus serocomplexes (*33*). Timing of sample collection probably explains high levels of DENV–Zika virus cross-neutralizing antibodies observed by only some groups. Barba-Spaeth et al. (*15*) and Priyamvada et al. (*16*) performed their studies with blood samples collected from patients a few days to weeks after recovery from acute DENV infection, when overall antibody response is known to be broadly cross-neutralizing. In contrast, our studies were performed with samples collected ≥6 months after infection when the neutralizing antibody response has become more specific to the viruses responsible for infection.

In essence, the question is whether Zika virus will behave as a "fifth serotype" of DENV or as a flavivirus outside the DENV serocomplex. Extensive cross-reactivity in IgG binding between these viruses clearly occurs, consistent with sequence homology and structural similarity among different flavivirus serocomplexes (*10*,*12*). However, binding does not

reliably predict functional outcomes, such as neutralization, enhancement, or protection, and our results suggest that most persons infected with DENVs do not maintain high levels of Zika virus neutralizing antibodies 6 months after DENV infection. Although Zika virus is genetically more closely related to the DENVs than to other flaviviruses, the long-lived antibody cross-neutralization within the DENV serocomplex does not extend to Zika virus in most of our cohort. Furthermore, our data indicate that a population of Zika virus–specific neutralizing antibodies develops after Zika virus infection, even in the presence of preexisting DENV immunity. The corollary is that cross-reactive antibodies and memory B cells from the prior DENV infection are not the major source of Zika virus neutralizing antibodies; rather, de novo priming of naive B cells by Zika elicits Zika type-specific antibody responses. Thus, we propose that Zika virus does not belong to the DENV serocomplex and that DENV-immune persons will remain susceptible to Zika virus infection. Moreover, live attenuated tetravalent DENV vaccines are being evaluated for safety and efficacy in Asia and Latin America. Our results indicate that DENV neutralizing antibody induced by these vaccines are unlikely to protect against Zika virus infection.

In the field, undifferentiated fever in the tropics can have a multitude of causes and often presents a diagnostic challenge (*37*). Molecular detection of Zika virus has good specificity but is not ideal for diagnosing it in persons who seek care many days after illness onset or have asymptomatic infection. Simple serologic assays based on binding antibody are difficult to interpret because of flavivirus cross-reactivity (*9*,*10*). This scenario, typified by DT144, 165, and 166, whereby flavivirus exposure history is uncertain with currently available assays (IgG or IgM ELISA), represents a critical challenge facing public health systems throughout

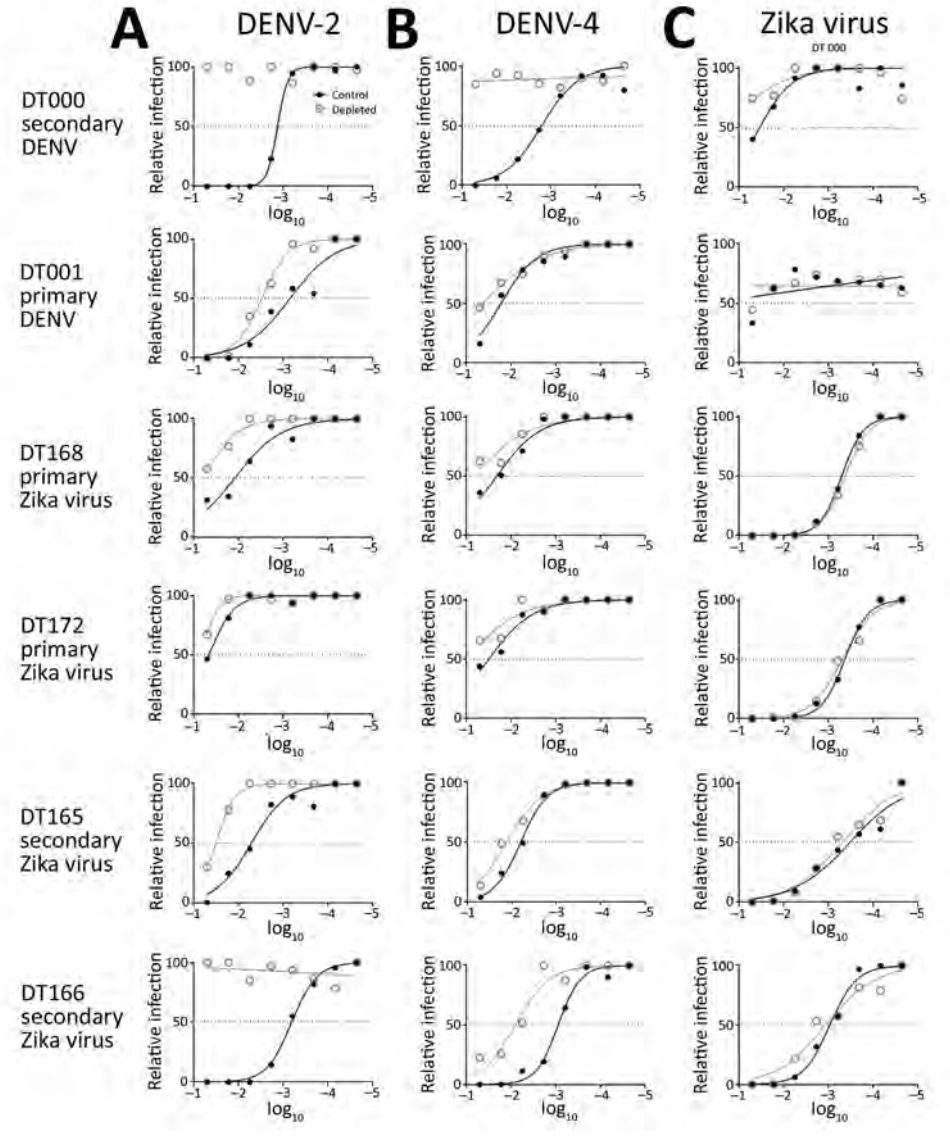

Figure 4. Lack of contribution of cross-reactive DENV antibodies to Zika virus neutralization. Immune serum was depleted with DENV-1 and DENV-2 antigens bound to polystyrene beads, and neutralization activity was measured against DENV-2 (A), DENV-4 (B), and Zika virus (C) for indicated serum. DENV, dengue virus. Dilutions are 1:the value given.

the tropics where billions of persons are at risk for DENV and Zika virus infections (*38*). Our results suggest that the classical flavivirus plaque/focus reduction neutralization test might be reliable for determining previous Zika virus infection, particularly in the setting of retrospective serologic surveys and vaccine trials. We also note that the neutralization test is not reliable for testing samples collected during or soon after recovery from a DENV or Zika virus infection because of temporarily broad cross-neutralization and poor specificity. Indeed, US public health laboratories and others have reported on the poor specificity of testing for Zika virus in acute or early convalescent samples using currently available tests and algorithms (*39*–*42*). On the basis of our studies using samples collected at late convalescence, we propose that the neutralization assay might retain utility for supporting Zika virus and DENV vaccine and other clinical trials; population-level serosurveillance; and clinical management of some patients, such as pregnant women, when blood samples are collected many months after a suspected infection.

Interest and investigation are ongoing regarding whether Zika virus strain–dependent factors might explain phenomena observed during the current epidemic. Although a more comprehensive and in-depth analysis of Zika virus genetic variation might reveal viral determinants of pathogenesis, our experiments suggest that epitopes conferring susceptibility to neutralization by human serum have not changed substantially over time. We do find that the prototype Zika virus strain MR766 is more readily neutralized than other Zika virus strains. MR766 might provide the most sensitive screen for cross-neutralizing antibody, but relevance of such antibody should be confirmed by an isolate more representative of contemporary circulating viruses.

In conclusion, the current Zika virus epidemic presents many urgent challenges but also great opportunities to dramatically expand our knowledge of humoral immunity to flaviviruses. Although our results argue for infrequent cross-neutralization of Zika virus by DENV-immune serum and for development of independent populations of neutralizing antibody to these 2 viruses, additional and larger studies are needed to determine whether the rate of Zika virus cross-neutralization varies in different populations, particularly in DENV-endemic areas where ongoing subclinical exposure to DENV could further broaden the range of cross-neutralization in polyclonal serum. That effective vaccines have been developed against several flaviviruses is encouraging (*43*–*46*), but much remains to be learned about Zika virus–specific antibody responses and the dynamics of cross-reactive antibody in persons with multiple flavivirus exposures. Knowledge on these fronts will better inform Zika virus vaccine development, rational design of serodiagnostic tests, and general understanding of antibody responses to related viruses.

This study was supported by National Institutes of Health grants R01-AI107731 (to A.d.S.) and P01 AI106695 (subcontract to A.d.S; principal investigator Eva Harris, University of California, Berkeley).

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Control of Malaria Vector Mosquitoes by Insecticide-Treated Combinations of Window Screens and Eave Baffles

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We assessed window screens and eave baffles (WSEBs), which enable mosquitoes to enter but not exit houses, as an alternative to indoor residual spraying (IRS) for malaria vector control. WSEBs treated with water, the pyrethroid lambda-cyhalothrin, or the organophosphate pirimiphos-methyl, with and without a binding agent for increasing insecticide persistence on netting, were compared with IRS in experimental huts. Compared with IRS containing the same insecticide, WSEBs killed similar proportions of *Anopheles funestus* mosquitoes that were resistant to pyrethroids, carbamates and organochlorines and greater proportions of pyrethroid-resistant, early exiting *An. arabiensis* mosquitoes. WSEBs with pirimiphos-methyl killed greater proportions of both vectors than lambda-cyhalothrin or lambda-cyhalothrin plus pirimiphos-methyl and were equally efficacious when combined with binding agent. WSEBs required far less insecticide than IRS, and binding agents might enhance durability. WSEBs might enable affordable deployment of insecticide combinations to mitigate against physiologic insecticide resistance and improve control of behaviorally resistant, early exiting vectors.

Vector control with long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) interventions account for 78% of the 663 million malaria cases and most of the 4 million deaths averted globally over recent years (*1*,*2*). LLINs and IRS can reduce malaria transmission by killing sufficient numbers of vector mosquitoes when they attack sleeping humans or rest indoors (*3*–*5*). However, as these approaches have been scaled up, physiologic resistance to insecticidal active ingredients has become increasingly common, threatening a "looming public health catastrophe" (*6*). Physiologic resistance to pyrethroids, the only class of insecticides suitable for use on LLINs, is now widespread and undermining vector control across Africa (*7*).

Only 4 directly lethal insecticide classes are recommended for control of adult malaria vectors with LLINs or IRS: pyrethroids (e.g., permethrin, deltamethrin, lambda-cyhalothrin); organochlorines (e.g., DDT); carbamates (e.g., bendiocarb, propoxur); and organophosphates (e.g., malathion, fenitrothrion, pirimiphos-methyl) (*8*). Mechanisms of cross-resistance against organochlorines and pyrethroids limit their utility for combined use in rotations, mosaics, or combinations (*7*,*8*). Organochlorines (especially DDT) and carbamates have a long history of use in agriculture and public health, and resistance to these classes is already emerging after only a few years of use in IRS at programmatic scales (*7*). However, these classes and organophosphates cannot be safely applied to LLINs at operationally effective doses (*8*), and are prohibitively expensive for routine IRS applications (*9*–*11*).

Year-round protection for the 40 million persons at risk of malaria in Tanzania, with IRS using the ideal recommended dose of the new capsule suspension formulation of the organophosphate pirimiphos-methyl, would cost US $157 million annually for insecticide procurement, exceeding the entire national malaria control budget of $114 million. Pirimiphos-methyl procurement for continuous IRS coverage of all at-risk populations would cost $3.3 billion annually across Africa and $12.5 billion worldwide, dwarfing the total global malaria control budget of $2.5 billion (*10*). As such expensive insecticides have become increasingly necessary because of pyrethroid resistance, IRS coverage has inevitably decreased (*9*–*11*) to only 3.4% globally (*12*). Although new insecticides are being developed for malaria vector control (*6*,*7*,*13*), these insecticides might also be similarly expensive. Unless new active ingredients are astutely delivered through

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DOI: http:// dx.doi.org/10.3201/eid2305.160662

rotations, mosaics, or combinations, they might not be any less prone to emergence of physiologic resistance (*6–8*).

Beyond physiologic resistance, effects of LLINs and IRS are also attenuated by the tendency of vectors to enter houses but then rapidly exit them, without resting on treated surfaces long enough to accumulate lethal doses of insecticide (*14–16*). Repeatedly entering and rapidly exiting several houses, until an unprotected human can be bitten, enables mosquitoes to mediate persistent residual malaria transmission by maximizing feeding opportunities while minimizing risks of exposure to LLINs and IRS when foraging indoors (*17*,*18*). Therefore, new insecticide delivery methods must target such evasive early exiting vectors (*14*,*16*), which might be described as behaviorally resilient (preexisting traits, typically with considerable phenotypic plasticity) or resistant (increasing frequency of selected heritable traits) (*17*,*19*). However, life history simulation analyses suggest such repeated visits to houses represent a vulnerability that can be exploited to great effect with improved methods for killing mosquitoes inside houses (*17*,*18*). Even for early exiting vectors that often feed outdoors instead, most mosquitoes old enough to transmit malaria have previously entered ≥1 house, where they could be targeted with lethal insecticides or traps (*18*).

Personal protection provided by LLINs and IRS can be superseded and improved by physically mosquito-proofing houses with screened windows, ceilings, and closed eaves (*20*). However, most of the overall effects of LLINs and IRS on malaria transmission are achieved by killing mosquitoes en masse to protect entire communities, with more obvious contributions of personal or household protection being far less equitable and of lower magnitude (*4*). Household protection measures, such as spatial repellents or physical mosquito-proofing, which merely deter mosquitoes from entering houses and force them to seek blood meals elsewhere, might have less overall effect than measures that directly kill mosquitoes (*21*). In many settings with highly efficient vectors, elimination of malaria transmission will probably require lethal measures that suppress (*3–5*) or eliminate (*22*) mosquito populations, rather than merely deter them from entering houses (*21*). Therefore, new insecticide delivery methods are urgently needed to enable affordable deployment of multiple active ingredients and more effective targeting of early exiting mosquitoes (*6*,*8*,*13*).

We describe a simple housing modification with widely available netting materials that traps mosquitoes inside houses after they enter, and forces them into lethal contact with insecticides when they attempt to exit (Figure 1). Eave baffles have been used for decades (*23*) in standardized experimental hut designs for assessing LLINs and IRS (*24*,*25*). These baffles consist of netting panels slanting inwards and upwards from the upper end of the wall toward the roof, but leaving a small gap so that mosquitoes can freely enter the hut but cannot leave by the same route (Figure 1, panel A). Eave baffles have been used to target house-entering mosquitoes with fungal entomopathogens (*26*). In our study, baffles were combined with netting window screens and evaluated as a targeted delivery format for off-the-shelf formulations of commonly used chemical insecticides (Figure 1, panel B). This combination, called treated window screens and eave baffles (WSEBs), required far less insecticide than IRS. We assessed whether WSEBs could achieve control of physiologically resistant *Anopheles funestus* mosquitoes and early exiting *An. arabiensis* mosquitoes equivalent to that of IRS.

Methods

All experiments were conducted in Lupiro village in the Kilombero Valley of rural southern Tanzania using commercially available IRS formulations of pyrethroids and organophosphates, which were combined with existing binding agent (BA) products for extending insecticide durability on LLINs. In this area of southern Tanzania, intense malaria transmission is mediated by 2 of the major malaria vectors in Africa. The first mosquito is *An. funestus*, which mediates rebounding (*14*) malaria transmission in this setting because it is physiologically resistant to pyrethroids, carbamates and organochlorines (*27*). The second mosquito is *An. arabiensis*, which mediates resilient residual transmission (*14*) because it is physiologically resistant to pyrethroids (*27*) and also exhibits early exiting behavior

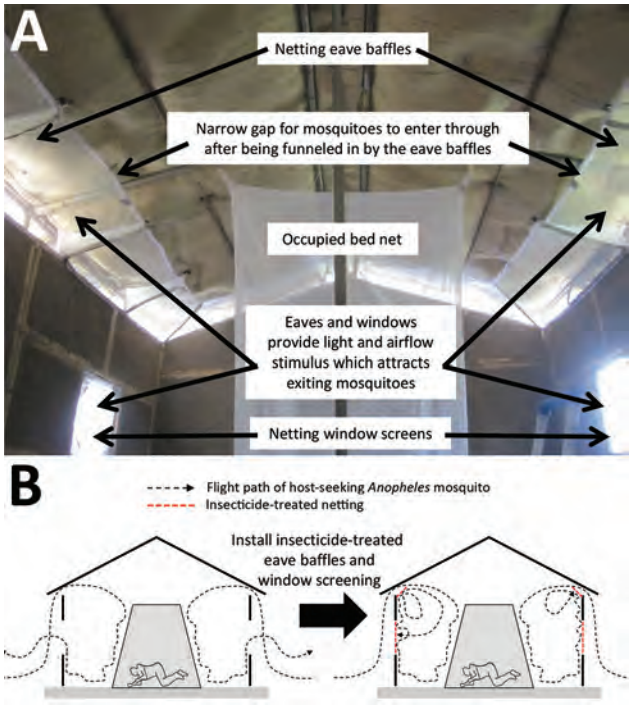

Figure 1. Design (A) and mechanism of action (B) of insecticide-treated window screens and eave baffles for control of malaria vector mosquitoes, Tanzania.

that renders it robust to indoor control with LLINs and IRS (*18*,*28*,*29*). All procedures were approved by the Institutional Review Board of the Ifakara Health Institute (IHI/IRB/34–2014) and the Medical Research Coordination Committee of the National Institute for Medical Research (NIMR/HQ/R.8a/Vol IX/1903).

We used 13 experimental huts of the Ifakara design (*24*,*29*,*30*) and standard methods (*31*) to assess effects of LLINs, IRS, and insecticide-treated WSEBs. Four of these huts were randomly selected, and their inner wall and roof surfaces were sprayed with 2 g/m^2 of a capsule suspension formulation of pirimiphos-methyl (Actellic 300CS) by using standard programmatic application procedures (*32*). Another 4 randomly selected huts were sprayed with 30 mg/m^2 of the pyrethroid lambda-cyhalothrin, which was also in a capsule suspension formulation (Icon 10CS). Both of these long-lasting, microencapsulated, insecticide formulations are manufactured by Syngenta AG (Basel, Switzerland) for IRS applications and are well characterized (*33*–*35*). The remaining 5 huts were sprayed only with water to serve as negative controls. After spraying, 2 mattresses and intact PermaNet LLINs (100-denier polyester multifilament mesh with 156 holes/inch^2, surface-treated with 45-55 mg/m^2 of deltamethrin in a resin foundation; Vestergaard, Lausanne, Switzerland) were installed in each hut.

Eave baffles are incorporated into experimental hut designs to ensure that mosquitoes can enter through approximately half of the eave gaps between the wall and the roof but are then all either retained in the hut or forced into interception traps fitted to the remaining exit points (*24*,*25*). In a conventional experimental hut study, those remaining exit points are windows and the remaining unbaffled half of eave gaps (*24*,*25*). However, the purpose of this study was to evaluate WSEBs as an insecticide delivery format. Therefore, all WSEB treatments, except for the negative control, included eave baffles fitted to all eave gaps, with and without exit traps, and identically treated screens fitted over all windows (Table; Figure 1). Treated WSEBs were fitted in front of exit traps, which were fitted immediately outside the hut (*24*), so that any mosquito attempting to exit through any eave gap or window would be forced into contact with these insecticidal netting barriers (Figure 1).

The only treatment without screens over the windows or eave baffles over the half of the eave gaps with exit traps immediately outside were the negative control (Table). These controls had untreated eave baffles fitted only to the half of the eave spaces lacking exit traps, thus enabling mosquitoes to enter and exit. The 2 partial negative controls had screens fitted over the windows and baffles fitted to all eave gaps, regardless of whether they acted as entry or exit points for mosquitoes, but were not treated with any insecticides (Table). One partial negative control was treated with the noninsecticidal BA that Syngenta AG includes along with lambda-cyhalothrin (the same Icon 10CS formulation we used for IRS) in their Icon Maxx product to extend its active life on polyester netting (*36*).

The first insecticidal WSEB treatment (Table) was this same long-lasting Icon Maxx product, this time including both BA and lambda-cyhalothrin (*36*). Although the manufacturer-recommended dose of lambda-cyhalothrin on netting treated with the Icon Maxx product (55 mg/m^2) is somewhat higher than that used for IRS (30 mg/m^2), it is

Table. Window screen and eave baffle treatments that were rotated through experimental huts with 3 IRS treatments for control of malaria vector mosquitoes, Tanzania*

Treatment no.	Description	Eaves baffled		Windows screened	Treatment of window screen and eave baffle netting		
		Entrances	Exits		LC, mg/m^2	PM, g/m^2	BA
1	Negative control: no trapping or insecticide	Yes	No	No	0	0	No
2	Partial negative control: trapping without insecticide	Yes	Yes	Yes	0	0	No
3	Partial negative control: trapping without insecticide	Yes	Yes	Yes	0	0	Yes
4	Trapping plus long-lasting LC and BA treatment	Yes	Yes	Yes	55	0	Yes
5	Trapping plus varying dose PM treatments	Yes	Yes	Yes	0	1	No
6		Yes	Yes	Yes	0	2	No
7		Yes	Yes	Yes	0	4	No
8	Trapping plus varying dose PM treatments with BA	Yes	Yes	Yes	0	1	Yes
9		Yes	Yes	Yes	0	2	Yes
10		Yes	Yes	Yes	0	4	Yes
11	Trapping plus varying dose PM treatments with BA and LC	Yes	Yes	Yes	55	1	Yes
12		Yes	Yes	Yes	55	2	Yes
13		Yes	Yes	Yes	55	4	Yes

*Indoor residual spraying treatments of experimental huts used lambda-cyhalothin (30 mg/m^2 in 4 huts), pirimiphos-methyl (2 g/m^2 in 4 huts), or a negative control (water diluent only: 5 huts), which was applied to all inner surfaces of walls and ceilings. All doses are per square meter of treated netting (window screening and eave baffles) or wall and ceiling surface (IRS), so that these doses can be directly compared in terms of lethality and cost per unit area treated. The 26-day schedule applied to complete 1 full replicate of evaluation for duplicates of these 13 treatments, by rotating them through all 13 IRS-treated experimental huts, is detailed in online Technical Appendix 1 (https://wwwnc.cdc.gov/EID/article/23/5/16-0662-Techapp1.xlsx). BA, binding agent; IRS, indoor residual spraying; LC, lambda-cyhalothin; PM, primiphos-methyl.

similar to that for deltamethrin on PermaNet LLINs used in this study (45-55 mg/m^2). WSEBs treated with pirimiphos-methyl were assessed at 3 doses that were comparable with typical IRS application rates per square meter treated (Table). These 3 pirimiphos-methyl doses were also assessed as a co-treatment with BA to potentially extend insecticide life, with and without lambda-cyhalothrin as a complementary second insecticide from a different chemical class (Table). Lambda-cyhalothrin was chosen, despite being a pyrethroid to which both vector species in the study area are resistant (*27*), to assess the potential of such combinations to select for restored pyrethroid susceptibility (*37*). Conceptually, this approach relies on selectively reducing mortality rates for insects that are susceptible to its lethal mode of action and responsive to its irritant/repellent effects on mosquito behavior (*37*). The mathematical modeling study that motivated assessment of this combination assumed that these 2 pyrethroid susceptibility and responsiveness phenotypes, and presumably their underlying genotypes, are closely associated and therefore co-selected (*37*).

Although all exit traps on eaves and windows were made of Teflon-coated fiberglass mesh (*24*), all eave baffles and window screens were made of 100-denier polyester netting (A to Z Textile Mills, Arusha, Tanzania) of the kind typically used for bed nets. All WSEBs were treated by soaking in aqueous suspensions of the insecticides, BA, or both and then drying in the shade.

To execute the experimental design of this study, duplicate sets of the 13 detachable, movable WSEB treatments (Table) were rotated nightly through the 13 huts over two 26-day rounds of experimental replication (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/5/16-0662-Techapp1.xlsx) during December 5, 2015-February 1, 2016. Each night, 2 men (volunteers) slept under the 2 LLINs inside each hut from 7:00 PM to 7:00 AM. These men then collected all mosquitoes inside the hut by using a Prokopak aspirator (John W. Hock Co., Gainesville, FL, USA) (*38*) and those inside the exit traps by using a mouth aspirator (*24*). Dead mosquitoes were then sorted taxonomically, classified by sex and abdominal status, and counted. Specimens collected alive were maintained in a field insectary for 24 h before separating live and dead specimens for sorting, classification, and counting. A random sample of 242 specimens from the *An. gambiae* complex was identified to sibling species by PCR (*39*).

Each pair of men remained assigned to a fixed experimental hut throughout the study so that variability associated with these volunteers and the huts could be analyzed as a single, consistent source of variance. After mosquito collection each morning, each pair of men was responsible only for installing the set of WSEBs assigned to their hut that evening and for removing that set from the hut it had been fitted to the previous night. All volunteers used a fresh pair of gloves each morning and were not allowed to handle any WSEBs other than those to be used in their hut that night. All WSEB sets were labeled and stored in labeled buckets during transfer between huts and the 13-day storage period of each 26-day replication cycle (online Technical Appendix 1).

All field data were collected on hard copies of the adult field collection (ED1) and sample sorting (SS3) forms, recently described for informatically robust collection of entomologic data (*40*). To ensure rigid compliance with the experimental design, all attributes defined by it were prefilled into the forms (online Technical Appendix 1). All statistical analysis was accomplished by using generalized linear mixed models with a binomial distribution and logit link function for the binary mosquito death outcome and fitted by using R version 3.2.1 (https://www.r-project.org/). WSEB treatments were included as categorical independent variables, and hut and night were included as random effects.

Results

A total of 1,318 specimens from the *An. funestus* group and 5,842 from the *An. gambiae* complex were captured. Molecular identification confirmed continued absence of nominate *An. gambiae* mosquitoes in the study area (*22*). All of the 176 specimens that were successfully amplified (73% of the 242 specimens from this complex) were identified as *An. arabiensis* mosquitoes. All WESBs, other than the negative control, clearly retained mosquitoes within the huts, because this is where most (>90%) were collected, rather than in exit traps.

Effects of WSEBs and IRS on *An. funestus* Mosquito Mortality Rates

When used alone, most (8/10) WSEB treatments that included insecticides killed similarly high proportions of *An. funestus* mosquitoes as did IRS alone with the same insecticide formulations (Figure 2, panel A). Mortality rates for lambda-cyhalothrin plus BA-treated WSEBs alone were indistinguishable from those for lambda-cyhalothrin IRS ($p = 0.363$). The only exceptions among the 10 WSEB treatments were the highest pirimiphos-methyl dose plus BA and the intermediate pirimiphos-methyl dose plus lambda-cyhalothrin and BA.

Both of these WSEB treatments alone killed lower proportions of *An. funestus* mosquitoes than IRS with lambda-cyhalothrin alone; a similar but nonsignificant pattern was observed for comparisons of the same WSEB treatments alone with pirimiphos-methyl IRS alone (Figure 2; online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/23/5/16-0662-Techapp2.xlsx). Nonetheless, mortality rates for pirimiphos-methyl–treated WSEBs alone were consistently high (Figure 2, panel A), regardless of treatment dose ($p \geq 0.156$), and were statistically indistinguishable from

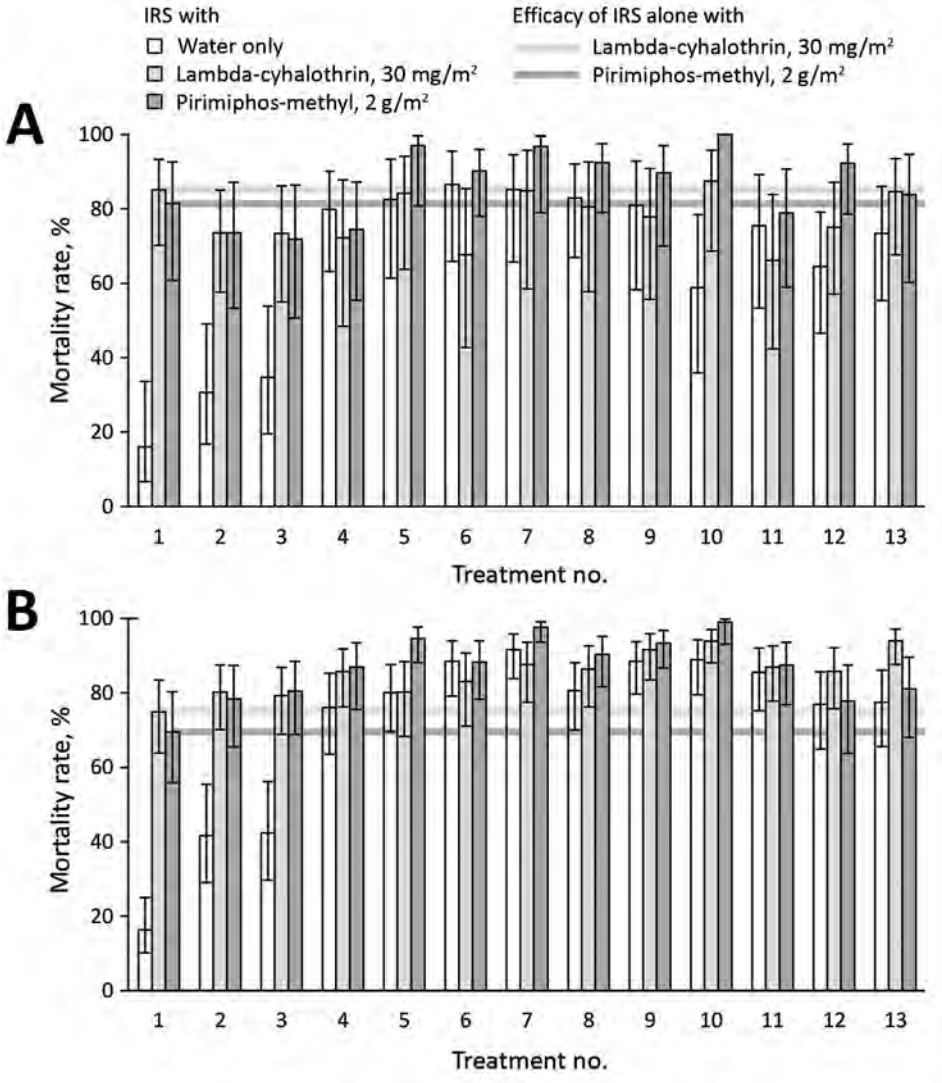

Figure 2. Effect of window screens and eave baffles treated with 13 combinations of insecticides and binding agents on malaria vector mosquito mortality rates inside experimental huts, Tanzania. A) *Anopheles funestus*. B) *An. arabiensis.* Huts were previously sprayed with 1 of 3 alternative indoor residual spraying regimens (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/5/16-0662-Techapp1.xlsx) and occupied by 2 volunteers sleeping under pyrethroid-treated, long-lasting insecticidal nets. IRS, indoor residual spraying. Error bars indicate 95 CIs. Estimated mean mortality rates and 95% CIs, as well as statistical contrasts between the most relevant treatment pairs, are indicated in online Technical Appendix 2 (https://wwwnc.cdc.gov/EID/article/23/5/16-0662-Techapp2.xlsx).

pirimiphos-methyl IRS alone (p≥0.713), even though the lowest WSEB dose per unit area treated was only half that for IRS. Although all combinations of pirimiphos-methyl–treated WSEBs with pirimiphos-methyl IRS resulted in higher mortality rates than pirimiphos-methyl IRS alone or pirimiphos-methyl–treated WSEBs alone, none of these differences were significant (p≥0.080) because too few mosquitoes survived either treated WSEBs alone or IRS alone.

Effects of WSEBs and IRS on *An. arabiensis* Mosquito Mortality Rates

Overall, insecticide-treated WSEBs either matched or were superior to IRS when used against *An. arabiensis* mosquitoes (Figure 2, panel B; online Technical Appendix 2). WSEBs treated with lambda-cyhalothrin plus BA showed similar mortality rates as IRS with the same lambda-cyhalothrin formulation (p = 0.345). WSEBs treated with the lowest dose of pirimiphos-methyl showed similar mortality rates for *An. arabiensis* mosquitoes as IRS with twice as much pirimiphos-methyl per square meter treated (p = 0.419). However, increasing the pirimiphos-methyl treatment dose from 1 to 2 or 4 g/m^2 increased the mortality rate for WSEBs (odds ratio [OR] 2.10, 95% CI 1.16–3.79, p = 0.0139; and 2.34, 95% CI 1.28–4.26, p = 0.0055, respectively), although there was no difference between intermediate and high doses (p = 0.758).

WSEBs with intermediate or high doses of pirimiphos-methyl killed more *An. arabiensis* mosquitoes (OR 5.9, 95% CI 1.4– 24.3, p = 0.0145; 10.8, 95% CI 1.6–74.8, p = 0.0157, respectively) than IRS, even though the intermediate pirimiphos-methyl dose was the same as for IRS per square meter treated. Supplementing pirimiphos-methyl–treated WSEBs with pirimiphos-methyl IRS increased *An. arabiensis* mosquito mortality rates for the lowest WSEB dose (OR 4.8, 95% CI 1.5–15.5, p = 0.0081), which was half that of IRS per unit area treated. However, supplementary

pirimiphos-methyl IRS did not increase mortality rates when WSEBs were treated with the same dose as IRS (p = 0.748) or twice that dose (p = 0.429).

Pirimiphos-Methyl Supplemented with BA and Lambda-Cyhalothrin as WSEB Co-treatments

Adding BA had no effect on the mortality rates for pirimiphos-methyl–treated WSEBs for *An. funestus* (p = 0.393) or *An. arabiensis* (p = 0.424) mosquitoes. Supplementing organophosphate pirimiphos-methyl plus BA treatment with the irritant pyrethroid lambda-cyhalothrin as a second active ingredient reduced *An. funestus* mosquito mortality rates for WSEBs (OR 0.64, 95% CI 0.46–0.89, p = 0.0076), presumably because the irritant properties of lambda-cyhalothrin reduced mosquito contact times with co-treated WSEBS, and therefore exposure to both insecticides. A similar but less dramatic, nonsignificant trend was observed for *An. arabiensis* mosquitoes (OR 0.88, 95% CI 0.73–1.06, p = 0.174).

Discussion

Although WSEBs had higher efficacy than IRS against early exiting *An. arabiensis* mosquitoes, the 2 delivery formats had similar efficacy against *An. funestus* mosquitoes. Therefore, the most striking advantage of WSEBs is that they reduced the surface area treated per hut by >5-fold. Furthermore, co-application with existing BAs that already extend durability of pyrethroids on LLINs (*36*) for as much as 3 years (*41*) suggests new opportunities for reducing reapplication frequency by up to 6-fold, relative to IRS.

These WSEBs are an experimental prototype that were evaluated in the necessarily homogenous and controlled environment of experimental huts. This short-term efficacy study did not address key issues regarding potential effectiveness and cost-effectiveness of WSEBs under programmatic operational conditions. It is encouraging that a set of these WSEBs for these experimental huts, specifically designed to match the dimensions of local houses (*24*), required only 11 m^2 of netting to manufacture, similar to a typical LLIN. However, this netting had to be carefully hand-tailored with hooks and Velcro to enable easy daily removal and reinstallation in experimental huts, at a manufacturing labor cost of $47 per set. More practical and affordable formats for operational use in a diversity of house designs must be developed and rigorously evaluated before WSEBs could be considered for routine, programmatic deployment by national programs.

Nevertheless, the potential of this approach merits consideration, even if only speculatively at this early stage. It takes almost an entire 833-mL bottle of the 0.3 g/mL pirimiphos-methyl formulation used here, costing ≈$24, to treat 1 typical rural house in Tanzania twice a year with IRS at the ideal recommended dose of 2 g/m^2. In comparison, a house of equivalent size with WSEBs installed could be treated with the same insecticide at the same dose per square meter of treated netting for only $2.15. Although greater quantities of BA might be required than applied here (*42*), it could extend the life of pirimiphos-methyl on netting to the same extent as for lambda-cyhalothrin on LLINs that are approved for 3 years of use. If BA-treated WSEBs were similarly durable, they could provide up to 3 years of protection for only $0.72 per year in recurrent insecticide procurement costs. Because scale-up nationally in Tanzania would cost only $4.8 million for insecticide procurement, a combination of 3 similarly expensive complementary insecticides would be affordable to the national program at a cost of <$15 million annually. Corresponding global costs would be <$1.2 billion annually for such a triple combination.

Changing deployment format for existing IRS formulations could also eliminate the need to apply them in potentially hazardous aerosol form. Although handling insecticides is always associated with some risks, and protective clothing, eyewear, and a breathing apparatus might be required, WSEBs may be impregnated by simply dipping them in an aqueous suspension, similarly to bed nets. Therefore, WSEB deployment formats might enable national programs to develop and manage their vector control platforms more flexibly than when using IRS.

Although these insecticide cost estimates are entirely speculative, assume that BA will be equally efficacious for extending longevity of pirimiphos-methyl, and do not consider costs of netting installation or maintenance, they outline the potential economic benefits that could be accrued by optimizing WSEB deployment formats, netting materials, and treatment formulations. In addition, such reduced insecticide requirements might make rational resistance management (*8*) feasible and affordable with existing budgets and off-the-shelf insecticide products.

The observation that supplementing pirimiphos-methyl–treated WSEBs with the irritant pyrethroid lambda-cyhalothrin reduced mortality rates for *An. funestus* mosquitoes, which were strongly resistant to pyrethroids but not organophosphates (*27*), suggests that WSEBs could be used in an affordable format with which to field-test the theory that such combinations might select for restored pyrethroid susceptibility (*37*). The underlying assumption of this hypothesis is that physiologic susceptibility and behavioral responsiveness to pyrethroids are genetically linked, so that insecticide combinations, such as the LC-PM mixture used here, would selectively kill insects that are both resistant and non-responsive to pyrethroids.

The case for assuming that physiologic susceptibility and behavioral responsiveness are at least phenotypically associated has recently been strengthened by laboratory studies of *Culex quinquefasciatus* mosquitoes, which

demonstrated that 4 pyrethroid-resistant field populations were all less responsive to the irritant properties of permethrin than a fully susceptible laboratory colony (*43*). These empirical studies (*43*) also suggest grounds for optimism regarding the recent theory that combining recently developed, low-technology emanators for airborne pyrethroid vapor (*44*,*45*) with complementary nonpyrethroid indoor control measures, such as IRS, WSEBs, or alternative technologies, such as eave tubes (*46*–*48*) and entry traps (*49*), could coselect for evolutionarily stabilized restoration of physiologic susceptibility and behavioral responsiveness to pyrethroids generally (*50*).

Genetic linkage between physiologic susceptibility and behavioral responsiveness to pyrethroids remains to be demonstrated. Also, both mathematical models predicting restoration of these preferred traits (*37*,*50*), by definition, merely illustrate the plausibility of these hypotheses in mathematically explicit terms. Alternatively, selection for physiologic resistance to both insecticides might be exacerbated by reducing contact exposure to sublethal levels. Although potential benefits and risks of combining irritant pyrethroids with nonirritant insecticides from complementary classes remain to be satisfactorily assessed, our results suggest that WSEBs might be a potentially scalable delivery format with which to test these hypotheses empirically through large-scale field studies.

Acknowledgments

We thank John Vontas and Hilary Ranson for their critical comments on the article.

This study was supported by the European Union through the Seventh Framework Program (FP7/2007-2013 grant 265660). F.O. is supported by a Wellcome Trust Intermediate Research Fellowship (grant WT102350/Z/13/Z).

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Use of Blood Donor Screening Data to Estimate Zika Virus Incidence, Puerto Rico, April–August 2016

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Puerto Rico has been heavily impacted by Zika virus, a mosquitoborne flavivirus that emerged in the Americas during 2015. Although most persons with Zika virus show no symptoms, the virus can cause neurologic and other complications, including fetal microcephaly. Local Zika virus transmission in Puerto Rico has been reported since December 2015. To prevent transfusion-associated transmission, local blood collection ceased in March 2016 but resumed in April 2016 after Zika virus screening of blood donations became available. Using data from screening of blood donations collected by the 2 largest blood centers in Puerto Rico during April 3–August 12, 2016, and assuming a 9.9-day duration of viremia, we estimated that 469,321 persons in Puerto Rico were infected during this period, for an estimated cumulative incidence of 12.9%. Results from blood donation screening during arboviral outbreaks can supplement routine clinical and surveillance data for improved targeting of prevention efforts.

Zika virus, a flavivirus transmitted primarily by *Aedes aegypti* mosquitoes, has rapidly spread in the Americas since it emerged in the region in 2015 (*1*). Although most infections are asymptomatic, Zika virus has been identified as a cause of adverse outcomes of pregnancy, including microcephaly and other congenital brain defects (*2*), and has been linked to Guillain-Barré syndrome (*3*) and severe thrombocytopenia (*4*,*5*). Zika virus also has been recognized as a potential threat to blood safety (*6*). In other arbovirus outbreaks, related mosquitoborne flaviviruses, such as West Nile virus and dengue virus, have been transmitted through blood transfusion; the high percentage of asymptomatic infections was a contributing factor (*7*). Retrospective nucleic acid testing (NAT) of blood donations after a large Zika virus outbreak in French Polynesia during 2013–2014 found detectable Zika virus RNA in 2.8% of blood donations (*8*), and cases of likely transfusion-transmitted Zika virus infection (through whole blood–derived platelets) were documented in Brazil (*9*,*10*).

Puerto Rico first reported local transmission of Zika virus in December 2015 (*11*) and has since been heavily affected. As of October 17, 2016, a total of 25,355 cases of locally acquired Zika virus infections had been reported from Puerto Rico to the Centers for Disease Control and Prevention (CDC) national arboviral surveillance system (ArboNET) (*12*).

To reduce the risk for transfusion-transmitted Zika virus infection, in February 2016, the Food and Drug Administration (FDA) recommended that all US areas with active Zika virus transmission cease blood collections unless donations are screened by NAT or treated with approved pathogen-reduction technology (*13*). Blood safety interventions in Puerto Rico were limited to importation of blood units from unaffected US areas and treatment of plasma and apheresis platelets with pathogen-reduction technology until early April 2016, when FDA authorized use of an individual donation NAT test (ID-NAT; cobas Zika, Roche Molecular Systems, Inc., Pleasanton, CA, USA) under an investigational new drug application (*14*).

Data from blood donor screening have been used during previous arbovirus outbreaks to supplement surveillance and guide the implementation of public health interventions. For example, in 2003, blood donor screening data were used to estimate the seasonal incidence of West Nile virus among the general US population (*15*). We describe the use of cobas Zika testing of blood donations from the 2 largest blood collection organizations in Puerto Rico to estimate the total number of incident Zika virus infections in Puerto Rico during April 3–August 12, 2016.

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DOI: http://dx.doi.org/10.3201/eid2305.161873

Methods

Since April 3, 2016, all blood donations collected in Puerto Rico have been screened for Zika virus by using the cobas Zika ID-NAT, which uses PCR amplification to detect Zika virus RNA in plasma specimens. A blood donor with a reactive cobas Zika test result on initial donation is considered to be a presumptive viremic donor (PVD). In this study, we used data on PVDs to estimate Zika virus incidence.

For these analyses, we used data from blood donations collected by the Banco de Sangre de Servicios Mutuos (BSIS; San Jose, PR) during April 3–August 12, 2016, and by the Banco de Sangre del Centro Médico de la Administración de Servicios Médicos (ASEM; San Jose, PR) during April 4–July 31, 2016. These organizations collect most blood donations in Puerto Rico (*16*), with collections throughout the main island. Information collected and reported to CDC included a unique donor identification number, donor sex and age, city and ZIP code of donor residence, date of donation, and cobas Zika test result. City and ZIP code of donor residence were used to identify a donor's municipality (i.e., county) and then health region as defined by the Puerto Rico Department of Health: Aguadilla, Arecibo, Bayamón, Caguas, Fajardo, Mayagüez, Metro/San Juan, and Ponce (*17*).

Because the minimum amount of time donors are required to wait between whole blood and plasma donations at blood centers is 56 and 28 days, respectively, the maximum number of donations per donor during the study period was 5. To estimate Zika virus incidence, all donations from any 1 donor were included in these analyses, except for repeat donations from donors who had a previous cobas Zika-reactive donation because such results could indicate infection and thus immunity. We also excluded donations from donors residing outside Puerto Rico.

To calculate the total number of incident Zika virus infections and the population incidence during the study period, we first calculated the proportions of cobas Zika-reactive donations to estimate the point incidence of Zika virus infection at the time of donation. The point incidence of cobas Zika-reactive donations, which we report aggregated to the week of collection, was then scaled to give estimates of Zika virus incidence during the referenced time frame. Estimates and 1-at-a-time 95% CIs of the number of incident Zika virus infections were computed weekly and cumulatively by week beginning April 3. The weekly values are estimates of the number of incident Zika virus infections during the given week; the weekly cumulative incidence values are aggregated estimates of the number of incident Zika virus infections from April 3 to the given week. The Zika virus incidence estimation process for April 3–August 12, 2016, followed the method of Busch et al. (*15*), although this approach was modified to incorporate the fact that donors are necessarily asymptomatic at time of donation. In brief, proportions of cobas Zika-reactive donations were multiplied by a factor given as the ratio of the duration of the period of collection to the average viremia duration, whereas Zika virus–infected persons are asymptomatic. Parameters used to characterize the average asymptomatic viremia duration were the overall average viremia duration, the average incubation period (i.e., duration from infection to symptom onset), and the proportion of asymptomatic infections. We used statistical computer simulation to account for uncertainty in these parameters.

Because demographic or geographic factors might have affected transmission rates across Puerto Rico, we compared the proportions of cobas Zika-reactive donations across these factors using Fisher exact test. Factors statistically significant at the 5% level were incorporated into the estimation procedure by simultaneously stratifying the donation and population data by these factors, using the procedure outlined earlier (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/16-1873-Techapp1.pdf) to compute separate estimates of the numbers of incident Zika virus infections during the period of interest for each stratum and summing these values for an estimate of the total number of incident Zika virus infections. We divided this summation by the total size of the population at risk to give the estimated incidence of Zika virus infection for this population during the 5-month study period (online Technical Appendix).

We used US Census estimates for 2014 for population totals by stratum (*18*). For the primary analyses, the estimates of the parameters used were 9.9 days (95% CI 6.8–21.6 days) for mean Zika virus viremia duration (*19*), 6.2 days (95% CI 5.3–7.1 days) for the mean Zika virus incubation period (Krow-Lucal ER, Biggerstaff BJ, Staples JE, unpub. data), and 0.79 (95% CI 0.73–0.90) for proportion asymptomatic (*8*). The key parameter was the mean duration of Zika virus viremia. We performed a sensitivity analysis to evaluate the influence of the specification of this parameter by computing estimates for the total number and percentage of Zika virus infections in the population for different values of Zika virus viremia duration, ranging from 7 to 21 days. Analyses were performed and graphics created in the R version 3.3.1 statistical software package (https://www.R-project.org/) by using purpose-written routines, and we used StatXact version Eleven (http://www.cytel.com) for Fisher exact test.

This study involved analyses of data collected as part of public health response activities. Therefore, the Office of the CDC Associate Director for Science considered it exempt from institutional review board review.

Results

Data on 21,643 blood donors from BSIS and ASEM were reported to CDC for April 3–August 12, 2016. Of these donors, 21,468 (17,850 from BSIS and 3,618 from ASEM)

were included in the analysis; 175 were excluded because of invalid data or residence outside of Puerto Rico. Included donors made 22,028 total blood donations during the study period. Of all included donors, 190 (153 BSIS and 37 ASEM) were PVDs; 21,278 had cobas Zika-nonreactive screening test results; 20,912 were first-time donors; and 14,407 (67%) were men (Table 1). Reported donor residence included all of the municipal health regions in Puerto Rico (Table 1). Among the 190 PVDs, 181 had reactive cobas Zika test results on their first donation, and 9 had nonreactive results at first donation but reactive results on repeat donation. Also among PVDs, 142 (75%) were men, 67 (35%) were 45–59 years of age, and 129 (68%) resided in either Metro/San Juan (44%) or Bayamón (24%) (Table 1). The overall rate of cobas Zika ID-NAT donor reactivity during the 5-month period was 89/10,000 donors.

Combining donation data from all health regions, we found no statistically significant difference in cobas Zika test reactivity by age group (p = 0.32), but the proportion of reactivity (number of reactive donations/number of donations) significantly differed by donor sex (women, 48 [0.67%] of 7,125; men, 142 [0.95%] of 14,903; risk ratio 1.41, 95% CI 1.02–1.96; p = 0.036) and by health region (p<0.001). By health region, the association between reactivity and sex was significant in only 1 (Ponce, in which all of the 14 reactive donations were from men).

Table 1. Characteristics of blood donors screened for Zika virus infection with cobas Zika ID-NAT at BSIS and ASEM, Puerto Rico, April 3–August 12, 2016*

Characteristic	Total donors screened, no. (%)	Presumptive viremic donors, no. (%)
Total	21,468 (100)	190 (100)
Sex		
M	14,407 (67.1)	142 (74.7)
F	7,061 (32.9)	48 (25.3)
Age at donation, y		
16–29	4,313 (20.1)	39 (20.5)
30–44	7,179 (33.4)	51 (26.8)
45–59	7,046 (32.8)	67 (35.3)
60–74	2,751 (12.8)	31 (16.3)
≥75	179 (0.8)	2 (1.1)
Month of donation		
April	4,339 (20.2)	14 (7.4)
May	4,891 (22.8)	33 (17.4)
June	5,602 (26.1)	67 (35.3)
July	4,773 (22.2)	56 (29.5)
August	1,863 (8.7)	20 (10.5)
Region of residence		
Aguadilla	751 (3.5)	9 (4.7)
Arecibo	1,883 (8.8)	15 (7.9)
Bayamón	4,797 (22.3)	45 (23.7)
Caguas	4,359 (20.3)	19 (10.0)
Fajardo	979 (4.6)	2 (1.1)
Mayagüez	917 (4.3)	2 (1.1)
Metro/San Juan	6,081 (28.3)	84 (44.2)
Ponce	1,701 (7.9)	14 (7.4)
Donation type		
First-time	20,912 (97.4)	168 (88.4)
Repeat	556 (2.6)	22 (11.6)

*Data for August 1–August 12, 2016 available only for BSIS. cobas Zika, Roche Molecular Systems, Inc., Pleasanton, CA, USA. ASEM, Banco de Sangre del Centro Médico de la Administración de Servicios Médicos; BSIS, Banco de Sangre de Servicios Mutuos; ID-NAT, individual nucleic acid testing.

Based on the 2014 US Census Puerto Rico population estimate of 3,639,000 residents and using a mean viremia duration of 9.9 days (SD ± 3.94 days) and stratifying by health region and sex, we estimated the number of incident Zika virus infections for April 3–August 12, 2016, to be 469,321 (95% CI 401,477–559,126). This number represents a Zika virus cumulative incidence of 12.9% (95% CI 11.0%–15.4%) for Puerto Rico for the 5-month period (Figures 1, 2). The estimated number of Zika virus infections for reproduction-aged women (16–44 years) was 69,675 (95% CI 48,226–117,578), which represents 9.7% (95% CI 6.7%–16.3%) of the total population of women of reproduction age in Puerto Rico.

Estimates of the total number and percentage of the population infected with Zika virus during the study period are given using mean viremia durations of 7–21 days (Table 2, Figure 3) Estimates for percentage of the population infected with Zika virus declined with increasing viremia duration, ranging from 16.1% for 7 days viremia duration to 5.9% for 21 days. The incidence estimate would be lower if we had used an estimated mean viremia duration of >9.9 days in our calculations (Figure 3).

Discussion

In this analysis of routine blood donation screening data from the 2 largest blood collection centers in Puerto Rico, we estimated that 469,321 persons were infected with Zika virus during April–August 2016, assuming a mean viremia duration of 9.9 days. The estimated cumulative incidence of Zika virus infection for the study period was 12.9%.

Among the parameters used in this estimation, mean duration of Zika virus viremia is most influential because it is inversely related to the overall estimate of the number of persons infected with Zika virus in Puerto Rico. To our knowledge, the mean duration of viremia in serum is still unknown but has been shown to range from 4–10 weeks in gravid women (*20*) to 3–18 days in asymptomatic, nonpregnant persons (*19*). We used the value of 9.9 days (95% CI 6.8–21.6 days) on the basis of a literature review of 25 cases that provided doubly interval-censored data (*19*). The wide 95% CI for the mean viremia duration estimates reflected the current paucity of data on viremia duration. To evaluate the influence of this key parameter in our analyses, we included a sensitivity analysis by varying the assumed mean viremia duration and computing corresponding incidence estimates of Zika virus infection.

Using the mean viremia duration of 9.9 days gave a substantially higher total number of incident Zika virus infections than the number of new laboratory-confirmed infections reported from Puerto Rico to ArboNET during

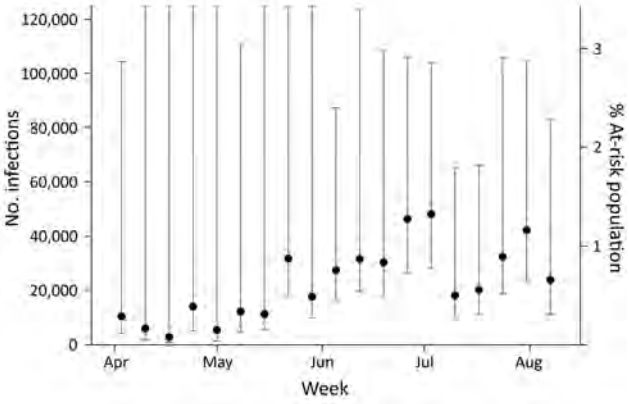

Figure 1. Individual weekly estimates of the number and percentage of at-risk population with incident Zika virus infections computed with cobas Zika (Roche Molecular Systems, Inc., Pleasanton, CA, USA) individual nucleic acid testing results from Banco de Sangre de Servicios Mutuos and Banco de Sangre del Centro Médico de la Administración de Servicios Médicos, Puerto Rico, April 3–August 12, 2016. These estimates assume a mean viremia duration of 9.9 days (SD ± 3.9). To retain readability of the point estimates, some of the confidence interval line segments extend beyond the vertical boundary. Data for August 1–August 12, 2016 available only for Banco de Sangre de Servicios Mutuos. Error bars indicate 95% CIs.

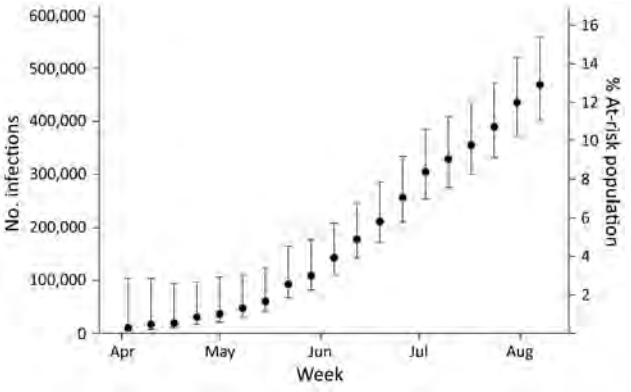

Figure 2. Cumulative weekly estimates of the number and percentage of at-risk population with incident Zika virus infections computed with cobas Zika (Roche Molecular Systems, Inc., Pleasanton, CA, USA) individual nucleic acid testing results from Banco de Sangre de Servicios Mutuos and Banco de Sangre del Centro Médico de la Administración de Servicios Médicos, Puerto Rico, April 3–August 12, 2016. These estimates assume a mean viremia duration of 9.9 days (SD ± 3.9). Each weekly estimate is computed from all donation data collected from April 3 to the given week. Data for August 1–August 12, 2016 available only for Banco de Sangre de Servicios Mutuos. Error bars indicate 95% CIs.

the same period (≈10,000 infections) (*21*). However, because of limitations in general population testing, this system reflects only symptomatic persons and a subset of asymptomatic pregnant women. One advantage of using blood donor screening as a surveillance tool is that it can rapidly capture real-time, cumulative incidence data from a large, diverse convenience sample of the general population; this information might otherwise be unattainable during a public health emergency. As observed during previous outbreaks of arbovirus diseases (e.g., West Nile, dengue, chikungunya) in the continental United States and territories, blood donation screening conducted during outbreaks can identify persons who are acutely infected and asymptomatic, which can aid in active case surveillance and enable characterization of viral and immunologic dynamics of clinical illness (*15*,22,*23*). Detection of Zika virus–infected asymptomatic blood donors is important not only for preventing transfusion-transmitted infections but also because the infection can be sexually transmitted and might result in adverse birth outcomes, even among pregnant women who do not have signs or symptoms. As US blood centers implement updated FDA recommendations for universal Zika virus blood donation screening (*24*), the coupling of prompt communication of reactive blood donor screening results to public health authorities with appropriate prevention messages and other public health interventions will become increasingly important in helping to mitigate the spread of Zika virus.

The findings of this study are subject to several limitations. First, the number of persons residing in Puerto Rico (estimated at 3.4 million in 2016 by the Puerto Rico Department of Health) might differ from the 2014 US Census population estimate of 3.6 million in our model. Second, the demographic composition of blood donors, specifically sex and age, does not match that of the general population. Data from this study show that men represented >67% of blood donors. Furthermore, data from persons <16 years of age were unavailable because of blood donor age restrictions, so the estimates we give for the whole population include an extrapolation to this age group. Although the data do not indicate a substantial difference in Zika virus incidence by age, whether the lack of data from the 0–15-year age group substantially affected our population incidence estimates is unknown. Alternatively, with regard to sex and infectivity, few data are available to

Table 2. Sensitivity analysis for Zika virus infections, computed from BSIS and ASEM cobas Zika ID-NAT results, Puerto Rico, April 3–August 12, 2016*

Mean viremia duration, d	Total no. infections (95% CI)	Population infected, % (95% CI)
7	684,937 (585,924–816,000)	18.82 (16.1–22.43)
14	323,525 (276,756–385,431)	8.89 (7.61–10.59)
21	214,563 (183,546–255,619)	5.89 (5.04–7.02)

*Data for August 1–August 12, 2016 available only for BSIS. cobas Zika, Roche Molecular Systems, Inc., Pleasanton, CA, USA. ASEM, Banco de Sangre del Centro Médico de la Administración de Servicios Médicos; BSIS, Banco de Sangre de Servicios Mutuos; ID-NAT, individual nucleic acid testing.

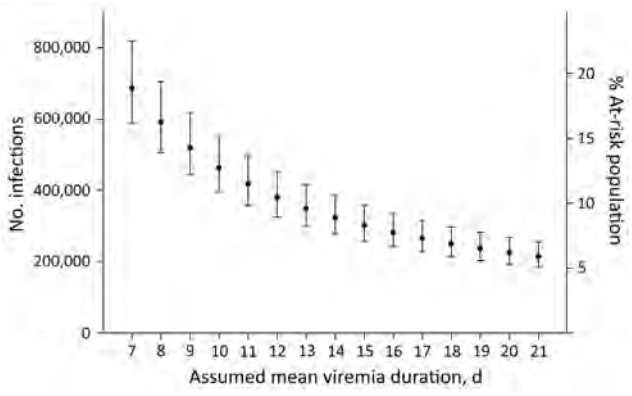

Figure 3. Estimated total number of incident Zika virus infections and percentage of the at-risk population infected with Zika virus during the study period by assumed mean viremia duration computed with cobas Zika (Roche Molecular Systems, Inc., Pleasanton, CA, USA) individual nucleic acid testing results from Banco de Sangre de Servicios Mutuos and Banco de Sangre del Centro Médico de la Administración de Servicios Médicos, Puerto Rico, April 3–August 12, 2016. Data for August 1–August 12, 2016 available only for Banco de Sangre de Servicios Mutuos. Error bars indicate 95% CIs.

support a predisposition for Zika virus infection in men; nevertheless, the statistically significant study finding of a male-to-female ratio of infectivity of 1.41 among donors suggests the need for further exploration of any possible interplay between sex and the length of viremia from Zika virus infection or Zika virus susceptibility. Third, blood donors are subjected to a medical examination and questionnaire to ascertain signs and symptoms of illness, and potential donors who are feeling ill are excluded from donation. Consequently, blood donor screening data might underestimate infection incidence because of the exclusion of symptomatic persons. Because our model adjusted for the exclusion of these persons, this limitation should not affect our analysis; however, this factor is an important consideration when blood screening data are used as a surveillance tool. Last, the duration of Zika virus viremia is unknown, and assumptions made for this model were based on limited data. Important research priorities will be to determine viremia duration through longitudinal follow-up of infected blood donors and studies of acute infection in animal models, resulting in more precise calculation of viral kinetics.

In summary, the findings of this study suggest that a much larger proportion of the population in Puerto Rico was infected with Zika virus during April–August 2016 than reported through surveillance. Although Puerto Rico mandates reporting of Zika virus infections, the conveyance of arboviral surveillance data across local, state, and national levels is often delayed and can affect strategic planning and interventions. Blood donation screening data can augment clinical Zika virus surveillance data to provide real-time communication of Zika virus incidence estimates to enable better ascertainment of the extent of outbreaks and improved targeting of prevention and response efforts.

Acknowledgments

We thank Sandra I. Santiago Montalvo, Edwin Velez, Jorge Pi, Ada M. Justiniano Soto, and Francisco Alvarado-Ramy for their support with data collection and preparation; Kalanthe Horiuchi for assistance with the population data; and the Biomedical Advanced Research and Development Authority for funding the blood donor screening at BSIS and ASEM.

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Invasive Nontuberculous Mycobacterial Infections among Cardiothoracic Surgical Patients Exposed to Heater–Cooler Devices[1]

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Invasive nontuberculous mycobacteria (NTM) infections may result from a previously unrecognized source of transmission, heater–cooler devices (HCDs) used during cardiac surgery. In July 2015, the Pennsylvania Department of Health notified the Centers for Disease Control and Prevention (CDC) about a cluster of NTM infections among cardiothoracic surgical patients at 1 hospital. We conducted a case–control study to identify exposures causing infection, examining 11 case-patients and 48 control-patients. Eight (73%) case-patients had a clinical specimen identified as *Mycobacterium avium* complex (MAC). HCD exposure was associated with increased odds of invasive NTM infection; laboratory testing identified patient isolates and HCD samples as closely related strains of *M. chimaera*, a MAC species. This investigation confirmed a large US outbreak of invasive MAC infections in a previously unaffected patient population and suggested transmission occurred by aerosolization from HCDs. Recommendations have been issued for enhanced surveillance to identify potential infections associated with HCDs and measures to mitigate transmission risk.

Nontuberculous mycobacteria (NTM) typically cause infection in patients who are immunocompromised or have chronic lung disease (*1*–*5*) but have also caused healthcare-associated infections related to water sources such as showers and ice machines (*6*–*8*). Outbreaks of NTM infections have occurred among patients undergoing cardiac surgery; these typically involve surgical site infections or infections associated with contaminated products, such as prosthetic implants and cardioplegia solutions (*6*,*7*,*9*). Pulmonary infections are the most common disease manifestation of NTM, but 10% of NTM infections are extrapulmonary (*2*). Disseminated infections are uncommon among immunocompetent patients (*10*–*15*) but are often serious and require treatment with a long, complicated regimen of antibiotic drugs (*2*).

During spring 2015, investigators in Switzerland reported an outbreak of invasive infections with *Mycobacterium chimaera*, a distinct species within the NTM category *M. avium* complex (MAC), associated with contaminated heater–cooler devices (HCDs) used during cardiopulmonary bypass for cardiac surgery (*16*). HCDs regulate the temperature of patient blood, cardioplegia solution, and warming/cooling blankets through a water circuit not intended to have contact with patients or their blood. Given this outbreak and similar outbreaks reported in other countries in Europe, European public health authorities have issued a warning regarding the risk for *M. chimaera* infections associated with HCDs (*17*).

In July 2015, a cluster of invasive NTM infections was identified among patients who underwent cardiothoracic surgery at Wellspan York Hospital in York, Pennsylvania, USA. The Pennsylvania Department of Health (PADOH) and the Centers for Disease Control and Prevention (CDC) conducted a field investigation to identify the extent of infections and determine associated risk factors and exposures to prevent further infections.

Methods

Setting

Wellspan York Hospital is a 585-bed community teaching hospital at which ≈650 cardiac surgeries are performed annually. Of these, ≈400 require cardiopulmonary bypass,

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DOI: http://dx.doi.org/10.3201/eid2305.161899

[1]Preliminary results from this study were presented at the Epidemic Intelligence Service conference, May 2–5, 2016, Atlanta, Georgia, USA; and the American Society for Microbiology Microbe conference, June 16–20, 2016, Boston, Massachusetts, USA.

which involves use of a HCD. Three operating rooms are used for cardiothoracic surgery.

Initial Case Finding

We searched a database of microbiology results to identify all NTM-positive blood, sputum, pleural fluid, and tissue specimens at this hospital during the previous 5.5 years (January 1, 2010, to July 1, 2015). We cross-referenced patients with an NTM-positive specimen with the hospital's surgical database to determine whether they underwent surgical procedures during an exposure period 30 days to 3.5 years preceding the NTM-positive specimen collection date. Surgical procedures occurring <30 days before an NTM-positive specimen was collected were excluded because of the likelihood that they were either diagnostic or therapeutic procedures for a suspected NTM infection (and therefore not responsible for NTM transmission). Surgical procedures occurring >3.5 years before an NTM-positive specimen was collected were excluded because available published reports suggested that most NTM infections were diagnosed within 3.5 years after cardiac surgery (*16*). To explore whether NTM infection rates differed by surgery category, we calculated the rate of NTM-positive patients (per 10,000 operations performed) for the 3 most common surgical categories (cardiothoracic, general surgery, or orthopedic) and compared these rates using the Fisher exact test.

Case–Control Study

We found that NTM-positive specimens occurred at a higher rate among cardiothoracic surgical patients than among patients in other major surgical categories. Given this finding and recent reports suggesting that HCDs are a potential risk factor for NTM infection, we conducted a case–control study to identify risk factors associated with invasive extrapulmonary NTM infections among patients who underwent cardiothoracic surgery at Wellspan York Hospital.

Case Definition

Inclusion criteria for case-patients were an extrapulmonary NTM-positive specimen collected during 2010–2015 and a cardiothoracic surgery during 2009–2014 occurring during the exposure period (30 days–3.5 years before collection of the NTM-positive specimen). We excluded patients with NTM-positive specimens collected before 2010 because acid-fast bacillus tissue cultures before 2010 were not included in the microbiology database; patients with a history of MAC infection (or a MAC-positive specimen) before cardiothoracic surgery, which suggests that their infection could not be temporally attributed to their cardiac surgery; patients whose cardiothoracic surgeries occurred before 2009, because surgical documentation in the electronic medical record at that time was less standardized and reliable; and patients with only NTM-positive pulmonary specimens, because patients with pulmonary infections have been shown to differ epidemiologically from patients with other types of NTM infections (*11*,*18*). All patients with NTM-positive specimens from an extrapulmonary sterile body site were included.

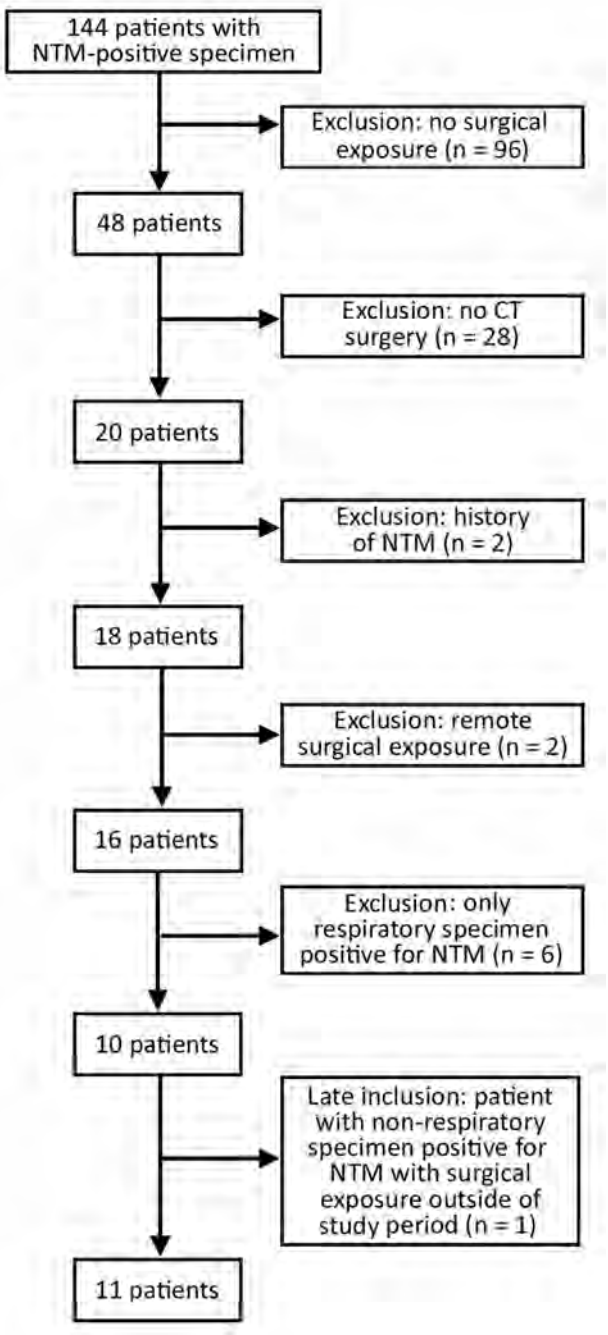

Figure 1. Inclusion criteria flowchart for case–control study of patients with NTM-positive specimens in investigation of invasive extrapulmonary NTM infections among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015. CT, cardiothoracic; NTM, nontuberculous mycobacteria.

Control Selection

We selected 48 unmatched controls at random from a list of all patients who underwent cardiothoracic surgery at this hospital during 2009–2014 and who had no history of MAC infection. Because controls did not have an NTM-positive specimen date to determine the surgical exposure period (30 days–3.5 years before the NTM-positive specimen collection date), we assigned an index date based on the median incubation period of all patients with NTM infection (397 days from cardiothoracic surgery to NTM-positive specimen) and used this date to determine a comparable exposure period.

Data Collection

We abstracted patient demographic characteristics, medical history or risk factors, outcomes, and NTM specimen information (for case-patients only) from patients' electronic medical records. We also collected perioperative and hospital exposures for every surgery that patients underwent during the exposure period. For the 1 control-patient who had 2 cardiothoracic surgical procedures that required a cardiopulmonary bypass machine to be operational in the room, we summed time of surgery and time connected to the bypass machine to reflect cumulative exposure for both operations.

Infection Control, Environmental, and Laboratory Assessment

We conducted interviews with healthcare personnel and directly observed operating room practices during cardiac surgery. We reviewed the facility's perioperative protocols and the HCD manufacturer's instructions for use. Before the field team's arrival, all 3 HCDs used for cardiothoracic surgery at Wellspan York Hospital had been removed from service and replaced with new ones. During the field investigation, we collected water samples from the decommissioned HCDs, the new HCDs introduced during the investigation, the nearest scrub sink, and 2 ice machines that supplied nonsterile ice to the operating rooms. We collected swab samples from the internal water reservoirs of the 3 HCDs. We disassembled 1 HCD to permit a more thorough inspection. Another HCD was operated in an empty cardiothoracic operating room during a simulation in which we collected water samples from the HCD and air samples from various locations within the operating room (18 inches from the HCD exhaust vent and next to the operating room exhaust vents located in the room corners) in 200-L and 500-L volumes using an impaction air sampler (SAS 90; Bioscience International, Rockville, MD, USA) before starting the HCD and then intermittently over 5 hours after starting the HCD.

Three case-patient isolates (2 from blood and 1 from bone marrow) were available for further characterization. Patient isolates and environmental samples were sent to CDC for testing, including culture isolation, acid-fast bacillus staining, identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and 16S and rpoB sequencing, as well as molecular typing by pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing.

Statistical Analyses

We compared patient demographic and clinical characteristics between case-patients and control-patients using the Fisher exact test for categorical variables and 2-sample *t* test and Wilcoxon 2-sample test for continuous variables. To assess the association between case status and surgical exposures, we initially conducted univariable logistic regression with Firth's penalized maximum likelihood method that accounts for small sample size, issues of data separability, and bias of the parameter estimates, and obtained crude odds ratios (ORs), 95% CIs, and p values. Several surgical exposure variables associated with increased odds of case status were further tested for association with exposure to cardiopulmonary bypass with HCD using the Fisher exact test. Because undergoing a surgery requiring cardiopulmonary bypass with HCD was associated with major cardiothoracic surgery ($p<0.0001$), presence of a central line ($p<0.0001$), and implantation of an artificial valve or graft ($p<0.0001$), we did not include these variables in the multivariable regression analysis that examines the association between case status and length of HCD exposure.

To examine the association between case status and length of HCD exposure, we used 2 different, but related, exposure length variables: surgical time with HCD (time a patient is in the operating room while an HCD is operated) and time on cardiopulmonary bypass (time when a patient's blood is routed through the bypass machine). We conducted multivariable logistic regression with Firth's penalized maximum likelihood method to evaluate such relationships and examined collinearity between factors considered in the multivariable models. Because of the notable correlation detected between surgical time with HCD and time on bypass (Pearson $\rho = 0.9$, variance decomposition proportion for surgical time with HCD = 0.92, and variance decomposition proportion for pump time = 0.88 at a given condition index of 10), we analyzed these 2 exposure-length variables in separate models. For each exposure-length variable, we began with a full saturated model that also included several potential patient factors, all of which were removed by using a backward elimination method owing to nonsignificance at an α of 0.05, except for immunocompromised status, which was retained because of the biological plausibility of this medical condition affecting the risk for invasive NTM infections. We used SAS statistical software version 9.3 (SAS Institute, Inc., Cary, NC, USA) for all analyses.

Table 1. Demographic and clinical details of case-patients with invasive extrapulmonary NTM infections following procedures using an HCD, York, Pennsylvania, USA, 2015*

Patient no.	Age, y	Infection latency, y†	Year of first NTM specimen collection	Infection type	Location of NTM specimens	NTM organism	Bypass (year of surgery)	Time on bypass, h	Death‡
1	80–90	2.3	2010	Thoracic	Pleural fluid	*M. kansasii*	Yes (2008)	2–3	Yes
2	70–80	0.1	2010	Thoracic	Pleural fluid	MAC	Yes (2010)	3–4	Yes
3	40–50	1.7	2011	Extrathoracic	Blood, tissue from port site	*M. mucogenicum/ M. fortiutum*	No	NA	Yes
4	60–70	0.7	2012	Extrathoracic	Bone marrow, spleen	*M. chimaera*	Yes (2012)	2–3	Yes
5	70–80	2.0	2012	Extrathoracic	Liver, bone marrow	*M. chimaera*	Yes (2010)	2–3	No
6	70–80	0.5	2012	Extrathoracic	Blood, bone marrow	MAC	Yes (2012)	2–3	Yes
7	60–70	0.8	2014	Thoracic	Deep sternal wound§	*Mycobacterium* species	No	NA	No
8	60–70	1.9	2014	Thoracic	Deep sternal wound§	MAC	Yes (2012)	<1	No
9	80–90	1.4	2014	Extrathoracic	Psoas abscess¶	MAC	Yes (2013)	1–2	No
10	60–70	0.5	2015	Extrathoracic	Bone marrow	*M. chimaera*	Yes (2014)	2–3	Yes
11	60–70	1.4	2015	Thoracic	Pleural fluid	MAC	Yes (2013)	2–3	Yes

*HCD, heater–cooler device; MAC, *Mycobacterium avium* complex; NA, not available; NTM, nontuberculous mycobacteria.
†Length of time in years between first NTM-positive specimen and most invasive cardiothoracic surgery
‡Death was not necessarily attributable to NTM infection.
§Communicating with pleural space.
¶With radiographic findings of adjacent vertebral osteomyelitis.

Results

Initial Case Finding

Among 144 patients with an NTM-positive specimen collected during January 1, 2010, to July 1, 2015, 48 (33%) underwent ≥1 surgery during the exposure period. The rate of NTM-positive specimens was noticeably higher among cardiothoracic surgical patients (20 patients/10,000 surgeries) than the rates among patients in other common surgical categories, including general surgery (8 patients/10,000 surgeries; p = 0.04) and orthopedic surgery (5 patients/10,000 surgeries; p = 0.004). Approximately 2,276 surgical procedures with HCDs were performed during this period.

Of the 20 NTM-positive patients who had ≥1 prior cardiothoracic surgery, we excluded 10 patients based on the case definition (Figure 1), which left 10 patients with invasive extrapulmonary infections, all of whom demonstrated clinical signs of infection at the time of specimen collection. One of the patients who was excluded because of a previous cardiothoracic surgery before 2009, when surgical documentation in the electronic medical record was less standardized and reliable, was later included in

Table 2. Descriptive characteristics of case-patients with invasive extrapulmonary NTM infections and control-patients among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015*

Characteristic	Case-patients, n = 11	Control-patients, n = 48	p value†
Demographics			
Age, y, mean (range)	69 (47–84)	64 (20–84)	0.33
Male sex	9 (82)	27 (56)	0.17
White race	11 (100)	45 (94)	1.00
Predisposing medical condition			
Chronic lung disease	4 (36)	8 (17)	0.21
COPD	1 (9)	8 (17)	1.00
Sarcoidosis	3 (27)	0	0.005
Diabetes	1 (9)	15 (31)	0.26
Immunocompromised	3 (27)	10 (21)	0.69
HIV	0	0	NA
Transplant	0	0	NA
Chemotherapy	2 (18)	9 (19)	1.00
Steroid treatment	0	2 (4)	1.00
Hematologic malignancy‡	2 (18)	1 (2)	0.09

*Values are no. (%) patients except as indicated. COPD, chronic obstructive pulmonary disease; NA, not applicable; NTM, nontuberculous mycobacteria.
†Fisher exact test for categorical variables or two 2-sample *t* tests with pooled variance.
‡Among cases, hematologic malignancy included chronic lymphocytic leukemia and myelodysplastic syndrome. Among controls, hematologic malignancy included Hodgkin's lymphoma.

Table 3. Surgical exposures of case-patients with invasive extrapulmonary NTM infections and control-patients among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015*

Exposures	Case-patients, n = 11	Control-patients, n = 48	Odds ratio (95% CI)	p value†
No. surgeries per patient				
All surgeries, median (IQR)	1 (1–4)	2 (1–3)		0.90
Cardiothoracic surgeries, median (IQR)	1 (1–3)	1 (1–2)		0.86
Type of procedure‡				
Major cardiothoracic procedure	11 (100)	31 (65)	12.8 (1.5–>999.9)	0.09
Major cardiac procedure	9 (82)	21 (44)	4.9 (1.2–27.5)	0.04
Coronary artery bypass grafting surgery	6 (55)	17 (35)	2.1 (0.6–7.9)	0.25
Cardiac valve surgery	4 (436)	8 (67)	2.9 (0.7–11.4)	0.15
Aortic surgery	5 (45)	0 (0)	82.1 (7.9–>999.9)	0.008
Pericardial window	0 (0)	2 (4)	0.8 (0.01–10.9)	0.91
Major thoracic procedure	2 (18)	11 (23)	0.9 (0.1–3.6)	0.85
Perioperative exposures§				
Central line¶	10 (91)	27 (56)	5.5 (1.1–53.5)	0.07
Chest tube	10 (91)	31 (65)	3.9 (0.8–38.2)	0.15
Shower during hospitalization	7 (64)	20 (42)	2.3 (0.7–9.2)	0.21
Artificial valve or graft	8 (73)	9 (19)	10.1 (2.6–48.1)	0.002
Topical medication	10 (91)	37 (77)	2.1 (0.4–21.4)	0.43
Topical antibiotic	10 (91)	31 (65)	3.9 (0.8–38.2)	0.15
Topical anticoagulant	5 (45)	15 (31)	1.8 (0.5–6.7)	0.37
Use of cardiopulmonary bypass with HCD exposure	9 (82)	20 (42)	5.3 (1.3–29.9)	0.03
Length of HCD-related exposures#				
Surgical time with HCD, min, median (IQR)	328 (164–360)	0 (0–222)		0.003
Time on bypass, min, median (IQR)	147 (46–175)	0 (0–74)		0.002

*Any surgery that occurred 30 d to 3.5 y before first NTM-positive specimen for cases or index date for controls. Values are no. (%) patients except as indicated. NTM, nontuberculous mycobacteria.
†Fisher exact test for categorical variables or Wilcoxon 2-sample test for continuous variables.
‡Major cardiac surgery: coronary artery bypass grafting, valve replacement, aortic graft, pericardial window; major thoracic surgery: lung removal, thoracotomy, esophagectomy.
§Exposure was considered present if any of the surgeries (≥1) had the exposure.
¶Present at any time during hospital stay. Did not include chronic indwelling lines, such as PICC lines or dialysis catheters.
#If a patient had multiple cardiothoracic surgeries, the time for the longest operative time was included.

the analysis to increase the number of cases, given the low sample size. Demographic and clinical details of these 11 case-patients are shown in Table 1. Most case-patients (8, 73%) had specimens positive for MAC. Five (45%) case-patients were considered to have a thoracic infection, with only specimens from sterile thoracic sites testing positive for NTM. The remaining 6 (55%) case-patients had extrathoracic infections with NTM-positive specimens obtained from sterile body sites outside the thoracic cavity, which likely represented disseminated infections. A large proportion (63%) of patients died, although the cause of death was not necessarily attributable to NTM infection. Of the 11 patients who met the case criteria, 0–3 cases occurred each year during 2010–2015. The median infection latency (length of time between patients' most invasive cardiothoracic surgery and NTM-positive specimen collection date) was 1.2 years (range 0.1–2.3 years).

Case–Control Study

Case-patients and control-patients did not differ noticeably on demographics (age, gender, or race) or predisposing medical conditions (chronic lung disease, diabetes, or immunocompromised state) (Table 2). However, sarcoidosis was more likely to have been diagnosed in case-patients than in control-patients (27% versus 0%; p = 0.005).

Overall, the number of surgical exposures, and specifically cardiothoracic surgical exposures, was similar among case-patients and control-patients (Table 3). Case-patients had 1–3 cardiothoracic surgeries during the exposure period, but none had >1 cardiothoracic surgery requiring cardiopulmonary bypass. Case-patients had greater odds of major cardiothoracic surgery compared with control-patients; all case-patients had undergone either major cardiac surgery or major thoracic surgery. Having major cardiac surgery was associated with increased odds of invasive NTM infection (OR 4.9, 95% CI 1.2–27.5; p = 0.04). Specifically, having aortic surgery increased the odds of being a case-patient 82-fold (95% CI 3.2–>999.9; p = 0.008). Whereas 45% of case-patients had aortic surgery, none of the control-patients had undergone this procedure, likely reflecting its rarity (n = 154, 1.6% of cardiothoracic surgeries). Nine (82%) case-patients had cardiothoracic surgery requiring cardiopulmonary bypass, and most (8, 89%) also had a specimen positive for MAC (Table 1). Having a central line placed during admission (OR 5.5, 95% CI 1.1–53.5; p = 0.07) and having an artificial valve or graft implanted during cardiothoracic surgery (OR 10.1, 95% CI 2.6–48.1; p = 0.002) were both associated with higher odds of invasive NTM infection. Use of cardiopulmonary bypass, which results in exposure to an operating HCD, was

Table 4. Results from logistic regression models to evaluate exposure length variables in investigation of invasive extrapulmonary NTM infections among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015*

Regression models	Odds ratio (95% CI)	p value
Univariable models†		
Patient characteristics		
Age, per 1-y increase	1.0 (1.0–1.1)	0.4
Male sex	3.0 (0.7–16.8)	0.2
White race	1.8 (0.2–244.3)	0.7
Chronic lung disease: yes	2.9 (0.7–11.4)	0.1
Diabetes: yes	0.3 (0.03–1.5)	0.2
Immunocompromised: yes	1.5 (0.3–6.0)	0.6
Length of surgical exposure		
Surgical time with HCD, h		
0	Referent	
>0 to <4	1.8 (0.2–15.4)	0.6
≥4 to <5	2.6 (0.2–23.6)	0.4
≥5	15.6 (3.2–103.9)	0.002
Time on bypass, h		
0	Referent	
>0 to <2	1.7 (0.2–12.2)	0.6
≥2	19.0 (3.7–133.0)	0.001
Final logistic model‡		
Model 1.1, only surgical time with HCD retained in the final model:		
Surgical time with HCD >5 h	13.2 (3.2–62.9)	0.0008
Model 1.2, includes surgical time with HCD and immunocompromised status		
Surgical time with HCD >5h	13.6 (3.3–68.8)	0.001
Immunocompromised: yes	2.2 (0.4–12.2)	0.4
Model 2.1, only time on bypass retained in the final model:		
Time on bypass >2 h	16.5 (3.8–84.0)	0.0004
Model 2.2, includes time on bypass and immunocompromised status		
Time on bypass >2h	16.6 (3.8–88.4)	0.0006
Immunocompromised: yes	2.1 (0.3–12.0)	0.4

*HCD, heater–cooler device; NTM, nontuberculous mycobacteria.
†Regression models for factors considered in the multivariable model; each regression model used Firth's penalized maximum likelihood method.
‡Each final regression model used Firth's penalized maximum likelihood method; other variables (sex, age, immunocompromised status, chronic lung disease) were removed because of nonsignificance.

also associated with significantly higher odds of invasive NTM infection (OR 5.3, 95% CI 1.3–29.2; p = 0.03). The use of cardiopulmonary bypass with an HCD was correlated with undergoing major cardiothoracic surgery (p<0.0001), placement of a central line (p<0.0001), and implantation of an artificial vale or graft (p<0.0001); these factors were not independently associated with case status. The mean surgical time when an HCD was required and mean time on bypass machine were both significantly longer for case-patients than for controls (surgical time, p = 0.003; time on bypass, p = 0.002).

No patient characteristics were significantly associated with increased odds of case status (Table 4). Odds of invasive NTM infection increased for progressively longer surgery times with HCD and longer time on bypass, although this reached statistical significance only for surgery time with HCD ≥5 hours and time on bypass ≥2 hours. Using the final logistic regression model in which surgery time with HCD was a dichotomous variable (models 1.1 and 1.2), the odds of NTM infection for surgical time with HCD ≥5 hours was 13.2 times and 13.6 times higher than for surgical time with HCD <5 hours, respectively, both without and with adjustment for immunocompromised status. Similarly, time on bypass for ≥2 hours (models 2.1 and 2.2) was associated with significantly higher odds of NTM infection both without (OR 16.5, 95% CI 3.8–84.0; p = 0.0004) and with (OR 16.6, 95% CI 3.8–88.4; p = 0.0006) adjustment for immunocompromised status.

Infection Control and Environmental Assessment

An infection control assessment focusing on perioperative practices did not identify any breaches related to operating room ventilation, water use, storage, operating practices, and patient care. The hospital had 3 HCDs, all LivaNova (formerly Sorin Group Deutschland GmBH, Munich, Germany) Stöckert Heater-Cooler 3T Systems (referred to as 3T HCDs); 1 was acquired in 2009 and 2 were acquired in 2012. The manufacturer revised its instructions for use in February 2015 and made additional revisions in a June 2015 field safety notice, including recommendations for more frequent and higher potency disinfection and for positioning the HCD so that its exhaust vent is directed away from the surgical field. Periodic updates to the manufacturer's disinfection recommendations may have resulted in inconsistencies between the hospital's HCD cleaning and disinfection practices before June 2015 and the manufacturer's recommendations at that time. Before the onsite investigation, sterilized water had been used in HCDs at the hospital,

Figure 2. Biofilm visualized on surfaces submerged in an internal water reservoir of a heater–cooler device during investigation of invasive extrapulmonary nontuberculous mycobacteria infections among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015.

but all 3 HCDs were replaced and appropriate changes made to ensure compliance with the most recent manufacturer's operating instructions. When a decommissioned HCD was dissembled for further inspection, biofilm was visible on tubing and surfaces submerged in an internal water reservoir (Figure 2).

Laboratory Assessment

Water samples from all 3 decommissioned HCDs and swab specimens of the biofilm from the 1 disassembled HCD tested positive for *M. chimaera* (Table 5). Water samples from a scrub sink near the cardiothoracic operating rooms and ice machines used for nonsterile purposes in the operating room tested positive for rapid-growing NTM species but not *M. chimaera*. Culture results from water samples collected from the new HCD before installation were also negative. However, the concentrations of non-NTM bacteria detected in HCD water samples were higher after operating the device than before (150,000 vs. 116 colony-forming units/mL).

Air samples collected 18 inches from the HCD exhaust vent during the operating room simulations were found to be positive for *M. chimaera* after 2, 3, and 4 hours of HCD operation (Table 5). All remaining air samples, including those collected before starting the HCD, were negative for all NTM.

All 3 available case-patient isolates were identified as *M. chimaera*; these and the environmental *M. chimaera* isolates (obtained from air and HCD samples) were found to be highly related by PFGE (Table 5; Figure 3). Subsequent whole-genome sequencing results confirmed the PFGE analysis; *M. chimaera* sequences from clinical isolates, the HCDs, and air samples were highly related (*19*).

Discussion

This investigation confirmed a prolonged outbreak of invasive MAC infections associated with cardiac surgery requiring cardiopulmonary bypass with exposure to 3T HCDs, similar to reports from Europe (*16*). The infection rate was low (8 cases/2,276 surgeries with HCDs) among those who were exposed, making recognition of this outbreak difficult. We describe a case–control study in which 8 case-patients likely had HCD-related *M. chimaera* and MAC infections. Laboratory testing suggested a common source of *M. chimaera* transmission from the HCDs through aerosolization, which is consistent with studies demonstrating NTM's high propensity for aerosolization (*20*).

Our investigation had several limitations. Presentation with nonspecific symptoms and the low clinical suspicion by providers make diagnosis of invasive NTM infections difficult in this patient population. Challenges in diagnosis and clinical follow-up may have resulted in decreased sensitivity of case detection and misclassification bias.

Table 5. Microbiologic test results for case-patient isolates and environmental samples in investigation of invasive extrapulmonary NTM infections among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015*

Description	Sample type	Species identified
Patient isolates		
Case-patient 3	Blood	*M. chimaera*
Case-patient 4	Blood	*M. chimaera*
Case-patient 9	Blood	*M. chimaera*
Environmental samples		
HCD 1	Water	*M. chimaera*
HCD 2	Water/swab specimen	*M. chimaera*
HCD 3	Water	*M. chimaera*
Ice machine	Ice	*M. mucogenicum/M. phocaicum; M. gastri*
Scrub sink	Water	*M. avium*
Air sampling of HCD exhaust	Air	*M. chimaera*

*HCD, heater–cooler device; NTM, nontuberculous mycobacteria.

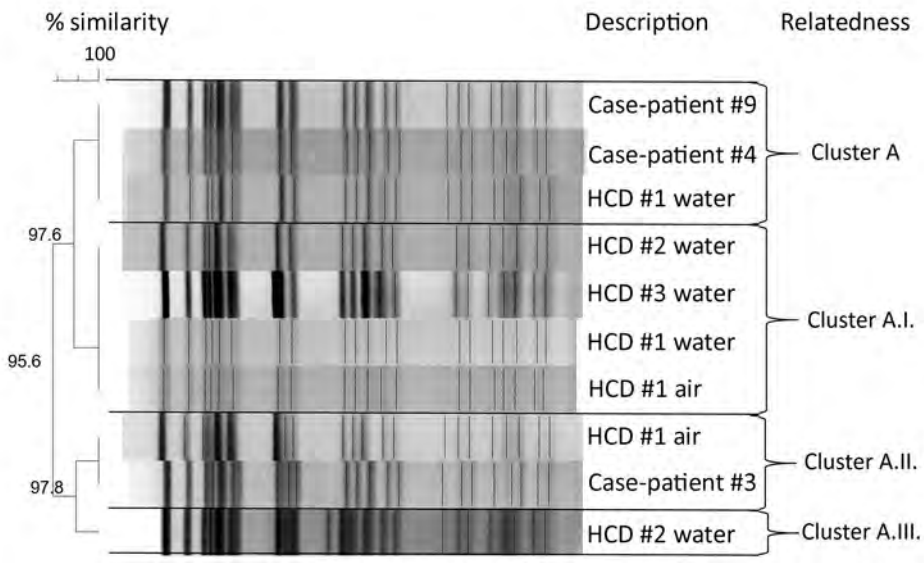

Figure 3. Molecular strain typing by pulsed-field gel electrophoresis of case-patients and environmental isolates of *Mycobacterium chimaera* in investigation of invasive extrapulmonary nontuberculous mycobacteria infections among patients who underwent cardiothoracic surgery, York, Pennsylvania, USA, 2015. HCD, heater–cooler device.

Misclassification of cases as controls is possible; patients may have received follow-up care at a different healthcare facility from the facility where surgery was performed. Because of the retrospective nature of this study and the prolonged period of the outbreak, it was not practical to obtain all potential health records from any healthcare facility at which patients may have been treated. Many clinical isolates from case-patients were unavailable, preventing further species identification of MAC specimens as *M. chimaera*. We used a maximum exposure window of ≈3.5 years for our analysis based on published reports available during the time of the investigation (*16*), but subsequent reports have suggested that the time between exposure and diagnosis can be as long as 6 years (*21*). However, preliminary review did not identify any additional patients with exposures >3.5 years before specimen collection who would have qualified for inclusion in this analysis. Our statistical analysis was limited by the small sample size and high correlation between various surgical exposures. Laboratory isolation of certain NTMs is hindered by NTM's slow growth. Additionally, many clinical laboratories are unable to perform species-level identification of MAC to *M. chimaera* and would not be able to identify a cluster of infections caused by this organism.

Based on this investigation, CDC and the PADOH made several recommendations to the hospital to enhance detection and surveillance of HCD-related NTM infections and to mitigate risk during future cardiac surgeries. These recommendations included increasing awareness among patients and providers to facilitate earlier diagnosis and treatment. Wellspan York Hospital notified 1,300 cardiac surgery patients and their providers of the potential exposure and established an NTM clinic to evaluate and monitor these patients. Subsequently, an additional 4 patients with likely HCD-associated infections were identified. CDC and the PADOH also issued recommendations to mitigate the risk for HCD-related NTM infections prospectively by ensuring compliance with the manufacturer's cleaning and disinfection instructions, positioning the HCD to minimize aerosolized particles from reaching patients, and using filter-sterilized water to decrease HCD contamination.

PADOH has been proactive in raising awareness of MAC infections related to the 3T HCDs, publicly reporting and investigating these initial cases and raising awareness of the issue among healthcare facilities and clinicians in Pennsylvania by issuing a statewide health advisory (*22*). Since October 2015, the Food and Drug Administration (FDA) and CDC have issued multiple nationwide communications to raise awareness, improve identification of contaminated HCDs and HCD-related infections, encourage notification of potentially exposed patients, and mitigate risk (*23–29*), including broad outreach to professional societies of providers caring for this patient population. CDC and FDA have continued to receive reports of NTM-contaminated devices and related infections with *M. chimaera*, and an FDA advisory panel was convened (*30*). Recent evidence suggests that contamination likely occurred during the manufacturing of the 3T HCDs; nearly identical strains of *M. chimaera* have been detected among samples from infected patients, HCDs from 3 different countries, and the 3T manufacturing plant (*31*). Whole-genome sequencing analysis of clinical and 3T HCD *M. chimaera* isolates from geographically distinct areas of the United States have demonstrated closely related strains, also suggesting point-source contamination of the 3T HCDs (*19*).

Additional areas for research include the effectiveness of disinfection practices given NTM's propensity to form biofilm, as well as device design issues to decrease

NTM growth and aerosolization (*30*). Hospitals and public health services should continue to raise provider and patient awareness about the risks of the 3T HCD (*25*). Short-term solutions to minimize risk, such as passing device exhaust through a HEPA filter or moving the device outside the operating room, may affect the design and functionality of the HCD and require careful examination (*28*). In addition, because clusters of extrapulmonary NTM infections may be a frequently underrecognized sentinel of medical device or environmental contamination that decreases the safety of surgical procedures, reporting of such infections to public health is a key patient safety measure.

In conclusion, our investigation confirmed an outbreak of MAC infections in which undergoing cardiac surgery requiring cardiopulmonary bypass with an HCD was associated with increased odds of infection, even in immunocompetent patients. Environmental sampling results suggest that airborne transmission occurred through aerosolization and dispersal of MAC while an HCD was operational. These findings highlight the need for increasing awareness of invasive NTM infection risk among cardiac surgery patients exposed to 3T HCDs; identifying best practices for notifying, evaluating, and managing potentially infected patients; and identifying options for mitigating infection risk from these devices.

Acknowledgments

We acknowledge the persons at Wellspan York Hospital who participated in the investigation, including Marlyse Charney and Amy Cook. We also acknowledge the persons in CDC's Clinical and Environmental Microbiology Branch of the Division of Healthcare Quality Promotion who conducted laboratory testing of specimens; these include Tom de Man, Terri Forster, Alison Laufer Halpin, Hollis Houston, Bette Jensen, Adrian Lawsin, James Matheson, Allison Perry, Nadege Toney, and Rolieria West-Deadwyler.

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Anthrax Cases Associated with Animal-Hair Shaving Brushes

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During the First World War, anthrax cases in the United States and England increased greatly and seemed to be associated with use of new shaving brushes. Further investigation revealed that the source material and origin of shaving brushes had changed during the war. Cheap brushes of imported horsehair were being made to look like the preferred badger-hair brushes. Unfortunately, some of these brushes were not effectively disinfected and brought with them a nasty stowaway: *Bacillus anthracis*. A review of outbreak summaries, surveillance data, and case reports indicated that these cases originated from the use of ineffectively disinfected animal-hair shaving brushes. This historical information is relevant to current public health practice because renewed interest in vintage and animal-hair shaving brushes has been seen in popular culture. This information should help healthcare providers and public health officials answer questions on this topic.

"Hopefully someone gave you a badger hair brush during the holidays…" begins a modern-era advertisement for a purveyor of high-end shaving supplies. Since the turn of the 21st century, there has been a resurgence of interest in luxury-brand, animal-hair shaving brushes, evocative of an idyllic premodern esthetic. In the spring of 2017, a Google search for "badger shaving brush shopping" produced ≈1.8 million hits; the same search limiting results through 2000 produced only ≈100 hits. But this luxury comes with a footnote, regarding an era when the sale of improperly prepared animal-hair shaving brushes caused dozens of sometimes fatal cases of cutaneous anthrax.

In 1915, British military officials began investigating cutaneous anthrax appearing on the heads and necks of soldiers newly recruited to serve in the Great War (*1*). Although the outbreak was initially attributed to the "diabolical tactics of the enemy," officials soon realized that the source of the problem lay closer to home: low-cost shaving brushes that were being supplied to the troops (*2*). We describe this outbreak on the basis of 3 sources of published data—outbreak summaries, surveillance data from the United States, and descriptions of individual cases—and discuss current risk.

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DOI: http://dx.doi.org/10.3201/eid2305.161554

Our first data source was early outbreak summaries from Europe and the United States. During 1915–1924, numerous shaving brush–associated cases were reported from the United States and England; 149 cases occurred in members of the US military; 28, in the British military; 17, in American civilians; and 50, in British civilians (*3*,*4*).

Our second data source was anthrax surveillance from the United States (Figure). Nationwide surveillance data for 1919–1924 (although noted at the time to be incomplete) suggest that contaminated shaving brushes accounted for at least 10% of all anthrax cases (*5*,*6*). The situation was worse in New York City (NY, USA), where during this period up to half of anthrax cases were linked to shaving brushes (*10*).

Our third data source was our recent review of systemic anthrax cases that were published in the English-language literature during 1880–2013 (Figure) (*7*). A total of 43 cases with anthrax definitely or possibly associated with shaving brushes were individually described in case reports, case series, or line lists during 1917–1989. Of these 43 cases, 20 (47%) were "possibly associated" on the basis of circumstantial evidence of usage of a recently purchased shaving brush and the absence of alternative exposure sources; the remainder were "definitely associated." Of the individually described cases, 37% died; 85% of the survivors and 56% of those who died had been given antiserum. Most (84%) of the case-patients were from the United States. Although most shaving-associated anthrax cases occurred during 1917–1923, another 2 cases occurred well outside the outbreak period. The first was a cutaneous case in a patient from Trinidad in 1935, confirmed by both culture and guinea pig inoculation from a new goat-hair shaving brush (*8*). The second was a meningitis case in a patient from India in 1989, thought to be linked to a ritual shaving of the head the day before symptom onset (*9*). The age of the shaving brush was described for 25 of the 43 cases; 76% of the brushes were new and another 16% were ≤2 months old.

We are now able to explain the etiology and denouement of this mini epidemic. The First World War seems to have changed the demand for and the type, source, and treatment of hair used in shaving brushes (Figure). The use of chlorine gas in 1915 and mustard gas in 1917 led to the provision of 2 million "khaki kits" with safety razors to American troops in 1918 because it was believed that gas masks would be more effective on clean-shaven soldiers. Before the war, shaving brushes were generally

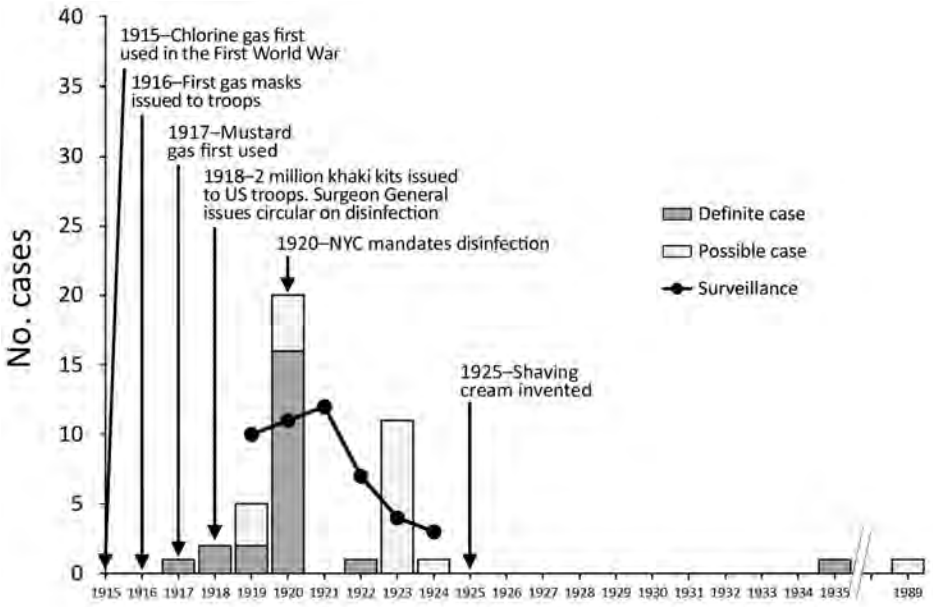

Figure. Timeline of use of shaving brushes and anthrax, 1915—1989. Case totals for the United States were reported in 1924 and 1930 and included 2 cases for 1927 through mid-1929, but the exact year of occurrence was unspecified (*5*,*6*). Data for English-language case descriptions were obtained from a systematic review of systemic anthrax cases published during 1880–2013 (*7*). Individual cases were reported from the United States, with the following exceptions: 1917, 1 definite case from England; 1918, 2 definite cases from Canada; 1920, 1 definite case from England; 1924, 1 possible case from South Africa; 1935, 1 definite case from Trinidad (*8*); and 1989, 1 possible case from India (*9*). NYC, New York City.

made from hair from badgers, horses, or boars, but badger-hair brushes were the most popular because of their ability to hold water. However, with the wartime disruption of commerce, badger hair from Russia—then its main exporter—became difficult to acquire. In response, imitation "badger" brushes made from horsehair from Russia, China, or Japan appeared in the United States. Before the war, bundles of hair used to make shaving brushes were cleaned and disinfected in France or Germany while en route to the United States. During the war, however, the bundles were shipped directly to the United States (*11*).

Anthrax risk during 1914–1917 seems to have varied by brush color and country of origin. Cases were more likely to be associated with light- than with dark-colored brushes, and brushes from horsehair from Japan were considered to be particularly risky. Public health officials investigating these outbreaks at the time speculated that at least some of these manufacturers used the hair as received, assuming it was already disinfected (*2*). They also speculated that high-temperature disinfection may have been avoided for brushes made from light-colored hair out of concern that this treatment might diminish their resemblance to badger hair. Thus, light-colored brushes may not have been as effectively disinfected as their dark-colored or darkly dyed counterparts (*12*).

At least in New York City, a "smoking brush" was easy to find. In 1921, Bellevue researchers described testing shaving brushes recently purchased from New York City street vendors; they were able to confirm *B. anthracis* by guinea pig inoculation for 8% and to culture "anthracoid" bacilli from another 78%. Given the high proportion of brushes that seemed to be contaminated, these reviewers concluded that the only reason there weren't more cases was "man's relatively high degree of immunity to anthrax" (*3*).

After health officials determined that inadequate disinfection of shaving brushes was the reason for the outbreak, they enacted a series of control measures. These included a 1918 Surgeon General report publicizing a method for disinfecting brush hair, followed by a slew of edicts in 1920 by the New York City Board of Health, which described a method for disinfection, required all brushes for grooming (shaving, tooth, hair, nail, or other brush for human use) to be disinfected by use of this method, mandated labeling with both the manufacturer's name and the word "sterilized," and restricted sales to "sterilized" brushes (*5*,*13*).

Today, anthrax is rarely seen in the United States or the United Kingdom or mentioned outside the realm of bioterrorism preparedness and response. However, anthrax remains a reportable medical condition, and a search of the ProMED outbreak monitoring Web service suggests the international scope of the problem; in 2015, at least 400 human anthrax cases were reported worldwide. In the outbreak we describe, most cases occurred in American military or civilians during 1919–1923 and were associated with new imitation "badger-hair" brushes of equine origin. The equine connection is not surprising; research has shown that herbivores, such as horses, are more susceptible to anthrax than omnivores, such as badgers and pigs (*14*), and contemporaneous information on anthrax in US livestock mentioned that horses were more frequently affected than pigs (*15*). It is possible that hair destined for shaving brushes originating from the new sources across the Pacific was harvested from horses that had died of anthrax and then bypassed the cleaning and disinfection steps that had been in place before the war.

Although the risk of acquiring anthrax from a shaving brush has been low since the mid-1920s, this article serves to remind those interested in a return to natural grooming that use of untreated hair from horses, pigs, badgers, or other animals poses a potential, and perhaps hypothetical, risk of inoculating anthrax spores into the abrasions and minor lacerations caused by shaving razors. Therefore, we emphasize the following points:

- Because of modern decontamination and import regulations (*16*,*17*), new animal-hair brushes are unlikely to be a source of anthrax.
- Risk from brushes manufactured in the United States after 1930 and well-used (even vintage) brushes would seem to be extremely low.
- We do not recommend trying to disinfect vintage brushes at home because the risks associated with various combinations of steam, pressure, and formaldehyde are likely to outweigh possible benefits.

This brief communication describes the history of anthrax and animal-hair shaving brushes. It should provide useful information for healthcare providers and public health officials answering questions on this topic.

Acknowledgment

We thank Stefan Katharios-Lanwermeyer for his unflagging effort in abstracting cases and cleaning the data for the original systematic review.

This study was supported by the Centers for Disease Control and Prevention.

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Increasing Macrolide and Fluoroquinolone Resistance in *Mycoplasma genitalium*

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Escalating resistance to azithromycin and moxifloxacin is being reported for *Mycoplasma genitalium* in the Asia-Pacific region. Analyzing 140 infections, we found pretreatment fluoroquinolone-resistance mutations in *parC* (13.6%) and *gyrA* (5%). ParC S83 changes were associated with moxifloxacin failure. Macrolide/fluoroquinolone-resistance mutations were in 8.6% of specimens, for which recommended therapies would be ineffective.

Mycoplasma genitalium infection is a major cause of urethritis in men and is associated with cervicitis, pelvic inflammatory disease, preterm birth, and spontaneous abortion in women (*1*). In the United States, Australia, and Europe, the recommended first-line treatment for *M. genitalium* infection is the macrolide azithromycin. However, a recent meta-analysis documented a rapid decline in its efficacy, from 85% before 2009 to 67% after 2009; the highest levels of resistance were in the Asia-Pacific region (*2*). The second-line therapy recommended by the US Centers for Disease Control and Prevention (https://www.cdc.gov/std/tg2015/default.htm) is the fluoroquinolone moxifloxacin. Quinolones target the DNA gyrase (comprising GyrA and GyrB) and topoisomerase IV (ParC and ParE). Quinolone binding involves serine at position 83 (*Escherichia coli* GyrA numbering) and the acidic amino acid 4 positions away (D87 or E87) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/16-1745-Techapp1.pdf) (*3*). Mutations affecting these residues or surrounding sequence (the quinolone resistance-determining region, QRDR) may confer resistance (*4*).

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DOI: http://dx.doi.org/10.3201/eid2305.161745

Moxifloxacin treatment failure is being increasingly reported, particularly in the Asia-Pacific region (*5*,*6*), along with increasing detection rates of resistance mutations (*7*). Although several studies have reported the prevalence of QRDR mutations in *M. genitalium*, most mutations have not been linked with treatment outcomes. Our aims with this study were to report the prevalence of mutations in the *parC* and *gyrA* genes in patients with *M. genitalium* infection, to correlate specific mutations with moxifloxacin outcomes, and to determine the prevalence of dual (macrolide/fluoroquinolone) resistance.

The Study

From July 1, 2012, through June 30, 2013, samples were collected from consecutive *M. genitalium*–infected participants at the Melbourne Sexual Health Centre in Australia (*5*). Detection of *M. genitalium*, load quantitation, and sequence analyses were performed as described previously (*5*,*8*). Overall, 155 patients (112 men, 43 women) with PCR-confirmed *M. genitalium* infection were recruited, representing 90% of patients with infections diagnosed at the Centre over the study period. We obtained adequate samples from 140 of the 155 patients to generate baseline *parC* and *gyrA* gene sequences; these 140 formed the study group.

Patients were initially given a single dose of 1 g azithromycin. The 54 for whom this treatment failed (positive by PCR test-of-cure at day 28 or persistent symptoms before day 28, with no identified reinfection risk) were given moxifloxacin (400 mg/d for 10 d). The 6 for whom moxifloxacin treatment failed were given pristinamycin (1 g 4×/d for 10 d). This study was approved by The Alfred Hospital Ethics Committee (no. 150/12), and informed consent was obtained from patients.

In pretreatment specimens, various single-nucleotide polymorphisms (SNPs) were observed in the *parC* and *gyrA* QRDR (Table 1; online Technical Appendix). Of the 19 (13.6%) of 140 samples with ParC substitutions, 16 had S83 mutations (14 S83I, 2 S83R) and 3 had D87N substitutions.

We found a significant association between detection of ParC S83 mutations and treatment failure. *M. genitalium* from all 6 patients for whom moxifloxacin failed but from only 3 of the 48 patients for whom moxifloxacin was effective had the ParC S83 mutation ($p<0.0001$ by Fisher exact test) (Table 2, patients 1–9). The 3 infections successfully treated despite a change at ParC S83 are of interest. For

Table 1. Pretreatment *gyrA* and *parC* SNPs detected according to moxifloxacin treatment from 140 samples collected from patients with *Mycoplasma genitalium* infection, Melbourne, Australia, July 2012 through June 2013*

Gene, SNP†	Amino acid change	Moxifloxacin failure, no. (%), n = 6‡	Moxifloxacin success, no. (%), n = 48	Not treated with moxifloxacin, no. (%), n = 86	Total prevalence, no. (%), n = 140
gyrA					
A229G§	K77E	–	–	1 (1.2)	1 (0.71)
G240A§	Silent (R80)	–	–	1 (1.2)	1 (0.71)
G285A	M95I	2 (33)¶	2 (4.2)¶	–	4 (2.9)
G295A§	D99N	1 (17)¶	–	–	1 (0.71)
G295T§	D99Y	1 (17)¶	–	–	1 (0.71)
A296G§	D99G	1 (17)	–	–	1 (0.71)
parC					
C184T§	P62S	–	–	13 (15)	13 (9.3)
C234T	Silent (H78)	–	6 (12.5)	10 (12)	17 (12)
A247C	S83R	2 (33)	–	–	2 (1.4)
G248T	S83I	4 (67)	3 (6.3)	7 (8.1)	14 (10)
G259A	D87N	–	1 (2.1)	2 (2.3)	3 (2.1)
T269A§	I90N	–	–	1 (1.2)	1 (0.71)
C324T§	Silent (N108)	–	–	1 (1.2)	1 (0.71)
No change	–	–	38 (79)	60 (70)	98 (68.5)

*SNP, single-nucleotide polymorphism; –, mutation absent.
†The *M. genitalium* G37 genome sequence (NC_000908) was the reference sequence.
‡All corresponding postmoxifloxacin treatment-failure samples contained the same SNP profiles. Multiple SNPs were detected in some samples.
§Sequence variations not previously described in *M. genitalium.*
¶A mutation was combined with an S83 change.

these patients, low bacterial load may have contributed to therapeutic success (*9*), led to spontaneous clearance, or resulted in false-negative follow-up PCR (Table 2). However, in contrast, treatment failed for 1 patient with a low anal load of *M. genitalium* and S83 change. Similar to the findings for this study, in the parent cohort of 155 patients, organism load influenced apparent azithromycin cure; 7% of infections carrying markers of azithromycin resistance were cured by azithromycin, and organism load was significantly lower than that among those with resistant infections for whom azithromycin treatment failed (*5*).

The prevalence of S83 changes in this study is higher than that detected in a study at Sydney Sexual Health Centre (Sydney, New South Wales, Australia) (8.4%, n = 143) (*10*). Studies in Japan reported prevalence ranging from 3.6% (n = 28) to 29.4% (n = 51) and 36.8% (n = 19) (*7*,*8*,*11*), although 1 study involved a cohort at higher risk (female sex workers). A low prevalence of S83 mutation has been observed in Europe (1.5% in France, 5% in England and Germany) (*12*–*14*). This mutation has been associated with moxifloxacin failure in 3/3 cases in the Sydney-based study (*6*).

Table 2. Samples containing changes in key amino acids of ParC and GyrA from 140 patients *with Mycoplasma genitalium* infection, Melbourne, Australia, July 2012 through June 2013*

Patient no./sex	Sample	Log_{10} load, GEQ†	Treatment (moxifloxacin) outcome	SNPs in *parC* A247C S83R	SNPs in *parC* G248T S83I	SNPs in *parC* G259A D87N	SNPs in *gyrA* G285A M95I	SNPs in *gyrA* G295A D99N	SNPs in *gyrA* G295T D99Y	SNPs in *gyrA* A296G D99G
1/F	Cervical swab	4.73	Failure	–	+	–	+	–	–	–
2/M	Urine	2.94	Failure	–	+	–	+	–	–	–
3/F	Anal swab	2.15	Failure	–	+	–	–	+	–	–
4/M	Urine	3.80	Failure	–	+	–	–	–	–	+
5/M	Urethral swab	5.10	Failure	+	–	–	–	–	+	–
6/M	Urine	4.66	Failure	+	–	–	–	–	–	–
7/F	Cervical swab	1.82	Success		+	–	+	–	–	–
8/M	Urine	2.47	Success	–	+	–	+	–	–	–
9/M	Urine	1.82	Success	–	+	–	–	–	–	–
10/M	Urine	4.07	Success	–	–	+	–	–	–	–
11/F	Urine	2.47	NT	–	+	–	–	–	–	–
12/M	Urine	3.46	NT	–	+	–	–	–	–	–
13/M	Urine	4.51	NT	–	+	–	–	–	–	–
14/M	Urine	3.51	NT	–	+	–	–	–	–	–
15/M	Urine	2.25	NT	–	+	–	–	–	–	–
16/M	Urine	1.11	NT	–	+	–	–	–	–	–
17/F	Urine	1.94	NT	–	+	–	–	–	–	–
18/M	Urine	2.50	NT	–	–	+	–	–	–	–
19/F	Cervical/swab	3.08	NT	–	–	+	–	–	–	–

*Dataset includes all failed treatments for moxifloxacin. GEQ, genome equivalent; NT, not treated with moxifloxacin because azithromycin treatment was successful; SNP, single-nucleotide polymorphism; +, sequencing of sample successful and SNP present; –, SNP absent.
†The log_{10} loads of *M. genitalium* were calculated per swab or 1 mL of urine. For all 155 patients in the parent cohort, loads varied from 0.84 to 6.17 (median 3.36).

SNPs that changed the ParC acidic residue (D87) were rare (2.1%) and because of low numbers could not be associated with treatment outcomes. Other studies found higher frequency of this change (3.5%–7.1%) (*7*,*8*,*10*,*15*); authors of 1 study reported an association with levofloxacin failure (*15*).

M. genitalium GyrA lacks the S83 residue common to GyrA of other bacteria, having instead a methionine at the equivalent position (M95). This enzyme is therefore probably partially resistant to quinolones. GyrA changes (at M95 or D99) occurred at a frequency of 5.0% (7/140) but could not be correlated with treatment outcome because they occurred concurrently with S83 changes in ParC. Previously, a GyrA M95I change was associated with *M. genitalium* treatment failure in 1 patient (*6*).

Patients who received moxifloxacin were followed up with a PCR test-of-cure at 14 and 28 days. For the 6 for whom treatment failed, the mutation profiles in follow-up specimens were unchanged from the initial premoxifloxacin sequence, suggesting lack of resistance selection in vivo after moxifloxacin.

A total of 60 (42.9%) of the 140 pretreatment samples had macrolide-resistance mutations (*5*). Both macrolide and *parC* fluoroquinolone mutations at S83 or D87 were present in 12 (8.6%) of the 140 samples. Prevalence of fluoroquinolone resistance markers was higher in samples with (20%, 12/60) than without (8.8%, 7/80) macrolide-resistance mutations, although this difference did not reach statistical significance ($p = 0.08$). This finding suggests that successive treatment failures with first-line, then second-line, antimicrobial drugs are generating strains resistant to 2 classes of drugs. Previous studies found lower levels of combined macrolide and fluoroquinolone mutations in men attending a urology clinic (3/51, 5.9%) (*7*) and higher levels in a high-risk population (female sex workers; 4/16, 25%) (*11*).

This study has limitations. The resistance profiles for the infecting strains of *M. genitalium* were not tested in in vitro culture. There may be other unknown changes in the genome that confer resistance to the drugs of interest. In addition, the resistance levels reported are probably underestimates because samples were collected in 2012–2013 and levels have probably risen since then (*7*).

Conclusions

We found high frequency of ParC S83 changes associated with fluoroquinolone resistance in a sexually transmitted infection clinic in urban Australia; these changes were associated with moxifloxacin failure. The high level of dual markers for macrolide/fluoroquinolone resistance suggests successive treatment failure after sequential monotherapy leading to the serious outcome that ≈10% of *M. genitalium* infections are not treatable with recommended or readily available antimicrobial drugs. In the absence of alternatives, treatment with pristinamycin cured all 6 patients with dual-class resistance infections (G.L. Murray et al., unpub data).

This study highlights the urgent need for antimicrobial drug resistance surveillance and the value of diagnostic assays that report the presence of resistance markers to optimize treatment. Our results suggest that it is time to reconsider the indications for azithromycin and invest in trials of different available as well as novel classes of antimicrobial drugs for *M. genitalium* treatment. They also raise serious concerns about sequential use of monotherapy and the need to evaluate combination therapies as we enter a new era of untreatable sexually transmitted infections.

This work was supported by the Australia National Health and Medical Research Council program grant APP#1071269 awarded to S.M.G. and C.K.F.

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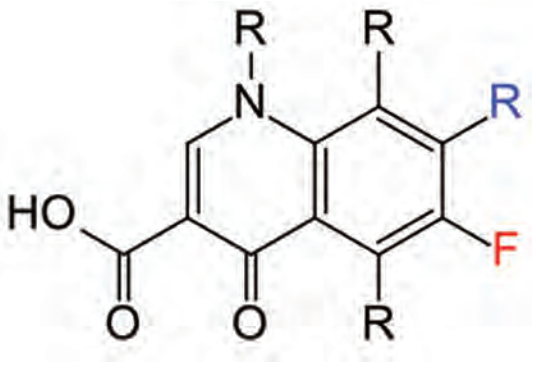
etymologia

Fluoroquinolone [floor"o-kwin'o-lōn]

Ronnie Henry

The first quinolone (*quinol*[ine] + *-one* [compound related to ketone]), nalidixic acid, was isolated as a byproduct of chloroquine (see "quinine," https://wwwnc.cdc.gov/EID/article/21/7/ET-2107_article) synthesis and was introduced in 1962 to treat urinary tract infections. In 1980, researchers at the Kyorin Pharmaceutical Company showed that the addition of a fluorine atom to the quinolone ring resulted in an antibiotic with broader antimicrobial activity, which was named norfloxacin, the first fluoroquinolone. In 1983, Bayer published data that showed adding a single carbon atom to norfloxacin—what would become ciprofloxacin—further increased activity. Fluoroquinolones are today among the most frequently used antimicrobial drugs to treat infections in humans and animals.



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DOI: http://dx.doi.org/10.3201/eid2305.ET2305

Population Responses during the Pandemic Phase of the Influenza A(H1N1)pdm09 Epidemic, Hong Kong, China

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During August 2009–July 2010, we conducted 7 longitudinal telephone surveys among 503 adults in Hong Kong, China, to explore changes in their behavioral and psychological responses to the influenza A(H1N1)pdm09 virus epidemic. Trends were examined using generalized estimating equations models. Findings showed that responses varied with the course of the pandemic.

On June 11, 2009, the World Health Organization declared the influenza A(H1N1)pdm09 (pH1N1) virus outbreak a pandemic (*1*). Previous studies have investigated community responses to the pandemic in different countries during early stages of the epidemic (*2*–*5*). The studies investigated persons' risk perceptions and knowledge related to the virus, perceived efficacy of preventive measures, and psychological and behavioral responses. However, because of intersample variations, these cross-sectional studies did not capture within-person changes. We conducted a longitudinal cohort study to investigate changes in responses among the general Hong Kong, China, population during the pH1N1 pandemic.

The Study

A cohort sample of 18- to 60-year-old adults in Hong Kong participated in 7 rounds of telephone surveys during August 2009–July 2010, which covered almost the entire pH1N1 pandemic period in Hong Kong. At baseline, we invited 677 adults to participate; 503 (74.3%) consented and completed the survey (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-0768-Techapp1.pdf). We measured the following variables: knowledge about the modes of pH1N1 virus transmission; risk perceptions associated with the virus (perceived susceptibility to and severity of infection); perceived efficacy and use of preventive measures (e.g., handwashing, using a facemask); psychological responses (worry about infection and emotional distress); and evaluations of the government's performance in pandemic control. Sample sizes for surveys 2–7 ranged from 452 to 481, yielding retention rates of 89.8%–95.6%.

Most participants were women (57.9%), 40–60 years of age (55.8%), employed full time (55.9%), and married (65%). Sex and age distributions were comparable to those in the local census data (*6*). We aimed to determine whether there were overall linear trends in participants' perceptions, psychological responses, and behavioral responses to the pandemic. We examined linear trends for these variables across the 7 time points by using generalized estimating equations (GEE) models. GEE models not only account for intracorrelated repeated measures data but also fit various data types using appropriate link functions. The analyses were conducted using PROC GENMOD (SAS Institute, Cary, NC, USA); 2-sided $p<0.05$ was considered significant.

Over time, >85% of the participants used a face mask and immediately visited a doctor when experiencing influenza-like symptoms. More than 50% of the participants washed their hands >10 times every day throughout the survey period ($p>0.05$). As the pandemic progressed, a decreasing percentage of participants wore masks in public areas; avoided touching their mouth, nose, and eyes; took antiviral drugs; and avoided crowded places ($p<0.001$) (online Technical Appendix Table 2). Percentages of participants feeling worried, depressed, or emotionally disturbed about pH1N1 virus decreased over time ($p<0.001$) (online Technical Appendix Table 3).

Over time, a decreasing percentage of participants recognized that touching infected persons or contaminated objects could result in virus transmission ($p<0.001$). Throughout the study period, a consistently high percentage of participants (>92%) recognized that the virus could be transmitted via respiratory droplets. Misconceptions about possible transmission through insect bites (26.1%) and water sources (34.5%) were prevalent. The percentage of participants reporting at least 1 misconception was stable over time ($p>0.05$). A consistently high percentage (>90%) of participants believed that using face masks in public areas, washing hands frequently, and avoiding crowded places could effectively prevent the spread of pH1N1 virus ($p>0.05$).

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DOI: https://dx.doi.org/10.3201/eid2305.160768

The percentage of participants believing that pH1N1 virus would be more harmful than seasonal influenza in terms of fatality and bodily damage increased over time ($p<0.001$). The percentages of participants who believed the population was highly susceptible to pH1N1virus infection and who perceived a high chance of having a large-scale local outbreak in the coming year dropped significantly ($p<0.001$), but some fluctuations were observed; for example, the percentage peaked during survey round 2 (around the September influenza season).

Throughout the study period, ≈12% 21% of the participants gave a failing score (<5 on a 0- to 10-point scale) for the governments overall performance in controlling the pandemic ($p>0.05$). However, during survey rounds 2 7, an increasing percentage of participants believed in the governments ability to control the pandemic ($p<0.001$) (online Technical Appendix). The percentage of participants who believed that Hong Kong would not have enough vaccine or medication to deal with the pandemic decreased over time ($p<0.001$).

Conclusions

This study investigated changes in community perceptions over the course of the pH1N1 pandemic in Hong Kong. Findings were highly comparable to those from other local cross-sectional surveys (*5*,*7*) and a systematic review (*8*). Knowledge regarding preventive measures and adherence to such measures was, in general, higher among our participants than among the general population in other countries (e.g., Australia, India, and the Netherlands) (*9*–*11*). The prevalence of misconceptions about some incorrect modes of transmission (e.g., insect bites) gradually declined. However, ≈50% of participants still held at least 1 of the 4 misconceptions regarding transmission (i.e., airborne transmission over a long distance and transmission through insect bites, water sources, and well-cooked pork). Furthermore, over time, a lower percentage of participants avoided touching their eyes, nose, and mouth to prevent virus transmission. A 2015 systematic review suggested that health authorities should provide more updated information about the virus (*8*). We also recommend using health campaigns to increase public awareness about different routes of pH1N1 virus transmission.

Perceived severity of pH1N1 virus infection decreased over time, which may partially explain the decline in distress and avoidance behaviors; this pattern was also observed in a recent review (*8*). However, an increasing proportion of participants believed that, compared with seasonal influenza, pH1N1 resulted in more deaths and more severe body damage. Perceived susceptibility to infection declined substantially as the epidemic progressed, suggesting that the public gradually perceived fewer risks from pH1N1 virus. Avoidance behaviors and use of facemasks in the absence of influenza-like symptoms became less prevalent over time, similar to a trend seen in Malaysia (*12*). Mental distress among persons in Hong Kong was lower during the pH1N1 pandemic than during the SARS (severe acute respiratory syndrome) pandemic (*13*), possibly due to the milder consequences of pH1N1 infection. Persons in Hong Kong seemed to remain rational during the pandemic, thereby avoiding possible pandemic-associated economic threats.

Public support for the government declined over time. During survey round 5, a total of 20.6% of the participants gave a failing score to the government's performance, and 13.5% perceived that the government would not be able to control the pandemic. The poll was split as to whether the government should use the same response for pH1N1 influenza and seasonal influenza. Our findings suggest that the public should be advised of the pros and cons of pH1N1 control policies; a watchful step-down may be better accepted if the policies are understood.

This study has limitations. First, telephone surveys may be subject to self-selection bias. However, participants' demographics were comparable to those in local census data (*6*). Second, Hong Kong's unique experience with the SARS outbreak may have influenced the population's response to the pH1N1 pandemic; thus, our findings may not be fully generalizable to other countries. Third, we treated time as a continuous variable in the GEE models. Ideally, polynomials should be added to the linear time variable; however, given the small number of time points and absence of theoretical shapes, that was not feasible.

Our findings provide valuable information regarding overall linear trends and changes in community responses toward the pH1N1 pandemic among a Hong Kong cohort. These findings should help inform other countries in formulating appropriate pandemic control plans for influenza and other emerging infectious diseases.

Acknowledgments

We thank the study participants, and special thanks are extended to Mei Wah Chan, Cheri Tong, and Albert Cheung for coordinating the telephone surveys.

This study was supported by the Research Fund for the Control of Infectious Diseases (PHE-1&10), Food and Health Bureau, the Hong Kong Special Administrative Region.

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Survey of Treponemal Infections in Free-Ranging and Captive Macaques, 1999–2012

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Survey results showed treponemal infection among pet macaques in Southeast Asia, a region with a high prevalence of human yaws. This finding, along with studies showing treponemal infection in nonhuman primates in Africa, should encourage a One Health approach to yaws eradication and surveillance activities, possibly including monitoring of nonhuman primates in yaws-endemic regions.

Yaws, an endemic tropical disease distinguished by bone and skin lesions, is caused by infection with *Treponema pallidum* subsp. *pertenue* treponemes. Successful yaws treatment campaigns during 1950–1965 were followed by a resurgence of disease, and the World Health Organization (WHO) consequently mounted a yaws eradication campaign (*1*). Although the agent of yaws is spread among humans via direct contact, research has shown that nonhuman primates (NHPs) may serve as mammalian host reservoirs with the potential for zoonotic transmission (*2*). Successful eradication campaigns depend on there being no reservoir shielding the agent from eradication efforts; thus, the role that NHPs play in yaws among humans must be determined (*3*).

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DOI: http://dx.doi.org/10.3201/eid2305.161838

African Old World primates (OWPs) can be infected by *T. pallidum* and exhibit symptoms of yaws (*2*). Of note, the *Treponema* Fribourg-Blanc strain (isolated from a baboon in western Africa in 1966) exhibits remarkable genetic similarity to strains that cause yaws in humans (*4*) and in experiments, was shown capable of infecting humans (*5*). More recently, studies focusing on treponemal infections among NHPs in eastern Africa and the Republic of Congo showed that the NHP geographic range overlaps considerably with areas having a formerly high prevalence of yaws in humans (*2*).

Macaques (*Macaca* spp.), OWPs native to Asia and northern Africa, are susceptible to and have been experimentally infected with *T. pallidum* (*6*). After the initial WHO eradication efforts, yaws was believed to be largely eliminated from countries of mainland Asia, although reporting and active case detection have not been uniform throughout the region (*7*). Several island nations in Asia, however, continue to report active human yaws cases (*8*,*9*).

Macaques, the most widely distributed and numerous NHPs in the world, are sympatric with humans throughout Asia, thriving in human-altered environments and commonly kept as pets. To further characterize the role NHPs might play in the maintenance of *T. pallidum* subspecies, we screened an extensive archive of serum samples collected from free-ranging and captive macaques.

The Study

As part of a project characterizing the pathogen landscape among macaques and humans, we collected blood samples from NHPs during 1999–2012 and stored them at −80°C (*10*). We retrospectively screened samples from 734 macaques representing 13 species distributed throughout the animal's natural geographic range (Table 1). Study protocols were approved by the University of Washington Institutional Animal Care and Use Committee (no. 4233–01) and adhered to the American Society of Primatologists Principles for the Ethical Treatment of NHPs (https://www.asp.org/society/resolutions/EthicalTreatmentOfNonHumanPrimates.cfm).

We used a Macro-Vue RPR Card Test Kit (BD, Franklin Lakes, NJ, USA) to screen the 734 blood samples; 11 (1.5%) were positive (Table 2). The RPR (rapid plasma reagin) test, a lipoidal test (nontreponemal) for IgG and IgM typically associated with treponemal infection, can

Table 1. Number and species of free-ranging and captive macaques tested for treponemal infection, by location, 2000–2014*

Country, species	Year(s) sampled	Total no. sampled	No. captive	No. free-ranging
Nepal	2003			
Macaca mulatta		28	0	28
Bangladesh	2008–2012			
M. mulatta		137	14	123
Thailand	2003			
M. arctoides		2	2	0
M. assamensis		5	5	0
M. fascicularis		2	2	0
M. mulatta		9	9	0
M. nemestrina		4	4	0
Cambodia	2011			
M. fascicularis		39	0	39
M. leonina		5	0	5
M. nemestrina		1	0	1
M. spp. (hybrid)		3	0	3
Singapore	2003, 2005–2006, 2009			
M. fascicularis		76	0	76
Gibraltar	2004, 2009, 2013–2014			
M. sylvanus		124	0	124
Indonesia				
Bali	2000–2003			
M. fascicularis		157	0	157
Java	2002			
M. fascicularis		25	25	0
Sulawesi	2000–2002			
M. balantak		5	5	0
M. fascicularis		5	5	0
M. hecki		7	7	0
M. maura		9	9	0
M. nemestrina		2	2	0
M. nigra		22	14	8
M. nigrescens		11	11	0
M. ochreata		1	1	0
M. tonkeana		40	40	0
Macaca spp. (hybrid)		15	15	0
Total	2003–2014	734	170	564

*The 734 tested macaques represented 13 species. Captive category included pets, macaques used in performances, and macaques in zoos; free-ranging included wild macaques, urban macaques, and those at temples, shrines, and reserve parks.

occasionally elicit nonspecific responses. To confirm RPR-positive samples, we used ESPLINE TP (Fujirebio, Tokyo, Japan), an enzyme immunoassay for measuring reactivity to 2 recombinant *T. pallidum* antigens, Tp47 and Tp17. ESPLINE TP and RPR tests have been validated for use in OWPs (*11*). Of the 11 RPR test–positive samples, 1 was from Singapore; 2 from Bali, Indonesia; and 8 from Sulawesi, Indonesia. Six samples (all from Sulawesi) yielded confirmatory positive results on the ESPLINE TP assay. Of note, in Sulawesi, the only positive macaques were pets sampled from South Sulawesi and West Sulawesi Provinces, which make up the island's southwestern peninsula (Figure). We also used ESPLINE TP to test the 28 RPR test–negative samples from Sulawesi's southwestern peninsula; none tested positive.

At the time of sampling, the macaques underwent a physical examination, including close inspection of head, trunk, extremities, oral cavity, and genitals. We conducted a retrospective review of the data and found that none of the macaques had lesions typical of treponemal infection (*4*). Of the 734 macaques, 13, including 2 seropositive macaques from Sulawesi's southwestern peninsula, had hypopigmentation on the palms of their hands, feet, or both. Hypopigmentation is rarely seen in yaws but is a common manifestation of pinta, which is caused by infection with *T. carateum*, a close relative of *T. p. pertenue*.

Conclusions

Our findings show that pet macaques in Southeast Asia can be infected with *Treponema* spp. related to those that infect humans. The overall prevalence of infection was low in our survey, but the pocket of infection detected among pets in Sulawesi's southwestern peninsula is noteworthy. The demonstration of reactivity in the serologic tests provides unequivocal evidence that the macaques had been infected with *T. pallidum* or a highly related pathogen. We had hoped to amplify a portion of *tp0548*, a locus in the *T. pallidum* genome used for molecular typing, but no amplifiable pathogen DNA was found in the whole-blood samples that had been held in storage for >10 years. Therefore, we could not determine whether the treponemal strains from NHPs in Sulawesi resembled strains that cause human yaws.

Table 2. Treponemal infections in blood samples from free-ranging and pet macaques, by geographic location, 1999–2012*

Location	No. macaques positive/no. negative (% reactive)†	No. macaques tested/no. positive‡
Indonesia		
Bali	2/155 (1.3)	2/0
Java	0/25 (0)	NA
Sulawesi	8/109 (7.3)	8/6
Nepal	0/28 (0)	NA
Singapore	1/75 (1.3)	1/0
Bangladesh	0/137 (0)	NA
Thailand	0/22 (0)	NA
Cambodia	0/48 (0)	NA
Gibraltar	0/124 (0)	NA
Total	11/734 (1.5)	11/0

*NA, indicates that samples in the region were not tested.
†Determined by using the Macro-Vue RPR (rapid plasma reagin) test (BD, Franklin Lakes, NJ, USA).
‡Determined by using ESPLINE TP (Fujirebio Inc., Tokyo, Japan), a reagent for the detection of *Treponema pallidum* antibodies.

Sulawesi, the third largest island in the Indonesian archipelago, has a population of ≈17 million persons and 7 endemic macaque species. The seropositive samples from South Sulawesi and West Sulawesi Provinces were collected in July and August of 2000, immediately predating an active yaws outbreak among humans in the region that caused 241 documented cases in the neighboring southeastern peninsula during 2001–2011 (WHO, http://apps.who.int/iris/bitstream/10665/75528/1/WHO_HTM_NTD_IDM_2012.2_eng.pdf) (Figure). During that outbreak, WHO characterized the South Sulawesi and West Sulawesi Provinces as "data deficient" regions in regard to the status of yaws among the human population. Most macaques whose samples were used in this study were free-ranging, but all of the macaques sampled in South Sulawesi and West Sulawesi Provinces had been captured at a young age for use as pets. The association between humans and pet macaques is often intimate, with the sharing of food; space; and physical contact through grooming, play, or aggression (*12*). Two of the *Treponema* spp.–infected pets were owned by the same person and housed together. Studies of pet macaques in Sulawesi and their owners have indicated that infectious agents can move between these populations (*12*,*13*). Although the treponemal serologic status of the pet owners in this study is unavailable, the fact that seropositive pet NHPs from a region neighboring an area with a high number of human yaws cases suggests that the NHP cases resulted from treponeme transmission from humans to pets.

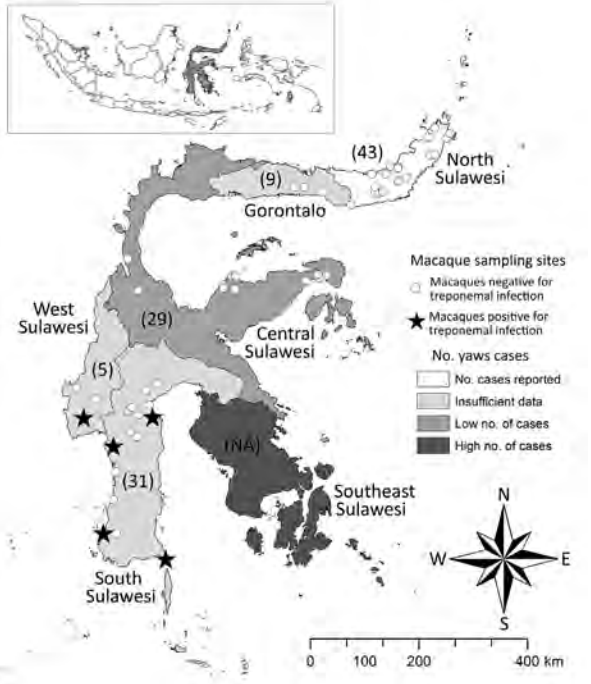

Figure. Individual sampling sites where macaques were tested for infection with *Treponema* spp. during 1999–2012 and the number of human yaws cases during 2001–2011, Sulawesi, Indonesia. Numbers in parentheses indicate number nonhuman primates sampled in each of the 6 provinces. ESPLINE TP (Fujirebio Inc., Tokyo, Japan) reagent for the detection of *T. pallidum* antibodies was used to determine whether macaque samples were positive for treponemal infection. The number of human yaws cases was determined by the World Health Organization (*1*). Inset map shows the location of Sulawesi in Indonesia (gray shading). NA, not available.

All macaques in this study, with the exception of *M. sylvanus* from Gibraltar, were from historically yaws-endemic areas where WHO conducted past yaws eradication campaigns. Much of Asia has a rich tradition of human–NHP commensalism, and macaques are common in villages, often as pets (*10*). Moreover, we previously showed that macaques can harbor an array of mammalian picornaviruses, astroviruses, and mycobacteria (*13*–*15*), underscoring the role of macaques in the ecology of these pathogens. However, as with our current study of treponemal infections, definitive evidence for transmission and the direction of transmission have not been established for these pathogens.

Our findings of treponemal infections among macaques in Southeast Asia, along with published work showing infection in NHPs in Africa (*4*), should encourage holistic and One-Health approaches to eradication and surveillance activities, including consideration of monitoring NHPs in yaws-endemic regions. Such approaches are particularly relevant for pet NHPs, which can easily be assessed and treated. The human–NHP interface is ancient and complex, and continued research, particularly in yaws-endemic regions, can help to ameliorate concerns as a second WHO yaws eradication campaign moves forward.

Acknowledgments

We are particularly grateful to all of the communities, temple committees, and government agencies in the areas where we

have been sampling monkeys for years: Lembaga Ilmu Pengetahuan, Indonesia; S. Chan and the staff of the Central Nature Reserve, National Parks Board, Singapore; M. Pizarro and the staff of the Gibraltar Ornithological and Natural History Society; S. Begum, M. K. Hasan, S. Akhtar, and the students and faculty of the Department of Zoology, Jahangirnagar University; and S. Son, C. Kimleng, S. Bunnary, T. Sothearos, M. Sisiket, H. Davun, and K. Pal in Cambodia and Nepal. We are also grateful to D. Cohn, A. Fuentes, J. Supriatna, R. Babo, Y. Paramastri, E. Iskandar, J. Froehlich, L. Engel, H. Engel, and L. Johnson for supporting and participating in this research.

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) (grant R01 AI078229 and R03 AI064865 to L.J.E.); National Center for Research Resources, NIH (grant P51 RR000166RR to L.J.E); US Public Health Services (grant R01 AI42143 to S.A.L.); Defense Advanced Research Projects Agency (grant N66001-02-C-8072 to L.J.E.); University of Toronto Connaught Fund (M.S.); the Chicago Zoological Society (to L.J.E.); and University of New Mexico Research Allocations Committee (L.J.E.).

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Phenotypic and Genotypic Shifts in Hepatitis B Virus in Treatment-Naive Patients, Taiwan, 2008–2012

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We examined the characteristic changes of hepatitis B virus (HBV) in antiviral drug treatment–naive patients referred for pretreatment evaluation in Taiwan during 2008–2012. Over time, we observed substantial decreases in the prevalence of HBV e antigen (HBeAg) and increasing prevalence of the precore G1899A mutation and HBV-DNA levels in HBeAg-positive patients.

Hepatitis B virus (HBV) replication is dependent on the activity of its reverse transcriptase, an error-prone enzyme, which results in the accumulation of genomic mutations. In the natural course of chronic hepatitis B, the prevalence of some mutations (e.g., precore stop codon mutations, basal core promoter mutations, deletions in the pre-S gene region) gradually increases with the progression of disease through the different clinical stages (*1*,*2*). This increase is largely caused by longer duration of chronic HBV infection, during which HBV has to adapt to environmental changes for better survival. Because of the availability of antiviral drug therapies, HBV replication can now be completely suppressed in most patients (*3*,*4*). However, drug resistance and suboptimal or no responses to antiviral drugs can occur (*5*), and there is a lag of few months between the start of treatment and complete virologic suppression. In addition, treatment noncompliance and intermittent treatment almost always result in virologic, or even clinical, relapses. In such cases, antiviral drug therapy imposes an iatrogenic selection pressure on HBV, and the selected viruses carrying mutations can cause infections.

HBV has been infecting humans for a long time; thus, it is possible that genotypic or phenotypic changes have occurred over time, especially since the introduction of HBV vaccine and antiviral drugs. We examined serologic changes and genotypic alterations of HBV in treatment-naive patients during 2008–2012 in Taiwan, where a universal vaccination program was launched in 1986.

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DOI: https://dx.doi.org/10.3201/eid2305.161894

The Study

During January 2008–December 2012, we reviewed the clinical and virologic data for 1,224 treatment-naive patients with chronic hepatitis B who were referred to our clinic at Chang Gung Memorial Hospital, Taoyuan, Taiwan, from all parts of Taiwan for pretreatment evaluation. We obtained patients' age and sex and assessed platelet count, cirrhosis status, HBV e antigen (HBeAg) and HB e antibody (anti-HBe) status, HBV genotype, HBV DNA level, basal core promoter mutations, and precore stop codon mutations. We conducted the study using previously described methods (*6*,*7*). The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Univariate regression analysis indicated a significant increase in patient age ($p = 0.001$) and a significant decrease in the number of HBeAg-positive patients ($p<0.001$) over the 5-year year period. Three factors were significantly associated with HBeAg status: an increased prevalence of anti-HBe ($p = 0.004$) and increased prevalence of precore G1896A ($p = 0.003$) and G1899A ($p = 0.019$) mutations. However, no significant changes occurred in the prevalence of 9 of 10 basal core promoter mutations (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1894-Techapp1.pdf). We noted a mild, but significant, decrease in the prevalence of G1730C mutations ($p = 0.034$).

Multivariate analysis showed that patient age and changes in the prevalence of HBeAg were independent of each other (adjusted $p = 0.025$ and 0.021, respectively). However, multivariate analysis that included G1730C, HBeAg, and age showed that the changing prevalence of G1730C was not independent of HBeAg and age (adjusted $p = 0.222$, 0.049, and 0.027, respectively).

These data indicate that the decreasing prevalence of HBeAg was not due to the increasing age of treatment-naive patients but due to an authentic phenotypic change of HBV over the years. It was unclear why patient age increased over the 5-year period; one possibility is the gradual acceptance of antiviral drug therapy by older patients, who may have been worried about possible side effects of new drugs. The cause of the decreasing prevalence of HBeAg over time was possibly due to the fact that a higher proportion of HBeAg-positive patients were treated in the early era of antiviral drug use and more HBeAg-negative patients received treatment at a later time. Alternatively,

because HBV infection has occurred in and expanded among the human population over the past decades, the serologic alterations may be attributed to a changing trend in the mode of transmission on a population scale; for example, in Taiwan, horizontal transmission has increased because of injection drug abuse and sexual transmission, and vertical transmission has decreased because of the neonatal vaccination program.

We subsequently separated patients into HBeAg-positive (n = 398) and HBeAg-negative (n = 826) groups. In the HBeAg-positive group, we saw a significant increase over time in the prevalence of precore G1899A mutations (p = 0.039) and level of HBV DNA (p = 0.013); however, these 2 factors were independent of each other (adjusted p = 0.009 and 0.003, respectively). Of note, we found no change in age over time in this subgroup (p = 0.281) (online Technical Appendix Table 2). Furthermore, because these patients were all HBeAg-positive, we could not explain the data by a differential proportion of HBeAg-positive patients being treated in the early and later periods of the antiviral drug era. Instead, other factors (e.g., changes in transmission routes, altered predominant risks of exposure, changes of HBV prevalence in different subpopulations) could be responsible. Alternatively, dependent on the scale of antiviral drug treatment received in this population, the therapeutic methods might also partly contribute to selection of mutation G1899A and HBV with higher replication efficiency.

In the HBeAg-negative treatment-naive patients (n = 826), we found a borderline increase in patient age (p = 0.046) and a borderline decreased prevalence of male patients (p = 0.044) over time. In addition, we noted a significant decrease in the prevalence of mutation A1752G over the 5-year period (p = 0.022). Multivariate analysis showed that these 3 changes were not independent (adjusted p = 0.062 for age, 0.067 for male sex, and 0.201 for mutation A1752G; data not shown).

Conclusions

Our findings showed a shift in the phenotypic and genotypic characteristics of HBV in treatment-naive patients in Taiwan, an area where chronic hepatitis B is endemic, after the widespread use of antiviral drugs. In Taiwan, because of a limited budget for national health insurance and a high prevalence of chronic hepatitis B, insurance coverage for hepatitis B treatment is not lifelong. Under the insurance plan, patients are provided with continuous nucleotide and nucleoside antiviral treatment for 3 years, after which the drugs are withdrawn to observe for durability of clinical remission. About half of patients have clinical relapses, and almost all have virologic relapses (*8*). Only patients with clinical relapses are retreated. After 3 years of retreatment, the antiviral drugs are withdrawn again to observe for durability; subsequent retreatments (for 3 years) are given only to patients with clinical relapses. The procedure is repeated until no clinical relapse has occurred. It is conceivable that the characteristics of HBV in these antiviral drug–treated, hepatitis B–relapsed patients have been selected by antiviral drugs. Antiviral drug–selected HBVs could potentially spread to treatment-naive patients and cause new infection or superinfection. However, the contribution of this therapeutic factor to the phenotypic and genotypic alterations of HBVs is unclear. Other environmental and social factors, such as altered transmission routes, changes of exposure risks, variations of HBV prevalence in subpopulations over time, and effects of neonatal vaccination, could lead to the changes in HBV characteristics.

Over the 5 years of this study, we found an increasing prevalence of G1899A mutations and an increasing concentration of serum HBV DNA in treatment-naive, HBeAg-positive patients. The mean HBV DNA concentrations increased from 7.7 to 8.3 $\log_{10}$/mL and then stabilized without further increases during the last 3 years of the study. On the other hand, prevalence of G1899A mutations increased from 2.5% to 17.4%. The clinical significance of this mutation in HBeAg-positive patients is unclear and requires further study. In conclusion, this study revealed that, in Taiwan, HBV characteristics have been changing in the era after introduction of antiviral drug treatment and HBV vaccination.

Acknowledgments

We thank all staff of the Liver Research Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan, for helpful discussions and technical assistance.

This study was supported by grants from Chang Gung Memorial Hospital Research Council (CRRPG3F0051 and CLRPG3C0012).

C.T.Y. designed and supervised the study; C.T.Y. and K.-H.L. drafted the manuscript; and M.-L.C., C.-W.H., Y.-C.C., C.-L.L., W.-R.L., and M.-W.L. collected the samples, performed all assays, and interpreted the data.

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Reassortant Clade 2.3.4.4 Avian Influenza A(H5N6) Virus in a Wild Mandarin Duck, South Korea, 2016

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A reassortant clade 2.3.4.4 avian influenza A(H5N6) virus was isolated from a fecal sample of a Mandarin duck (*Aix galericulata*) in South Korea during October 2016. This virus was genetically similar to H5N6 subtype virus isolates from China, Vietnam, Laos, and Hong Kong, including human isolates.

Highly pathogenic avian influenza viruses (HPAIVs) have caused major economic losses in poultry industries and represent a serious threat to public health. The H5N1 subtype of these viruses was first detected in 1996 from a domestic goose in Guangdong, China (Gs/GD), and its H5 hemagglutinin (HA) gene has subsequently evolved into 10 genetically distinct virus clades (0–9) and multiple subclades (*1*). Since 2008, novel reassortant HPAIVs bearing the HA gene of the Gs/GD lineage H5 clade 2.3.4 and neuraminidase (NA) gene subtypes N1, N2, N5, N6, N8, and N9 have been identified in China (*2*).

Although clade 2.3.4 of influenza A(H5N8) virus caused influenza outbreaks in eastern Asia and was subsequently disseminated into Europe and North America by wild aquatic birds in late 2014 (*3*,*4*), clade 2.3.4.4 of this virus has caused continuous outbreaks in China since 2013 (*5*). This virus disseminated into Laos and Vietnam in 2014 and Hong Kong in 2015 (*6*,*7*). Since the first influenza case in Sichuan Province, China, 15 human cases of influenza caused by this subtype have been reported in China during April 2014–May 2016 (*8*).

We report detection of an H5N6 subtype HPAIV in a fecal sample obtained from a wild bird sampled in South Korea during the fall 2016. We sequenced and genetically analyzed the complete genome of this virus isolate.

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DOI: http://dx.doi.org/10.3201/eid2305.161905

The Study

On October 28, 2016, we isolated an H5N6 subtype HPAIV from 1 of 391 fecal samples collected from wild birds in Gokgyo-cheon, South Korea (36°45′12.3″N, 127°07′12.7″E). Gokgyo-cheon is a wild bird habitat for wintering of migratory waterfowl, including mallard (*Anas platyrhynchos*), spot-billed duck (*Anas poecilorhyncha*), Mandarin duck (*Aix galericulata*), and common teal (*Anas crecca*). The species of the positive fecal sample was identified as Mandarin duck on the basis of DNA barcoding technique as described (*9*). There were no detectable clinically ill or dead wild birds at the sampling site.

Full-length genome sequencing and phylogenetic analysis were conducted to trace the origin of A/Mandarin_duck/Korea/K16-187-3/2016(H5N6) virus, hereafter referred to as MD/KR/2016. Methods used are detailed in online Technical Appendix 1 (https://wwwnc.cdc.gov/EID/article/23/5/16-1905-Techapp1.pdf). We entered genome sequences in the GISAID (Global Initiative on Sharing All Influenza Data) EpiFlu database (https://www.gisaid.org) under accession nos. EPI861480–EPI861488. Strains used in analysis are shown in online Technical Appendix 2 (https://wwwnc.cdc.gov/EID/article/23/5/16-1905-Techapp2.xlsx).

The isolate was identified as an HPAIV on the basis of multiple basic amino acids at the HA proteolytic cleavage site (PLRERRRKR/G). GISAID BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches indicated that H5 and N6 genes had high nucleotide identity in HA (99.17%) and NA (99.24%) with A/great_egret/Hong_Kong/00032/2016 (H5N6) (Table 1). Internal gene segments, except the polymerase basic 1 (PB1) gene, had high nucleotide identity with other H5N6 subtypes isolated in Guangdong and Jiangxi, China (PB2, 99.09%; polymerase acidic, 98.96%; nucleoprotein, 99.16%; matrix, 98.98%; and nonstructural protein [NS], 98.31%). However, the PB1 gene had high nucleotide identity (97.01%) with H4 low pathogenicity avian influenza viruses (LPAIVs).

In previous phylogenetic analyses, the HA gene of clade 2.3.4.4 viruses was divided into 4 distinct subgroups (online Technical Appendix 1 Figure 1) (*10*). Group intercontinental A (icA) contains H5N8 subtype virus and

[1]These authors contributed equally to this article.

Table 1. Nucleotide identities between reassortant clade 2.3.4.4 avian influenza A(H5N6) virus isolated from a wild Mandarin duck, South Korea, 2016, and nearest virus homologs in the GISAID database*

Gene	Virus	GISAID accession no.	% Identity
PB2	A/feline/Guangdong/2/2015(H5N6)	EPI760095	99.09
PB1	A/duck/Guangdong/S4040/2011(H4N2)	EPI692414	97.01
PA	A/Syrrhaptes paradoxus/Guangdong/ZH283/2015(H5N6)	EPI839169	98.96
HA	A/great_egret/Hong_Kong/00032/2016(H5N6)	EPI687156	99.17
NP	A/Syrrhaptes paradoxus/Guangdong/ZH283/2015(H5N6)	EPI839171	99.16
NA	A/great_egret/Hong_Kong/00032/2016(H5N6)	EPI687157	99.24
M	A/feline/Guangdong/2/2015(H5N6)	EPI760101	98.98
NS	A/duck/Jiangxi/NCDZT1123/2014(H5N6)	EPI590810	98.31

*GISAID, Global Initiative on Sharing All Influenza Data (http://www.gisaid.org); HA, hemagglutinin; MP, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.

its reassortant viruses identified in China, South Korea, Japan, Taiwan, Canada, the United States, and countries in Europe during 2013–2016. Group B contains H5N8 subtype viruses identified in China and South Korea during 2013–2014, and in Russia in late 2016. Group C contains H5N1 and H5N6 subtype viruses identified in China, Vietnam, Laos, and Hong Kong, including isolates from humans in Guangdong, Yunnan, and Hunnan Provinces, China. Group D contains H5N6 subtype viruses identified in China and Vietnam, including an isolate from a human in Sichuan Province, China. The HA gene of MD/KR/2016 virus belonged to group C and clustered with H5N6 subtype viruses isolated from humans, cats, and the environment in Guangdong during 2014–2015 and a migratory aquatic bird in Hong Kong during January 2016 (A/great_egret/Hong_Kong/00032/2016 [H5N6]) (online Technical Appendix 1 Figure 1).

A previous study reported that A/environment/Guangdong/GZ693/2015 (H5N6), hereafter referred to as GZ693/2015(H5N6), is a 7:1 gene reassortant virus between H5N6 HPAIV and LPAIVs found in southern China (*7*). MD/KR/2016 clustered with GZ693/2015(H5N6) virus for all 8 genes (online Technical Appendix 1 Figure 2). In particular, the HA, NA, PB2, polymerase acidic, nucleoprotein, matrix, and NS protein genes clustered with GZ693/2015(H5N6) and other clade 2.3.4.4 group C H5N6 viruses. The PB1 gene clustered with GZ693/2015(H5N6) (nucleotide identity 92.79%) and LPAIVs, such as H3N2 and H4N2 subtype viruses, from southern China. Phylogenetic analysis and BLAST search collectively sug-

Table 2. Amino acid analysis of avian influenza A(H5N6) virus from a wild mandarin duck, South Korea, 2016, and reference strains of clade 2.3.4.4 H5N6 subtype virus*

	HA (H5 numbering)†							PB2‡			NA del,§	NS¶		
												80–84		
Group, strain	123	126	129	133	156	222	224	591	627	701	59–69	42	del	PDZ
South Korea H5N6 subtype and closely related avian isolates														
A/Mandarin_duck/Korea/K16-187-3/2016	P	E	Del	S	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
A/great_egret/Hong_Kong/00032/2016	P	E	Del	A	A	Q	G	Q	?	?	Yes	?	?	No
A/environment/Guangdong/GZ693/2015	P	E	L	A	A	Q	G	Q	E	D	No	S	No	ESEV
C, human isolates														
A/Shenzhen/1/2016	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/_Guangdong_/ZQ874/2015H5N6	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Del	ESEV
A/_Guangdong_/SZ872/2015H5N6	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	No	No
A/Shenzhen/1/2015	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	No	No
A/Yunnan/14563/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/Yunnan/14564/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	No	No
A/Yunnan/0127/2015	P	Del	S	A	A	Q	G	Q	K	D	Yes	S	Yes	No
A/Guangzhou/39715/2014	P	E	L	A	T	Q	G	Q	K	D	Yes	S	Yes	ESEV
A/Changsha/1/2014	P	Del	S	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
D, human isolate														
A/Sichuan/26221/2014	T	E	L	A	A	Q	G	Q	E	N	No	S	Yes	ESEV
C, mammalian isolates														
A/swine/Guangdong/1/2014	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	EPEV
A/swine/Guangdong/2/2014	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	EPEV
A/feline/Guangdong/1/2015	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV
A/feline/Guangdong/2/2015	P	E	L	A	A	Q	G	Q	E	D	Yes	S	Yes	ESEV

*Del, deletion; HA, hemagglutinin; NA, neuraminidase; NS, nonstructural protein; PB2, polymerase basic 2; PDZ, PDZ binding motif.
†S123P, S133A, T156A, Q222L, and G224S mutations in HA have been associated with increased binding to human-like receptor (α-2–6 sialic acid).
‡Q591K, E627K, and D701N mutations have been associated with improved replication of avian influenza virus in mammals.
§NA stalk deletion has been associated with enhanced pathogenicity in mice.
¶42S, 80–84 deletion, and ESEV PDZ binding motif have been associated with increased virulence in mice.

gest that MD/KR/2016 virus had an identical genotype to GZ693/2015(H5N6).

Most of clade 2.3.4.4 group C viruses have leucine or serine at position 129 (H5 numbering) in HA protein. However, MD/KR/2016 had a single amino acid deletion at position 129 (Table 2), as did A/great_egret/Hong_Kong/00032/2016 (H5N6). This deletion at position 129 and phylogenetic network analysis suggested that MD/KR/2016 is closely related to H5N6 subtypes isolated from wild birds in Hong Kong in 2016 (online Technical Appendix 1 Figure 3). MD/KR/2016 contained the mutation associated with increased virulence in mammals and mammalian transmissibility (S123P and T156A mutations in the HA gene; P42S and D92E mutation, and elongated C-terminus with PDZ binding motif in NS gene). However, this isolate lacked the Q226L and G228S mutations in HA, which have been associated with increased binding to human-type receptor (α-2,6–linked sialic acid) and lacked Q591K, E627K and D701N mutations in PB2, which have been associated with enhanced pathogenicity and adaptation to mammalian hosts (*11*). All of the 9 H5N6 subtype human isolates of group C lacked the Q226L and G228S mutations in HA, but 5 viruses contained the E627K mutation in PB2 (Table 2), suggesting that some purported mammalian adaptation amino acid substitutions were not necessary for sporadic virus infection of H5N6 HPAIV in humans.

Conclusions

Wild aquatic birds have been suspected to play a key role in dissemination of HPAIVs to various regions, as seen with clade 2.2 H5N1 HPAIV in 2005, clade 2.3.2.1 H5N1 HPAIV in 2009, and clade 2.3.4.4 H5N8 HPAIV in 2014 (*4*,*12*). Some populations of Mandarin ducks are year-round residents in South Korea and Japan; others populations migrate between Russia and eastern Asia (*13*). In South Korea, HPAIV was detected from Mandarin duck samples in 2010 (H5N1) and 2014 (H5N8) (*14*,*15*) and again in 2016 during this study, suggesting that Mandarin ducks are a major host species for clade 2.3.4.4 H5 HPAIV and can disseminate the virus throughout South Korea and into other countries. Detection of the H5N6 HPAIV clade 2.3.4.4 in a migratory bird species in South Korea; reports of H5N6 outbreaks in poultry from China, Laos, and Vietnam; and diagnosis of lethal human cases of highly homologous H5N6 viruses in China raise a concern over the potential for broad geographic dissemination of zoonotic H5N6 HPAIV by wild birds outside eastern Asia.

Acknowledgments

We thank Junbeom Kim, Kyujik Kim, Jiho Lee, and Yujin Kim for assistance in collecting samples and excellent technical assistance.

This study was supported by the Korea Institute of Science and Technology Open Research Program (Project no. 2E25720-15-P030) and partially supported by the Animal Production and Health Section of the Joint Food and Agriculture Organization/International Atomic Energy Agency Division for Nuclear Applications in Food and Agriculture (project officers Ivancho Naletoski and Gerrit Viljoen).

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Amoxicillin and Ceftriaxone as Treatment Alternatives to Penicillin for Maternal Syphilis

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There is no proven alternative to penicillin for treatment of maternal syphilis. We report 2 case-patients with maternal syphilis who were successfully treated without penicillin. We used amoxicillin and probenecid for the first case-patient and amoxicillin, probenecid, and ceftriaxone for the second case-patient.

Syphilis is caused by the bacterium *Treponema pallidum*. Untreated maternal syphilis can lead to serious complications, including congenital syphilis, stillbirth, and neonatal death (*1*). In 2012, the estimated numbers of worldwide cases of maternal and congenital syphilis were 930,000 and 350,000, respectively (*1*). Recently, the United States reported increasing rates of congenital syphilis (*2*).

Penicillin G is recommended for treatment of maternal syphilis (*3*). Benzathine penicillin G is used in many countries but is unavailable in others, such as Japan. As a result, guidelines in Japan of treatment sexually transmitted diseases recommend benzylpenicillin benzathine hydrate (1.2 million units/d) and oral amoxicillin or ampicillin (1.5 g/d) as alternatives (*4*). However, there is little evidence to support use of these regimens. We report 2 case-patients with maternal syphilis who were successfully treated without penicillin.

The Study

Case-patient 1 was a 20-year-old woman who came to the hospital at the National Center for Global Health and Medicine (Tokyo, Japan) because of positive results for treponemal and nontreponemal tests in December 2014. Eight months earlier, she was given a diagnosis of trichomonal vaginitis, which resolved after a course of metronidazole. Three months before coming to the hospital, she was examined in a clinic because of a rash on her abdomen and back, for which she was given topical treatment, which resulted in resolution of the rash within a few weeks. A rapid plasma reagin (RPR) and *T. pallidum* hemagglutination assay (TPHA) were not performed at that time.

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DOI: http://dx.doi.org/10.3201/eid2305.161936

Two months before coming to the hospital, she missed her menstrual period and showed a positive result for a home pregnancy test. Serologic tests for treponemal and nontreponemal antibodies were performed at a nearby clinic, and she was referred to our hospital 13 weeks into her pregnancy. Her RPR titer was 1:16, and TPHA showed positive results. The patient was given a diagnosis of early latent syphilis and prescribed a 14-day course of amoxicillin (6 g/day) and probenecid (1 g/d). The RPR titer decreased to 1:8 and 1:4 at 3 and 6 months after treatment, respectively. RPR was the last test performed before delivery.

She gave birth to a boy at 41 weeks gestation. The baby did not have any signs or symptoms of congenital syphilis, and his serum RPR and TPHA titers were 1:1 and 1:640, respectively (Table). The baby was not treated for congenital syphilis and has not shown any signs of congenital syphilis infection. At 15 months of age, results for RPR and TPHA were negative for a serum sample from the infant.

Case-patient 2 was a 31-year-old woman who came to the same hospital because of a fever in July 2015. One month earlier, she went to another hospital for investigation of a genital ulcer. The day before coming to our hospital, she was given a diagnosis of syphilis and prescribed amoxicillin (1.5 g/d).

A few hours after she took the first dose of amoxicillin, a fever developed, and the patient came to the emergency department of our hospital, where she was given a diagnosis of a Jarisch–Herxheimer reaction. She was also found to be 6 weeks pregnant. Her RPR titer was 1:32 and TPHA titer was 1:160, and she was given a diagnosis of primary syphilis. Three days later, she again came to our hospital for additional evaluation. Treatment was changed to amoxicillin (3 g/d) and probenecid (750 mg/d). Three days after this change in treatment, she could no longer tolerate the medication because of hyperemesis gravidarum, and she was admitted to our hospital. She was given ceftriaxone because she could not tolerate frequent administration of penicillin. Intravenous ceftriaxone (2 g/d) was given for 8 days. Her RPR titer decreased to 1:4 and 1:4 at 6 and 7 months after treatment, respectively. RPR was the last test performed before delivery.

In March 2016, she gave birth to a girl at 39 weeks gestation. The baby did not have any signs or symptoms of congenital syphilis, her RPR titer was negative, and the TPHA titer was 1:320 in a serum sample (Table). The baby was not treated for congenital syphilis, and RPR and TPHA results at 4 months of age showed negative results.

Table. Characteristics of 2 newborns of women with maternal syphilis, Japan*

Characteristic	Newborn for case-patient 1	Newborn for case-patient 2
Leukocytes, ×1,000 cells/μL	13.78	22.88
Neutrophils, %	76.0	68.0
Lymphocytes, %	13.0	18.0
Monocytes, %	7.0	4.0
Eosinophils, %	3.0	7.0
Hemoglobin, g/dL	14.7	19.2
Hematocrit, %	41.6	54.3
Platelets, ×10,000/μL	18.8	30.2
Total bilirubin, mg/dL	6.1	3.0
Direct bilirubin, mg/dL	0.1	0.2
Blood urea nitrogen, mg/dL	7.7	8.3
Serum creatinine, mg/dL	0.64	0.50
Aspartate aminotransferase, U/L	37	49
Alanine aminotransferase, U/L	10	23
Lactate dehydrogenase, U/L	595	560
Alkaline phosphatase, U/L	315	493
C-reactive protein, mg/dL	0.35	<0.01
Rapid plasma reagin titer	1:1	Negative
Chest radiograph	Unremarkable	Unremarkable
Bone radiograph	ND	Normal
Hepatomegaly	No	No

*ND, not done.

Conclusions

In 2012, the World Health Organization estimated that 930,000 cases of maternal syphilis resulted in cases of 350,000 congenital syphilis (*1*). In Japan, the National Institute of Infectious Diseases reported that the number of patients with syphilis is increasing (*5*). As the incidence of women with syphilis increases in Japan, incidence of congenital syphilis also increases (*6*). The efficacy of penicillin for treatment of syphilis is well established by clinical experience and is the only treatment option with documented efficacy (*3*).

Both case-patients described in this report were given amoxicillin and probenecid. A pharmacokinetic study reported that oral amoxicillin and probenecid could attain treponemicidal concentrations in cerebrospinal fluid; therefore, these drugs were considered alternative agents for treatment of neurosyphilis (*7*). Tanizaki et al. (*8*) reported that treatment with oral amoxicillin (3 g) and probenecid (750 mg) was highly effective in and well tolerated by syphilis patients with HIV infection. However, in their report, all patients were men.

For case-patient 2, we changed treatment to ceftriaxone, which is active against *T. pallidum* and has an effective concentration in cerebrospinal fluid. Marra et al. (*9*) reported that ceftriaxone is an alternative to penicillin for treatment of neurosyphilis or early syphilis among HIV-infected patients. US Centers for Disease Control and Prevention guidelines recommend ceftriaxone as an alternative treatment of syphilis in nonpregnant women (*3*). However, data regarding the use of ceftriaxone for treatment of maternal infections and prevention of congenital syphilis are insufficient (*3*).

Because RPR titers for both case-patients became nonreactive, treatment with amoxicillin plus probenecid and ceftriaxone successfully prevented syphilis in both fetuses. Amoxicillin and probenecid are not routinely prescribed for pregnant women because of little evidence of their efficacy in preventing congenital syphilis. Because benzathine penicillin G is not available in Japan, intravenous penicillin G is used to treat maternal syphilis. However, this treatment option requires hospitalization for frequent administration; admission of all maternal syphilis patients is not feasible.

Although ceftriaxone can be administered once a day, it requires daily hospital visits. Azithromycin is not recommended for use during pregnancy (*3*), and treatment failures for fetuses have been reported (*10*). Tetracyclines are contraindicated during pregnancy (*3*). Therefore, we used amoxicillin in accordance with guidelines for Japan (*4*).

One study reported the effect of probenecid during pregnancy on fetal outcomes (*11*). Because probenecid can cross the placental barrier, its use in pregnancy must follow careful consideration of anticipated benefits and possible hazards (*12*). Probenecid was prescribed to increase serum levels of penicillin. Amoxicillin monotherapy might be considered for treatment maternal syphilis if an appropriate dose is given.

The World Health Organization estimates that 5.6 million doses of 2.4 million units of benzathine penicillin are needed annually to treat all syphilis cases, and 930,000 doses are needed to prevent all cases of congenital syphilis (*13*). In May 2016, the 69th World Health Assembly reported that benzathine penicillin had been in short supply for several years (*14*). Therefore, during shortages of penicillin, it is prudent to consider alternative treatment regimens.

In conclusion, amoxicillin and ceftriaxone should be considered as alternatives to penicillin for treatment of maternal syphilis. Further studies evaluating the efficacy of amoxicillin and ceftriaxone are warranted.

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Azithromycin Resistance and Decreased Ceftriaxone Susceptibility in *Neisseria gonorrhoeae*, Hawaii, USA

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During 2016, eight *Neisseria gonorrhoeae* isolates from 7 patients in Hawaii were resistant to azithromycin; 5 had decreased in vitro susceptibility to ceftriaxone. Genomic analysis demonstrated a distinct phylogenetic clade when compared with local contemporary strains. Continued evolution and widespread transmission of these strains might challenge the effectiveness of current therapeutic options.

Neisseria gonorrhoeae is a sexually transmitted pathogen that has progressively developed resistance to the antimicrobial agents recommended for treatment (*1*). Third-generation cephalosporins are among the last class of antimicrobial agents that are still effective, and ceftriaxone is the foundation of treatment options recommended by the United States (*2*) and other countries. The diminished cache of drugs to treat gonorrhea has led most countries to recommend a combination of ceftriaxone and azithromycin in an attempt to ensure effective therapy and slow the emergence of resistance by decreasing the likelihood that a *N. gonorrhoeae* isolate would survive concomitant exposure to 2 antimicrobial agents with distinct mechanisms of action (*2*). However, sporadic treatment failures have been reported (*2*), and gonorrhea is considered a global health concern by the World Health Organization and the Centers for Disease Control and Prevention (CDC) because of the few remaining treatment options.

Surveillance for antimicrobial susceptibility of *N. gonorrhoeae* was established by the CDC in the United States in 1986 as penicillin and tetracycline resistance was becoming widespread. The CDC Gonococcal Isolate Surveillance Project (GISP; Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention) collects ≈5,000 isolates per year from men with urethritis seeking care at sexually transmitted disease clinics across the United States and assesses the isolates for antimicrobial susceptibility (*3*). The findings are used by CDC to formulate national treatment recommendations and develop research and disease intervention priorities. Ceftriaxone remains highly effective in treating gonorrhea in the United States; 99.9% of isolates were inhibited by <0.125 μg/mL in 2014 (*4*). However, the percentage of isolates with decreased azithromycin susceptibility (azithromycin MIC ≥2 μg/mL) rose sharply from 0.6% in 2013 to 2.5% in 2014. Fortunately, none of the 2014 isolates demonstrated clinical resistance or decreased susceptibility to both azithromycin and ceftriaxone.

The Hawaii Department of Health (HDOH) State Laboratories Division maintains nucleic acid amplification, culture, and antimicrobial drug susceptibility testing by Etest for *N. gonorrhoeae*. During 2016, the HDOH and CDC became aware of several *N. gonorrhoeae* isolates with high-level resistance to azithromycin and decreased susceptibility to ceftriaxone in Hawaii as a result of routine laboratory testing and jointly initiated an enhanced laboratory investigation of the isolates.

The Study

The HDOH confirmed the identification of 61 isolates of *N. gonorrhoeae*, collected during February 2016–May 2016, and antimicrobial drug susceptibility testing was performed on all of them. Isolates were identified as *N. gonorrhoeae* by using the API NH test kit (bioMérieux, Marcy l'Etoile, France), and the MICs for azithromycin, ceftriaxone, and cefixime was assessed by Etest (bioMérieux) on GC II agar supplemented with 1% IsoVitaleX (bioMérieux). Etest carried out at the HDOH State Laboratories Division found that 8 *N. gonorrhoeae* isolates had extremely high MICs (>256 μg/mL) for azithromycin and MICs of 0.125–0.25 μg/mL for ceftriaxone and cefixime (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/17-0088-Techapp1.pdf). The 8 isolates,

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DOI: http://dx.doi.org/10.3201/eid2305.170088

which were collected from 7 patients and included 2 isolates (urethral and urine) from the same patient (GCWGS_0182 and GCWGS_0322), were sent to CDC for confirmatory testing using agar plate dilution (*5*).

All 61 *N. gonorrhoeae* isolates were sequenced (paired-end; 2 × 250-bp read length) on an Illumina MiSeq sequencer (Illumina Denmark ApS, Copenhagen, Denmark) at the HDOH State Laboratories Division. De novo assembly was conducted at CDC by using SPAdes 2.5.1 (http://www.cab.spbu.ru/software/spades), and the core genome single-nucleotide polymorphism alignment was generated by using Parsnp 1.2 (http://www.cbcb.umd.edu/software/harvest), with the FA19 genome (GenBank accession no. CP012026) as the reference. The maximum-likelihood phylogeny was reconstructed by using RAxML 8.0.0 (http://sco.h-its.org/exelixis/web/software/raxml) with 1,000 bootstrap replicates. Whole-genome sequencing data were also used to determine the multilocus sequence typing (MLST) and *N. gonorrhoeae*–multiantigen sequence typing (NG-MAST) allelic profiles for the targeted isolates.

Results of the phylogenetic analysis indicated that the 8 isolates were closely related and formed a single clade (Figure) with 223 single-nucleotide polymorphism differences. MLST analysis revealed 1 unique profile, sequence type (ST) 1901 (online Technical Appendix), which is a highly successful lineage associated with multidrug resistance that probably originated in Japan (*6*). The results of the NG-MAST analysis indicated that all 8 isolates shared 1 novel profile, ST14121. Epidemiologic investigations did not associate sexual network transmission among the 7 patients, although 2 patients reported sex with the same partner. However, the consistent MLST and NG-MAST profiles, in combination with the strongly supported clade, suggest the circulation of a single strain within the population.

To assess the contribution of known mutations to macrolide and cephalosporin resistance, we examined mutations in *penA, ponA, mtrR*, and 23S rRNA genes. Regarding azithromycin resistance, a deletion in the *mtrR* promoter associated with low-level resistance (*7*) and 4 mutated 23S rRNA copies with the A2059G mutation that confers high-level resistance (*8*) were identified in all 8 isolates. The *ponA* L421P mutation and mosaic *penA* alleles have been associated with reduced susceptibility to cephalosporins (*7*,*9*). The *ponA* L421P mutation was found in all 8 isolates; however, only the nonmosaic *penA* XVIII allele was detected.

The first *N. gonorrhoeae* isolate (H11S8) with high-level azithromycin resistance (HL-AziR) in the United States was identified in Hawaii in 2011 (*10*). More recently, Public Health England characterized 7 *N. gonorrhoeae* HL-AziR isolates that were collected in northern England during November 2014–March 2015 (*11*). Isolate H11S8 and those from England were more susceptible to ceftriaxone (MIC range 0.004–0.03 μg/mL) than the cluster of *N. gonorrhoeae* HL-AziR isolates identified in Hawaii. Genetic comparisons of the 2011 Hawaii isolate placed it in a distinct clade on the phylogenetic tree (Figure). The NG-MAST of H11S8 was ST649, and those from England were ST9768. Three HL-AziR *N. gonorrhoeae* strains were

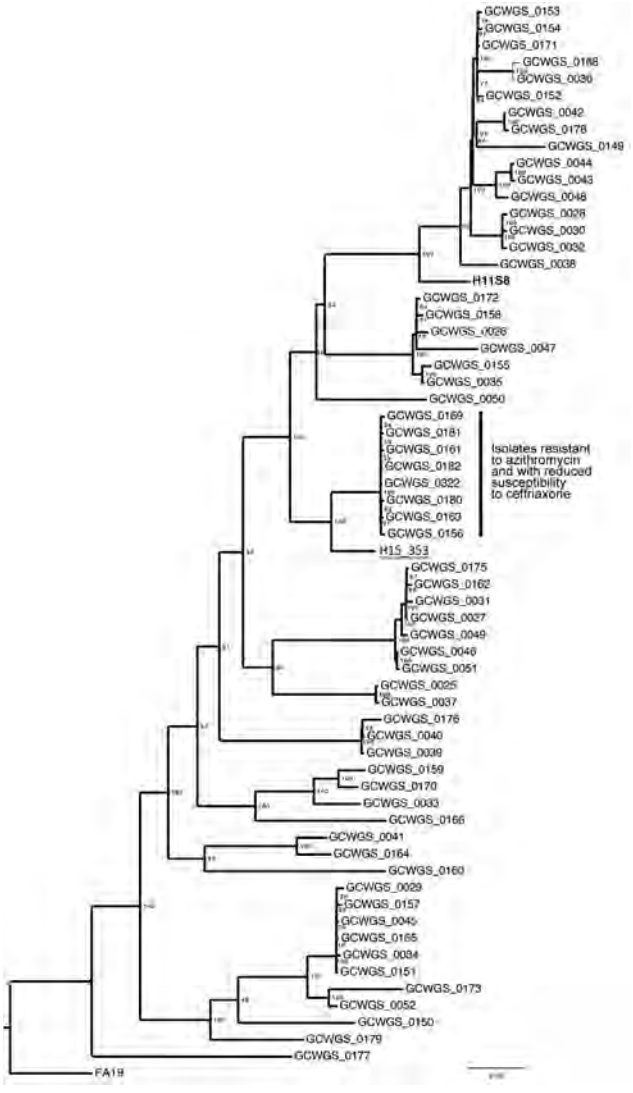

Figure. Maximum-likelihood phylogeny of *Neisseria gonorrhoeae* samples (N = 62) collected in Hawaii during February–May 2016, 1 isolate collected in Hawaii in 2011, and 1 isolate collected in the United Kingdom in 2015. The clade denoted with the black vertical bar contains 8 samples that exhibited resistance to azithromycin (MIC ≥256 μg/mL by Etest) and reduced susceptibility to ceftriaxone (MIC range 0.094–0.125 μg/mL). The 2011 isolate from Hawaii (H11S8, bold) also exhibited resistance to azithromycin. The United Kingdom isolate (underlined) was associated with failed dual antimicrobial therapy of ceftriaxone and azithromycin. The phylogeny is based on the core genome single nucleotide polymorphism alignment of the 62 genomes and the FA19 reference genome. Values on the nodes of the phylogeny (based on 1,000 bootstrap replicates) represent the support for each node and the corresponding clade. Scale bar indicates substitutions per site.

isolated in 2011 and 2012 in Sweden with slightly higher ceftriaxone MICs (range 0.032–0.064 μg/mL) and were identified as either NG-MAST ST285 or ST8727 (*12*).

All patients infected with the HL-AziR isolates in our study were successfully treated with 250 mg ceftriaxone plus 1 g azithromycin. In contrast, a recent pharyngeal *N. gonorrhoeae* isolate, resistant to azithromycin and ceftriaxone, was recovered from a patient in the United Kingdom following treatment with dual antimicrobial therapy of 500 mg ceftriaxone plus 1 g azithromycin (*13*). Although the isolate was genetically distinct from the 8 isolates in Hawaii, it was more closely related to those 8 isolates than to the other 53 contemporary isolates from Hawaii.

Conclusions

The combination of ceftriaxone and azithromycin remains the hallmark for the treatment of gonorrhea worldwide on the basis of surveillance data that monitors antimicrobial susceptibility (*2*,*14*,*15*). Slight fluctuations have been observed in ceftriaxone MICs, but rarely have isolates been recovered with a MIC >0.5 μg/mL. However, a growing body of evidence suggests that azithromycin is becoming less effective and should not be used as a monotherapeutic agent for gonorrhea. The observation of increased MICs for ceftriaxone and azithromycin in a cluster of strains from Hawaii might be the harbinger that the effectiveness of current treatment options will be challenged. It is critical that countries expand systematic surveillance for drug-resistant *N. gonorrhoeae* and that laboratories maintain culture capacity to support rapid response activities to confirm suspected treatment failures and mitigate transmission through contact tracing. Expansion of laboratory capacity to conduct genetic analysis in real time would further benefit clinicians and sexually transmitted disease public health programs by identifying novel mechanisms of resistance that could be used to develop nonculture antimicrobial resistance tests and rapidly identify resistant *N. gonorrhoeae* strains in sexual networks.

Acknowledgments

We thank Kevin Pettus and Samera Sharpe for excellent technical assistance in testing the *N. gonorrhoeae* isolates for antimicrobial susceptibility.

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Regional Transmission of *Salmonella* Paratyphi A, China, 1998–2012

Xin Lu, Zhenpeng Li, Meiying Yan, Bo Pang, Jialiang Xu, Biao Kan

To explore transmission patterns and genetic relationships of *Salmonella enterica* serovar Paratyphi A in China, we conducted a genome-wide single-nucleotide polymorphism analysis on the strains in the 4 provinces in which incidence was highest during 1998–2012. Markedly phylogeographic clustering suggested regional virus circulation after introduction from areas in southeastern China.

In Asia, incidence of paratyphoid fever remains high (*1*). In the mid-1990s, the number of paratyphoid fever cases in Asia caused by *Salmonella enterica* serovar Paratyphi A started to increase (*2*–*4*). In 2000, an estimated 5.41 million cases occurred; areas where incidence was highest (i.e., >100 cases/100,000 population per year) included south-central and Southeast Asia (*5*). Since 1998, the incidence of paratyphoid fever in Asia and the world has been highest in China, ranging from 0.08 to 192.5 cases/100,000 population annually (*6*); the provinces in which incidence is highest are Guangxi, Guizhou, Yunnan, and Zhejiang (*7*).

Information about the transmission routes and risk factors for infection could be used to improve the control strategies and measures for paratyphoid fever. Laboratory-based pathogen molecular subtyping, particularly genome-wide single-nucleotide polymorphism (SNP) analysis, can markedly improve outbreak detection, source tracing, and understanding of the epidemic modes. In this study, we analyzed genome-wide SNP and epidemiologic data from *Salmonella* Paratyphi A strains isolated from the China provinces where incidence was highest over a long period (1998–2012) and detected region-limited clone expansion in the epidemic provinces.

The Study

In 1998, the incidence of typhoid/paratyphoid fever in China was 4.82 cases/100,000 population (60,146 cases reported); this measure has since decreased annually to 0.88/100,000 (11,890 cases) in 2012 (China Information System for Disease Control and Prevention, unpub. data). Typhoid/paratyphoid fever cases in Guizhou, Yunnan, Zhejiang, and Guangxi Provinces accounted for 45.8% (in 1998) to 76.5% (in 2001) of all cases in China (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/5/15-1539-Techapp1.pdf).

To analyze the genomic epidemiology of paratyphoid fever in these provinces, we first selected 96 *Salmonella* Paratyphi A strains circulating in 15 provinces in China during 1998–2011 (online Technical Appendix Table 1). Strains were isolated from hospitalized patients suspected of having typhoid/paratyphoid fever and were maintained in the strain bank of the Chinese Center for Disease Control and Prevention. We then conducted genome-wide SNP genotyping by using the iPLEX Gold assay (Sequenom Inc., San Diego, CA, USA) with 2,343 SNPs obtained from 7 genomes sequenced in a previous study (*8*) and 17 genomes of *Salmonella* Paratyphi A strains sequenced in this study. We obtained 112 phylogenetically informative SNPs (including 57 nonsynonymous SNPs) (online Technical Appendix Table 2), which were further analyzed in 335 *Salmonella* Paratyphi A strains (online Technical Appendix Table 1) isolated from the provinces where incidence was highest (i.e., Guangxi, Guizhou, Yunnan, and Zhejiang) during 1998–2012 by using the iPLEX Gold assay. The population history of *Salmonella* Paratyphi A was estimated by using BEAST version 2.1.3 (http://beast.bio.ed.ac.uk/), and the maximum clade credibility tree was summarized by using TreeAnnotator and visualized by using FigTree version 1.4.2 (both within BEAST). The consensus tree (Figure 1) showed that all strains fell into 2 main clades: clade 1 consisted of 16 strains isolated from Yunnan, Guizhou, and Guangxi Provinces during 1998–2007; clade 2 consisted of the strains that were most common and widespread in these 4 provinces during 1998–2012. In clade 2, at least 3 subclades were formed, which were markedly characterized by geographic clustering according to province (Figure 1), suggesting intraprovince transmission of the different clones. In addition, the earlier strains in the root of each major subbranch were isolated mainly from Zhejiang, and in the years before 2005, some strains from Guangxi were also mixed in the Guizhou branch.

On the basis of the trees, we further determined from/to transmission of *Salmonella* Paratyphi A by using

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DOI: http://dx.doi.org/10.3201/eid2305.151539

Circos (*9*) (Figure 1). The same SNP genotypes of *Salmonella* Paratyphi A strains were preferentially transmitted within a single province from year to year, whereas the strains from Zhejiang were frequently transmitted to Guizhou and Guangxi, particularly during 1998–2002. The transmission between Guangxi and Guizhou was markedly more frequent before 2004 and decreased after 2005. After 2005, we found no transmission from Yunnan to other provinces.

We also extracted information for 112 SNPs from 127 genomes of the worldwide *Salmonella* Paratyphi A isolates in GenBank (*10*) and constructed a phylogenetic tree by combining these data with data from the 335 strains from China obtained in this study (Figure 2). The

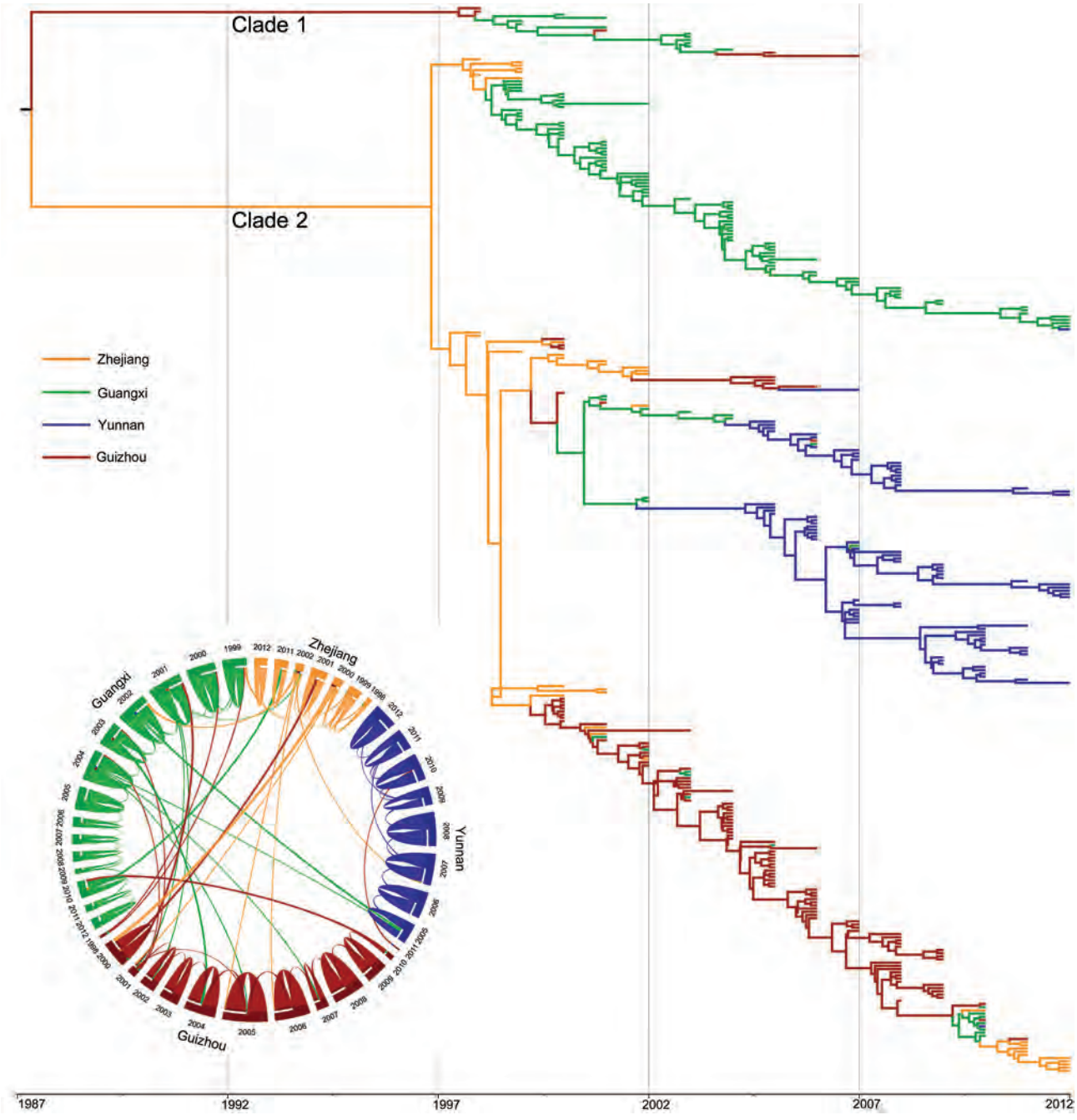

Figure 1. Phylogenetic tree of *Salmonella enterica* serovar Paratyphi A strains isolated from China, 1998–2012. The branches are colored according to inferred location. Inset: potential transmission of *Salmonella* Paratyphi A strains isolated from 4 provinces (Zhejiang Guangxi, Guizhou, and Yunnan). The flow bars indicate the source of transmission; 1 end of the bar directly touches the province of origin, and the other end of the bar exhibits a small gap before the province of destination.

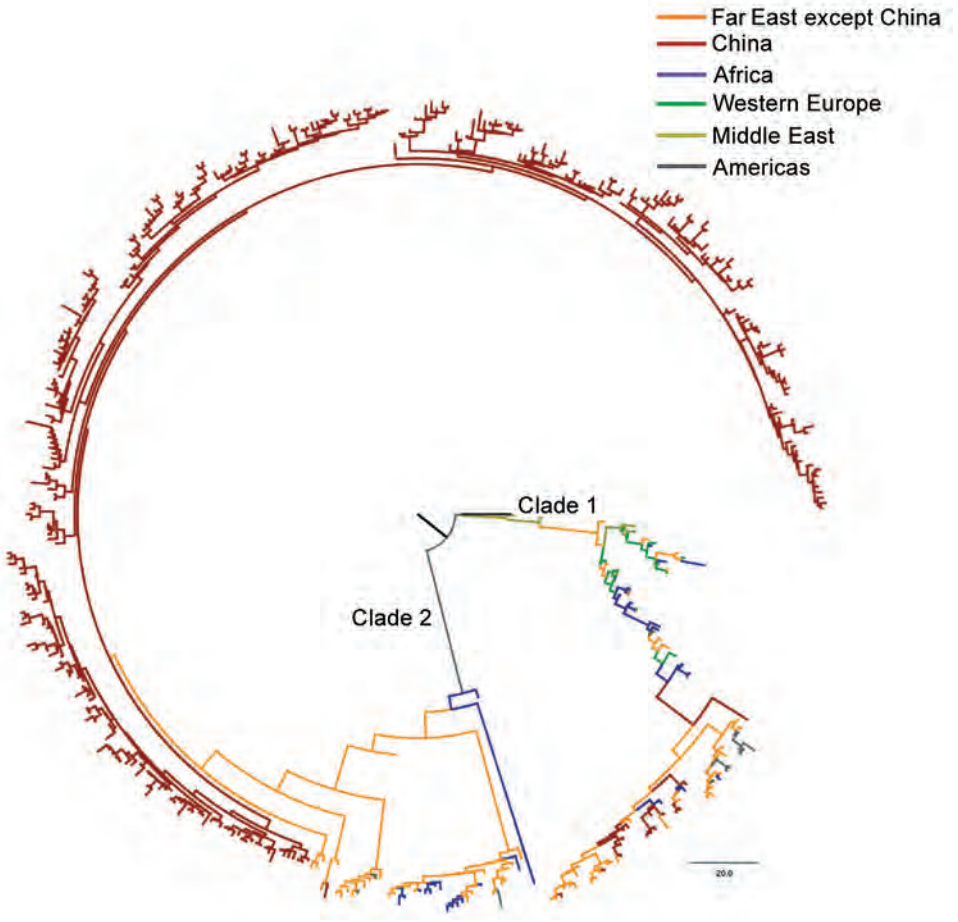

Figure 2. Phylogenetic tree of *Salmonella enterica* serovar Paratyphi A strains in China and worldwide. The branches are colored according to the inferred location. Scale bar indicates number of years.

international strains fell into 2 clades, and the strains from Southeast and southern Asia were positioned much closer to the root of the strains from China, suggesting that the potential source of *Salmonella* Paratyphi A in China might be India or Indonesia.

Conclusions

The genome-wide SNP phylogeny provided more accurate insights into the variation of *Salmonella* Paratyphi A strains in China. In Guizhou, Guangxi, and Yunnan Provinces, which are geographically adjacent, *Salmonella* Paratyphi A has existed for many years. Although we had speculated that the organism might show a mixture of genetic patterns, the phylogenetic tree showed that epidemic strains from different provinces gradually accumulated their own mutations to evolve and form obvious geographic branches. In earlier years of the study period (1998–2002), the epidemic strains from Guangxi and Guizhou Provinces might have originated from early epidemic strains from Zhejiang Province. The level of economic development in Zhejiang Province is high, whereas in Guangxi, Guizhou, and Yunnan Provinces it is lower; the rural population from these 3 provinces migrates frequently to work in the economically developed southeastern coastal areas in China, including Zhejiang (*11*), Jiangsu, and Guangdong Provinces. According to the fifth national census conducted in 2000 (http://www.stats.gov.cn/tjsj/pcsj/rkpc/5rp/index.htm) and the sixth conducted in 2010 (http://www.stats.gov.cn/tjsj/pcsj/rkpc/6rp/indexch.htm), the migration data within the 4 provinces showed this population movement trend (online Technical Appendix Table 3). At irregular intervals, migrant workers, mainly those who are young and middle-aged, return to their hometown for family reunions.

In the mid-1990s, paratyphoid fever became an emerging problem in Zhejiang Province; during 1997–2005, incidence was 8.61 cases/100,000 population (*12*). In those years, managing ex situ healthcare and medical treatments in China was problematic. When migrant workers got ill, they seldom sought medical treatment at the hospital in the city in which they worked; rather, they bought medicine at a chemist's shop or returned to their hometown for treatment (Zhang Q. The study on the health seeking behavior of migrant workers [master's thesis]. China: Shaanxi Normal University; 2012).

Because of lack of medical treatment in hospitals, migrant workers who become infected with *Salmonella* Paratyphi A easily become chronic carriers. Therefore, *Salmonella* Paratyphi A might be transmitted to Guangxi, Guizhou, and Yunnan Provinces via a migrating infected population, including patients and carriers. In addition, these 3 provinces are mainly mountainous, and the population flow among these provinces is limited by their lower economic development and inaccessibility. Therefore, the transmission pattern in these regions could be closely associated with the southeastern coastal areas, where the level of economic development is higher, and transmission among these 3 provinces could be absent. Moreover, in these paratyphoid-epidemic provinces, most of the overall population lives in rural agricultural areas. Given the combination of poor water and food hygiene with a hot and humid climate, the epidemic clones of *Salmonella* Paratyphi A could persist for a long time after being introduced into these areas.

In summary, we identified the evolution and transmission mode of paratyphoid fever in the China provinces where incidence is highest. Populations migrating to southeastern China probably mediated the transmission of *Salmonella* Paratyphi A. Considering the obvious regional clone expansion in these provinces, the local natural, social, and economic conditions need to be investigated for their potential roles in the spread of paratyphoid fever and for the development of intervention strategies.

This work was supported by the National Key Basic Research Program (2015CB554201), the National Key Research and Development Program (2016YFC1200103) of the Ministry of Health of China and by the Science Foundation (2014SKLID203) of the State Key Laboratory of Infectious Disease Prevention and Control, China.

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Exposure Risk for Infection and Lack of Human-to-Human Transmission of *Mycobacterium ulcerans* Disease, Australia

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We conducted epidemiologic and genetic analyses of family clusters of *Mycobacterium ulcerans* (Buruli ulcer) disease in southeastern Australia. We found that the incidence of *M. ulcerans* disease in family members was increased. However, the risk for exposure appeared short-term and not related to human-human transmission.

Mycobacterium ulcerans is a slow-growing organism that causes necrotizing infections of skin and soft tissue, often requiring reconstructive surgery and resulting in long-term disability (*1*,*2*). Prevailing opinion is that humans are infected from the environment; insects, such as mosquitoes (*3*,*4*), and water-residing biting arthropods (*5*,*6*), have been proposed as vectors for transmission. In Victoria, Australia, there is evidence that native opossums might be involved in transmission (*7*). However, despite extensive research, the environmental reservoir of the organism and mode of transmission remain unknown.

We postulated that examination of *M. ulcerans* disease (Buruli ulcer) family clusters might provide useful new information about disease epidemiology. Theoretically, genetically related first-degree relatives have similar susceptibility to disease, and families share the same environment and therefore a similar exposure risk. Thus, we examined the epidemiology of *M. ulcerans* disease in family clusters managed in a large prospective observational cohort from the Bellarine Peninsula in southeastern Australia. We used data collected from all confirmed *M. ulcerans* cases managed during January 1, 1998–April 12, 2016, at Barwon Health, a tertiary referral hospital in Geelong, Australia (*8*).

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DOI: http://dx.doi.org/10.3201/eid2305.160809

The Study

For this study, only initial *M. ulcerans* lesions were analyzed. A family cluster was defined as multiple family members independently given a diagnosis of *M. ulcerans* disease who were living at the same residence at the time of diagnosis. Data was collected by using Epi Info 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and analyzed by using Stata 12 (StataCorp LLC, College Station, TX, USA).

To determine the genetic relatedness of isolates derived from family clusters, we performed whole-genome sequencing and single-nucleotide polymorphism (SNP) analysis for 6 isolates derived from 3 family cluster pairs (Tables 1, 2). We sequenced DNA as 300-bp paired-end reads by using an MiSeq Sequencer (Illumina, Inc., San Diego, CA, USA). Resulting reads were mapped against the *M. ulcerans* Agy99 genome (*9*), including plasmid pMUM001 (*10*), by using Bowtie2 (*11*). Raw sequence reads for the 6 isolates have been deposited in the National Center for Biotechnology Information (Bethesda, MD, USA) Sequence Read Archive under BioProject accession no. PRJNA321660. We also performed whole-genome SNP analysis for 6 additional unrelated previously sequenced human *M. ulcerans* isolates (Sequence Read Archive accession no. SRP004497) obtained from the same disease-endemic region.

A total of 324 patients with *M. ulcerans* disease from the Bellarine Peninsula, Victoria, Australia, were managed in the Barwon Health observational cohort during January 1, 1998–April 12, 2016. Median age was 57 years (IQR 34–74 years), and 164 patients (50.6%) were men. For the whole cohort, a combined time of 1,968.5 years had elapsed from diagnosis of the initial *M. ulcerans* lesions until the time of study analysis (April 12, 2016). The median duration elapsed from initial diagnosis until study analysis was 4.7 years (IQR 2.8–9.7 years).

Twenty-one (6.5%) patients were part of a family cluster (Table 1), 9 genetically related and 12 related by marriage. All family clusters were diagnosed after the beginning of 2008. We found that significantly fewer family clusters were diagnosed during the first half of the study period (0 of 92 cases during 1998–2007) than in the second half (21 of 232 cases during 2008–2016) ($p<0.01$). The median time between diagnoses of *M. ulcerans* lesions in an additional family member, after the initial family

Table 1. Characteristics of 21 patients associated with family clusters of *Mycobacterium ulcerans* disease, Bellarine Peninsula, Victoria, Australia, 1998–2016*

Cluster	Isolate	Date of diagnosis	Time between lesions, mo	Location	Relationship	Patient age at diagnosis, y,/sex	Site of lesion	Type of lesion	WHO stage
1a	mu179	2008 Jul 21	0.4	PTL	Mother	54/F	Right thigh	Ulcer	1
1b	mu180	2008 Aug 4		PTL	Daughter	26/F	Left calf	Ulcer	1
2a	mu248	2010 Oct 24	20.6	PTL	Husband	84/M	Right forearm	Ulcer	1
2b	mu394	2012 Jul 4		PTL	Wife	84/F	Right forearm	Ulcer	1
3a	NT	2011 Jul 25	0.1	QUE	Husband	76/M	Right ankle	Ulcer	3
3b	NT	2011 Jul 28		QUE	Wife	75/F	Right elbow	Ulcer	1
4a	mu294	2011 Aug 22	1.3	PTL	Wife	65/F	Right knee	Ulcer	1
4b	mu308	2011 Sep 29		PTL	Husband	65/M	Left calf	Ulcer	1
5a	NT	2011 Aug 25	1.1	BH	Father	56/M	Right leg	Ulcer	1
5b	NT	2011 Sep 26		BH	Son	26/M	Right leg	Ulcer	1
6a	NT	2012 Jun 19	22.7	PTL	Wife	34/F	Left knee	Ulcer	1
6b	NT	2014 Apr 30		PTL	Husband	37/M	Right ankle	Ulcer	1
7a	NT	2012 Aug 14	22.9	QUE	Wife	74/F	Left ankle	Ulcer	1
7b	NT	2014 Jul 3		QUE	Husband	76/M	Left leg	Ulcer	1
8a	NT	2012 Oct 16	15.9	BH	Sister	20/F	Right foot	Ulcer	1
8b	NT	2014 Feb 14		BH	Brother	18/M	Left leg	Ulcer	1
9a	NT	2013 Apr 27	12.7	QUE	Wife	85/F	Right ankle	Ulcer	1
9b	NT	2014 May 12		QUE	Husband	90/M	Left forearm	Ulcer	1
10a	NT	2013 Dec 10	2.8	PTL	Father	34/M	Left hand	Ulcer	1
10b	NT	2014 Mar 4		PTL	Daughter	4/F	Right knee	Nodule	1
10c	NT	2014 Mar 5	0.0	PTL	Son	7/M	Right ankle	Nodule	1

*BH, Barwon Heads; NT, not tested; PTL, Point Lonsdale; QUE, Queenscliff; WHO, World Health Organization.

member was given a diagnosis, was 2.8 months (IQR 1.1–20.6 months). The rate of new diagnosis of an *M. ulcerans* lesion in another family member was 5.69/1,000 person-years (95% CI 3.15–10.29/1,000 person-years). We determined the cumulative proportion of patients given a diagnosis who had an affected family (Figure 1).

Core SNPs based on common variable nucleotide positions were identified for the 6 examined family isolates by whole-genome sequencing. A total of 4,918 core SNPs ascribed to the African Agy99 reference genome were identified according to strict filtering criteria. Only 8 SNPs were specific to ≥1 of the 6 isolates (Table 2). Of the 8 SNPs that differed among the isolates, only 3 were nonsynonymous substitutions. The remaining 5 SNPs were either intergenic or synonymous mutations.

Pairwise comparisons of family cluster isolates showed that isolates from the 4a/4b pair were genetically identical. In contrast, isolates from the 2a/2b and 1a/1b pairs contained several isolate-specific SNPs (Table 2; Figure 2). SNP analysis of unrelated *M. ulcerans* isolates from the same disease-endemic area showed that 3 of the 6 isolates were also genetically identical (Figure 2), which demonstrated that unrelated isolates can share a common genotype. The remaining 3 isolates contained 1–3 unique SNPs. Thus, family cluster isolates were not any more closely genetically related than 6 random isolates from the same geographic region.

Table 2. Description of 8 single-nucleotide polymorphisms specific to ≥1 of 6 family cluster isolates of *Mycobacterium ulcerans* disease, Bellarine Peninsula, Victoria, Australia, 1998–2016*

Position	Loci	Protein	Substitution	Amino acid change	Isolate	Coverage statistics
398430	Intergenic	–	G/A	–	mu179	T: 0, A: 35, G: 0, C: 1
398430	Intergenic	–	G/A	–	mu180	T: 0, A: 67, G: 0, C: 0
398430	Intergenic	–	G/A	–	mu248	T: 0, A: 100, G: 0, C: 1
398430	Intergenic	–	G/A	–	mu294	T: 0, A: 75, G: 0, C: 0
398430	Intergenic	–	G/A	–	mu308	T: 0, A: 58, G: 0, C: 0
1758272	MUL_1618	Membrane protein	C/T	Synonymous	mu248	T: 91, A: 1, G: 0, C: 0
2153447	MUL_1947	Thiamine pyrophosphate	A/G	Lys→Arg	mu294	T: 0, A: 1, G: 58, C: 0
2153447	MUL_1947	Thiamine pyrophosphate	A/G	Lys→Arg	mu308	T: 0, A: 0, G: 40, C: 0
2462577	MUL_2205	Hypothetical protein	T/C	Asp→Gly	mu179	T: 1, A: 1, G: 0, C: 47
4359638	MUL_3902	Membrane protein	C/A	Ala→Ser	mu180	T: 0, A: 60, G: 1, C: 0
4359638	MUL_3902	Membrane protein	C/A	Ala→Ser	mu248	T: 0, A: 108, G: 0, C: 1
5189291	Intergenic	–	G/T	–	mu248	T: 76, A: 0, G: 4, C: 0
5354966	MUL_4830	Putative GTPase	T/C	Synonymous	mu180	T: 2, A: 0, G: 1, C: 18
5354966	MUL_4830	Putative GTPase	T/C	Synonymous	mu248	T: 0, A: 0, G: 0, C: 20
5577431	MUL_5032	Immunogenic protein mbt64	A/G	Synonymous	mu394	T: 0, A: 0, G: 28, C: 0

*A total of 4,918 core single-nucleotide polymorphisms were identified for all 6 isolates compared with the African Agy99 reference genome. –, not applicable (mutations were not within a coding region).

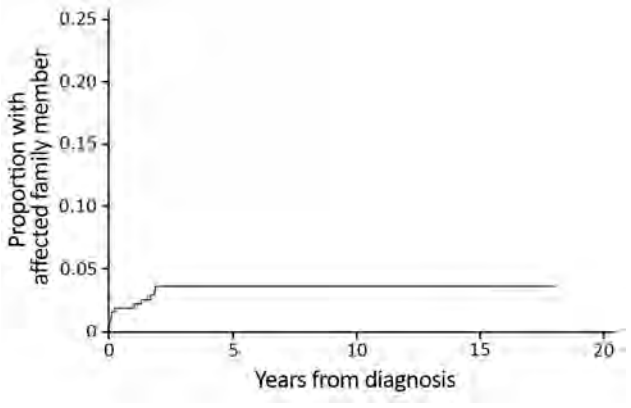

Figure 1. Cumulative proportion of patients with a family member affected by *Mycobacterium ulcerans* disease, Barwon Health cohort, Bellarine Peninsula, Victoria, Australia, 1998–2016.

Conclusions

Our examination of family clusters of *M. ulcerans* disease provides useful insights into the environmental reservoir and mode of transmission of this organism. First, the median time to diagnosis between family members was short (2.8 months), and no family members were given a diagnosis of an *M. ulcerans* lesion >23 months apart in a cohort spanning 18 years and nearly 2,000 combined years of elapsed time since diagnosis. This finding suggests that family members have been exposed to a source in the family's environment that persists only for a short period.

Second, with an incubation period for *M. ulcerans* disease estimated to be a median of 4.5 months (*12*), the observation that the median time between diagnoses in family clusters was <3 months suggest that infections were not being transmitted between family members. Further evidence against human-to-human transmission is apparent from whole-genome SNP analysis, which showed that pairs of isolates from 2 (2a/2b and 1a/1b) of 3 family clusters were not genetically identical. These findings support previous suggestions that *M. ulcerans* is unlikely to be transmitted from person to person (*13*).

Unknown is the type of short-term exposure that leads to the close temporal relation of family clustered infections. Opossums have been proposed as a source, either through contamination of the environment by infected feces or by an intermediate vector, such as mosquitoes, which transfer the infection from infected opossums to humans by a bite (*7*). Infected opossum(s) in the family environment might cause cases of human infection, then subsequently die of the disease (*14*), removing the source of infection. Alternatively, transmission could be related to a short-term change in the environment involving soil or foliage as a result of such events as home construction and renovation, or planting and removing trees or grasses (*13*). Mosquitoes in the area might be transiently infected/contaminated with *M. ulcerans* and infect humans through bites during this time (*15*).

In summary, the incidence rate of lesions in another family member (5.69/1,000 person-years) was higher than reported incidence rates during 2005–2009 in the general population of the Bellarine Peninsula (0.85–4.04 cases/year/1,000 population) (*7*). This finding suggests that genetic susceptibility or, more likely, localized exposure risk increases the likelihood of infection.

The incidence of *M. ulcerans* disease family clusters in an observational cohort in southeastern Australia was higher than in the general population of the disease-endemic area. However, when clusters occur, they are closely temporally related, which suggests a short-term risk for exposure and infection. Epidemiologic and genetic evidence suggests human-to-human transmission is not the source of infection.

Acknowledgment

We thank Janet Fyfe for providing the 6 isolates for whole-genome sequencing.

This study was supported by the Barwon Health Education, Training and Research Profile Fund.

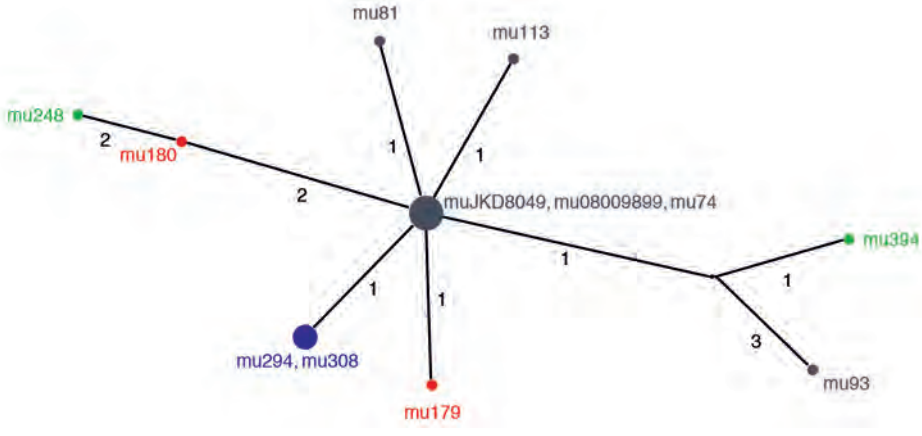

Figure 2. Median joining network of 12 SNPs of 12 *Mycobacterium ulcerans* isolates from patients with *Mycobacterium ulcerans* disease, Barwon Health cohort, Bellarine Peninsula, Victoria, Australia, 1998–2016. Node colors indicate clusters. Blue, cluster 4a/4b; red, cluster 1a/1b; green, cluster 2a/2b. Black nodes represent 6 unrelated isolates. The size of each node is proportional to the number of genetically identical isolates with identical genotypes. Values indicate number of SNPs between each node. SNP, single-nucleotide polymorphism.

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Estimated Incubation Period for Zika Virus Disease

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Information about the Zika virus disease incubation period can help identify risk periods and local virus transmission. In 2015–2016, data from 197 symptomatic travelers with recent Zika virus infection indicated an estimated incubation period of 3–14 days. For symptomatic persons with symptoms >2 weeks after travel, transmission might be not travel associated.

Zika virus is a mosquito-borne flavivirus transmitted primarily through the bite of infected *Aedes* spp.mosquitoes. Transmission can also occur through occupational laboratory exposure and by intrauterine, intrapartum, or sexual routes (*1*–*3*).

In May 2015, Zika virus disease cases were identified in Brazil, representing the first local transmission in the Americas (*4*). Subsequently, Zika virus spread rapidly, resulting in >463,000 suspected and laboratory-confirmed cases in the Americas as of June 30, 2016 (*5*). This rapid expansion highlighted key knowledge gaps, including incubation period. Characterizing the incubation period for Zika virus is needed for defining periods of risk and identifying local virus transmission. To estimate the incubation period, we used data from symptomatic persons who had traveled to an area with ongoing Zika virus transmission and for whom laboratory evidence indicated recent infection.

The Study

We included in our analysis persons for whom samples tested at the Centers for Disease Control and Prevention from January 1, 2015, through June 23, 2016, gave positive results, indicating recent Zika virus infection (defined as Zika virus RNA positivity by real-time reverse transcription or Zika or dengue virus positivity by IgM capture ELISA and confirmed by plaque reduction neutralization test with a Zika virus–specific neutralizing antibody titer ≥10 and Zika virus titer ≥4-fold higher than dengue virus titer) (*6*,*7*). We restricted our analysis to persons who were symptomatic, had known symptom onset date (onset of first symptom), had known travel dates from/to the continental United States, and were probably infected through a mosquito bite. We excluded from analysis those for whom disease was congenital or sexually transmitted and those reporting illness onset >2 months after travel (because of the typically shorter incubation periods for other flavivirus diseases).

To estimate the incubation period distribution, we first defined the exposure period as either the duration of travel if a person experienced illness after return from travel or the time from beginning of travel to the onset of illness if the traveler became ill during travel (Figure 1, panel A). We then fit various probability distributions in R (https://cran.r-project.org/) by using the dic.fit function in the coarseDataTools package, which uses methods detailed by Reich et al. (*8*). We selected the best model by using the Akaike information criterion. In addition to reporting fitted cumulative distribution function and associated 95% CIs, we reported certain quantiles and means. All analyses were conducted by using R.

For our primary analysis, we used all persons with evidence of a recent Zika virus infection (primary case set). We then performed a secondary analysis of persons with confirmed Zika virus infection and <2 weeks of travel (secondary case set), enabling evaluation of our estimates by using more stringent case definition requirements. A confirmed case of Zika virus disease was illness in a symptomatic person with a sample that was either Zika virus RNA positive or Zika or dengue virus IgM positive with neutralizing antibodies against Zika virus only.

From January 1, 2015, through June 23, 2016, we identified 337 persons with evidence of recent Zika virus infection. Of these, we excluded 140 (42%) because they did not meet the study criteria (Figure 2). Among the remaining 197 persons, median age was 42 (range 1–81) years, most (119/197; 60%) were female, and 11 (6%) were pregnant (Table). Median length of travel was 11 (range 2–177) days. The diagnosis of recent Zika virus infection was made by serologic testing for 134 (68%) persons, by molecular testing for 57 (29%), and by molecular and serologic testing for 6 (3%).

The Weibull distribution fit our data best (parameter estimates in online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1715-Techapp1.pdf). For the primary case set, our estimates for incubation period were median 6.2 (95% CI 5.7—6.6) days (Figure 1, panel B) and mean 6.4 (95% CI 5.7–7.0) days. We estimated that, among persons in whom symptoms would develop,

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DOI: http://dx.doi.org/10.3201/eid2305.161715

they would develop in 5% by 2.1 (95% CI 1.7–2.4) days and in 99% by 13.6 (95% CI 13.0–14.2) days (Figure 1, panel B; online Technical Appendix Table 2).

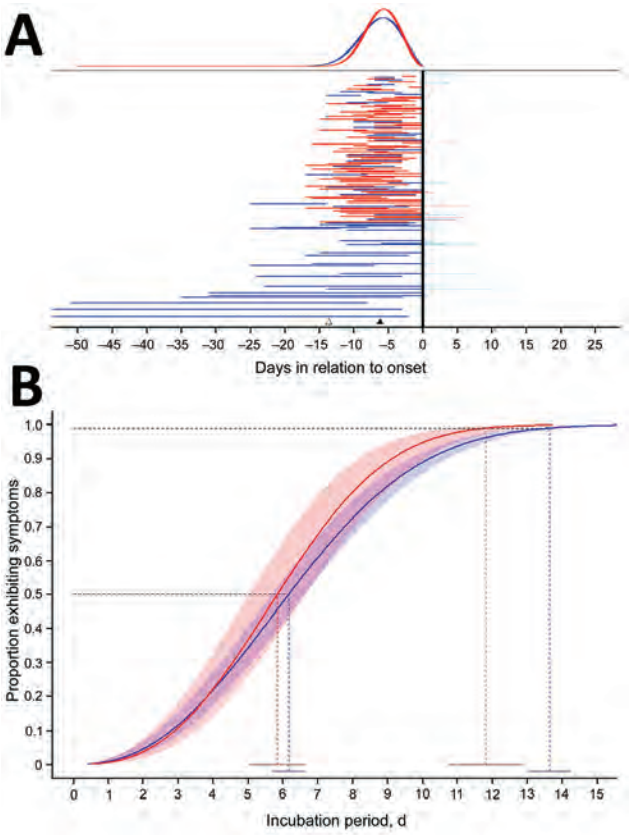

Figure 1. Estimated distribution of incubation period in days since infection for persons with evidence of recent Zika virus disease. A) Representation of individual interval censored travel data based on time of exposure relative to symptom onset (n = 197). Horizontal lines represent exposure times relative to onset. Vertical black line indicates symptom onset; red indicates persons with confirmed Zika virus disease; blue indicates all persons with Zika virus diseases; pink indicates exposure durations after symptom onset; and light blue indicates that these times did not contribute to the analysis. Individual data are sorted from bottom to top by exposure duration; to ease visible interpretation, we truncated long durations. The black triangle marks the estimated median incubation period for all Zika virus disease cases; the white triangle marks the estimated 95th quantile. The top panel shows the fitted Weibull density function; the blue line represents the distribution for all Zika virus disease cases; and the red line represents only those with confirmed Zika virus disease. B) Estimated distribution of time from infection to symptom onset (incubation period) for 197 persons with evidence of recent Zika virus infection (blue) and with confirmed Zika virus disease (red). The heavy line represents the estimated Weibull cumulative distribution function for the incubation period; 95% confidence bands are shown in red and blue shading. The 2 dotted lines represent the 50th and 99th quantiles; blue represents all cases; and red represents confirmed cases only. The solid horizontal line near the *x*-axis gives the point estimates and 95% CIs for the quantiles. Additional quantiles and CIs are shown in online Technical Appendix Table 2 (https://wwwnc.cdc.gov/EID/article/23/5/16-1715-Techapp1.pdf).

Of the 112 (57%) persons who had traveled for <2 weeks, cases were confirmed for 79 (71%). The age and sex distributions for these patients did not differ significantly from those of the primary case set (p = 0.67 and 0.44, respectively) (Table). The median length of travel was 8 (range 3–13) days. Zika virus diagnosis was confirmed by serologic testing for 47 (59%) patients, by molecular testing for 31 (39%), and by both methods for 1 (1%).

For patients with confirmed cases, we estimated the median incubation period to be 5.8 (95% CI 5.0–6.7) days (Figure 1, panel B; online Technical Appendix Table 2) and the mean to be 6.0 (95% CI 5.2–6.8) days. The quantile estimates (5%–95%) for these patients were similar to those for all travelers; however, among travelers with shorter travel durations and confirmed Zika virus infections, symptoms developed within 11.8 (95% CI 10.8 –12.9 days) days for 99%, compared with 13.6 days for all travelers.

On the basis of our analysis, we estimate that the incubation period for Zika virus is 3–14 days. We expect symptoms to develop within 1 week of infection for 50% and within 2 weeks for 99%. Our estimates for Zika virus incubation period are similar to those reported for other flaviviruses (*9*–*12*). The incubation period for Zika virus has

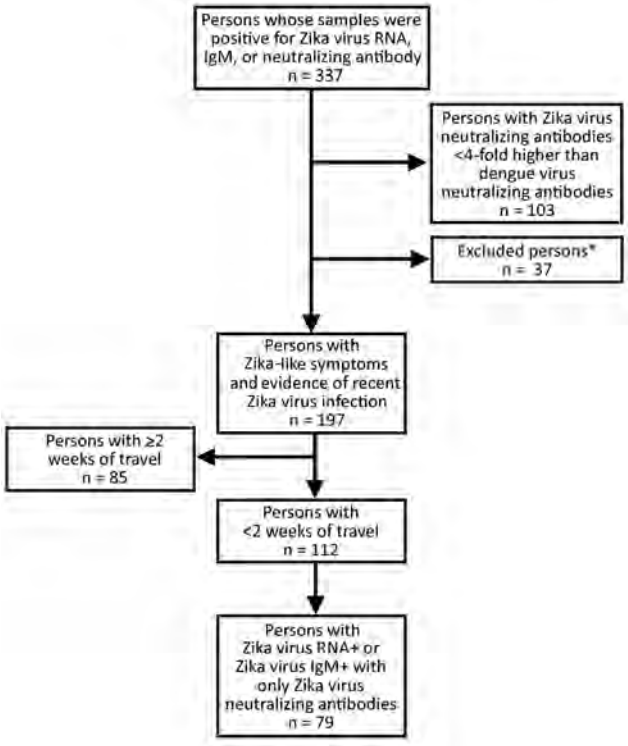

Figure 2. Persons with Zika virus–like symptoms and positive test results for Zika virus infection identified from samples received and tested for Zika virus infection at the Centers for Disease Control and Prevention. *Excluded for being asymptomatic, having congenital infection, having sexually transmitted infection, history of travel originating outside the United States, no date of symptom onset, symptom onset >2 months after travel return.

Table. Demographics, travel data, and laboratory testing results for Zika virus disease patients, United States, January 1, 2015, through June 23, 2016

Patient characteristic	All cases, no. (%), n = 197*	Confirmed cases, no. (%), n = 79†
Age, y		
0–19	19 (10)	10 (13)
20–39	71 (36)	29 (37)
40–59	79 (40)	32 (40)
≥60	27 (14)	7 (9)
Unknown	1 (<1)	1 (1)
Sex		
M	77 (39)	26 (33)
F	119 (60)	52 (66)
Unknown	1 (1)	1 (1)
Pregnant		
Yes	11 (6)	2 (3)
No	161 (82)	60 (76)
Unknown	25 (13)	17 (22)
Travel duration, d		
<7	24 (12)	15 (19)
7–13	88 (45)	64 (81)
14–20	31 (16)	0
21–27	12 (6)	0
≥28	42 (21)	0

*Persons with Zika virus–like symptoms and positive results for Zika virus RNA by real-time reverse transcription PCR or positive results for Zika or dengue virus IgM and Zika virus plaque reduction neutralization test (PRNT) results ≥10 and Zika virus titer ≥4-fold higher than dengue virus titer.
†Persons who traveled <2 weeks, experienced Zika virus–like symptoms, and had positive Zika virus RNA results by real-time reverse transcription PCR or positive Zika or dengue virus IgM results and PRNT ≥10 and dengue PRNT <10.

been estimated by Lessler et al., who reported data from 25 patients with variable exposure and laboratory evidence of infection (*13*). Their estimated median incubation period was similar to ours, 5.9 days, but the upper limit from that study was 18 days, which is 6 and 7 days longer than our estimates for the primary and secondary case sets, respectively. The difference in the upper limit was probably the result of the lower number of cases and higher variability in travel durations for their cohort.

Our analysis has several limitations. First, samples were submitted to the Centers for Disease Control and Prevention for all patients in this analysis, although guidance for testing recommended testing only persons with symptom onset <2 weeks after travel (*14*). Testing of all patients could have biased our sample population. Second, we included persons who were Zika virus IgM positive, considered as having recent infection. However, because the duration of IgM after Zika virus infection is not known, we might have included persons who had a prior infection unrelated to their most recent travel. Third, our analysis does not include other modes of transmission, such as sexual or congenital, for which incubation periods might differ. Fourth, we cannot be sure that all cases included in the analysis were caused by vector transmission because sexual transmission may have occurred during travel. Similarly, our primary case set included 11 pregnant women. Data suggest that the immunologic response to Zika virus infection might differ during pregnancy (*15*); however, in our analysis, the incubation periods of the pregnant women did not differ qualitatively from those of nonpregnant travelers.

Conclusions

According to our analysis, among Zika virus–infected travelers who will become symptomatic, 99% will experience symptoms within 2 weeks of exposure and 50% within 1 week. Persons for whom symptoms develop >2 weeks after travel and test results for a recent Zika virus infection are positive should be evaluated for alternative modes of transmission (e.g., sexual transmission) or local vectorborne transmission.

Acknowledgments

We thank the Zika Virus Response Epidemiology and Laboratory Teams and vectorborne disease surveillance coordinators in state and local health departments for their efforts with collection and sharing of data used in this analysis.

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Virulence Analysis of *Bacillus cereus* Isolated after Death of Preterm Neonates, Nice, France, 2013

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After the deaths of 2 preterm neonates with *Bacillus cereus* systemic infection in the same intensive care unit, we investigated the pathogenic potential of this bacterium. Genetic and virulence analysis indicated the neonates were infected with 2 different strains with a virulence potential similar to environmental strains, indicating likely patient immune response failure.

Bacillus cereus is a gram-positive, spore-forming bacterium that is widespread in the environment. In adults, *B. cereus* is involved mainly in gastrointestinal infection and is the third most common cause of food poisoning (*1*). Rarely, this bacterium causes invasive or fatal infections in high-risk patients, such as immunocompromised adult patients and preterm neonates who have an immature immune system that is mostly restricted to innate immunity (*2*–*4*).

In 2013, two preterm infants with *B. cereus* infection died in the same intensive care unit. As part of the investigation of these deaths, we conducted genetic and virulence analyses of *B. cereus* strains from the patients and from the environment.

The Study

In September 2013, tracheobronchial aspiration and blood cultures positive for *B. cereus* were obtained from 2 premature newborns hospitalized in the same intensive care unit. An unfavorable outcome led to the infants' deaths despite an appropriate treatment with wide-spectrum antibiotic drugs.

The first premature infant was female, born at 27 weeks and 2 days of gestation, and weighed 880 g. An emergency cesarean delivery was performed because of the mother's preeclampsia. The Apgar score at birth was 1-2-10, with bagging ventilation and intubation at 5 min after birth. No evidence of maternal–fetal transmission of infection was retrieved. On day 4, signs of infection were noted in the newborn, including respiratory distress, tachycardia, and a gray skin complexion. Investigations revealed elevated inflammatory markers (C-reactive protein level 88 mg/L). Empirical intravenous antimicrobial drug therapy (cefotaxime, gentamicin, and vancomycin) was started.

Tracheobronchial aspiration was performed and, a sample grew 10^6 CFU/mL of *B. cereus* identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MicroFlex LT; Bruker Daltonics, Billerica, MA, USA) (log score value of 2.07 matching with *B. cereus* reference strain DSM 31T, MALDI Biotyper v2.3). The blood culture remained sterile after 14 days. During her stay, the neonate had refractory hypoxemia due to a diffuse pulmonary lung parenchymal necrosis that required high-frequency ventilation and continuous thoracic drain. Despite an appropriate antimicrobial drug treatment (15 days of vancomycin followed by fluoroquinolone), the neonate had chronic hypoxemia and died at 26 days of age.

The second premature neonate, born 2 days after the first, was male, born at 29 weeks and 4 days of gestation, and weighed 1,480 g. A cesarean section was performed to enable the mother to start chemotherapy for a maternal malignancy, diagnosed at 26 weeks of gestation. The Apgar score at birth was 10. Physical examination indicated no sign of maternal or neonatal infection. On day 4, signs of infection were observed in the newborn, along with respiratory distress. The infant was reintubated, and antimicrobial drug therapy (cefotaxime, gentamicin, and vancomycin) was started.

Blood cultures were positive after 9 hours, and subcultures grew with *B. cereus* (log score 2.02). Catheter cultures were positive and grew 10^6 CFU/mL of *B. cereus* (log score 2.1). On day 5, despite appropriate care and sepsis control, the newborn showed severe neurologic impairment. Control cranial ultrasound revealed brain empyema, cerebral necrosis, and cranial hemorrhages (Figure 1). An unfavorable outcome led to the patient's death at 8 days of age from multiple organ failure and cerebral abscesses.

The hospital's infection control team looked for environmental reservoirs as potential sources of contamination

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DOI: http://dx.doi.org/10.3201/eid2305.161788

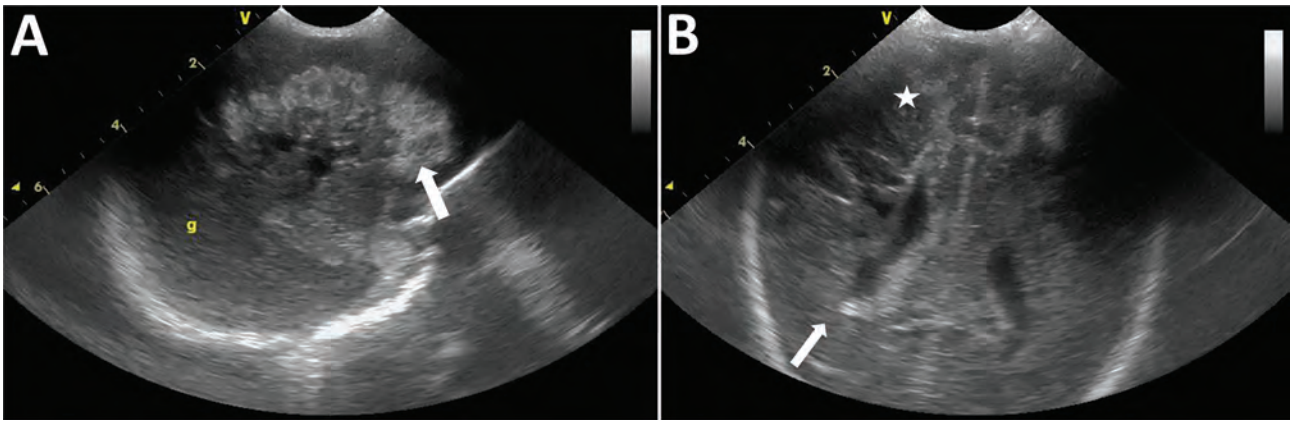

Figure 1. Standard echography cranial ultrasound of premature infant with *Bacillus cereus* sepsis, Nice, France, 2013. A) Left sagittal section showing large hemorrhagic hyperechogenic area of white material (white arrow). B) Frontal section showing right periventricular kystic hypoechogenic lesions (white arrow) with associated bilateral hemorrhagic hyperechogenic lesions (white star).

of the 2 newborns. Therefore, ventilation equipment, balloons used in manual ventilation, intravenous umbilical catheters, ultrasonic probes, linens (including towels and bedsheets), breast milk, and freeze-dried breast milk were collected and sent for microbiological analysis (Table 1). *B. cereus* cultures were positive for 5 environmental samples, including the surface of the incubator used for the first newborn (3 samples), ultrasonic probes (5 samples), and a bench surface used for bottle-feeding (5 samples). We compared all *B. cereus* strains, including those isolated from the 2 newborns, by using M13-PCR methods (*5*). This analysis revealed that the patients were infected by 2 different strains and that the environmental strains were different from strains isolated from patients. These data excluded a clonal transmission between the 2 patients and the hypothesis of a nosocomial outbreak caused by an emerging virulent strain (Figure 2, panel A). Nevertheless, a common source of infection for the 2 newborns by polyclonal strains cannot be excluded.

Table 1. Microbiological results of environmental sampling after deaths of 2 preterm neonates with *Bacillus cereus* infection, Nice, France, 2013*

Environmental site	No. positive/no. samples tested
Incubator, first newborn	3/7
Incubator, second newborn	0/7
Ultrasonographic probe	1/1
Bench surface used for bottle feeding	1/1
Control incubator 1	0/4
Control incubator 2	0/4
Control ultrasonographic probe	0/1
Heated humidifier for 2 control incubators	0/2
Wet bulb for 2 control incubators	0/2
Heating ramp for control incubator 1	0/1
Control incubator mattresses	0/2
Babies' sheets	0/1
Towels	0/1
Sterile field	0/1
Ultrasound gel dispenser bottles	0/2
Used ultrasound gel	0/1
Air filtration/ventilation equipment	0/1
Air flow sensor for ventilator	0/1
Heating unit for ventilator	0/1
Food refrigerator	0/1
Various cutlery	0/2

We screened the isolated strains for *B. cereus* main virulence factor genes hemolysin BL, nonhemolytic enterotoxin, cytotoxin K, and hemolysin II (Table 2) by using PCR and toxin production assay methods (*6*–*9*). Both patient and environmental isolates produced toxins. We further assessed the virulence potential using an in vivo model of *Drosophila melanogaster* infection. To validate the capacity of this model to detect the virulence of various bacterial strains, we first infected wild-type flies with *Escherichia coli* CIP 102181, *Staphylococcus aureus* CIP 110856, and *B. cereus* CIP 66.24T. We grew bacteria in Luria-Bertani broth overnight at 37°C and subcultured them up to an optical density of 0.8 at 600 nm. We dipped a tungsten needle into an equal volume of bacterial suspension or phosphate-buffered saline (control) and used it to prick 20–30 adult male flies (*10*). All flies infected with *S. aureus* and *B. cereus* died after 20 and 12 hours, respectively. Flies infected with *E. coli* displayed a survival rate similar to that of control flies, enabling us to validate *D. melanogaster* as a model for evaluating the strains' virulence potential (Figure 2, panel B).

We used the same protocol to compare all *B. cereus* strains. We found no statistical difference in survival between flies infected with the different *B. cereus* strains, including *B. cereus* CIP 66.24T (Figure 2, panel C). These data correlate with the absence of a specific virulence signature for those strains (Table 2).

Conclusions

Considering the fatal outcome of the 2 infections despite appropriate antimicrobial drug therapy, we addressed the question of a high virulence potential of the patients'

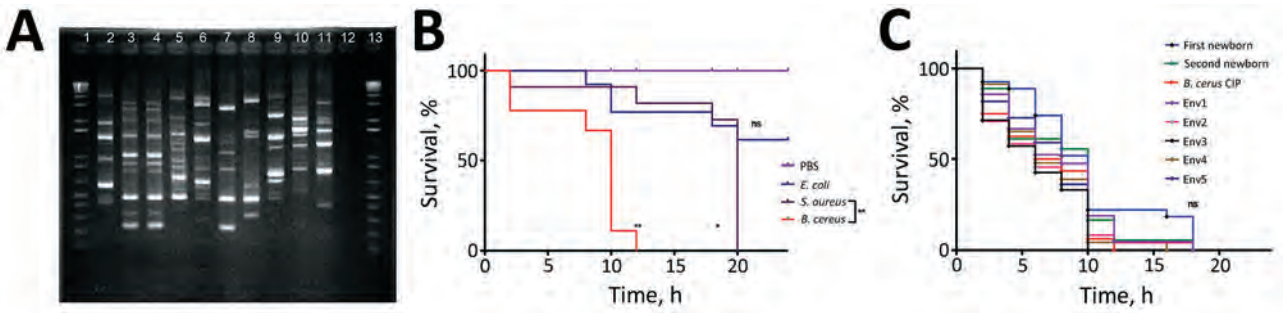

Figure 2. Genetic and virulence analyses of *Bacillus* spp. strains isolated from 2 preterm neonates with *B. cereus* infection and environmental sampling from intensive care unit, Nice, France, 2013. A) Molecular typing using M13-PCR methods, as described by Guinebretiere et al. (*5*). Lane 1, DNA ladder; lane 2, tracheobronchial fluid, first newborn; lane 3, blood culture, second newborn; lane 4, catheter, second newborn; lane 5, incubator surface, first newborn (Env1); lane 6, incubator surface, first newborn (Env2); lane 7, incubator surface, first newborn (Env3); lane 8, ultrasonographic probe (Env4); lane 9, bench surface used for bottle feeding (Env5); lane 10, incubator surface, second newborn (1); lane 11, incubator surface, second newborn (2); lane 12, negative control; lane 13, DNA ladder. B) Survival of flies infected with *Escherichia coli* CIP 102181, *Staphylococcus aureus* CIP 110856, and *B. cereus* CIP 66.24 T, compared with survival of control flies injected with phosphate-buffered saline (PBS). *p <0.01; **p <0.001; ns, not significant (all by Gehan-Breslow-Wilcoxon χ^2 test). C) Survival of flies infected with the different strains of *B. cereus* tested. Env, environmental; ns, not significant by Gehan-Breslow-Wilcoxon χ^2 test.

B. cereus strains by testing for the presence of virulence factor genes and expression levels. We found a similar virulence factor profile in the patients and in the environmental strains. This profile suggested that the outcome of the infection was probably not linked to the virulence potential of the strains.

We then used *D. melanogaster* as an infection model. We chose this model because flies rely only on innate immunity to survive infections, similar to preterm newborns, who have immature immune systems. We found that all isolated *B. cereus* strains (patient and environmental strains) displayed a similar killing potential, suggesting that the fatal outcome in both newborns was due not to the emergence of a hypervirulent strain but rather to a similar pathogenic potential for all *B. cereus* strains toward at-risk patients.

Given that *B. cereus* is ubiquitous in the environment and potentially fatal in preterm neonates, it appears critical to determine how these 2 neonates were infected and why they died, as well as why other preterm neonates hospitalized concurrently in the same room of the intensive care unit remained uninfected. Further investigations would be necessary to determine whether the deaths were a consequence of an innate immune defect, a high bacterial load at time of contamination, or a combination of both parameters.

Our study, along with previous ones (*3*,*4*,*11*–*13*), reinforces the idea that *B. cereus* is an underestimated emerging pathogen that can be involved in fatal healthcare-associated infections in premature newborns. Our results indicate that all *B. cereus* strains display potentially pathogenic properties toward at-risk patients. Considering that *B. cereus* is ubiquitous in the environment, it is essential to emphasize the necessity of strict hygiene measures and protocols to prevent bacterial transmission. Evaluating an immune response capacity in at-risk patients must be considered to avoid a fatal outcome from *B. cereus* infection.

Table 2. Virulence factor analysis of strain characteristics of bacteriologic samples obtained after deaths of 2 preterm neonates with *Bacillus cereus* infection, Nice, France, 2013*

Tested strain	Source of sample	Gene				Genotype group (%)	Nhe production inde)†	Hbl detection limit
		cytK1	*cytK2*	*ces*	*hlyII*			
First newborn	Tracheobronchial aspiration	–	+	–	–	III (99.72)	+++	–
Second newborn	Blood culture	–	+	–	–	IV (100)	+	1/64
Second newborn	Catheter	–	+	–	–	IV (100)	+	1/64
Environmental isolate 1	Incubator surface, first newborn	–	–	–	–	II (97.71)	++	–
Environmental isolate 2	Incubator surface, first newborn	–	+	–	–	III (99.72)	+++	–
Environmental isolate 3	Incubator surface, first newborn	–	+	–	+	IV (100)	+++	1/64
Environmental isolate 4	Ultrasonographic probe	–	+	–	–	IV (100)	+++	1/32
Environmental isolate 5	Bench surface used for bottle feeding	–	–	–	–	III (100)	+++	–

*Hbl, hemolytic BL toxin; Nhe, nonhemolytic enterotoxin; +, positive; –, negative.
†Nhe production level: +, low; ++ moderate; +++, high.

Acknowledgments

We thank the Department of Bacteriology of Nice University Hospital, Nice, France, for technical support, and Elsa Garcia, Pierre Leopold, and Julien Colombani for their help with fly experiments. We also thank Orane Visvikis and Nicolas Degand for careful reading of the manuscript and insightful comments.

This study was supported by Fondation Recherche Médicale (FRM grant #FDM20150632804 to R.L.). L.B. is supported by Inserm and the Fondation L'Association pour la Recherche sur le Cancer (RAC15014AAA).

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The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use

Robert Gaynes

After just over 75 years of penicillin's clinical use, the world can see that its impact was immediate and profound. In 1928, a chance event in Alexander Fleming's London laboratory changed the course of medicine. However, the purification and first clinical use of penicillin would take more than a decade. Unprecedented United States/Great Britain cooperation to produce penicillin was incredibly successful by 1943. This success overshadowed efforts to produce penicillin during World War II in Europe, particularly in the Netherlands. Information about these efforts, available only in the last 10–15 years, provides new insights into the story of the first antibiotic. Researchers in the Netherlands produced penicillin using their own production methods and marketed it in 1946, which eventually increased the penicillin supply and decreased the price. The unusual serendipity involved in the discovery of penicillin demonstrates the difficulties in finding new antibiotics and should remind health professionals to expertly manage these extraordinary medicines.

According to British hematologist and biographer Gwyn Macfarlane, the discovery of penicillin was "a series of chance events of almost unbelievable improbability" (*1*). After just over 75 years of clinical use, it is clear that penicillin's initial impact was immediate and profound. Its detection completely changed the process of drug discovery, its large-scale production transformed the pharmaceutical industry, and its clinical use changed forever the therapy for infectious diseases. The success of penicillin production in Great Britain and the United States overshadowed the serendipity of its production and the efforts of other nations to produce it. Information on penicillin production in Europe during World War II, available only in the last 10–15 years, provides new insights into penicillin's story.

Dawn of Chemotherapy and the "Magic Bullet"

At the beginning of the 20th century, Paul Ehrlich pioneered the search for a chemical that would kill a microorganism and leave the host unaltered—the "magic bullet." Ehrlich also coined the term chemotherapy: "There must be planned chemical synthesis: proceeding from a chemical substance with recognizable activity, making derivatives from it, and then trying each to discover the degree of its activity and effectiveness. This we call chemotherapy" (*2*). After extensive testing, he found a drug with activity against the bacterium *Treponema pallidum*, which causes syphilis. The introduction of this drug, arsphenamine (Salvarsan), and its chemical derivative neoarsphenamine (Neosalvarsan) in 1910 ushered in a complete transformation of syphilis therapy and the concept of chemotherapy. Unfortunately, despite exhaustive searches, the promise of more magic bullets for microbial therapy remained elusive. For 20 years, Salvarsan and Neosalvarsan were the only chemotherapy for bacterial infections.

Alexander Fleming's Discovery

A chance event in a London laboratory in 1928 changed the course of medicine. Alexander Fleming, a bacteriologist at St. Mary's Hospital, had returned from a vacation when, while talking to a colleague, he noticed a zone around an invading fungus on an agar plate in which the bacteria did not grow. After isolating the mold and identifying it as belonging to the *Penicillium* genus, Fleming obtained an extract from the mold, naming its active agent penicillin. He determined that penicillin had an antibacterial effect on staphylococci and other gram-positive pathogens.

Fleming published his findings in 1929 (*3*). However, his efforts to purify the unstable compound from the extract proved beyond his capabilities. For a decade, no progress was made in isolating penicillin as a therapeutic compound. During that time, Fleming sent his *Penicillium* mold to anyone who requested it in hopes that they might isolate penicillin for clinical use. But by the early 1930s, interest had waned in bringing to life Paul Ehrlich's vision of finding the magic bullet.

Discovery of Prontosil and Sulfa Drugs

This dismal outlook on chemotherapy began to change when Gerhard Domagk, a German pathologist and bacteriologist, found bacteriologic activity in a chemical derivative from oil dyes called sulfamidochrysoïdine (also known as Prontosil). This compound had bacteriologic activity in animals, but strangely, none in vitro. Prontosil had limited but definite success when used to treat patients with bacterial infections, including Domagk's own child. A German company patented the drug, and ultimately, Domagk won a Nobel Prize in 1939. The paradox of Prontosil's in vivo success but lack of success in vitro was explained in 1935, when French scientists determined that only part of Prontosil was active: sulfanilamide. In animals, Prontosil was metabolized into sulfanilamide. Within 2 years, sulfanilamide

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DOI: http://dx.doi.org/10.3201/eid2305.161556

and several derivative sulfa drugs were on the market. The success of sulfanilamide changed the cynicism about chemotherapy of bacteria (*1*).

Isolation of Penicillin at Oxford University

The success of sulfa drugs sparked interest in finding other agents. At Oxford University, Ernst Chain found Fleming's 1929 article on penicillin and proposed to his supervisor, Howard Florey, that he try to isolate the compound. Florey's predecessor, George Dreyer, had written Fleming earlier in the 1930s for a sample of his strain of *Penicillium* to test it for bacteriophages as a possible reason for antibacterial activity (it had none). However, the strain had been saved at Oxford. In 1939, Howard Florey assembled a team, including a fungal expert, Norman Heatley, who worked on growing *Penicillium* spp. in large amounts, and Chain, who successfully purified penicillin from an extract from the mold. Florey oversaw the animal experiments. On May 25, 1939, the group injected 8 mice with a virulent strain of *Streptococcus* and then injected 4 of them with penicillin; the other 4 mice were kept as untreated controls. Early the next morning, all control mice were dead; all treated mice were still alive. Chain called the results "a miracle." The researchers published their findings in The Lancet in August 1940, describing the production, purification, and experimental use of penicillin that had sufficient potency to protect animals infected with *Streptococcus pyogenes, Staphylococcus aureus,* and *Clostridium septique* (*4*).

After the Oxford team had purified enough penicillin, they began to test its clinical effectiveness. In February 1941, the first person to receive penicillin was an Oxford policeman who was exhibiting a serious infection with abscesses throughout his body. The administration of penicillin resulted in a startling improvement in his condition after 24 hours. The meager supply ran out before the policeman could be fully treated, however, and he died a few weeks later. Other patients received the drug with great success. The Oxford team then published their clinical findings (*5*). At the time, however, pharmaceutical companies in Great Britain were unable to mass produce penicillin because of World War II commitments. Florey then turned to the United States for assistance.

Penicillin and US Involvement

In June 1941, Florey and Heatley traveled to the United States. Concerned about the security of taking a culture of the precious *Penicillium* mold in a vial that could be stolen, Heatley suggested that they smear their coats with the *Penicillium* strain for safety on their journey. They eventually arrived in Peoria, Illinois, to meet with Charles Thom, the principal mycologist of the US Department of Agriculture, and Andrew Jackson Moyer, director of the department's Northern Research Laboratory. Thom corrected the identification of Fleming's mold to *P. notatum*; it was initially identified as *P. rubrum* (*1*).

Thom also recognized the rarity of this *P. notatum* strain because only 1 other strain in his collection of 1,000 *Penicillium* strains produced penicillin. The strain that was eventually used in mass production was a third strain, *P. chrysogenum,* found in a moldy cantaloupe in a market, which produced 6 times more penicillin than Fleming's strain. When a component of the media that Heatley used to grow the mold in England was unavailable, A.J. Moyer suggested using corn steep liquor, a waste product from the manufacture of cornstarch that was available in large quantities in the midwestern United States. With corn steep liquor, the investigators produced exponentially greater amounts of penicillin in the filtrate of the mold than the Oxford team had ever produced. Heatley remained in Peoria for 6 months to work on methods of growing *Penicillium* strains in large quantities. Florey headed east to interest the US government and multiple drug companies in penicillin production. The US government took over all penicillin production when the United States entered World War II. Researchers at drug companies developed a new technique for producing enormous quantities of penicillin-producing *Penicillium* spp.: deep-tank fermentation. This process adapted a fermentation process performed in swallow dishes to deep tanks by bubbling air through the tank while agitating it with an electric stirrer to aerate and stimulate the growth of tremendous quantities of the mold. Unprecedented United States/Great Britain cooperation for penicillin production was incredibly successful. In 1941 the United States did not have sufficient stock of penicillin to treat a single patient. At the end of 1942, enough penicillin was available to treat fewer than 100 patients. By September 1943, however, the stock was sufficient to satisfy the demands of the Allied Armed Forces (*6*).

Public Awareness: The Fleming Myth

Early in 1942, Florey and Heatley went back to England. Because of the shortage of penicillin supplies coming from the United States, the Oxford group still had to produce most of the penicillin they tested and used. In August 1942, Fleming obtained some of the Oxford group's supply and successfully treated a patient who was dying of streptococcal meningitis. When the patient recovered, the cure was the subject of a major article in The Times newspaper in Great Britain, which named Oxford as the source of the penicillin. However, neither Florey nor Fleming was acknowledged in the article, an oversight quickly corrected by Fleming's boss, Sir Almroth Wright. He wrote a letter to

The Times expounding on Fleming's work and suggested that Fleming deserved a "laurel wreath." Fleming happily talked to the press. Florey not only did not speak with the press but prohibited any member of the Oxford team from giving interviews, leading many to erroneously believe that Fleming alone was responsible for penicillin.

Secrecy in Wartime England

The British government went to great lengths to prevent the means for producing penicillin from falling into enemy hands. However, news about penicillin leaked out. A Swiss company (CIBA, Basal, Switzerland) wrote to Florey requesting *P. notatum*. Concerned about responding, Florey contacted the British government. Agents attempted to track down where Fleming's *Penicillium* cultures had been distributed. Fleming wrote, "During the past 10 years I have sent out a very large number of cultures of *Penicillium* to all sorts of places, but as far as I can remember NONE have gone to Germany" (*7*). Florey believed that, without the mold, no one in Germany could produce penicillin even though his publication had provided a "blueprint" for its small scale manufacture. Florey was wrong, and so was Fleming.

Fleming had sent a culture of *Penicillium* strains to "Dr. H. Schmidt" in Germany in the 1930s. Schmidt was unable to get strain to grow, but even though the Germans did not have a viable strain, other Europeans did.

Production during World War II

France

Someone at Institut Pasteur in France, had Fleming's strain. In 1942, efforts began at Institut Pasteur and Rhone-Poulenc to produce penicillin. Eventually, German officials found out and, in early 1944, the Germans asked the French for their *P. notatum*. They were given a false strain that did not produce penicillin. With limited supplies, the French produced only enough penicillin to treat ≈30 patients before the wars end.

The Netherlands

The situation in the Netherlands was different. The Centraalbureau voor Schimmelcultures (CBS) near Utrecht had the largest fungal collection in the world. A published list of their strains in 1937 included *P. notatum*. A letter found at CBS shows that in February 1942 the Nazis asked CBS to send their strain of *P. notatum* to Dr. Schmidt in Germany, mentioning penicillin in the letter. CBS told the Germans they did not have Fleming's strain of *P. notatum*. In fact, they did. In the 1930s, Fleming had sent his strain to Johanna Westerdijk, the CBS director. Westerdijk could not refuse the German request for their strain of *P. notatum* but sent them the one that did not produce penicillin.

Efforts to produce penicillin in the Netherlands went underground at a company in Delft, the Nederladsche Gist-en Spiritusfabriek (the Netherlands Yeast and Spirit Factory, NG&SF). After the German occupation in 1940, NG&SF was still allowed to function. Because Delft was not bombed in the war, NG&SF's efforts were unaffected. In early 1943, NG&SF's executive officer, F.G. Waller, secretly wrote to Westerdijk at CBS, asking for any *Penicillium* strains that produced penicillin. In January 1944, Westerdijk sent all of CBS' *Penicillium* strains to NG&SF.

Four reports in NG&SF records detailed their efforts (*8*). In the first report, NG&SF scientists tested 18 *Penicillium* strains from CBS; they found 1 strain with the greatest antibacterial activity, which was coded P-6 and was identified as *P. baculatum.* The second report discussed how NG&SF scientists then isolated an extract from P-6. They gave the substance in the extract the code name Bacinol after the species from which it was derived and to keep the Germans unaware of what they were doing (Figure). As Waller wrote, "When we first started looking, in 1943, only one publication was available, that of Fleming in 1929. It was on that basis we started our research" (*6*). NG&SF researchers then had help from an unanticipated source. In 1939, Andries Querido was employed by NG&SF as a part-time advisor. By January 1943, however, his Jewish background limited his visits. On his last visit in the summer of 1944, Querido met someone in Amsterdam's Central Train Station who gave him a copy of the latest Schweizerische Medizinische Wochenschrift (Swiss Medical Journal), which he passed on to the NG&SF scientists. The June 1944 issue contained an article entirely devoted to penicillin, showing the results that the Allies had achieved, including details of penicillin growth in corn steep extract, the scaling up of penicillin production, the measurement of strength by the Oxford unit, results of animal and human studies, and identification of the bacteria known to be susceptible to penicillin. The third report described how NG&SF scientists isolated Bacinol from the extract using the information supplied secretly by Querido.

Large-scale production would be difficult to do and to keep secret from the Germans, especially with a German guard on site. However, NG&SF scientists used an obvious ploy to keep the German guard, who knew nothing about microbiology, at bay: they kept him drunk. "We did have a German guard whose job it was to keep us under surveillance, but he liked gin, so we made sure he got a lot. He slept most afternoons" (*6*). NG&SF scientists used milk bottles for growing large quantities of *Penicillium* mold. From July 1944 until March 1945, production of Bacinol continued, as detailed in the fourth report. At the end of the war, the NG&SF team still did not know if Bacinol was actually penicillin until they tested it

Figure. Bacinol 2, building named in honor of the site of efforts in the Netherlands to produce penicillin during World War II and the drug produced by the Netherlands Yeast and Spirit Factory in Delft. Bacinol was a code name for penicillin. Source: https://commons.wikimedia.org/wiki/File:Delft_-_Gevel_Bacinol_2.jpg

against some penicillin from England, proving it to be the same compound. NG&SF began marketing the penicillin they produced in January 1946. Although the original building where Bacinol was produced was demolished, NG&SF named a new building in honor of their WWII efforts (Figure).

The Nazis eventually succeeded in making penicillin by October 1944. However, Allied air raids crippled mass production of the drug (*9*).

Patents

The issue of a patent for penicillin was a controversial problem from the beginning. Chain believed that obtaining a patent was essential. Florey and others viewed patents as unethical for such a life-saving drug. Indeed, penicillin challenged the basic notion of a patent, considering it was a natural product produced by another living microorganism. The prevailing view Great Britain at the time was that a process could be patented, but the chemical could not. Merck (New York, NY, USA) and Andrew Jackson Moyer each filed patents on the process of penicillin production with no opposition. Eventually, at war's end, British scientists were faced with paying royalties for a discovery made in England. The penicillin production at NG&SF turned out to be more than of historical interest. Because NG&SF had researched and developed their own penicillin using their own mold culture, *P. baculatum*, and used their own production methods, they were not embroiled in any patent clash; the marketing of their penicillin eventually increased penicillin supply and decreased prices.

Nobel Prize in 1945

Penicillin's colossal effects led to the awarding of the Nobel Prize in Medicine and Physiology in 1945 to Fleming, Chain, and Florey. Penicillin was isolated from other microorganisms, which led to a new term, antibiotics. Using similar discovery and production techniques, researchers discovered many other antibiotics in the 1940s and 1950s: streptomycin, chloramphenicol, erythromycin, vancomycin, and others.

Conclusions

Lessons can be learned from the circumstances surrounding the discovery of penicillin. The US government's successful takeover of penicillin's production and the unprecedented cooperation among drug companies (and nations) should strongly encourage public/private partnerships as we search for additional effective antimicrobial drugs. In addition, despite their essential value in modern medicine, antibiotics are also the only class of drugs that lose their efficacy with large-scale use as bacteria develop antibiotic resistance. We now are struggling with resistant bacteria that cause infections that are virtually untreatable. Infections such as those occurring after transplantation and surgical procedures, caused by these highly antibiotic-resistant pathogens, are threatening all progress in medicine. Yet, drug companies, some of the same companies that helped develop penicillin, have nearly abandoned efforts to discover new antibiotics, finding them no longer economically worthwhile. The dry pipeline for new antibiotics has led

the Infectious Diseases Society of America and others to call for a global commitment to the development of new agents (*10*). We also must expertly manage the drugs that are currently available. The noteworthy serendipity involved in the discovery of penicillin should remind us that new antibiotics are difficult to find and, more important, should make us mindful when using these limited medical treasures.

Acknowledgment

The author thanks Monica Farley for her helpful review of the manuscript.

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Persistence of Zika Virus in Breast Milk after Infection in Late Stage of Pregnancy

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DOI: http://dx.doi.org/10.3201/eid2305.161538

We detected Zika virus in breast milk of a woman in Brazil infected with the virus during the 36th week of pregnancy. Virus was detected 33 days after onset of signs and symptoms and 9 days after delivery. No abnormalities were found during fetal assessment or after birth of the infant.

Zika virus belongs to the family *Flaviviridae* and was first described in 1947. The first outbreak of infection with this virus was on Yap Island, Micronesia, in 2007 (*1*). The largest outbreak was in French Polynesia in 2013 (*2*).

The first cases of infection in Brazil were reported in Bahia State in 2015. Zika virus has since spread to ≥14 states in Brazil. Recently, the World Health Organization concluded that Zika virus is a cause of congenital brain abnormalities, including microcephaly; growth restriction and other damage, such as ophthalmologic alterations, also have been observed in neonates (*3*–*7*). We report a case of Zika virus infection in Brazil in an advanced stage of pregnancy and persistence of virus in breast milk 33 days after onset of signs and symptoms and 9 days after delivery.

A 28-year-old pregnant woman in the 36th week of gestation and living in Manaus, Brazil, reported mosquito bites and local infestation by *Aedes aegypti* mosquitoes in her neighborhood. She became ill and had a low-grade fever (temperature 38°C), rash (Figure), myalgia, and joint pain in the hands and wrists. PCR of blood samples showed a positive result for Zika virus (*8*). On the 4th day after illness onset, her clinical symptoms worsened, and she went to São Paulo, Brazil, for clinical evaluation. A timeline of symptoms and results of radiographic and laboratory studies is shown in the Figure.

General examinations were requested, and a PCR for Zika virus was repeated for blood and urine samples. Virus was detected only in urine. Serologic analysis detected dengue virus IgM and IgG; no antibodies against nonstructural protein 1 of this virus were detected. Results of reverse transcription PCR (RT-PCR) were negative for chikungunya virus. These findings were compatible with acute or recent Zika virus infection.

Fetal assessment was performed by using morphologic ultrasound at 35, 36, 37, and 38 weeks of gestation. We found no evidence of growth restriction, microcephaly, or cerebral calcifications. On the 22nd day after illness onset, blood and urine samples were tested by RT-PCR for Zika virus; results were negative. However, a colostrum sample was tested by RT-PCR and contained Zika virus (244×10^4 copies/mL) (*8*).

The baby was delivered during the 38th gestational week and had Apgar scores of 9 at 1 minute and 10 at 5 minutes. Birthweight was 2,860 g, and the newborn had a normal cranial circumference. RT-PCR for Zika virus was performed for amniotic fluid, umbilical cord blood, and placenta samples; results were negative. A urine sample from the newborn also showed a negative result for virus. However, breast milk remained positive for Zika virus.

Analysis of the placenta showed maturation compatible with the third trimester of gestation, preservation of chorioamniotic membranes, and no signs of infection or malignancy. No viral inclusions were observed. After birth, the mother and baby remained in good clinical condition and showed no signs or symptoms of infection. Breastfeeding was not recommended because of persistence of virus detected by PCR in breast milk. The mother and baby were discharged 2 days after birth.

We performed viral culture on Vero cells of breast milk and colostrum samples (Figure). A cytopathic effect was observed, which demonstrated viability and infectivity of the virus.

The most recent RT-PCR for Zika virus was performed for breast milk 33 days after onset of signs and symptoms and 9 days after delivery. RT-PCR results remained positive, and a high virus load (216,000 copies/mL) was observed. The mother and the medical team supported a decision to avoid breast-feeding once RT-PCR confirmed presence of the virus.

No studies have confirmed Zika virus transmission by breast-feeding or provided knowledge about the pathophysiology of infection. Our report describes a case of Zika virus infection in a patient at 36 weeks of pregnancy. The patient and baby remained well after delivery, with no evidence of transmission of Zika virus to the newborn. However, we detected persistence of virus by RT-PCR in breast

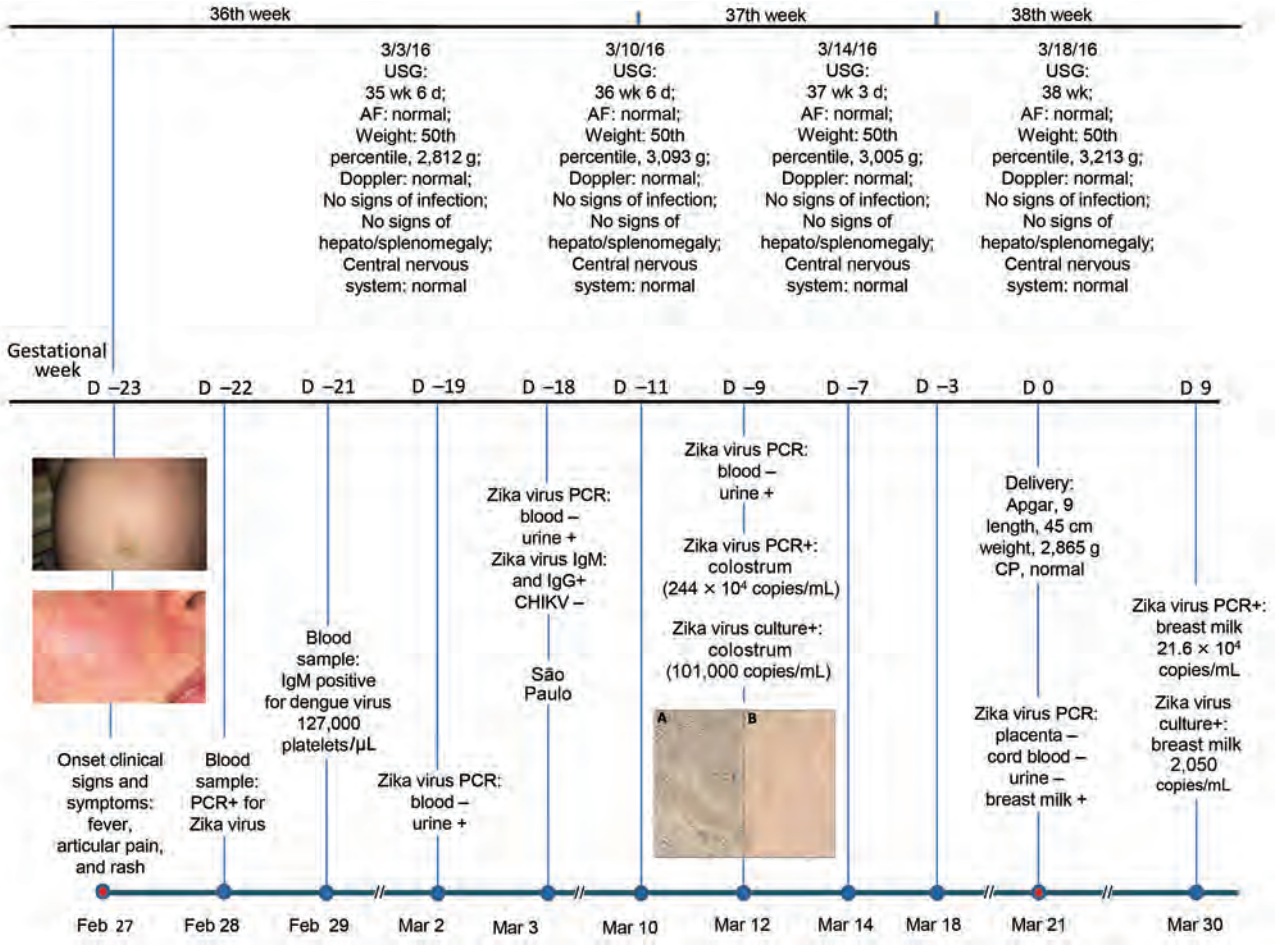

Figure. Timeline and clinical findings for a 28-year-old woman in the 36th week of pregnancy who had persistence of Zika virus in breast milk after infection in late stage of pregnancy and for her newborn, Manaus, Brazil. Top: ultrasound result for mother, fetus, and newborn. Bottom: follow-up test results for mother, fetus, and newborn. The 2 panels on the left show abdominal (top) and facial (bottom) rashes on the mother at the time of illness onset. Middle panels: Vero cell culture of breast milk and colostrum. A) Cells not infected with Zika virus. Original magnification ×10. B) Cells infected with Zika virus. Original magnification ×10. AF, amniotic fluid; CHIKV, chikungunya virus; CP, cranial perimeter; USG, ultrasound guidance.

milk from samples during the pregnancy (in colostrum) 33 days after onset of signs and symptoms (in breast milk).

Two studies have reported Zika virus in breast-feeding–related fluids. One study reported a virus load of 2.9×10^4 copies/mL by RT-PCR but no replicative virus in culture (*9*). A second study reported a virus load of 8.5×10^4 copies/mL and infective viral particles 3 days after birth (*10*).

We detected Zika virus in colostrum (2.44×10^6 copies/mL) and breast milk 9 days after birth (216,000 copies/mL) by PCR. We also observed cytopathic effect in virus culture, which showed infectivity of the virus. Our data provide evidence that Zika virus can persist in some tissues for a long period. Moreover, viral culture showed potential infectivity of the virus.

The World Health Organization does not recommend that mothers avoid breast-feeding in cases such as the one mentioned in this report. However, with indications that virus might be present and persistent in breast milk, further studies should be performed to elucidate the potential transmission of Zika virus to the newborn.

Acknowledgments

We thank Claudia Nunes dos Santos for assisting with immunohistochemical analysis.

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No Such Thing as Chronic Q Fever

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DOI: https://dx.doi.org/10.3201/eid2305.151159

Modern diagnostic methods enable clinicians to look beyond a diagnosis of chronic Q fever and discern whether patients instead have persistent focalized *Coxiella burnetii* infection(s). Use of these methods and development of criteria to define and treat such infections, especially cardiovascular infections, will improve the prognosis for patients previously thought to have chronic Q fever.

We read with interest the article by Kampschreur et al. (*1*), which in our opinion conveys a perspective that is incorrect concerning the diagnostic algorithm and treatment of Q fever. Kampschreur et al.'s article characterizes the understanding and management of Q fever by the Dutch Q Fever Consensus Group. However, this consensus opinion may be erroneous if developed without input from disease experts with long clinical experience.

Kampschreur et al.'s use of the term chronic Q fever is misleading because it may lead to inadequate treatment of persistent focalized *Coxiella burnetii* infection(s). The obsolete term chronic Q fever should be abandoned to prevent confusion between endocarditis, vascular infections, osteoarticular infections, lymphadenitis, genital infection, and pericarditis, which occurred in 68%, 20%, 7%, 6%, 3%, and 1%, respectively, of 494 patients with persistent focalized infection(s) who we followed during 2007–2015 at the French National Referral Center for Q Fever in Marseille, France (unpub. data).

Another example of the deleterious effect of the Dutch Q Fever Consensus Group guidelines is the absence of screening echocardiography in the standard work-up for patients with Q fever in the Netherlands (*2*). Because endocarditis has been reported in patients with clinically silent, undiagnosed valvulopathies (*3*), we recommend systematic echocardiography for all persons with acute Q fever. Endocarditis develops in most untreated Q fever patients who have extensive valvulopathy; however, Million et al. (*4*) showed that it did not develop in patients who received prophylaxis. This finding led us to recommend prophylaxis for acute Q fever patients with valvulopathy at the French National Referral Center for Q Fever; over the past 10 years, this strategy has reduced the incidence of Q fever endocarditis in patients at the center (*5*). Despite these observations, which were confirmed in the Netherlands in 2015 (*6*), the standard work-up for Q fever patients in that country has not included screening echocardiography since 2010 (*2*), leaving patients with clinically silent valvulopathy untreated.

Specific defining criteria for endocarditis (*7*) are needed to enable comparison of clinical series. Use of the term chronic to define cardiovascular infections in patients with Q fever is misleading. Indeed, valvular vegetations were recently reported in acute Q fever (*8*). Q fever vascular infections must be distinguished in the context of mycotic aneurisms, small saccular and embolic consequences of endocarditis that may go unnoticed, and underlying vascular disease. Positron emission tomography (PET) scanning has been used effectively in the Netherlands to systematically detect the localization of infection in patients with elevated serologic test results (*9*). PET scanning dramatically improves the diagnosis of cardiovascular infections (*10*). However, because the Dutch criteria lack clinical relevance (*7*), many cases of endocarditis were missed, and diagnoses of vascular infection were retained in the presence of mycotic aneurysms. These misdiagnoses

explain the low proportion of endocarditis cases compared with vascular infections in the Dutch series (15% vs. 36%, as reported by Kampschreur et al. (*1*)) compared with the series in our center (68% vs. 20%; unpub. data).

Endocarditis and vascular infections, whose first symptoms may be fatal decompensation or stroke, can be prevented in Q fever patients by implementing systematic screening echocardiography, phase I IgG monitoring, and PET scanning of patients with vascular disease (*10*). In our experience, only 1 patient with uncontrolled Q fever endocarditis has died since 2006, when we began following this protocol (*3*). The patient had a cardiac valve replacement 1 year before dying, but his phase I IgG titer was low (1:200), and *C. burnetii* PCR for his valve was negative, so no treatment was prescribed.

Reanalysis of the Q fever literature by different teams has brought challenging concepts to light (*7*). In a series from the Netherlands (*1*), 4 patients were shown to have died from endocarditis and 2 from vascular infections. These patients may have had better outcomes if the methods we propose here had been followed. Conversely, high serologic titers are not definite proof of persistent focalized infection, as illustrated in an outbreak in French Guiana, where exceptionally high serologic titers have been observed, but persistent focalized infections have rarely been diagnosed (*10*).

Accurate identification of persistent focalized *C. burnetii* infections will improve patient outcomes by preventing long-term, organ-specific, lethal complications (e.g., vascular infections are a risk for vascular rupture, lymphadenitis is a risk for lymphoma) and by avoiding drug side effects in patients with isolated elevated serologic test results. Clinicians should look beyond a diagnosis of chronic Q fever to determine whether a patient might have persistent focalized infection(s). The term fever in Q fever has evolved from a pathologic picture per se to a clinical epiphenomenon; it is now time to evolve from the concept of chronic Q fever to one of persistent focalized *C. burnetii* infection(s) (*10*).

Funding was provided by the French National Referral Center for Q Fever.

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ESBL- and Carbapenemase-Producing *Enterobacteriaceae* in Patients with Bacteremia, Yangon, Myanmar, 2014

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DOI: http://dx.doi.org/10.3201/eid2305.161100

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Among 42 gram-negative bloodstream isolates from inpatients in 3 hospitals in Yangon, Myanmar, admitted during July–December 2014, 16 (38%) were extended-spectrum β-lactamase–producing *Enterobacteriaceae* and 6 (14%) produced carbapenemase. The high prevalence of multidrug-resistant gram-negative bacteria raises concerns about the empiric treatment of patients with sepsis in Yangon.

Infections with extended-spectrum β-lactamase (ESBL)–producing gram-negative bacteria and carbapenem-resistant *Enterobacteriaceae* (CRE) have been reported worldwide (*1*). Little is known about the occurrence of ESBL-producing and CRE bacteria in Yangon, Myanmar. Therefore, we characterized 42 gram-negative organisms isolated from routine blood cultures from adult inpatients in Yangon.

All bacteria had been isolated at the microbiology laboratories of 3 hospitals in Yangon during July–December 2014. During the study period, 592 blood cultures were processed, 536 from Yangon General Hospital (YGH) and 56 from 2 private hospitals. YGH is a 2,000-bed tertiary referral and teaching hospital in Yangon, providing free hospital care to civilians. The 2 private hospitals have 350 and 100 beds and provide secondary-level medical and surgical services to paying patients.

Of the 592 blood cultures, 42 (7.8%) yielded gram-negative bacteria, 28 (67%) from YGH and 14 (33%) from the 2 private hospitals. No clinical information was available about the patients from whom the cultures were taken. The identity and antimicrobial drug susceptibility of isolates were confirmed at Southern Community Laboratories (Dunedin, New Zealand) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex LT; Bruker Daltonics, Billerica, MA, USA), disc diffusion testing using the Clinical and Laboratory Standards Institute method (*2*), and the Phoenix Automated Microbiology System (Bruker Daltonics) (panel NMIC/ID-95).

We conducted phenotypic confirmation of ESBL production on cefpodoxime-resistant isolates using cefotaxime and ceftazidime with and without clavulanic acid and that of carbapenmase production on meropenem-resistant isolates by modified Hodge test according to Clinical and Laboratory Standards Institute criteria (*2*). We performed PCR for β-lactamase genes on all ESBL- and potential carbapenemase-producing organisms (*3*,*4*). We conducted bidirectional Sanger sequencing of amplicons and identified DNA sequences by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and by comparison with known β-lactamase gene sequences.

Of the 42 isolates, 34 (81%) were *Enterobacteriaceae* (20 *Escherichia coli*, 7 *Klebsiella pneumoniae*, 6 *Salmonella enterica*, and 1 *Enterobacter cloacae*) and 8 (19.0%) were nonfermenting gram-negative bacilli. Of the *Enterobacteriaceae*, 20 (59%) were multidrug resistant (MDR), with resistance to ≥3 classes of antimicrobial drugs, and 7 (21%) were extensively drug resistant, with susceptibility to ≤2 classes of antimicrobial drugs (*5*). All MDR *Enterobacteriaceae* were susceptible to polymyxin (Table). Phenotypic testing

Table. Antimicrobial drug susceptibility of *Enterobacteriaceae* and *Acinetobacter* spp. isolated from blood cultures of inpatients from 3 hospitals in Yangon, Myanmar, 2014*

	Organism, no. (%) susceptible				
Agent	*Escherichia coli*, n = 20	*Klebsiella pneumoniae*, n = 7	*Salmonella enterica*, n = 6	*Enterobacter cloacae*, n = 1	*Acinetobacter* spp., n = 3
Ampicillin	2 (10)	0	6 (100)	0	NT
Amoxicillin/clavulanic acid	5 (25)	1 (14)	6 (100)	0	NT
Piperacillin/tazobactam	13 (65)	2 (29)	6 (100)	0	2 (67)
Cefuroxime	5 (25)	1 (14)	NT	0	NT
Ceftriaxone	6 (30)	1 (14)	6 (100)	0	1 (33)
Ceftazidime	6 (30)	1 (14)	6 (100)	0	0
Cefipime	7 (35)	1 (14)	6 (100)	0	2 (67)
Cefoxitin	11 (55)	4 (57)	NT	0	NT
Aztreonam	6 (30)	1 (14)	6 (100)	0	NT
Ertapenem	17 (85)	4 (57)	6 (100)	1 (100)	NT
Imipenem	17 (85)	4 (57)	6 (100)	1 (100)	3 (100)
Meropenem	17 (85)	4 (57)	6 (100)	1 (100)	3 (100)
Gentamicin	14 (70)	2 (29)	NT	1 (100)	2 (67)
Tobramycin	9 (45)	1 (14)	NT	0	3 (100)
Ciprofloxacin	1 (5)	1 (14)	3 (43)	0	2 (67)
Chloramphenicol	11 (55)	2 (29)	6 (100)	0	NT
Colistin†	20 (100)	7 (100)	6 (100)	1 (100)	3 (100)
Nitrofurantoin	16 (80)	1 (14)	NT	0	NT
Trimethoprim/ sulfamethoxazole	2 (10)	2 (29)	6 (100)	0	2 (67)

*NT, not tested.
†Because there are no Clinical and Laboratory Standards Institute for susceptibility testing criteria for colistin for *Enterobaceriaceae*, European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria were used (susceptible if MIC <2 mg/L, per EUCAST criteria version 6.0, 2016; http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf).

suggested the presence of an ESBL in 16 (38%) and a carbapenemase in 6 (14%) of all gram-negative isolates. Molecular analysis showed that 13 (81%) ESBL-producing isolates contained a group 1 CTX-M gene; all were confirmed as CTX-M-15 by sequencing. Carbapenemase-producing isolates, including 3 *E. coli* and 3 *K. pneumoniae*, contained the New Delhi metallo-β-lactamase (NDM) gene, sequenced as NDM-4 in 5 (83%) and NDM-7 in 1 (17%).

Our study revealed a high proportion of ESBL- and carbapenemase-producing organisms among gram-negative bloodstream isolates during the study period from hospital inpatients in Yangon. Half of *E. coli* isolates and 43% of *K. pneumoniae* isolates produced ESBLs. This finding is consistent with the high proportion of ESBL production reported in isolates from India (>80%), China (>60%), and other Asia and Southeast Asia countries (>30%) (*6*). Carbapenemase production (15% in *E. coli* and 43% in *K. pneumoniae*) in this study was comparable to those previously reported from clinical isolates in India (*7*).

CTX-M-15 ESBL and NDM carbapenemase were the most prevalent mechanisms of resistance to β-lactams in our study. This finding is consistent with the current global dissemination of CTX-M-15 among *E. coli* isolates (*8*). All CRE isolates were NDM-4 or NDM-7. Two previous case reports have indicated the presence of NDM-producing *Enterobacteriaceae* from travelers to Myanmar: 1 NDM-7 (*9*) and 1 NDM-4 (*10*).

Two thirds of all isolates included in this study originated from YGH, the largest public hospital in Myanmar. All CRE isolates and 14 (88%) of 16 ESBL producers were isolated from YGH; this may reflect a higher prevalence of colonization with MDR organisms among patients in YGH or they may be healthcare-associated infections. Of concern, at YGH 11 (73%) of 15 *E. coli* and 6 (100%) of 6 *K. pneumoniae* isolates produced either an ESBL or carbapenemase; among these, 3 (20%) of 15 *E. coli* and 3 (50%) of 6 *K. pneumoniae* isolates were NDM producers.

Whereas all 23 (100%) of the *Enterobacteriaceae* at YGH were susceptible to treatment with colistin, an empiric treatment regimen of meropenem plus gentamicin would have covered only 18 (78%) isolates. This finding highlights the difficulties with designing an effective empiric antimicrobial regimen for patients with suspected gram-negative sepsis in a setting of a high prevalence of antimicrobial resistance, without providing further selective pressure for the spread of CRE and the emergence of colistin resistance.

Our study has limitations. First, clinical data were not prospectively collected, and it was not possible to obtain data retrospectively because of poor recording systems. Second, we cannot be certain that study isolates represent the population of organisms causing gram-negative sepsis in Yangon. However, the high proportion of ESBL- and carbapenemase-producing gram-negative bacteria among bloodstream isolates from hospitalized patients in Yangon raises concern for the treatment of patients with gram-negative sepsis and suggests a need to reduce selective pressure and control the spread of resistant organisms.

This study was supported in part by a grant from the University of Otago Development Office, New Zealand.

Dr. Myat completed this work while she was working as a lecturer at the Department of Microbiology, University of Medicine 1 in Yangon, Myanmar. Currently, she is undertaking doctoral study from the University of Otago, New Zealand. Her research focuses on bacterial causes of febrile illness in Yangon.

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Reemergence of African Swine Fever in Zimbabwe, 2015

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DOI: http://dx.doi.org/10.3201/eid2305.161195

Zimbabwe is the only country in southern Africa with no reported African swine fever (ASF) outbreaks during 1993–2014. However, the 2015 discovery of genotype II ASF virus in Zimbabwe indicates the reemergence of ASF in this country and suggests that this viral genotype may be spreading through eastern and southern Africa.

In many countries in Africa, pig farm production is restrained by outbreaks of African swine fever (ASF), a highly contagious and rapidly spreading disease that causes high rates of death among infected pigs and for which there is no vaccine (*1*). In southern Africa, the epidemiology of the causative agent, ASF virus (ASFV), is further complicated by the presence of sylvatic and domestic transmission cycles. The sylvatic cycle has been implicated in most outbreaks reported near national parks where infected warthogs (*Phacochoerus africanus*) and soft tick (*Ornithodoros* spp.) vectors are present (*1*). The virus can be horizontally transmitted between pigs via the uncontrolled movement of virus-contaminated pig products and the feeding of swill containing infectious pig meat; transmission is also facilitated by the lack of adequate biosecurity measures (*2*).

Genotyping is used to determine relationships between ASFV strains (*3*). ASF became endemic in Eurasia after the spread of a genotype II ASFV from eastern Africa to Georgia in 2007 (*4*,*5*). The virus has continued to spread in Eurasia, and in 2014, it was detected in Lithuania and Poland, putting the European Union at risk (*6*). In eastern and southern Africa, genotype II ASFV has been described in Tanzania, Mauritius, and Mozambique (*7*). Zimbabwe had outbreaks caused by ASFV genotype I in 1990 and 1992 and by genotype VIII in 1961. No further outbreaks were reported in Zimbabwe until July 2015, when ASF was detected in domestic pigs in Mashonaland Central Province in northern Zimbabwe, where it was confined to the area along the border with Mozambique. The virus spread locally because of the salvage slaughter of infected pigs, the selling of infected pig meat at discounted prices, the movement of pigs between villages, and the inappropriate disposal of infectious carcasses.

During the 2015 outbreak in Zimbabwe, pigs with ASF exhibited fever (temperature 41°–42°C), dullness, anorexia, and swaying gait. Light-colored pigs also showed reddening of the skin on the ears and abdomen, but redness was not evident on the indigenous black breed of pigs. Postmortem examinations revealed bloody discharge from the anus and nostrils; edema of the lungs; and hemorrhages in the mesenteric lymph nodes, kidney, and heart.

We pooled the organs from 3 infected pigs from 3 separate villages in the outbreak area and extracted DNA. We

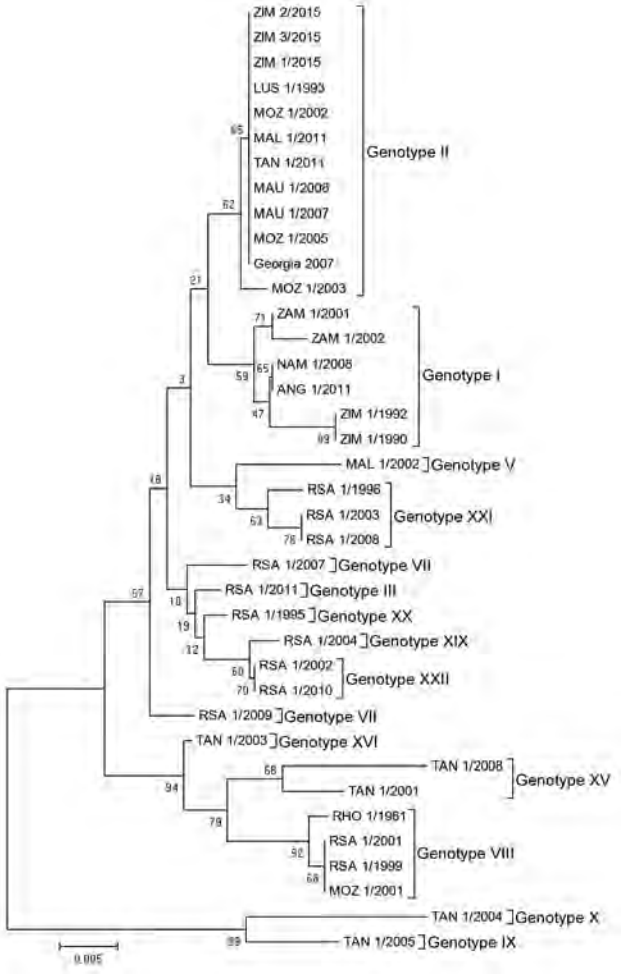

Figure. Neighbor-joining phylogenetic tree based on the partial B646L (p72) gene sequences of African swine fever virus (ASFV) isolates from a 2015 outbreak in Zimbabwe. The outbreak strains (ZIM/1/15, ZIM/2/15, and ZIM/3/15 [GenBank accession nos. KX090921–KX090923]) grouped with genotype II ASFV strains isolated in Mozambique (MOZ), Tanzania (TAN), Malawi (MAL), Mauritius (MAU), and Georgia, sharing 100% nucleotide identity with those strains. Phylogeny was inferred after 1,000 bootstrap replications; values at nodes indicate the percentage of bootstrap support. Scale bar indicates nucleotide substitutions per site.

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amplified the variable 3′ end of the B646L (p72) gene by using 2 oligonucleotide primers, p72-U and p72-D (*3*). To amplify the entire E183L gene (p54), we used primers described by Oviedo et al. (*8*), and to amplify the tetramer amino acid repeats within the hypervariable central variable region of the B602L gene, we used primers described by Gallardo et al. (*5*). Our results showed that sequences for all ASFV isolates from this outbreak and those for isolates previously collected in eastern Europe and eastern Africa were 100% homologous over the p72, p54, and central variable region gene-coding regions. The p72 sequences clustered in genotype II (Figure).

During the 2015 ASFV outbreak in Zimbabwe, a total of 3,427 pigs were at risk for infection in the affected area. Of those, 2,836 (≈83%) became infected, and all infected pigs died. The 591 pigs that did not become infected had been confined in pens and did not have exposure to infected pigs or their products. A follow-up study is under way in the region to genetically characterize the viruses in this outbreak, focusing on the p54, p30 and, central variable region genes.

All villages affected during the 2015 outbreak in Zimbabwe were along the northern border with Mozambique, where genotype II has been found before. It is essential that more of the ASFVs circulating in eastern and southern Africa be sequenced so that their relatedness can be determined. This knowledge will enable the establishment of an epidemiologic link between outbreaks in the region and underscore the need for adequate quarantine measures to prevent ASF from becoming endemic in southern and eastern Africa.

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Management of *Bartonella* Prosthetic Valve Endocarditis without Cardiac Surgery

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DOI: http://dx.doi.org/10.3201/eid2305.161238

Two cases of *Bartonella* prosthetic valve endocarditis were cured when treated for 2 weeks with gentamicin and 3 months with doxycycline. Clinical cure correlated with decreased *Bartonella* antibody titers. This report suggests a strategy to monitor, treat, and cure *Bartonella* prosthetic valve endocarditis.

Bartonella, a causative agent of blood culture–negative endocarditis (BCNE) that usually requires valve surgery, was first attributed to endocarditis over 20 years ago (*1–4*). We report 2 cases of *Bartonella* prosthetic valve endocarditis that were successfully treated with antimicrobial drugs alone.

Case 1 involved a 62-year-old man admitted to the hospital in 2011 with left-flank pain and a 3-year history of anorexia, weight loss, night sweats, and recent diagnosis of anemia. He kept pet cats. He underwent mechanical aortic valve replacement and patent foramen ovale closure in 1992 and a repeat aortic valve replacement and an aortic root replacement for a chronic type A dissection in 1996.

On physical examination, he was afebrile and had subconjunctival hemorrhages, normal prosthetic heart sounds, and 10-cm splenomegaly. Blood tests showed a hemoglobin concentration of 9 g/dL (reference range 14.0–17.5 g/dL), serum creatinine of 2.76 mg/dL (reference range 0.6–1.2 mg/dL), C-reactive protein of 48 mg/L (reference range 0.08–3.1 mg/L), rheumatoid factor of 742 U/mL (reference range 0–30 U/mL), and c-ANCA (cytoplasmic antineutrophil cytoplasmic antibody) positivity (proteinase 3 autoantibody concentration, 18.3 U/mL). Urine dipstick (Combur 7 Test; Roche Diagnostics Ltd, Basel, Switzerland) revealed the presence of blood (4+), and renal biopsy demonstrated necrotizing crescentic glomerulonephritis. Five blood cultures were sterile. Transesophageal echocardiography showed no evidence of endocarditis. *Bartonella* serologic testing was conducted with an indirect immunofluorescence assay by using the manufacturer's instructions (MRL, Cypress, CA, USA); results showed high IgG titers to both *B. henselae* and *B. quintana* (Table), but the infecting species could not be determined. The patient was treated for 2 weeks with intravenous gentamicin (2 mg/kg/d) and for 3 months with oral doxycycline (100 mg 2×/d). Nine months after completing treatment, he was well: splenomegaly had resolved, and hemoglobin (15.6 g/dL) and creatinine (1.31 mg/dL) concentrations approached normal levels. He was well when last reviewed in 2014.

Case 2 involved a 29-year-old woman with inflammatory bowel disease and primary sclerosing cholangitis. Her symptoms began in 2011 with fever, rigors, night sweats, and anorexia for 2 weeks. She had no pets but recalled contact with a kitten 8 months previously. In 2002, BCNE developed, requiring mechanical aortic and mitral valve replacements. In 2003, BCNE was again diagnosed but was complicated by an ascending aorta to left atrial fistula, requiring an aortic root replacement, a homograft, and a repeat mechanical mitral valve replacement.

Physical examination revealed fever, a splinter hemorrhage, and an ejection systolic murmur. Blood tests revealed a low hemoglobin concentration (10.9 g/dL), a high C-reactive protein concentration (26 mg/L), normal renal function, and positivity for rheumatoid factor (114 U/mL). Transesophageal echocardiograms revealed no evidence of endocarditis. One of 20 blood culture tests grew *B. henselae* after a 19-day incubation (Public Health England, identified by partial sequencing of 16S rDNA). A serologic test for *Bartonella* was strongly positive (Table). Three days after starting treatment with oral doxycycline (100 mg 2×/d) and intravenous gentamicin (3 mg/kg/d), she became afebrile; she received 14 days of gentamicin and 3 months of doxycycline in total. Two months later, she remained well, and her C-reactive protein concentration was <5 mg/L. Because of symptomatic stenosis caused by structural deterioration of the replacement aortic valve, she underwent another aortic valve and root replacement 19 months after completing antimicrobial drugs. No evidence of active endocarditis was found during surgery.

Bartonella antibody titers dropped slowly over a period of 3 years in both patients (Table). Only case 1 had definite infective endocarditis when using the modified Duke diagnostic criteria. However, because the Duke criteria are insensitive for BCNE diagnosis, it has been proposed that a *Bartonella* IgG titer of ≥1:800 and a positive Western blot or PCR analysis when using valve or blood specimens should be considered major Duke criteria (*5*).

Most reported cases of *Bartonella* endocarditis involve native valves; the first prosthetic valve infection was reported in 2002 (*6*). Although >80% of patients require valve replacement, infection with *Bartonella* is not in itself a recognized indication for surgery. Because our patients responded to medication, we did not need to consider cardiac surgery. The optimal antimicrobial drug therapy and duration for *Bartonella* endocarditis is undetermined. The recommended regimen of gentamicin for 14 days and doxycycline for 4 weeks (*7*) has limited evidence supporting its use (*8,9*). We found only 1 case of *Bartonella* prosthetic valve endocarditis

Table. *Bartonella* antibody titers in cases 1 and 2 by month after diagnosis*

Month after diagnosis	*B. henselae* IgM	*B. henselae* IgG	*B. quintana* IgM	*B. quintana* IgG
Case 1				
0	40	32,768	<20	1,024
8	<20	16,385	<20	512
11	20	8,096	<20	512
16	<20	8,096	<20	256
25	<20	4,096	<20	64
43	<20	2,048	<20	64
Case 2				
0	<20	8,192	<20	512
16	<20	≥512	<20	64
41	<20	64	<20	<64

*All time points were assayed in parallel. Antibody titers are the inverse of the greatest dilution that exhibited a reaction.

cured without valve surgery; it was cured with a 30-month antimicrobial drug regimen (*10*).

The role for serial serologic testing in assessing cure of *Bartonella* endocarditis is unknown. In our cases, as in a previous report (*10*), a drop in *Bartonella* titers occurred over a 3-year period in those who were cured, suggesting follow-up serologic testing might be useful to assess *Bartonella* endocarditis clinical cure.

Our findings suggest that a simple, inexpensive drug regimen is optimal therapy for *Bartonella* endocarditis and that serial serologic testing can confirm adequate treatment and cure. Further research is needed to validate this approach to managing *Bartonella* endocarditis.

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Zika Virus Infection and Prolonged Viremia in Whole-Blood Specimens

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DOI: http://dx.doi.org/10.3201/eid2305.161631

We tested whole-blood and plasma samples from immunocompetent patients who had had benign Zika virus infections and found that Zika virus RNA persisted in whole blood substantially longer than in plasma. This finding may have implications for diagnosis of acute symptomatic and asymptomatic infections and for testing of blood donations.

Since cases of severe neurologic disorders among adults (*1*) and fetal abnormalities (*2*) linked to Zika virus infections were initially reported, the World Health Organization has deemed the Zika virus outbreak a "public health emergency of international concern" and has raised Zika virus to the same level of concern as Ebola virus. In response, medical authorities from many countries have released advice and guidelines regarding prevention and diagnosis to contain the spread of this virus and guidelines regarding safety of whole blood and blood components. In August 2016, the Food and Drug Administration announced universal testing for Zika virus RNA in donated whole blood and blood components taken in the United States and its territories using a qualitative molecular assay on plasma specimens (*3*).

In Europe, advice on Zika virus regarding the safety of substances of human origin (*4*) has been applied in France since February 15, 2016. A qualitative individual molecular test for Zika virus RNA in plasma specimens is being used on whole-blood specimens from blood donors living in Guadeloupe and Martinique, 2 overseas administrative areas where Zika virus is autochthonous. Furthermore, in mainland France and in French overseas areas where no active Zika virus transmission exists, and since the beginning of the Zika virus outbreak in 2015, blood donors who have recently visited areas or countries with ongoing Zika

virus transmission are subject to a 28-day temporary deferral after their departure from these areas, a period twice the assumed maximum incubation period for Zika virus. Similarly, temporary deferral applies to blood donors who have a sex partner who has been recently infected or potentially exposed to a confirmed or suspected Zika virus infection within the previous 3 months.

We report results from a 2016 longitudinal follow-up of Zika virus RNA quantification in EDTA whole-blood and plasma samples taken from 5 immunocompetent patients (2 men, 33 and 70 years of age, and 3 women, 55, 58, and 67 years of age) and results from a point-to-point comparison of Zika viral loads on both EDTA whole-blood and corresponding plasma samples (27 pairs). We extracted RNA by using the MagNA Pure 96 instrument with the DNA and Viral NA Small Volume Kit (Roche Diagnostics, Meylan, France) (input and output volumes 200 and 100 µL). We quantified RNA by using the RealStar Zika RNA RT-PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany) (limit of selection 2.48 log copies/mL). We always successfully detected the manufacturer's internal control. All samples were collected from patients who had returned from the Caribbean or South and Central America and had had a benign form of Zika virus infection.

Results from the follow-up (18 whole-blood and 21 plasma samples) showed that the median duration of Zika virus was 22 (range 14–100) days in whole blood and 10 (range 7–37) days in plasma (p = 0.058). Mean viral loads of positive samples were 3.39 log copies/mL in whole blood (n = 13) and 2.52 log copies/mL in plasma (n = 6; p = 0.001). Viral loads in the last positive samples varied from 2.7 to 3.9 log copies/mL in whole blood and 2.2 to 2.8 log copies/mL in plasma (p = 0.06). Whole-blood samples from 2 patients remained positive at 14 and 63 days after their plasma samples had become negative (Figure, panel A).

The point-to-point comparison (18 pairs from the follow-up and 9 additional pairs) showed that Zika virus RNA was quantifiable in 23 whole-blood specimens but in only 10 plasma samples. Mean viral load was 3.50 (range 2.75–4.17) log copies/mL in whole blood and 3.01 (range 2.21–4.10) log copies/mL in plasma (p< = 0.018) (Figure, panel B).

These data show that Zika virus RNA persisted in whole blood after it disappeared in plasma. Similar results have been reported previously for West Nile virus, also a member of the *Flaviviridae* family (*5*,*6*), and for Zika virus with a qualitative in-house PCR (*7*).

Our data have 3 main consequences. First, for acute symptomatic infection, the use of whole blood extends the period of diagnosis. Second, for asymptomatic infections with a high likelihood of low viral load, virus detection in plasma might be less sensitive than detection in whole-blood specimens. Third, according to our data that show that viremia can persist for >28 days after symptom onset, recommendations used to reduce the risk for Zika virus transmission through blood or blood components should be modified. Potential options such as extending the deferral period or testing blood donations for Zika virus RNA in whole blood should be considered.

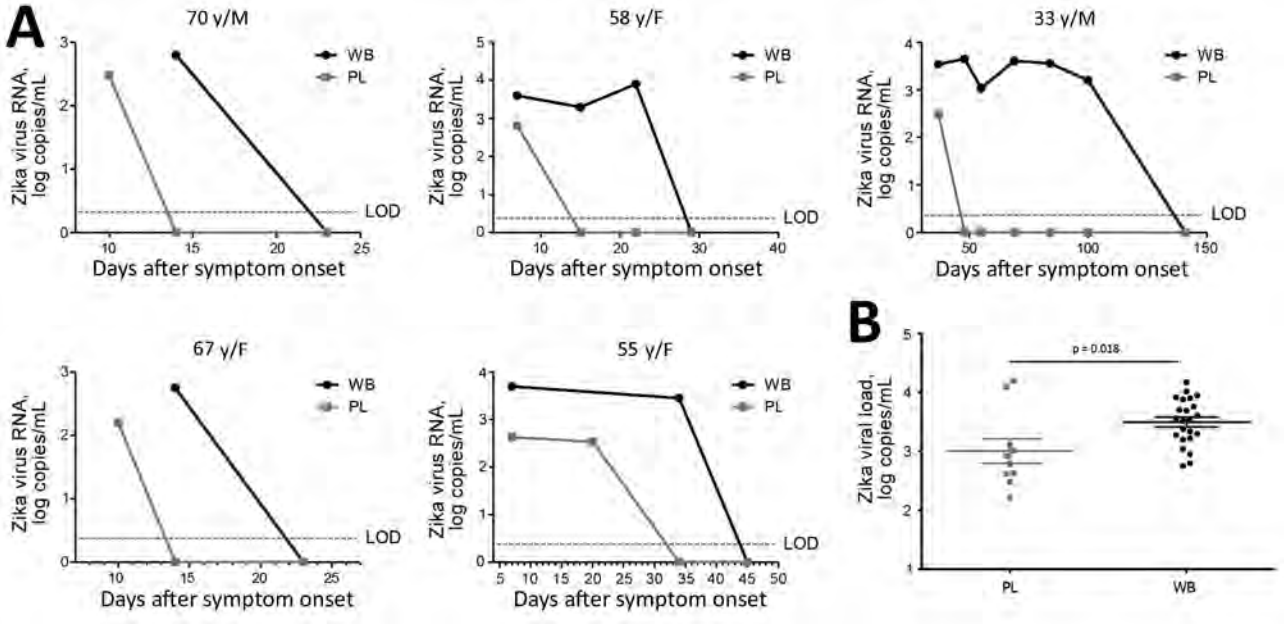

Figure. A) Zika virus viremia in whole blood and plasma from 5 immunocompetent patients in France (identified by sex and age, y) who had traveled to Central or South America or the Caribbean. B) Zika viral load in whole-blood (n = 23) and plasma (n = 10) samples from a point-to-point comparison of positive samples. Horizontal lines indicate mean ± SE. LOD, limit of detection; PL, plasma; WB, whole blood.

Overall, our data show that use of whole-blood specimens is much more sensitive than use of plasma samples to detect Zika virus RNA. These data could be useful in recommending the use of whole blood instead of plasma for the molecular diagnosis of acute symptomatic and asymptomatic Zika virus infections and for the safety of whole blood and blood components from donors, as well as for the safety of organs, tissues, and cells from deceased and living donors.

Acknowledgment

We thank Isabelle Da Silva for her technical assistance.

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Severe MRSA Enterocolitis Caused by a Strain Harboring Enterotoxins D, G, and I

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DOI: http://dx.doi.org/10.3201/eid2305.161644

We describe a case of methicillin-resistant *Staphylococcus aureus* (MRSA) enterocolitis in a healthy adult with previous antibiotic exposure. Colonoscopy revealed diffuse colitis and mild ileitis without ulceration. Stool cultures demonstrated abundant growth of MRSA and absent normal flora. Oral vancomycin treatment was effective and seems to be the consensus choice for therapy.

Staphylococcus aureus was recognized as a cause of antibiotic-associated colitis (AAC) in the mid-20th century (*1*,*2*). *Clostridium difficile* was later identified as the primary cause of AAC, and appreciation of *S. aureus* as a potential etiology declined (*2*). Methicillin-resistant *S. aureus* (MRSA) has also been implicated as a cause of AAC, with most reports coming from Japan. We report a case of MRSA enterocolitis in Canada caused by a strain harboring multiple enterotoxins.

In 2014, a 22-year-old woman sought care after 10 days of acute and profuse diarrhea, abdominal cramping, nausea, and weight loss of 5 lbs. She had 10–30 bowel movements a day and had observed blood-tainted stool. The patient reported a history of migraine and depression but was otherwise healthy. She worked in a pet store and had not been hospitalized. In the previous 2 months, she had been treated for chlamydia with a single course of azithromycin and cefixime. Subsequently, she received a 10-day course of azithromycin followed by 10 days of moxifloxacin for bronchopneumonia. Her family history revealed Crohn's colitis in her father.

The patient was afebrile; blood pressure was 104/58 mm Hg and pulse 91 bpm. Her abdomen was soft without rebound tenderness. Laboratory test results revealed a normal leukocyte count but a C-reactive protein level of 76 mg/L. Her initial diagnosis was with bacterial enteritis, and she was sent home with an order for stool cultures and oral ciprofloxacin to be started after stool collection. On

follow-up 3 days later, she felt better but was still having 6 bowel movements a day and bloody stool. She remained afebrile, but her blood pressure was 86/57 mm Hg and pulse 104 bpm. Colonoscopy with ileoscopy was performed to explore the possibility of inflammatory bowel disease; results revealed diffuse acute colitis with an inhomogeneous pattern (Figure, panels A–D) and minimal focal erythema in the terminal ileum. *C. difficile* testing was negative for glutamate dehydrogenase antigen and toxins by Quik-Chek Complete (Alere, San Diego, CA, USA). Testing of the 3 stool cultures demonstrated absent normal flora and abundant growth of MRSA. Eight days after initial consultation, she was seen by an infectious disease specialist and prescribed oral vancomycin (125 mg 4×/d for 10 d). She had an excellent and rapid clinical response to treatment, and her symptoms did not recur. The patient was found to have nasal colonization with MRSA.

Colonic biopsy confirmed colitis without chronicity (Figure, panel E), and ileal biopsy revealed focal active ileitis. Because MRSA colitis is rare, the strain was sent to the Laboratoire de Santé Publique du Québec for identification and further characterization (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/16-1644-Techapp1.pdf). Genetic typing was positive for *nuc* and *mecA* genes, confirming MRSA. The strain was negative for Panton–Valentine leukocidin, toxic shock syndrome toxin 1, enterotoxins A, B, C, E, and H, and exfoliative toxins A and B, but it harbored genes for enterotoxins D, G, and I. The *spa* type was t067, corresponding to the epidemic type in Canada, CMRSA2 (also known as USA100/800, New York, sequence type 5). This strain was resistant to levofloxacin, erythromycin, and clindamycin but susceptible to all remaining antibiotics (online Technical Appendix).

S. aureus enterocolitis has been recognized as a cause of AAC since 1948, and MRSA colitis is a variant of this disease. Most cases were reported from Japan (*1*); the validity of these reports has been questioned because of insufficient effort to rule out *C. difficile*, but recent reviews support the existence of the syndrome (*3*). In North America, few cases of MRSA colitis have been identified; however, this cause of enteritis might be underreported because few physicians order stool cultures for hospitalized patients with AAC (*1*,*4*). Cases of staphylococcal enteritis might occur when dysbiosis permits *Staphylococcus* overgrowth and toxin secretion causes bowel inflammation (*1*). The multiple antibiotics that had been taken by this patient presumably induced dysbiosis, MRSA overgrowth, and toxin-mediated enterocolitis. The relative contribution of the different toxins to the syndrome are unknown. Gut colonization is believed to originate from the upper respiratory tract of colonized persons (*5*).

In previous reported cases of MRSA enterocolitis, few authors reported the presence of *Staphylococcus*

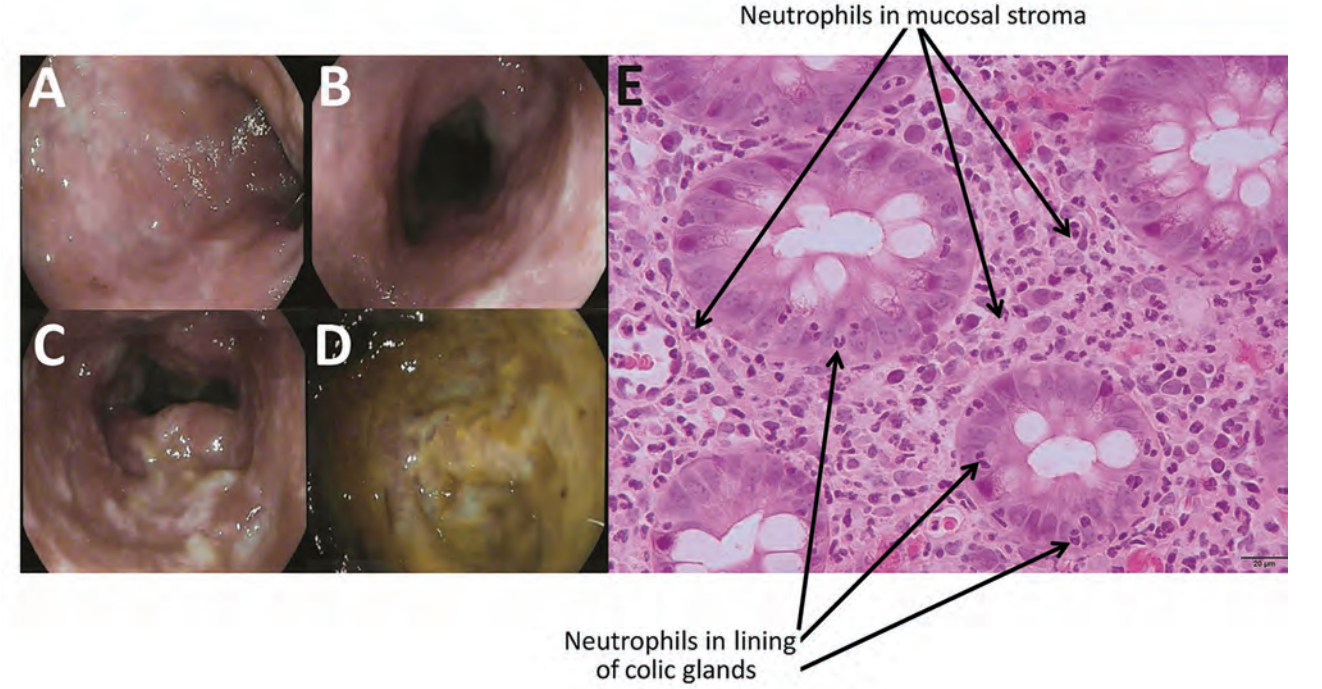

Figure. Endoscopic imagery of the distal sigmoid colon (A), proximal sigmoid colon (B), descending colon (C), and base of cecum (D), revealing diffuse colitis with mucosal erythema, edema, and mucopurulent exudate without ulceration. Colonic biopsy (E) demonstrating neutrophilic infiltrates (indicated with arrows) in the epithelial lining of the colic glands and the mucosal stroma compatible with mild active colitis without signs of chronicity.

toxins, mainly enterotoxin A. Although enterotoxin A is the most common toxin in *Staphylococcus*-related food poisoning, the enterotoxin D we found is reported to be the second most common, supporting its role in this patient's enterocolitis (*6*). Enterotoxins G and I are not as well-studied but were associated with a food poisoning outbreak in Taiwan (*7*). Staphylococcal enterotoxins G and I induce enterocolitis by a combination of direct enterocyte cytopathy mediated by epidermal cell differentiation inhibitor toxins (disrupting the epithelial barrier) and enterotoxin superantigen-induced mucosal T-cell activation (*8*).

The risk factors for *S. aureus* enterocolitis include age, hospitalization, abdominal surgery, immunosuppression, gastric acid suppression, MRSA colonization, and previous antibiotic therapy. Fluoroquinolone use seems to be particularly associated with this complication (*1*). MRSA enterocolitis might be an underrecognized cause of AAC because stool culture in patients hospitalized for >72 hours has been discouraged (*9*). MRSA enterocolitis should be considered once *C. difficile* colitis and *Klebsiella oxytoca* colitis have been ruled out (*10*). Physicians should consider stool cultures for severe or prolonged AAC, and microbiology laboratories should report *S. aureus* overgrowth in stool. Oral vancomycin was effective in this case and seems to be the consensus choice for therapy based on previous reports.

This work was supported by internal funding from the Laboratoire de Santé Publique du Québec.

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Serogroup B Meningococcal Disease Vaccine Recommendations at a University, New Jersey, USA, 2016

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DOI: http://dx.doi.org/10.3201/eid2305.161870

In response to a university-based serogroup B meningococcal disease outbreak, the serogroup B meningococcal vaccine Trumenba was recommended for students, a rare

instance in which a specific vaccine brand was recommended. This outbreak highlights the challenges of using molecular and immunologic data to inform real-time response.

In 2016, two undergraduate students at a large state university in New Jersey were hospitalized for suspected meningitis. The New Jersey State Public Health Laboratory (Trenton, NJ, USA) identified serogroup B *Neisseria meningitidis* in cerebrospinal fluid specimens from both patients by culture and slide agglutination. Both patients recovered without sequelae.

The isolates were sent to the Centers for Disease Control and Prevention (CDC) Bacterial Meningitis Laboratory for species and serogroup confirmation and whole-genome sequencing. Isolates were confirmed by real-time PCR as serogroup B *N. meningitidis* and were classified by multilocus sequence typing as clonal complex 11, sequence type 11, which is typically associated with serogroups C and W. Whole-genome sequencing revealed that the isolates were genetically indistinguishable from one another but different from all previously characterized meningococcal isolates in the United States.

Two serogroup B meningococcal (MenB) vaccines are licensed in the United States: MenB-4C (Bexsero, GlaxoSmithKline Biologicals, Inc., Philadelphia, PA, USA [*1*]), licensed as a 2-dose series; and MenB-FHbp (Trumenba, Pfizer, Inc., New York, NY, USA [*2*]), licensed as a 2- or 3-dose series. Either Bexsero or the 3-dose series of Trumenba is preferred for outbreak response (*3*). Both MenB vaccines are expected to help protect against most circulating serogroup B meningococcal strains, and in general, no brand preference exists (*3*). However, because the vaccines target different antigens (Table), they are not interchangeable; the same brand must be used for all doses (*5*). The vaccines might also have different effectiveness against specific *N. meningitidis* strains (*5*).

Whole-genome sequencing identified a mismatch between the antigens in the outbreak strain and those targeted by Bexsero (Table), prompting concern that Bexsero might not provide optimal protection against the outbreak strain. Although the outbreak strain antigens also did not exactly match those included in Trumenba, cross-protection with Trumenba was expected based on prior testing by the manufacturer (Table) (*2*). The outbreak isolates were sent to an independent laboratory for flow cytometry to measure antigen expression and serum bactericidal activity testing using human complement (hSBA) to evaluate whether stored serum from healthy adults previously

Table. Molecular profile and flow cytometry analysis of the university outbreak strain with reference to MenB vaccine antigens, New Jersey, 2016*

Antigen	Outbreak strain	MenB-4C vaccine (Bexsero)	MenB-FHbp vaccine (Trumenba)	Interpretation
FHbp	A22†/2.19‡	B24†/1.1‡	A05†, B01†	The 2 FHbp subfamilies (A and B) are not expected to be cross-reactive. The outbreak strain has a subfamily A FHbp. MenB-FHbp contains FHbp from subfamilies A and B. Although the subfamily A FHbp contained in MenB-FHbp (A05) is not the same peptide allele as that in the outbreak strain (A22), some level of cross-reactivity is expected based on prior testing by the manufacturer (*2*). However, based on flow cytometry, the strain had relatively low expression of FHbp, which would be expected to decrease susceptibility to anti-FHbp bactericidal activity (*4*). MenB-4C contains a subfamily B FHbp, which is not expected to provide protection against the outbreak strain FHbp.
PorA	P1.5–1,10–1	P1.7–2.4	Not included	The PorA present in MenB-4C and in the outbreak strain are 2 different PorA variable region sequence types and are not expected to be cross-reactive; therefore, no protection based on PorA is expected for either vaccine.
NHba	p0020	p0002	Not included	The NHba present in MenB-4C and in the outbreak strain are 2 different peptide alleles. The extent of cross-reactivity is not known. By flow cytometry, low binding to the outbreak strain was observed by using antisera against NHba p0002, but it is unclear whether this low binding is attributable to the difference in sequence between p0020 and p0002, low NHba expression in the outbreak strain, or both. Low binding with NHba p0002 antisera is consistent with decreased susceptibility to anti-NHba bactericidal activity in persons vaccinated with MenB-4C, but the correlation between flow cytometric binding and hSBA response has not been established. No protection based on NHba is expected for MenB-FHbp.
NadA	Negative	2/3.8	Not included	The outbreak strain does not contain NadA; therefore, no protection based on NadA is expected for either vaccine.

*FHbp, factor H binding protein; hSBA, serum bactericidal activity testing using human complement; MenB, serogroup B meningococcal; NadA, Neisserial adhesion A; NHba, Neisserial heparin binding antigen.
†Pfizer classification scheme.
‡GlaxoSmithKline classification scheme.

vaccinated with Bexsero or Trumenba could kill the outbreak strain bacteria (*6*).

By flow cytometry, the outbreak strain had low expression of factor H binding protein and low binding with antisera to the Neisserial heparin binding antigen included in Bexsero (Table) (*7*). Nevertheless, preliminary hSBA results suggested that 2 doses of either Bexsero or Trumenba would provide some short-term protection against the outbreak strain. Among persons with preimmunization titers of <1:4, most had hSBA titers of ≥1:4 at 1 month after the second dose (13/13 for Bexsero [*7*] and 9/10 for Trumenba). However, by 4 months after the second dose, hSBA titers fell back to <1:4 for some persons vaccinated with Bexsero (4/8) and Trumenba (4/5). The third dose of Trumenba, administered at 6 months after the first dose and 4 months after the second, boosted hSBA titers against outbreak strain bacteria to ≥1:4 for 9/9 persons 1 month after completion of the 3-dose series. On the basis of consideration of the laboratory data, the best and longest-lasting protection against the outbreak strain was expected with the 3-dose series of Trumenba. Accordingly, the New Jersey Department of Health and the university, with support from CDC, recommended vaccination with 3 doses of Trumenba for ≈35,000 persons at the university.

Serum bactericidal antibodies are used as a serologic correlate of protection for meningococcal vaccines (*8*) and have been correlated with clinical efficacy for serogroup C (*9*). For the purposes of US licensure, the effectiveness of MenB vaccines was inferred by using hSBA. Although hSBA titers probably correlate with protection against serogroup B meningococcal disease, this link has yet to be directly demonstrated through postlicensure effectiveness data.

Although hSBA results informed the vaccination strategy in this outbreak response, this experience underlines the challenges in obtaining hSBA testing and interpreting molecular and immunologic data on meningococcal outbreak strains. The hSBA results for this outbreak strain show that, because of variable protein expression and unknown cross-protection, using the molecular profile of a serogroup B meningococcal strain to reliably predict hSBA response in vaccinated persons is difficult. Additionally, to what extent hSBA results from a limited number of test subjects correspond to real-life protection in a different population is not clear. Furthermore, hSBA testing is time-consuming, a limited amount of serum from immunized humans is available for hSBA testing, and few laboratories are able to routinely perform this testing. These challenges make it difficult to routinely use either molecular typing or hSBA results to guide real-time outbreak response.

This recommendation for a specific brand of MenB vaccine was a rare exception to the general recommendation that either brand of MenB vaccine can help protect persons at increased risk during a serogroup B meningococcal disease outbreak. The use of molecular and immunologic data as potential tools to inform meningococcal outbreak response requires further investigation before being routinely implemented.

Acknowledgments

The authors thank Stephen Hadler, Amanda Cohn, Stacey Martin, and Xin Wang.

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Meningococci of Serogroup X Clonal Complex 181 in Refugee Camps, Italy

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DOI: http://dx.doi.org/10.3201/eid2305.161713

Four cases of infection with serogroup X meningococci (MenX) (1 in 2015 and 3 in 2016) occurred in migrants living in refugee camps or reception centers in Italy. All MenX isolates were identified as clonal complex 181. Our report suggests that serogroup X represents an emerging health threat for persons arriving from African countries.

Outbreaks of *Neisseria meningitidis* serogroup X meningococcal (MenX) infections in the African meningitis belt caused by isolates of clonal complex (CC) 181, including an outbreak in 2006 in Niger and one during 2007–2010 in Togo and Burkina Faso (*1*,*2*), were characterized by high rates of illness and death. Sporadic infections caused by MenX of different CCs have also been identified in Italy (*3*), Spain (*4*), and China (*5*).

Moreover, serogroup X invasive isolates from other European countries reported and available in the PubMLST database (http://pubmlst.org/neisseria/) showed high heterogeneity among themselves and with the MenX isolates of the African meningitis belt. Because of the lack of a specific herd immunity against this serogroup in Europe, non-African MenX isolates may be associated with increased host susceptibility (*4*).

Recently, the Italian Reference Laboratory for Invasive Meningococcal Disease (IMD) surveillance of the Istituto Superiore di Sanità, Rome, Italy, received samples from 4 unlinked case-patients with serogroup X IMD that occurred among migrants living in refugee camps or reception centers. The first case was reported in 2015 in a 15-year-old girl from Eritrea (ID2683) who had arrived in a refugee camp in Lombardy, Italy, 3 days before onset of disease, which manifested as septicemia. The other 3 cases were reported in 2016, two in Lombardy (in a 20-year-old man from Mali, ID2820, and a 31-year-old man from Niger, ID2849) and another in a Tuscany camp (in a 24-year-man from Bangladesh, ID2805). These cases were characterized by meningitis with fever >40°C and loss of consciousness. All patients were treated with ceftriaxone and survived. Chemoprophylaxis with rifampin or ciprofloxacin was administered to all persons directly exposed to the index case-patients. The man from Bangladesh lived in a camp with other Africa refugees for several months before disease onset, but symptoms developed in the other 3 patients shortly after their arrival in Italy.

Sample ID2849 was culture negative and characterized only by finetype (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/23/5/16-1713-Techapp1.pdf). We performed whole-genome sequencing and assembly on the other 3 isolates by using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) (*6*). Genomes are available through the PubMLST database, which runs on the Bacterial Isolate Genome Sequence Database platform (*7*).

The 4 isolates were further analyzed by core genome multilocus sequence typing (cgMLST) and compared with all serogroup X genomes (n = 36) in the PubMLST database (as of December 28, 2016) (*7*). The isolates bifurcated into 2 main groups, of which CC181 genomes clustered in a single branch (Figure, panel A). Of 18 CC181 genomes, 17 were resolved in 3 main groups (Figure, panel B), according to 3 main finetypes: group 1, finetype X:P1.5–1,10–1:F1–31:ST181 (CC181); group 2, finetype X:P1.5–1,10–1:F4–23:ST5789 (CC181); group 3, finetype X:P1.5–1,10–1:F4–23:ST181 (CC181). One CC181 genome (ID LNP13407) was positioned in a branch far from the 3 main groups. Two of the strains identified in Italy in 2016 (ID2805 and ID2820) clustered in group 1 with 7 MenX strains isolated from 2005 and 2016 in Niger, Burkina Faso, Benin, and the United Kingdom (mean distance 3 loci). Notably, the UK MenX CC181 strain (ID M16_240550) clustered close to the Italy strains (mean distance 24 loci). The strain diagnosed in Italy in 2015 (ID2683) clustered in group 2 with 3 strains isolated in 2006 in Niger (mean distance 36 loci). Group 2 was strictly related to group 3, comprising 4 meningococci strains isolated during 1996–2002 in Niger.

As described by Agnememel et al. (*8*), MenX isolates from Africa were genetically related: they belonged to CC181and formed a single main lineage. Our genome analyses confirmed the presence of MenX strains with similar characteristics to those already described. In particular,

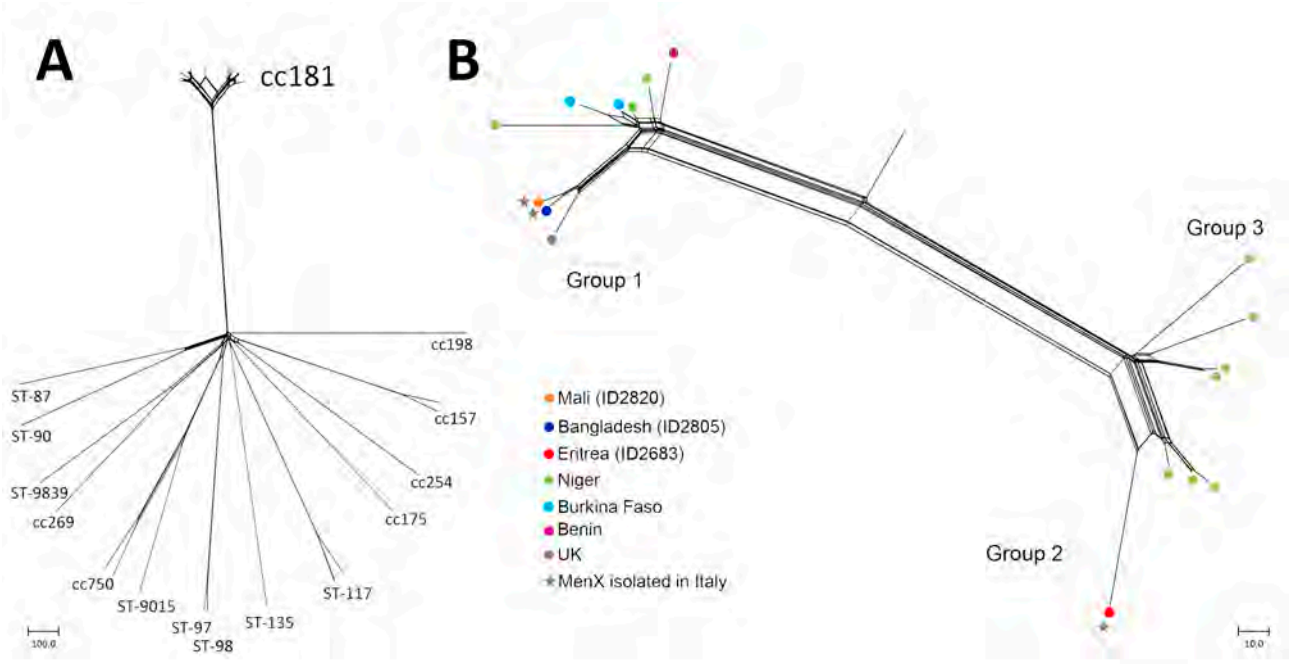

Figure. Analysis of *Neisseria meningitidis* serotype X (MenX) isolates from 3 refugees in Italy and comparison isolates from the *Neisseria* PubMLST database (http://pubmlst.org/neisseria/), as of December 28, 2016). A) Neighbor-net phylogenetic network based on a comparison of core genome loci of all MenX genomes (n = 36) available in PubMLST database. For each strain, the available designation by clonal complex (CC) or sequence type (ST) is indicated. B) Neighbor-net phylogenetic network showing 3 isolates from Italy (stars) compared with core genome loci (MenX CC181 genomes (n = 18) available in the *Neisseria* PubMLST database. Source locations for comparison isolates are indicated. Scale bars indicate number of differences among the loci compared.

these isolates harbored *lpt3* allele 45, previously described as a high virulence marker in the mouse model (*8*).

The analysis of meningococcal serogroup B vaccines antigens (PorA, fHbp, NadA and NHBA) identified the variants PorA VR2 10–1, fHbp-1.74 (Pfizer family B, variant B49), and NHBA-359 for samples ID2805, ID2820, and ID2849 and fHbp-1.391 (Pfizer family B) and NHBA-358 for isolate ID2683. fHbp and NHBA variant patterns had been associated with MenX CC181 isolated in Africa. NadA was absent in all analyzed MenX CC181 meningococci.

The probability of a migrant developing an infectious disease, such as IMD, after arriving in the country of destination may depend on a series of factors, such as the prevalence and incidence of the infectious diseases in the country of origin, the specific characteristics of the infectious diseases (incubation period), the number of contacts that the migrant had during the journey, and the duration of the journey. These factors should be taken into account when assessing the risk of developing specific infectious diseases, such as IMD. Our report suggests that MenX represents an emerging health threat for persons arriving in Italy from Africa. Early diagnosis, treatment, and prophylaxis should be ensured to protect vulnerable populations, including migrants, refugees, and the host community.

Acknowledgments

We thank Anna Maria Bartolesi, Eleonora Riccobono, Gaetana Sterrantino, Michele Trotta, Michele Spinicci, and Francesca Rinaldi, who performed the microbiological confirmation of *Neisseria meningitidis.*

This work was partly funded by the Italian Ministry of Health- Centro nazionale per la prevenzione e il controllo delle malattie (CCM) Project "Sorveglianza delle malattie invasive da *Neisseria meningitidis, Streptococcus pneumoniae* ed *Haemophilus influenza*" 2015.

This publication made use of the Neisseria Multi Locus Sequence Typing website (http://pubmlst.org/neisseria/) developed by Keith Jolley and sited at the University of Oxford (7). The development of this site has been funded by the Wellcome Trust and the European Union.

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Clinical Manifestations of Punta Toro Virus Species Complex Infections, Panama, 2009

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DOI: https://dx.doi.org/10.3201/eid2305.161925

An investigation in Panama found that Punta Toro virus species complex (PTVs) may contribute to febrile illnesses with symptoms mirroring those of dengue fever. However, further studies are needed to determine if PTV infection causes only a mild disease or if it can have more serious manifestations in some patients.

Acute febrile illness in the New World tropics has a broad differential diagnosis largely dependent on locale and seasonal outbreaks. In Central America, most febrile illnesses have historically been attributed to dengue or malaria. However, recent evidence from Panama suggests varied differential diagnoses, including hantavirus, chikungunya virus, and Zika virus infection (*1*,*2*). In 2009, a dengue outbreak was reported in Panama City, Panama. The Gorgas Memorial Institute in Panama City tested dengue-negative samples from this outbreak for alphaviruses, flaviviruses, and phleboviruses and detected Punta Toro virus species complex (PTVs) in some samples. PTV (genus *Phlebovirus*, family *Bunyaviridae*), a member of the sand fly fever group, was initially described in humans in 1966 after being isolated from a soldier in Panama who had fever, headache, myalgia, and leukopenia (*3*). The phylogenetics of PTV have been thoroughly characterized (*4*–*6*), but our search of the literature did not reveal reports of other PTV cases in humans.

The signs and symptoms of sand fly–associated phlebovirus infection vary, but most infections cause a mild febrile illness characterized by retroorbital headache, weakness, back pain, and leukopenia. However, infection with 2 other phleboviruses, mosquitoborne Rift Valley fever virus and tick-associated severe fever with thrombocytopenia syndrome virus, causes severe disease. Little is known regarding the signs, symptoms, and clinical course of PTV infection in humans.

During the 2009 investigation, the Gorgas Memorial Institute analyzed 4,852 samples from persons in Panama with suspected acute dengue; 1,667 (34.4%) of the samples were dengue-negative. We further analyzed 201 of these samples for phlebovirus (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/5/16-1925-Techapp1.pdf). In brief, we extracted viral RNA from the samples and evaluated it by using *Phlebovirus* genus–specific reverse transcription PCR (RT-PCR) based on the

highly conserved L (large) gene that detects Toscana, Naples, Sicilian, Aguacate, Punta Toro, and Rift Valley fever viruses (*7*). We also screened samples using panflavivirus and panalphavirus RT-PCRs.

Of the 201 samples, 27 (13.4%) were RT-PCR–positive for phlebovirus. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) nucleotide sequence comparison suggested all were PTVs; 1 was previously described as Cocle virus (*4*). We conducted phylogenetic analyses on 11 of the phlebovirus-positive samples, using a 482-nt sequence and MEGA7 software (*8*). An optimal maximum-likelihood tree confirmed the samples were PTVs; all samples from 2009 (GenBank accession nos. KY43355–KY435365) clustered together close to Cocle virus (online Technical Appendix Table 2) (*4*). Our attempts to isolate virus were unsuccessful. To determine if PTV had been previously detected, we tested 202 randomly selected dengue virus–negative samples from 2008; none was phlebovirus-positive.

Clinical data sheets were available for 92.6% (25/27) of the PTV-positive samples. After de-identifying the data, we entered it into a dataset. A control group consisted of 90 dengue virus–positive patients from 2009 who were frequency matched by age and randomly selected from available records. The PTV-positive case-patients were largely located in the Panama City metropolitan area (Figure). Case-patients and controls were compared primarily with regard to reported symptoms (online Technical Appendix Table 3). Case-patients were significantly less likely than controls to have exanthema (22% vs. 54%; odds ratio 0.23, 95% CI 0.08–0.66; p = 0.01). We found no major clinical differences between case-patients and controls with regard to other symptoms. No patients in either group had shock or hemorrhagic syndrome.

Febrile syndromes in the tropics are often treated empirically; clinical decisions are often made on the basis of epidemiologic information and concurrent outbreaks. In Central America, dengue fever and malaria are treated without confirmatory testing because testing is costly and time-consuming. However, an increasing number of agents responsible for causing febrile illnesses have been identified in recent years. The variety of clinical outcomes observed with hantavirus and dengue, chikungunya, and Zika virus infections underscores the need for more accurate diagnostics to differentiate between causative agents. Clinical decisions must rely on accurate diagnoses because symptomatology is not an accurate predictor of the true etiology of a febrile illness.

Our findings suggest that, in Panama, PTVs may be a major contributor to febrile illnesses with symptoms mirroring those of dengue fever. However, the clinical course and range of disease caused by PTVs are unknown. Prospective studies are needed to determine if PTV infection causes only mild disease or if it can have serious manifestations in some patients.

PTVs are assumed to be sand fly–borne, and sand flies are usually present in rural or forested areas (*9*). However, most cases of PTVs infection in Panama in 2009 were in urban and periurban areas, raising questions about the vector, the vector's habitat, and the mode of virus transmission. Panama City, however, is home to two thirds of the country's population and has improved healthcare infrastructure, which may explain the higher number of confirmatory

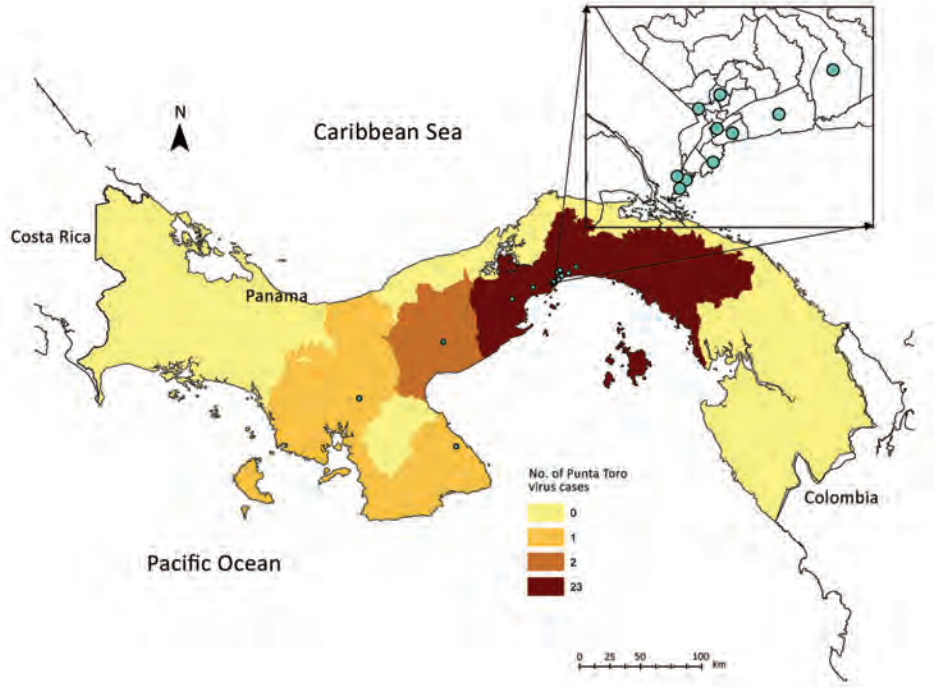

Figure. Distribution of confirmed Punta Toro species complex infections, Panama, 2009. Dots indicate cases. Inset shows enlargement of Panama City area.

tests from Panama City versus other areas of Panama and might result in a sampling bias. Despite these limitations, the recent Zika outbreak has shown the speed at which vectorborne diseases can spread and highlights the importance of detecting emerging viruses like PTVs.

Acknowledgments

We thank staff in the Department of Research in Virology and Biotechnology at the Gorgas Memorial Institute for Health Studies in Panama and in the Ministry of Health National Epidemiology Department for the surveillance data and outbreak response during 2009. We also thank Meghan Tipre for help creating the epidemiologic map.

This work was done in compliance with the Gorgas Bioethics Committee (1010/CBI/ICGES/15).

Funding was provided by the Panama Ministry of Economy and Finance (09.044.051 to S.L.-V. and 09.044.050 to J.M.P.); the Secretaría Nacional de Ciencia, Tecnología, e Innovación (SENACYT; FID-09-103 to J.-P.C); and Gorgas Memorial Institute, University of Alabama at Birmingham (to N.D.G.). J.M.P. and S.L.-V. are members of the Sistema Nacional de Investigación (SNI) of SENACYT in Panama.

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mcr-1 Colistin Resistance in ESBL-Producing *Klebsiella pneumoniae*, France

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DOI: http://dx.doi.org/10.3201/eid2305.161942

We report intestinal carriage of an extended-spectrum β-lactamase–producing *Klebsiella pneumoniae* strain with high-level resistance to colistin (MIC 24 mg/L) in a patient in France who had been hospitalized for fungal meningitis. The strain had the *mcr-1* plasmid gene and an inactivated *mgrB* gene, which are associated with colistin resistance.

Resistance to colistin in gram-negative bacteria stems mainly from structural modifications of bacterial lipopolysaccharide. These modifications include addition of 4-amino-4-deoxy-L-arabinose or phosphoethanolamine caused by chromosomal mutations in genes encoding the 2-component systems PhoPQ and PmrAB, or mutations in the *mgrB* gene, a negative regulator of PhoPQ (*1*).

The recent discovery of a horizontally transferable plasmid-mediated *mcr-1* gene encoding a phosphoethanolamine transferase is a cause for concern, but few *mcr-1*–positive clinical strains of *Klebsiella pneumoniae* have been reported so far in Europe (*2*). Colocalization of carbapenemases or extended-spectrum β-lactamase (ESBL) genes and the *mcr-1* gene on the same plasmids is of concern because it might lead to pandrug resistance (*1*,*3*). We report *mcr-1* colistin resistance in ESBL-producing *K. pneumoniae* isolated from a patient in France.

The patient was a 38-year-old man who had chronic granulomatous disease that was diagnosed when he was 8 months old. Since then, he has had several minor and major diseases and conditions, including primitive femoral osteitis, hepatic abscesses, disseminated candidiasis, and bacteremia, which required several treatments with antimicrobial drugs. However, the patient was never given colistin.

In April 2016, he was hospitalized for surgical removal of a thyroid abscess. Fungal cultures of the abscess grew *Aspergillus fumigatus*. Despite antifungal treatment with amphotericin B and flucytosine, fungal meningitis, cerebral arterial vasospasm at the Willis polygon, and hydrocephalus developed. The patient also received immunosuppressive therapy (methylprednisolone and anakinra) and empiric antimicrobial drug therapy, including cotrimoxazole, clindamycin, meropenem, and vancomycin successively.

In August 2016, systematic culture of a rectal swab specimen showed an ESBL-producing strain of *K. pneumoniae*; 2 previous rectal screenings showed negative results. The strain was resistant to colistin (MIC >4 mg/L). Resistance was determined by using a broth microdilution assay (BD Phoenix Instrument; Becton Dickinson, Franklin Lakes, NJ, USA) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/5/16-1942-Techapp1.pdf). An MIC of 24 mg/L was found for polymyxin B by using the E-test method (Polymyxin B E-test strip; bioMérieux, Marcy L'Etoile, France).

The strain was sent to the French National Reference Center for Antibiotic Resistance in *Enterobacteriaceae* (Hôpital Gabriel Montpied, Clermont-Ferrand, France), which confirmed phenotypic resistance to colistin and identified the *mcr-1* gene by using PCR and previously described primers (*2*). Whole-genome sequencing showed that the *K. pneumoniae* strain had genotype ST15 and confirmed the presence of the *mcr-1* gene on a 33,303-kb transferable plasmid of incompatibility group IncX4 (online Technical Appendix). This plasmid differed by only 4 mutations from *mcr-1.2*–encoding plasmid pMCR-1.2.IT (GenBank accession no. KX236309) previously characterized in Italy (*4*). Conjugation of the plasmid into *Escherichia coli* K12 conferred colistin resistance (MIC increased from 0.25 mg/L to 4 mg/L) to the *E. coli* strain.

Other resistance genes were also identified (Table), including the ESBL-encoding gene bla_{SHV106} (online Technical Appendix). None of them were localized with the *mcr-1* gene on the IncX4 plasmid. Moreover, insertion of mobile element *IS5* in the *mgrB* gene was detected, which is also associated with colistin resistance (*5*). No mutations were found in the *prmA, prmB, phoP,* and *phoQ* genes.

There is currently no commercial medium to screen gram-negative bacteria harboring the *mcr-1* gene. Nordmann et al. (*6*) described an in-house SuperPolymyxin medium composed of eosin methylene blue agar, 3.5 mg/L of colistin sulfate, 10 mg/L of daptomycin, and 5 mg/L amphotericin B, which showed excellent sensitivity and specificity. Colistin resistance can be confirmed within 2 h by using an in-house rapid polymyxin Nordmann-Poirel test (*7*). The *mcr-1* gene can be rapidly detected by real-time PCR of DNA extracts obtained from bacterial strains or directly from stool samples (*2*,*8*,*9*).

We obtained subcultures of the strain from the patient on Columbia CNA agar containing 10 mg/L of colistin and 15 mg/L of nalidixic acid and 5% sheep blood (CNA^+; bioMérieux) but not on Thayer-Martin agar medium containing unknown concentrations of vancomycin, colistin, amphotericin B, and trimethoprim (VCA3; bioMérieux). Lack of growth on this medium might be related to a high colistin concentration or the presence of vancomycin, which can potentiate colistin activity (*6*). Further investigations using

Table. Resistance genes identified by whole-genome sequencing of an ESBL-producing *mcr-1*–positive *Klebsiella pneumoniae* strain isolated from a 38-year-old man, France*

Resistance gene	Target antimicrobial drug
mcr-1 and inactivation of *mgrB* by *IS5* insertion	Colistin
$bla_{SHV-106}$	β-lactams
aac(3)-IId and *aad*A16-like	Aminoglycoside
aac(6′)Ib-cr	Quinolone and aminoglycoside
*fos*A5	Fosfomycin
sulI and *folP*	Sulfonamide
*dfr*A27	Trimethoprim
tetD	Tetracycline

*ESBL, extended-spectrum β-lactamase.

CNA+ medium did not identify intestinal carriage of ESBL-negative but *mcr-1*–positive enterobacteria in the index case-patient. On the basis of these results, rectal screening of 39 contacts was performed by using an ESBL-screening medium (BLSE agar [MacConkey agar and Drigalski agar]; bioMérieux). All of the tests showed negative results.

The origin of the *mcr-1* strain remains unknown. Nosocomial acquisition cannot be ruled out because colistin-resistant strains harboring the *mcr*-1 gene might have been isolated in the hospital but not identified because this resistance mechanism was initially reported in February 2016. Food might also be incriminated (*1*); one study identified a 21% *mcr-1* prevalence among ESBL-producing *E. coli* in calves in France (*10*).

Multiple antimicrobial drug therapy for this patient might have selected for this multidrug-resistant bacteria. The presence of a plasmid containing the *mcr-1* and ESBL or other resistance genes in the same strain might be involved in selection of colistin-resistant strains during administration of any ineffective antimicrobial drug (*3*). Development of efficient tools for rapid detection of *mcr-1*–harboring strains should be a priority to prevent dissemination of these strains in hospital settings.

Dr. Caspar is a clinical microbiologist in the Bacteriology Laboratory, Grenoble-Alpes University Hospital, and a probationary lecturer in the Medicine Faculty, Grenoble-Alpes University, Grenoble, France. His primary research interests are bacterial resistance or treatment failures for tularemia and evaluation of novel antimicrobial compounds.

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Chromosomal 16S Ribosomal RNA Methyltransferase RmtE1 in *Escherichia coli* Sequence Type 448

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DOI: http://dx.doi.org/10.3201/eid2305.162000

We identified *rmtE1*, an uncommon 16S ribosomal methyltransferase gene, in an aminoglycoside- and cephalosporin-resistant *Escherichia coli* sequence type 448 clinical strain co-harboring bla_{CMY-2}. Long-read sequencing revealed insertion of a 101,257-bp fragment carrying both resistance genes to the chromosome. Our findings underscore *E. coli* sequence type 448 as a potential high-risk multidrug-resistant clone.

RmtE (RmtE1 and its variant RmtE2) is an uncommon plasmid-mediated 16S rRNA methyltransferase (16S RMTase) found in gram-negative bacteria; only 4 strains have been reported to produce RmtE, all *Escherichia coli*, including 1 from the University of Pittsburgh Medical

Center (Pittsburgh, PA, USA) (*1*–*3*). We report the genetic context of *rmtE* (*rmtE1*) in another *E. coli* clinical strain identified at this hospital.

E. coli YDC774 was identified in 2016 in the urine of a local elderly man with a history of bladder cancer for which he had undergone transurethral resection of the bladder and completed chemotherapy. He had *E. coli* urinary tract infection treated with ciprofloxacin 3 months earlier; further details were unavailable. *E. coli* YDC774 was resistant to cefotaxime, levofloxacin, ciprofloxacin, and trimethoprim/sulfamethoxazole and susceptible to ceftazidime, cefepime, piperacillin/tazobactam, imipenem, meropenem, minocycline, and colistin. The strain was highly resistant to amikacin (MIC >32 μg/mL), gentamycin (MIC >16 μg/mL), and tobramycin (MIC >8 μg/mL). Because the positive culture was believed to represent asymptomatic bacteriuria, the patient was not treated with antimicrobial drugs.

We aimed to elucidate the genetic context of *rmtE* in *E. coli* YDC774. Although *rmtE* has been identified exclusively on plasmids, neither broth conjugation with *E. coli* J53 nor transformation of *E. coli* TOP10 with purified plasmids mobilized *rmtE*, leading us to speculate the gene might be located on the chromosome. We therefore sequenced the YDC774 genome with PacBio RS II sequencing instrument (Pacific Biosciences, Menlo Park, CA, USA) as described (*4*). Sequencing with a single SMRT cell yielded 64,878 reads averaging 10,991 bp. De novo assembly generated 8 contigs; the largest was ≈4.3 Mbp, which had ≈122× coverage and was consistent with a large portion of the *E. coli* chromosome.

E. coli YDC774 belonged to sequence type (ST) 448 by in silico multilocus sequence typing. *E. coli* ST448 has been reported in recent years among extended-spectrum β-lactamase– and New Delhi–type metallo-β-lactamase–producing strains (*5*,*6*). The chromosomal contig contained *rmtE* (*rmtE1* allele), bla_{CMY-2}, *aac(3)-VIa*, *aadA*, *strA/B*, *floR*, *sul1*, *sul2*, *tet*(A), and *dfrA7* as determined by ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/). We identified no resistance genes on the other contigs, including those representing a 96-kb IncY plasmid resembling p12579_1 (GenBank accession no. CP003110.1) in enteropathogenic *E. coli* strain RM12579 (99% identity over 83% coverage). Several other 16S RMTase genes, such as *rmtB*, *rmtC*, and *rmtF*, have been found on the chromosome of gram-negative bacteria (*7*,*8*).

The region surrounding *rmtE1* was annotated with Rapid Annotations by using Subsystem Technology server (http://rast.nmpdr.org) and curated manually by using blastn and blastp (http://blast.ncbi.nlm.nih.gov/blast) to elucidate the context of its chromosomal integration. Using *E. coli* ATCC 25922 as the reference genome, we determined that a 101,257-bp sequence was inserted in an intergenic region between the 4′-phosphopantetheinyl transferase gene and the NAD(P)H nitroreductase gene on the *E. coli* chromosome.

This inserted sequence can be divided into 2 regions. The first comprises several inserted sequences, such as IS*186*, IS*CR1*, and 1 antimicrobial resistance gene, *aadA*. Downstream of this first region, the inserted fragment is similar to that in pYDC637, an IncA/C plasmid carrying *rmtE1* also found at the University of Pittsburgh Medical Center in 2012 (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/5/16-2000-Techapp1.pdf) (*2*). However, the second region comprises 3 small fragments. The first contains *aadA1-bx*, 4 mobile elements, and several other genes and is in reverse orientation from that of pYDC637. The second small fragment harboring bla_{CMY-2} is identical to that found in the core region in pYDC637 (online Technical Appendix Figure) and also is in reverse orientation from the corresponding region of pYDC637. The third small fragment harboring *rmtE* is located in the acquired region of pYDC637. This finding suggests that, on mobilization into the chromosome, gene rearrangements occurred among these fragments. The region between 2 hypothetical proteins appears to have been deleted at or after integration, which includes genes involved in plasmid replication and conjugative transfer (online Technical Appendix Figure).

rmtE1 is bound by an IS*CR20*-like element and an IS*1294*-like insertion sequence. This immediate unit is identical to that found in pYDC637. IS*CR20* and IS*1294* belong to IS*91* family, which is considered related to some antimicrobial drug resistance genes, including 16S RMTase genes, which appears to have been the case in the mobilization of *rmtE1* as well. We could not identify direct repeats upstream and downstream of the unit that would define the exact boundary of this unit. In comparing the genetic context of *rmtE1* and *rmtE2*, IS*CR20*-like transposase is located upstream of *rmtE1* and *rmtE2* (GenBank accession nos. KT428293 and NZ_LRIX01000127). However, the transposase genes located downstream of the 2 16S RMTase genes are distinct. The genetic environment of *rmtE2* is identical between the 2 plasmids from China (GenBank accession no. KT428293) and Canada (GenBank accession no. NZ_LRIX01000127).

In summary, we identified chromosomal integration of *rmtE1*, an unusual 16S RMTase, and bla_{CMY-2}, a commonly observed acquired AmpC β-lactamase, in an *E. coli* ST448 clinical strain, an event that generated stable co-resistance to aminoglycosides and oxyiminocephalosporins. We found no evidence of further spread of this strain in the hospital. Nonetheless, the findings underscore *E. coli* ST448 as a potential high-risk multidrug-resistant *E. coli* clone.

Y.D. was supported by research grants from the National Institutes of Health (R01AI104895, R21AI123747).

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Carbapenem-Resistant *Enterobacter cloacae* in Patients from the US Veterans Health Administration, 2006–2015

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DOI: http://dx.doi.org/10.3201/eid2305.162034

We analyzed carbapenem-resistant *Enterobacteriaceae* (CRE) trends among patients from the US Veterans Health Administration (VHA). After the emergence of CRE in the eastern United States, resistance rates remained stable in *Klebsiella pneumoniae* but increased in *Enterobacter cloacae* complex, suggesting a "second epidemic." VHA offers a vantage point for monitoring nationwide CRE trends.

Carbapenem-resistant *Enterobacteriaceae* (CRE) have become a global public health threat. The epidemic of CRE began in the early 2000s with an outbreak of carbapenem-resistant *Klebsiella pneumoniae* harboring *K. pneumoniae* carbapenemase (KPC) in the eastern United States. Since then, KPC-producing *K. pneumoniae* have emerged in various communities across the country (*1*). Carbapenem resistance also occurs in other *Enterobacteriaceae* species and can be mediated by other enzymes, such as OXA-48 and metallo-β-lactamases, especially New Delhi metallo-β-lactamase and Verona integron–encoded metallo-β-lactamase (*1*). Carbapenem-resistant *Escherichia coli* occurs infrequently, but recent outbreaks of KPC-producing *Enterobacter cloacae* raise concerns about the emergence of carbapenem resistance in the *E. cloacae* complex (*1–4*).

The Veterans Health Administration (VHA) is the largest integrated healthcare system in the United States. Clinical and microbiologic data for the entire VHA network are accessible through its informatics platforms (*5*). We used this infrastructure to observe national trends of carbapenem resistance and nonsusceptibility in *K. pneumoniae* and *E. cloacae* complex during the past decade.

We identified 224,651 *K. pneumoniae* and 71,462 *E. cloacae* complex (*E. cloacae, E. asburiae, E. kobei,*

E. hormaechei, E. xiafangensis) isolates from patients hospitalized during 2006–2015. To minimize bias introduced by variability in susceptiblity reporting, we excluded isolates obtained within 30 days of another isolate from the same patient and isolates from facilities with nonstandard reporting, facilitites that identified <30 isolates in a 2-year period, and facilities that reported carbapenem susceptibilities for <90% of isolates. After these exclusions, 128,431 *K. pneumoniae* and 38,219 *E. cloacae* complex isolates from 140 facilities in 40 states, District of Columbia, and Puerto Rico remained for study. We obtained carbapenem (i.e., meropenem, imipenem, ertapenem, doripenem) susceptibility test results (i.e., susceptible, intermediate, or resistant) and calculated rates of resistance and nonsusceptibility to any carbapenem over time, looking at 2-year windows and grouping facilities into 10 regions designated by the US Department of Health and Human Services (https://www.hhs.gov/about/agencies/regional-offices/index.html).

Our data capture the chronologic and geographic spread of carbapenem-resistant *K. pneumoniae* and *E. cloacae* complex within VHA (Figure). Before 2010, carbapenem-resistant *K. pneumoniae* was observed primarily in the eastern United States, but by 2014–2015, the rate of carbapenem resistance detected in *K. pneumoniae* was >1% in all regions except Regions 8 (South Dakota, North Dakota, Montana, Wyoming, Colorado, Utah) and 10 (Washington, Oregon, Idaho, Alaska). In 2006–2007, carbapenem-resistant *E. cloacae* complex also had a focus in the East, but in 2008–2009, it was also observed in Region 8. By 2014–2015, carbapenem-resistant *E. cloacae* complex was centered in the Southwest and Pacific Coast. These regions also had higher rates of carbapenem-nonsusceptible *E. cloacae* complex, although this phenotype was present in all regions.

The carbapenem-nonsusceptibility rate among *K. pneumoniae* isolates remained at 3%–4% throughout 2006–2015; however, as with a 2016 study (*6*), we detected a recent decrease in the rate of carbapenem resistance in *K. pneumoniae* in Region 2, which includes New York. In contrast, the rates of carbapenem resistance and nonsusceptibility in *E. cloacae* complex increased nationwide during the last decade, reaching >4% nonsusceptibility and 2.5% resistance in 2014–2015. Also, the proportion of intermediate carbapenem-nonsusceptible isolates was higher for *E. cloacae* complex (46%) than *K. pneumoniae* (9%) isolates.

CRE trends during 2006–2015 in the VHA recapitulate the epidemic of carbapenem-resistant *K. pneumoniae* in the United States and indicate that a "second epidemic" of carbapenem-resistant *E. cloacae* complex appears to be unfolding. In the United States, the predominant carbapenem-resistant *K. pneumoniae* genotype is sequence type (ST) 258, which is associated with Tn*4401*, a mobile genetic element containing bla_{KPC} (*7*). In contrast, the genetic background of carbapenem-resistant *E. cloacae* complex is not well defined. Analysis of carbapenem-resistant *E. cloacae* from the US Midwest and New York, NY, demonstrated dissemination of *E. cloacae* complex ST171 harboring the bla_{KPC-3} gene (*2*,*3*,*8*). Further analysis demonstrated that ST171 was associated with a Tn*4401* variant within a pBK30683-like plasmid; however, various other plasmids in *Enterobacter* spp. also harbor bla_{KPC-3} (*4*). Of note, in a northeastern US hospital, one third of carbapenem-resistant *E. cloacae* contained carbapenemases and the rest harbored

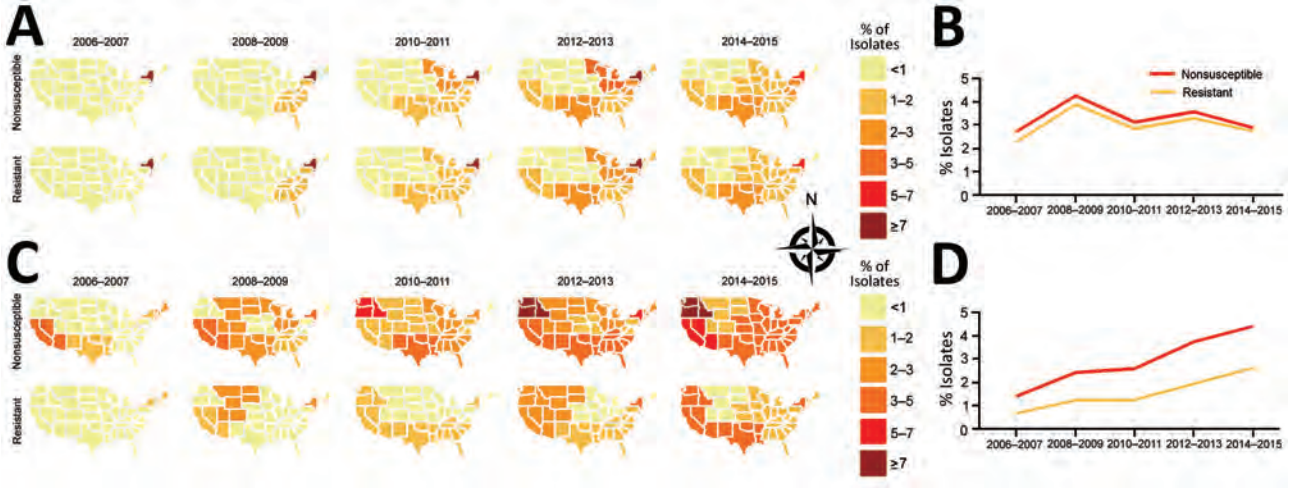

Figure. Geographic and temporal trends of carbapenem nonsusceptibility and resistance in *Enterobacteriaceae* seen at Veterans Health Administration facilities, United States, 2006–2015. A) Dissemination of carbapenem-resistant *Klebsiella pneumoniae* after an initial focus in the eastern United States. B) Nationwide percentage of carbapenem nonsusceptibility and resistance in *K. pneumoniae*. C) Emergence and dissemination ("second epidemic") of carbapenem-nonsusceptible and -resistant *Enterobacter cloacae* complex. D) Nationwide percentage of carbapenem nonsusceptibility and resistance in *E. cloacae* complex. Isolates from patients in Puerto Rico were not included in the maps.

cephalosporinases, usually only AmpC (*9*). Nevertheless, we hypothesize that *E. cloacae* complex contains genotypes with epidemic potential associated with increasing rates of carbapenem resistance observed in the VHA.

The scope of this study did not include molecular characterization, so we could not determine emerging genotypes or detect outbreaks at individual facilities. Also, nonuniform susceptibility testing and interpretation throughout the VHA may affect reporting of CRE. Although criteria for interpretation of carbapenem susceptibility changed during the past decade, the revised breakpoints do not appear to have a major effect on resistance rates in *Klebsiella* and *Enterobacter* spp., according to other surveillance data (*10*). Despite these limitations, the VHA may serve as a vantage point for detecting nationwide trends in antimicrobial drug resistance. Integration of susceptibility testing with molecular characterization at the VHA may help elucidate the changing epidemiology of CRE in the United States.

This work was approved by the Institutional Review Board at the Louis Stokes Cleveland Department of Veterans Affairs Medical Center.

This work was supported in part by the National Institutes of Health through the Clinical and Translational Science Collaborative of Cleveland (UL1TR000439), National Institute for Allergy and Infectious Disease (R01AI100560, R01AI063517, R21AI114508, and R01AI072219), and VA Research and Development Office (BX001974) and by the VISN 10 Geriatrics Research, Education and Clinical Center. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs.

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Vertical Transmission of Zika Virus by *Aedes aegypti* and *Ae. albopictus* Mosquitoes

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DOI: http://dx.doi.org/10.3201/eid2305.162041

To determine the potential role of vertical transmission in Zika virus expansion, we evaluated larval pools of perorally infected *Aedes aegypti* and *Ae. albopictus* adult female mosquitoes; ≈1/84 larvae tested were Zika virus–positive; and rates varied among mosquito populations. Thus, vertical transmission may play a role in Zika virus spread and maintenance.

Following the 2007 outbreak in Micronesia, Zika virus (*Flaviviridae, Flavivirus*) has continued to expand its distribution throughout the Pacific region and, since 2014, the Americas (*1*,*2*). The virus is primarily maintained by horizontal transmission between *Aedes aegypti* mosquitoes and humans, yet other *Aedes* spp. are also competent vectors (*3*). The extent to which Zika virus can utilize vertical transmission between mosquitoes (i.e., transmission from an infected adult female mosquito to her progeny) has not been adequately assessed after peroral infection. Such studies are required to accurately determine the potential role of vertical transmission in Zika virus expansion and maintenance.

Although previous studies have found that other flaviviruses, including West Nile (*4*), dengue (*5*), yellow fever (*6*), and St. Louis encephalitis (*7*), can undergo vertical transmission, such transmission is generally relatively inefficient, with filial infection rate (FIR) estimates ranging from 1/36 to 1/6,400 (*8*). A previous study estimated rates for Zika virus vertical transmission in *Ae. aegypti* mosquitoes to be 1/290, yet a reliable estimate for transmission in *Ae. albopictus* mosquitoes was not achieved (*8*). In addition, these estimates were based on intrathoracic inoculation of Zika virus rather than on assessment after infectious blood meal acquisition.

We exposed laboratory colonies of *Ae. aegypti* mosquitoes (collected in Posadas, Argentina, or Poza Rica, Mexico) and *Ae. albopictus* mosquitoes (obtained from Suffolk County, New York) to Zika virus through infectious blood meals and evaluated the mosquitoes' capacity to transmit the virus to progeny. For this study, we used the Zika virus strain ZIKV HND (Honduras 2016, GenBank accession no. KX906952), passaged once on C6/36 cells, and Zika virus PR (Puerto Rico 2015, GenBank accession no. KX087101.3), passaged 4 times on Vero cells and twice on C6/36 cells. Zika virus was propagated on C6/36 cells for 4 days, and freshly harvested supernatant was mixed 1:1 with sheep blood (Colorado Serum Company, Denver, CO, USA) and 2.5% sucrose.

Infectious blood meals were offered to 4- to 7-day-old female mosquitoes, and weekly noninfectious blood meals were offered after the first oviposition. Eggs laid during the second oviposition and beyond were collected and hatched for subsequent testing. Third- to fourth-instar larvae were collected in pools of 5 and processed by homogenization and centrifugation. After RNA extraction, we used Zika virus–specific quantitative reverse transcription PCR (*9*) to determine adult infection (indicated by positive bodies), dissemination (indicated by positive legs), viral load, and

Table. Vertical transmission of Zika virus in *Aedes* spp. mosquitoes*

Species/ population	Zika virus strain	Cycle	Blood meal titer, $\log_{10}$ PFU/mL	% Infected (diss)†	Mean body titer, $\log_{10}$ PFU/mL	Total no. pools	No. individual mosquitoes	dpi	Zika virus positive	FIR‡ (95% CI)
Ae. aegypti										
Mexico	ZIKV HND	All§	8.9	90.9 (95.0)	7.6	26	130	11–18	1	7.7 (0.5–36.9)
Argentina	ZIKV HND	All§	9.3	100 (100)	6.6	28	136	11–38	2	14.9 (2.7–8.3)
Combined¶	ZIKV HND	OV2				29	141	11–22	1	7.1 (0.4–4.0)
		OV3				23	115	18–38	2	17.7 (3.2–57.2)
		OV4				2	10	38	0	<94.6 (6.6–495.8)
Combined¶	ZIKV HND	All§	9.1#	95.5 (97.5)	7.4	54	266	11–38	3	11.5 (3.0–30.8)
Argentina	ZIKV PR	OV1				24	120	36–38	2	17.0 (3.1–54.8)
		OV2				15	75	43–52	0	<13.3 (0.8–63.6)
		OV3				4	18	60–62	0	<55.8 (3.4–262.5)
		OV4				7	35	63	1	28.5 (1.7–34.8)
	ZIKV PR	All§	9.1	100 (100)	7.7	50	248	36–63	3	12.3 (3.3–33)
Combined	Combined**	All§	9.1#	96.9 (98.3)	7.5	104	514	11–63	6	11.9 (4.9–4.6)
Ae. albopictus										
New York	ZIKV HND	All§	8.9	100 (93.3)	7.1	17	85	11–63	1	11.8 (0.7–56.2)

*Diss, disseminated; dpi, days post infection; FIR, filial infection rate; ZIKV HND, Zika virus Honduras 2016; OV, oviposition; ZIKV PR, Zika virus Puerto Rico.
†Percentage of infected with Zika virus–positive legs.
‡No. Zika virus positive/1,000 larvae.
§Combines data from all hatched eggs.
¶Data for both mosquito populations are combined.

FIR, which was calculated by using a maximum-likelihood estimate (PoolInfRate 4.0; Centers for Disease Control and Prevention, Atlanta, GA, USA).

We tested 104 *Ae. aegypti* pools; 6 were Zika virus–positive, indicating a FIR of 11.9 (range 4.9–24.6; Table). This value equates to a ratio of ≈1:84, which is substantially higher than that found by Thangamani et al., as well as ratios historically measured for flaviviruses (*4*–*8*). Although just 17 pools of *Ae. albopictus* were tested, 1 pool was positive, which equates to a similar FIR (11.8 [range 1.7–134.8]; Table) and establishes that *Ae. albopictus* mosquitoes are capable of vertical transmission of Zika virus in the laboratory.

Although the bypassing of the midgut during inoculation generally results in higher levels of vertical transmission, we fed mosquitoes high virus doses (8.9–9.3 $\log_{10}$ PFU/mL), resulting in >93% of disseminated infections and development of high viral titers in individual mosquitoes, averaging 7.1 (*Ae. albopictus*) to 7.5 (*Ae. aegypti*) $\log_{10}$ PFU/mosquito (Table). Although the likelihood that eggs were derived from mosquitoes with disseminated infections is high, the rate of vertical transmission (proportion of infected mosquitoes transmitting to progeny) could not be determined. Future studies assessing infection status and FIR of individual mosquitoes will help clarify the extent of individual variability in vertical transmission efficiency. In addition, we tested larvae rather than adults, and it is likely that transtadial transmission is not completely efficient, so further studies are required to fully evaluate transmission potential of adults infected via vertical transmission. We observed a trend of increasing vertical transmission with time and additional egg laying, similar to what has been reported for West Nile virus (*10*). This finding suggests that survival and gonotropic cycles could be key determinants of success of vertical transmission in nature. Finally, our results demonstrate population-specific differences, with the FIR of the population from Argentina more than twice that of the population from Mexico (Table), suggesting that particular populations may have increased capacity for maintenance through vertical transmission. Although we did not measure differences between ZIKV HND and ZIKV PR, evaluating additional strains could help clarify the influence of viral genotype on vertical transmission efficiency.

Together, these results indicate that Zika virus has a relatively high capacity for being transmitted vertically by both *Ae. aegypti* and *Ae. albopictus* mosquitoes. Although the mechanism of vertical transmission with flaviviruses is generally thought to be infection of eggs during oviposition, rather than transovarial transmission (*5*), these rates suggest that further investigation into Zika virus tropism in mosquitoes is warranted.

Acknowledgments

We thank the members of the Wadsworth Center Arbovirus Laboratory insectary staff for assistance with this project and the Wadsworth Center tissue and media facility for supplying cells and media for these studies. We are also grateful to V. Micieli, Centro de Estudios de Parasitología y Vectores, for supplying *Ae. aegypti m*osquitoes collected in Posadas, Argentina; to G. Ebel, Colorado State University, for *Ae. aegypti* mosquitoes collected in Poza Rica, Mexico; and to I. Rochlin, Suffolk County Health Department, for *Ae. albopictus* mosquitoes obtained from Suffolk County, New York.

The construction of the Wadsworth Center Arbovirus Laboratory insectary facilities was partially funded by the National Institutes of Health (NIH) grant C06-RR-17715.

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Diagnosis and Management of *Borrelia turicatae* Infection in Febrile Soldier, Texas, USA

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DOI: http://dx.doi.org/10.3201/eid2305.162069

In August 2015, a soldier returned from field exercises in Texas, USA, with nonspecific febrile illness. Culture and sequencing of spirochetes from peripheral blood diagnosed *Borrelia turicatae* infection. The patient recovered after receiving doxycycline. No illness occurred in asymptomatic soldiers potentially exposed to the vector tick and prophylactically given treatment.

Tickborne relapsing fever (TBRF) was first reported in the United States >100 years ago but is often difficult to identify, given its rarity and variety of clinical presentations (*1*,*2*). We describe a case of culture-confirmed TBRF caused by *Borrelia turicatae* acquired by an Army soldier during a military training exercise in Texas, USA.

The patient was a 31-year-old white man with no relevant medical history. In August 2015, he sought care at the Eglin Air Force Base Hospital (Valparaiso, Florida, USA) with a 5-day history of fever (102°F), chills, and myalgias. He reported headache and nausea but no vomiting or diarrhea. He denied localized joint pain, redness, or swelling but had discomfort in both popliteal fossae.

The patient had recently returned to Florida after a 30-day Army exercise under austere conditions in western Texas (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/5/16-2069-Techapp1.pdf). Potential exposures included sleeping nude in a sleeping bag on the floor of an abandoned barn that had been cleared of infestation with rabbits, rodents, birds, and bats; having eaten boar that had been slaughtered, dressed, and cooked over an open flame; and consuming water procured from a well, bottled sources, and at times through a LifeStraw (http://lifestraw.com/). Approximately 1 week before admission, he had noted multiple skin lesions, including scattered, presumed insect bites along his left leg and a small lesion at his urethral meatus. He denied any history of genital lesions and had not seen any biting insects. After 6 days, the lesions spontaneously resolved.

In a Texas emergency department, the initial diagnosis was viral syndrome, and a rapid influenza test result was negative. The fever persisted despite administration of antipyretics. After 2 days, the patient returned to the hospital, where he received only symptomatic treatment. No tests were ordered. After another 2 days, he sought care from his unit physician. Laboratory tests showed marked thrombocytopenia with 16×10^9 platelets/L (reference range 150–400×10^9 platelets/L). Spirochetes were seen on peripheral blood smear (online Technical Appendix Figure 2). He was referred for hospital admission. Physical examination findings were unremarkable: no splenomegaly, hepatomegaly, or rash. Blood cultures and serologic testing for rickettsiae, HIV, dengue virus, *Treponema pallidum,* and plasmodia produced negative results. Erythrocyte sedimentation rate (58 mm/h) and C-reactive protein level (>19 mg/L) were elevated. Electrolytes and transaminase levels were within reference ranges.

Serum samples collected at admission and 3 weeks later (≈5 and 26 days after illness onset, S1 and S2, respectively) were tested in parallel at the Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Division of Vector-Borne Infectious Diseases (Fort Collins, CO, USA) by enzyme immunoassay and Western blot (IgM and IgG) for TBRF antibody reactivity. Seroconversion was demonstrated by rising enzyme immunoassay values (S1 = 0.79, S2 = 2.41; equivocal range 0.64–0.91) and separate IgM and IgG Western blots (online Technical Appendix Figure 3). In addition, the samples demonstrated seroconversion (S1 = 0.91, S2 = 3.23; equivocal range 0.90–1.09) against C6, an immunogenic antigen conserved among *Borrelia* spp. (*Borrelia burgdorferi* ELISA; Immunetics, Inc., Boston, MA, USA).

Spirochetes were successfully cultured, and genomic sequencing determined that *B. turicatae* was the causative agent (*3*). The patient improved rapidly with doxycycline, and platelet count normalized within 2 weeks. Ten asymptomatic soldiers with similar exposure were identified and prophylactically given doxycycline; 24 asymptomatic soldiers who had been in the area but not in the same barn as the patient were monitored closely. No additional illnesses were detected.

TBRF is a neglected and probably underdiagnosed disease. The vector, the *Ornithodoros turicata* tick, is endemic to Texas and Florida (*4*); but although published cases in Texas have been supported by serology for the TBRF group, exposure location, and tick collections (*4*,*5*), to the best of our knowledge, successful identification of *B. turicatae* in a human has not been reported. Previously,

B. turicatae has been isolated only from ticks and canids in several areas of Texas (*4*–*6*).

The ecologic setting of the military exercises was predictable for high-risk exposure to the tick vector. TBRF attack rates >22% have been reported for group settings with sequelae severe enough to warrant hospitalization (*7*,*8*). Military training groups in Israel have declared certain caves off limits because of heavy tick presence (*9*) and have prophylactically administered doxycycline to those suspected to have been exposed (*7*). There has not been an association of Jarisch-Herxheimer reaction in asymptomatic patients receiving doxycycline (*7*), although this reaction is common during treatment of patients with active illness (*9*).

We identified several difficulties in epidemiologic awareness and diagnosis. There is overlap of bacterial, viral, and parasitic pathogens in location and nonspecific symptom presentations. The *O. turicata* tick bite is rarely noticed or reported because the vectors are rapid nocturnal feeders, attachment is painless, and often no lesions or ticks are discovered (*10*). This case report with successful isolation and genetic characterization of *B. turicatae* from the soldier (*3*) confirms that this spirochete species is a zoonotic pathogen. The initial misdiagnosis further indicates the neglected nature of this disease, especially in the military population.

Acknowledgment

We acknowledge the help of Dolli Lane, Christopher Boyd, and Michael McFall in identifying bacteria on peripheral smears and definitively identifying this case.

Dr. Christensen is a second-year family medicine resident and medical officer in the US Air Force, stationed at Eglin Air Force Base, Florida. Her research interests include infectious diseases as they affect military and operational medicine.

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Correction

Vol. 16 No. 1

The name of author Emerald Stewart was omitted from Rapid Displacement of Dengue Virus Type 1 by Type 4, Pacific Region, 2007–2009 (D. Li et al.). The article has been corrected online (https://wwwnc.cdc.gov/EID/article/16/1/09-1275_article).

CTX-M-27–Producing *Escherichia coli* of Sequence Type 131 and Clade C1-M27, France

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DOI: http://dx.doi.org/10.3201/eid2305.161865

To the Editor: We read with great interest the Matsumura et al. paper describing extended-spectrum β-lactamase (ESBL) CTX-M-27–producing *Escherichia coli* of sequence type (ST) 131 clonal group, an emerging clade called C1-M27 (*1*). ST131 *E. coli* having $bla_{CTX\text{-}M\text{-}27}$ has been described in several countries. We observed an alarming increase of this clonal group in the fecal carriage of children in France (0% in 2010 to 65% in 2015 among ESBL-producing ST131 *E. coli*) (*2*).

We wondered whether this clonal group's expansion in France was attributable to the same clade (C1-M27). For that, we designed primers (M27PP1-B-F, 5′-TTACTCC-GACTATGCGTTCAC-3′; M27PP1-B-R, 5′-CAAACTT-GCCCCTGATAGCG-3′; amplicon length, 1.5 kb) to amplify the insertion site of the structure comprising the direct repeat and prophage-like genomic island of *E. coli* PCN033, as previously described (*1*). PCR was performed on our recently described collection of 39 ESBL-producing ST131 *E. coli*, including 16 CTX-M-27–producing *E. coli*: 13 of subgroup O25b with *fimH*30 allele and 3 of O16 subgroup with *fimH*41 allele (*2*). Results showed that 81% (13/16) of the CTX-M-27–producing *E. coli* ST131 had the M27PP1 structure, similar to strain PCN033, and thus belong to the C1-M27 clade. Therefore, the C1-M27 clade found in Asia and America is also present in Europe in the fecal flora of young children. The 3 isolates belonging to the O16 subgroup with *fimH*41 lacked M27PP1, suggesting that $bla_{CTX\text{-}M\text{-}27}$ might diffuse among non-H30 ST131 *E. coli* without this prophage-like genomic island. Finally, the non–CTX-M-27–producing ST131 *E. coli* of our collection were negative for M27PP1 elements.

Our results show that CTX-M-27–producing *E. coli* ST131 subgroup O25b with *fimH*30 allele (one third of the ESBL-producing ST131 carriage isolates) isolated from children in France belong to C1-M27 and that CTX-M-27–producing O16 strains display distinct genetic characteristics. Altogether, our data confirm the worldwide distribution of C1-M27 and its high prevalence in children in France.

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Antimicrobial Drug Resistance among Refugees from Syria, Jordan

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DOI: http://dx.doi.org/10.3201/eid2305.170117

To the Editor: The Kassem et al. article regarding high rates of multidrug-resistant (MDR) bacteria colonizing Syrian children highlights the challenge of choosing empiric antimicrobial drugs to treat war-injured refugees from Syria (*1*). The findings mirror other reports (*2*,*3*) and our own experience in a charitable hospital in Amman, Jordan, which manages war-injured refugees from Syria. As part of a program of antimicrobial drug stewardship and infection prevention and control, empiric antimicrobial drug protocols were introduced. For antimicrobial

drug–naive patients, the first-line choice for prophylaxis and treatment of skin and soft-tissue infections, including those involving open fractures, was a narrow-spectrum cephalosporin, as recommended by the Infectious Diseases Society of America guidelines (*4*); however, clinical failure was common.

We retrospectively reviewed the clinical microbiology data of 75 patients admitted in January 2015 with a history of suspected post-trauma infection. All these patients were first treated in field hospitals in Syria; 82.7% were male, and 33% were <16 years old. Twenty-four percent had multiple injuries, 20% had osteomyelitis, and 53% had metal prosthetic implants.

Thirty bacterial isolates were identified, mostly from deep wound swabs of 21 (28%) injured patients; 9/21 were infected with 2 isolates. Twenty-nine (97%) isolates were gram-negative bacteria: 10 *Proteus* spp., 10 *Escherichia coli*, 5 *Pseudomonas* spp., and 4 *Klebsiella* spp. Disk diffusion susceptibility testing showed that 20 (66%) isolates were MDR and 11 (36.7%) were carbapenem resistant.

The hospital laboratory did not have the capacity to perform further testing and confirmation of the resistant strains in line with international quality standards because they lacked suitable equipment and financial resources. Preventing further dissemination of MDR organisms among war-injured refugees from Syria at hosting healthcare facilities requires an effective surveillance system, investment in infection prevention and control, appropriate antimicrobial drug stewardship, and urgent laboratory capacity building inside Syria and in the refugee-host countries.

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Pontormo (Jacopo Carucci) (1494–1557), **Portrait of a Halberdier (ca. 1528–1530)** (detail). Oil (or oil and tempera) on panel transferred to canvas, 37 1/2 in × 28 3/4 in/95.3 cm × 73 cm. Digital image courtesy of the Getty's Open Content Program, The J. Paul Getty Museum, Los Angeles, CA, USA.

Naïve Arrogance and Vulnerability

Byron Breedlove and Paul M. Arguin

Florentine painter Jacopo Pontormo, who took his surname from the village of his birth, was considered second only to Michelangelo among his 16th century peers. In Italy, Pontormo was among the first exponents of Mannerism, a style of painting that stressed artifice over realism. The National Gallery of Art offers this view: "Mannerism's artificiality—its bizarre, sometimes acid color, its illogical compression of space, the elongated proportions and exaggerated anatomy of figures in convoluted, serpentine poses—frequently creates a feeling of anxiety."

"Portrait of a Halberdier," this month's cover image, is considered Pontormo's masterpiece. The artist completed this painting during the tumultuous 10-month siege of the Florence Republic from October 1529 through August 1530. The J. Paul Getty Museum, which purchased this work in June 1989 notes that as a portraitist, Pontormo "was renowned for his subtle, complex psychological studies; here he conveyed the naïve arrogance and vulnerability of youth."

In the painting, Pontormo positions this young foot soldier, holding a combination spear and battle-ax called

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DOI: https://dx.doi.org/10.3201/eid2305.AC2305

a halberd, before a bastion. "His direct stare and swaggering pose are strikingly poignant, given the smooth unlined face and slim body that betray him as no more than a teenager," according to the Getty. The painting conveys ambivalence and tension, for the young man's display of physical confidence clashes with his anxious countenance and delicate features.

The authors of *Masterpieces of Painting in the J. Paul Getty Museum* state, "Our unbloodied fighter stares into the unknown, his expression suggesting he has just become aware of the myth of the immortality of youth." This ambivalent message is reinforced by his attire. This young foot soldier casually wears a vivid crimson cap and breech-hose and a fine white padded doublet, replete with a gold chain and cap badge, which depicts Hercules besting the giant Antaeus.

According to art history professor Elizabeth Cropper, this young soldier[1] displays "none of the brutal qualities that made it worth paying a man to fight, and his elegant costume conveys none of the exaggerated panache of the mismatched gear of a ruffian far from home, a type with which Pontormo was all too familiar in both his life and art." It is clear that despite having the trappings of strength, this defenseman lacks combat experience and remains quite vulnerable. With this image, Pontormo foreshadows the eventual capture of Florence.

Despite valiant, innovative, and creative tactics, the city of Florence, considered the birthplace of the Renaissance, was outmatched by the large show of force from Spain and the Holy Roman Empire and surrendered after a 10-month siege. Nearly 36,000 of the residents of Florence, about a third of the city's population, died during the siege, including many young people pressed to defend the city. Florence's citizens sustained other losses as well, including the ruin of their various export businesses and confiscation of their wealth and possessions.

It is tempting to view the issue of antimicrobial resistance—the ability of a microorganism to stop an antimicrobial from working against it—in terms of a siege. Standard treatments become ineffective over time, infections persist, and organisms mutate and propagate. Antimicrobials are armed with the ability to interfere with bacterial cell wall synthesis, to prevent pathogenic protein formation, or to alter bacterial metabolic processes. Those mechanisms of action are akin to the armaments of our stalwart halberdier.

However, whether by the naïve arrogance of overuse and misuse or by evolutionary bad luck, such weapons in the fight against infections are losing their effectiveness, potentially leaving us as vulnerable as Florence. To withstand the siege of antibiotic resistance, innovative strategies for preserving the effectiveness of our existing antimicrobials and for developing new drugs with novel mechanisms of action are needed.

A besieged city that finds its resources dwindling, allies absent, and enemies undeterred will eventually fall. In public health, putting on a brave face will not save the day. Public health must have the capacity to detect, respond to, and prevent antibiotic-resistance threats from a host of bacteria, fungi, viruses, parasites, and other microorganisms. Mounting effective responses across healthcare settings and communities across the world requires investments, infrastructure, and collaboration, including a One Health strategy, which involves partners representing the human, animal, and environmental determinants of health.

[1]A long-running debate over the identity of the youth who posed for this portrait lies outside the scope of this essay. Cropper argues that he is a young man named Francesco Guardi in *Pontormo: Portrait of a Halberdier*.

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Article Title

Exposure Characteristics of Hantavirus Pulmonary Syndrome Patients, United States, 1993–2015

CME Questions

1. You are seeing a 40-year-old man for his second clinic visit in 3 days because of symptoms of fever, myalgia, and nausea with vomiting. He was diagnosed with a nonspecific viral infection 2 days ago, and further questioning today reveals that he has been working on repairing an old, rat-infested barn for the past several weeks. You consider whether he has infection with hantavirus. What should you consider regarding the epidemiology and clinical presentation of infection with hantavirus?

A. Hantavirus illness is limited to the cardiopulmonary system
B. Hantavirus is found in North America, Asia, and Europe
C. The usual mode of transmission of hantavirus is fecal-oral
D. Hantavirus infection in deer mice is highest in the winter

2. Which of the following hantavirus subtypes is responsible for most infections in the United States?

A. Sin Nombre virus
B. New York/Mononghela virus
C. Black Creek Canal virus
D. Bayou virus

3. You assess this patient's risk factors for hantavirus infection. Which of the following statements regarding the epidemiology and outcome of hantavirus pulmonary syndrome (HPS) in the current study is most accurate?

A. American Indians were disproportionately represented among cases of infection
B. Most cases of HPS were fatal
C. There were no differences in mortality rates of HPS based on race or ethnicity
D. More than 90% of cases were among individuals with occupations judged to be at high risk for exposure to rodents

4. You question the patient further about exposure to rodents. What was the most common exposure setting to hantavirus in the current study?

A. Home
B. Recreational setting
C. Work
D. Cars, trailers, or mobile homes

Earning CME Credit

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Article Title

Increased Neurotropic Threat from *Burkholderia pseudomallei* Strains with a *B. mallei*–Like Variation in the *bimA* Motility Gene, Australia

CME Questions

1. Based on the mouse model of neurologic melioidosis studied by Morris and colleagues, which of the following statements about differences in virulence among *Burkholderia pseudomallei bimBm* sequence variants is most accurate?

A. Virulence of *bimBm* variants was similar to that of isolates with typical *bimA* alleles when delivered intranasally
B. Virulence of *bimBm* variants was similar to that of isolates with typical *bimA* alleles when delivered subcutaneously
C. Central nervous system (CNS) infection occurred with increased frequency and at lower inoculating doses after infection of mice with *bimBm* than with *bimBp* strains of *B. pseudomallei*
D. Virulence defined by 50% infectious dose (ID50) values was significantly greater for *bimBp* strains than for bimBm strains when delivered intranasally to BALB/c mice

2. Based on the mouse model of neurologic melioidosis studied by Morris and colleagues, which of the following statements about differences in progression among *B. pseudomallei bimBm* sequence variants is correct?

A. Compared with isolates with typical *bimA* alleles, those with the *B. mallei*–like variation had more rapid systemic dissemination and replication within brain, spinal cord, and other tissues
B. *bimBm* vs *bimBp* strains had greater bacterial colonization of the brain, but without meningeal involvement
C. Sites of abscessation within the CNS were consistent among infected animals
D. Phagocytic cells are not involved in rapid systemic dissemination of *B. pseudomallei* to the CNS and other distant sites

3. Based on the mouse model studied by Morris and colleagues, which of the following statements about implications for clinical disease of these differences among *B. pseudomallei bimBm* sequence variants in the progression and severity of experimental melioidosis is correct?

A. Route of infection, infecting dose, and host risk factors for melioidosis are the only predictors of melioidosis occurrence, manifestations, and severity
B. *bimBm* variation is a predictor for severe forms of melioidosis, including neurologic involvement
C. The study proves that *bimA* is the only gene that has a strong association with neurologic melioidosis
D. The study proves that *B. pseudomallei* uses only 1 mechanism to enter the CNS

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

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

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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. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. 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. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 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. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

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

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

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 not exceed 800 words in the main body of the text 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.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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ISSN 1080-6040

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