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Antonio de Francisci (1887–1964). US Silver Peace Dollar, 1935. Silver (90%), copper (10%), 1.5 in/38.1 mm. Photography by James Gathany.

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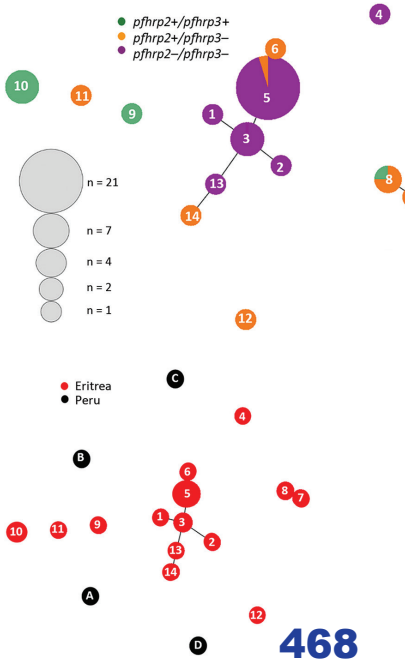

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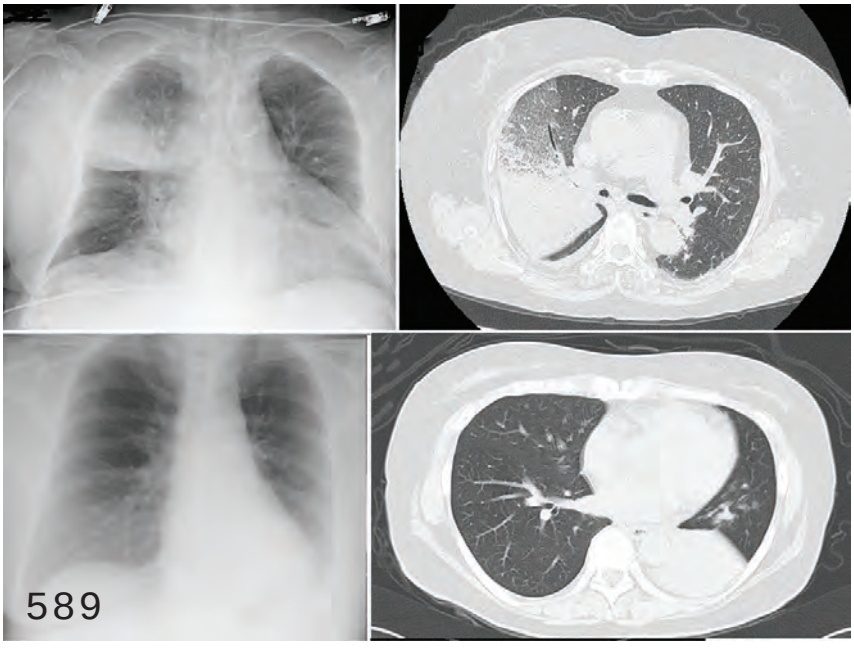

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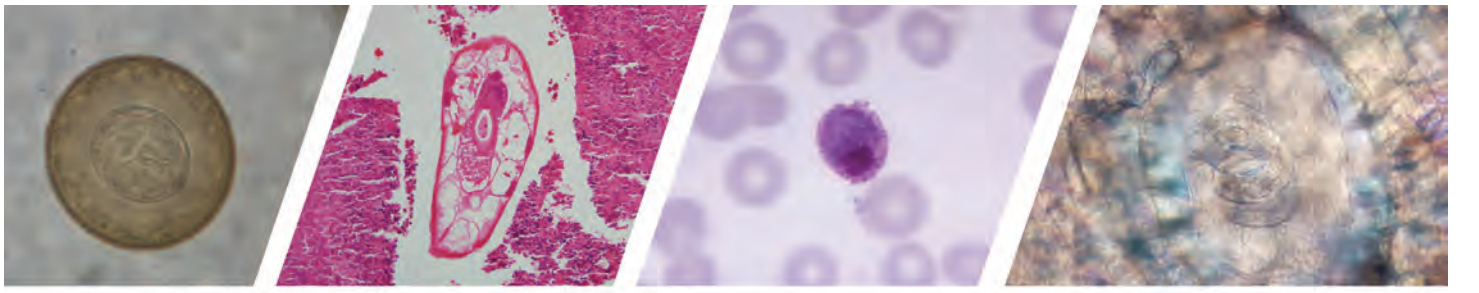

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Ending the HIV/AIDS Pandemic[1]

Robert Walter Eisinger,[2] Anthony S. Fauci[2]

The goal of ending the HIV/AIDS pandemic is theoretically achievable and would require addressing this global health catastrophe on individual and global levels by providing optimal prevention strategies and treatment regimens for individual persons living with or at risk for HIV, as well as ending the pandemic as an epidemiologic and global health phenomenon. However, from a practical standpoint, the pathway to ending the HIV/AIDS pandemic will be difficult and will require aggressive implementation of the biomedical research advances that have been made in the areas of treatment and prevention; development of additional tools, such as a moderately effective HIV vaccine; and attention to critical behavioral and social determinants. An end to the HIV/AIDS pandemic can be achieved only with provision of sustained and additional resources at the local, regional, national, and global levels.

Ending the HIV/AIDS pandemic continues to represent a realistic goal that demands that we address numerous considerations and obstacles at the local, regional, national, and global levels. One can view the attainment of this goal from at least 2 separate, but interrelated, vantage points: 1) optimizing treatment and prevention for individual persons living with or at risk for HIV, and 2) ending the HIV/AIDS pandemic as an epidemiologic and global health phenomenon.

In this article, we describe how the pathway to ending the HIV/AIDS pandemic will involve implementation of each of these elements. The phrase "HIV/AIDS pandemic" indicates that there is a global HIV epidemic, which may be generalized in some countries, such as South Africa, and localized in other countries, such as the United States. The phrase "end of HIV/AIDS" does not refer to the eradication of HIV; rather, it refers to the end of HIV/AIDS as an epidemiologically defined term that meets other criteria. Specifically, the number of new HIV infections would be fewer than the number of AIDS-related deaths; HIV infection and AIDS incidence would decrease to <1 AIDS case/1,000 population; 73% of persons living with HIV and AIDS would be receiving treatment; 86% would experience virus suppression; and mother-to-child transmission would be eliminated to <5% (*1*). Although our comments focus primarily on biomedical research advances and the effects of their implementation, an end to the HIV/AIDS pandemic will require their implementation within the broader socioeconomic, cultural, demographic, and political context at the local, regional, and national levels.

Science and the implementation of its discoveries has and will continue to benefit persons living with HIV and at risk for acquisition of HIV and will drive the process of ending HIV/AIDS as a global pandemic. Since the first reports in 1981 of the unusual cluster of Kaposi sarcoma and *Pneumocystis* pneumonia among young, previously healthy homosexual men in New York, NY, and California, USA (*2*,*3*), substantial scientific advances have been made in the fight against HIV/AIDS. The co-discovery of HIV as the causative agent of AIDS by the Institut Pasteur team led by Montagnier and Barré-Sinoussi (*4*) and the US National Institutes of Health team lead by Gallo (*5*) was merely the beginning of the process. Their seminal findings launched an intensive global multidisciplinary research effort that has led to, and continues to result in, the development and implementation of innovative treatment regimens, prevention interventions, and behavior strategies to extend the healthy lives of those who are already infected, to prevent infection of those at high risk, and to halt and reverse the trajectory of the pandemic.

Optimizing Treatment and Prevention for Individual Persons Living with or at Risk for HIV

Treatment

Since the early 1980s, major advances have been made in treatment for persons living with HIV. During the subsequent years since the recognition of this new and devastating disease, numerous landmark studies have resulted in availability of >30 US Food and Drug Administration–approved antiretroviral drugs for the treatment of HIV infection, along with an extensive group of strategies for the prevention and treatment of HIV-associated co-infections and comorbidities.

Over the past 4 decades, implementation of treatment modalities has faced several challenges. The challenges have been associated with drug toxicities, inconsistent

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[1]This article is based on a lecture given by Anthony S. Fauci on September 9, 2017, in Washington, DC, USA.

[2]The authors contributed equally to this article.

adherence to complex treatment regimens, drug resistance, decisions when to initiate treatment, pill fatigue, and limited access to treatment by special populations (*6*,*7*). For persons living with HIV who have access to these drugs, these regimens extended their life expectancy. In 1981 and 1982, when the first AIDS patients were seen in the United States, the median survival time for a person with AIDS was 1–2 years (*8*,*9*). In contrast, for a person of 20-some years of age with HIV infection being treated with combination antiretroviral therapy (ART) today, the projected life expectancy is ≈53 years (*10*). This major accomplishment resulted from public–private partnerships among academia, industry, the US government, international collaborators, and the affected community. According to recent reports from the Joint United Nations Programme on HIV/AIDS (UNAIDS) and largely as a result of the efforts of the US President's Emergency Plan for AIDS Relief and the Global Fund to Fight AIDS, Tuberculosis and Malaria, 19.5 million persons are now receiving lifesaving ART (representing 53% of all persons living with HIV globally), and AIDS-related deaths have been halved since 2005 (*11*). Increasing the number of persons living with HIV who receive ART will require continued optimization of treatment regimens and implementation of strategies to ensure prompt diagnosis, access to ART, adherence to drug regimens, and retention in care.

Prevention

Substantial progress has also been made in the development of strategies to prevent HIV transmission/acquisition, beyond the interventions that were available early in the AIDS epidemic (e.g., use of condoms, clean syringes, behavioral interventions, and blood supply screening). The HIV Prevention Trials Network 052 clinical trial, which involved 1,763 HIV-serodiscordant couples in 9 countries, clearly validated the concept of treatment as prevention as an effective HIV prevention strategy. The initial findings from this landmark clinical study showed a 96% reduction in HIV transmission (from 350–550 to <250 CD4+ T cells/μL) when the HIV-infected partner began ART early (*12*). These results were sustained in a follow-up study 4 years later (*13*). The results from the recent PARTNER study further demonstrated the benefits of suppressive ART in preventing HIV transmission during sexual activity (*14*). From this study, Rodger et al. reported that, after ≈58,000 condomless sex acts among HIV-serodiscordant couples, no linked HIV transmissions occurred while the HIV-infected partner was receiving suppressive ART. In addition, the findings from the Opposites Attract study (358 HIV-serodiscordant couples in Australia, Thailand, and Brazil) recently indicated no HIV transmissions among 591 couple-years of follow-up when the HIV-infected partner had an undetectable viral load (results based on 16,889 acts of condomless anal intercourse) (*15*). Together, these studies demonstrate that when ART effectively suppresses a person's viral load to undetectable levels, the risk for sexual transmission of HIV to an uninfected sexual partner is essentially zero. These clinical trials provided the crucial scientific evidence for the recent consensus statement that undetectable = untransmittable is achievable if the viral load of a person living with HIV and receiving ART is undetectable (*16*). The US Centers for Disease Control and Prevention recently stated, "When ART results in viral suppression, defined as less than 200 copies/mL or undetectable levels, it prevents sexual HIV transmission" (*17*). Accomplishing the undetectable = untransmittable strategy worldwide will require that everyone who is HIV-infected is receiving ART and that their viral load is suppressed to an undetectable level.

Another valuable HIV-prevention strategy is preexposure prophylaxis (PrEP). Results from several key clinical trials, including iPrEX (*18*), TDF2 (*19*), Partners PrEP (*20*), and the Bangkok Tenofovir Study (*21*), have demonstrated that emtricitabine and tenofovir disoproxil fumarate (Truvada; Gilead Sciences, Inc., Foster City, CA, USA) taken as a single pill daily is ≈95% effective for preventing HIV acquisition. Marcus et al. recently reported that no HIV infections were acquired during 5,104 person-years of PrEP use (*22*). Although PrEP is highly effective as a prevention intervention, it is currently underutilized in the United States and worldwide; thus, public health officials worldwide need to continue to develop, optimize, and implement HIV prevention strategies for persons at risk for HIV. Clinical trials in several countries are evaluating alternative approaches for mitigating the adherence challenges of daily oral dosing prevention regimens; these approaches include long-acting, injectable antiretroviral drugs such as cabotegravir and other long-acting agents as well as passive transfer of antibodies (*23*).

The comprehensive portfolio of interventions in our HIV prevention toolbox served as the basis for a recent commentary in the lay press stating that there are "No more excuses. We have the tools to end the HIV/AIDS pandemic" (*24*). Despite these valuable advances in prevention of HIV infection, several challenges have been encountered in the optimal implementation of these modalities. These challenges include structural, legal, and social barriers resulting in inequalities of access to and uptake of HIV testing and treatment; lack of retention in care; social networks; stigma and discrimination; poor adherence to PrEP; limited access to special populations; and difficulty meeting the UNAIDS targets for enrolling persons living with HIV into treatment programs (*25*,*26*).

Ending the HIV/AIDS Pandemic as an Epidemiologic and Global Public Health Phenomenon

In contrast to addressing the needs of the individual person living with or at risk for HIV infection, achieving an end to the HIV/AIDS pandemic as an epidemiologic and global public health phenomenon requires a somewhat different approach. According to the latest UNAIDS statistics, in 2016, there were ≈1.8 million new HIV infections, 1.0 million AIDS deaths, and 36.7 million persons living with HIV (*27*). In 2014, with a goal of achieving an end to the pandemic, UNAIDS issued the 90-90-90 targets for HIV treatment scale-up by 2020. These targets mean that 90% of persons with HIV/AIDS have had their infection diagnosed, 90% of those with a diagnosis are receiving ART, and 90% of those receiving ART have achieved virus suppression (*28*). In real numbers, these targets translate to 33.0 million persons with HIV receiving a diagnosis, 29.7 million persons receiving ART, and 26.8 million persons receiving ART acheiving virus suppression (*26*).

UNAIDS-sponsored modeling exercises predict an end of the HIV/AIDS pandemic by 2030 if the 90-90-90 targets are achieved by 2020 and 95-95-95 targets are achieved with a decrease to 200,000 new infections among adults annually (*29*,*30*). Although substantial progress has been made, with certain countries meeting the 90-90-90 targets (*31*) and 53% of all persons living with HIV accessing ART in 2016, a critical global HIV treatment gap remains: of the 36.7 million HIV-infected persons, an estimated 17.2 million are not receiving ART. Of those receiving ART, virus is suppressed in only 44%. In addition, the Fast-Track Target, agreed on by the United Nations General Assembly of reducing the number of new HIV infections to <500,000 per year by 2020 and 200,000 per year by 2030 is not being met and is substantially off target. All subpopulations must be included in this targeting. Specifically, UNAIDS notes that from 2010 to 2016, the average annual decrease in HIV incidence worldwide was only 2.3% (*26*). UNAIDS recently issued its HIV Prevention 2020 Road Map, which outlines a 10-point action plan that focuses on the need for country-level actions to achieve a 75% reduction in new HIV infections and country-level achievement of that specific goal of the 90-90-90 targets (*25*).

Summary

From a theoretical standpoint, the goal of ending the HIV/AIDS pandemic is achievable; however, it will require additional and sustained resources to make available the already existing scientific advances on local, regional, national, and global levels. From a practical standpoint, accomplishing this goal will be a substantial challenge. In this regard, the essential components in the effort toward achieving this most challenging goal would probably be development of a moderately effective HIV vaccine together with optimal implementation of existing treatment and prevention modalities (*32*). As persons living with HIV or at risk of acquiring HIV who have access to treatment and prevention continue to benefit from the fruits of scientific advances, we must not take our sights off or waver in our pursuit of the ultimate goal of ending the epidemic as a global health catastrophe.

About the Authors

Dr. Eisinger is special assistant for scientific projects in the Office of the Director of the National Institute of Allergy and Infectious Diseases. He has previously served as the acting associate director for AIDS Research and the acting director of the Office of AIDS Research at the National Institutes of Health. His primary area of research is the discovery and development of HIV therapeutics and diagnostics.

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Coccidioidomycosis Outbreaks, United States and Worldwide, 1940–2015

Michael Freedman, Brendan R. Jackson, Orion McCotter, Kaitlin Benedict

Coccidioidomycosis causes substantial illness and death in the United States each year. Although most cases are sporadic, outbreaks provide insight into the clinical and environmental features of coccidioidomycosis, high-risk activities, and the geographic range of *Coccidioides* fungi. We identified reports published in English of 47 coccidioidomycosis outbreaks worldwide that resulted in 1,464 cases during 1940–2015. Most (85%) outbreaks were associated with environmental exposures; the 2 largest outbreaks resulted from an earthquake and a large dust storm. More than one third of outbreaks occurred in areas where the fungus was not previously known to be endemic, and more than half of outbreaks involved occupational exposures. Coccidioidomycosis outbreaks can be difficult to detect and challenging to prevent given the unknown effectiveness of environmental control methods and personal protective equipment; therefore, increased awareness of coccidioidomycosis outbreaks is needed among public health professionals, healthcare providers, and the public.

Coccidioidomycosis, also known as Valley fever, is a disease caused by dimorphic fungi of the genus *Coccidioides* (*1*). The arthroconidia persist in soil, and environmental factors, including season, temperature, precipitation, and soil salinity, influence the organism's life cycle (*2*–*4*). Coccidioidomycosis is known to be endemic to the southwestern United States, south-central Washington, northern Mexico, and parts of Central and South America (*1*). Infection occurs primarily by inhalation of environmental arthroconidia, and symptoms develop in ≈40% of infected persons typically within 1–3 weeks after exposure (*5*). However, determining the time and place of exposure is often difficult unless a notable exposure occurred or the illness is part of an outbreak. Symptomatic patients frequently have an influenza-like syndrome characterized by cough, shortness of breath, fever, and fatigue that is commonly diagnosed as community-acquired pneumonia. In ≈1% of infections, disseminated extrapulmonary disease (e.g., meningitis, osteomyelitis, or soft tissue and subcutaneous infections) develops (*1*,*6*,*7*). Risk factors for disseminated infection include immunocompromised status and African and Filipino ancestry (*1*). Serologic methods are the mainstay of diagnosis in coccidioidomycosis-endemic areas; diagnostic confirmation with identification of *Coccidioides* by culture or histopathology is less common (*8*). Treatment for mild coccidioidomycosis is often supportive; however, severe infections require treatment with antifungal agents (*1*).

Approximately 10,000 coccidioidomycosis cases are reported in the United States annually through reportable disease surveillance, and the substantial year-to-year variation that occurs reflects changing environmental conditions and testing practices (*9*). The disease is widely underdiagnosed, however, and these cases most likely represent a small fraction of the true number (*10*). Much remains unknown about its epidemiology. Although most reported cases represent sporadic infections (i.e., non–outbreak-associated), outbreak investigations can provide insight into coccidioidomycosis epidemiology because the exposure sites and circumstances are known, along with dates of exposure and illness. Data from outbreaks have informed much of the knowledge about the disease's incubation period, environmental sources, geographic range, and high-risk activities, but coccidioidomycosis outbreaks have not been systematically studied. In 2016, we reviewed documented coccidioidomycosis outbreaks to identify common features and prevention opportunities.

Methods

Literature Review

We searched PubMed, Medline, Embase, Global Health, and Scopus without date or language restrictions for articles using combinations of the terms "coccidioides" or "coccidioidomycosis" and "epidemic," "outbreak," or "cluster." We also searched the digital archive of scientific literature produced by the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) Stacks database (http://stacks.cdc.gov) for any reports with the words "coccidioidomycosis" or "coccidioides" published in Morbidity and Mortality Weekly Report before 1981. We reviewed proceedings from annual Coccidioidomycosis Study Group

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meetings held during 1980–2015 for abstracts describing possible outbreaks (*11*) and searched National Institute for Occupational Safety and Health Hazard Evaluation reports using the terms "coccidioidomycosis" or "coccidioides" (*12*). We reviewed all references pertaining to outbreaks in all relevant articles and reviewed any references cited within the initial article if they alluded to additional outbreaks or additional information for a known outbreak. We included in the analysis English-language articles published during January 1940–August 2016. We abstracted clinical and epidemiologic data of interest from reports of outbreaks that met these inclusion criteria.

Definitions

We defined an outbreak as ≥2 human coccidioidomycosis cases linked to a common source, event, or activity in space and time. For an outbreak to be included, laboratory evidence of coccidioidomycosis was required for ≥1 case. However, to capture older literature describing outbreaks most likely attributable to coccidioidomycosis, we also included outbreaks if cases had a compatible clinical syndrome and unambiguous diagnostic terms, such as "coccidioidomycosis" or "primary pulmonary coccidioidomycosis," and were described in a report that also mentioned ≥1 patient with 1) laboratory evidence of coccidioidomycosis, 2) hospitalization, or 3) identification of *Coccidioides* from the environmental source.

We reviewed laboratory and radiologic evidence of infection. Specific laboratory tests documented as positive were serology (including immunodiffusion, precipitins, complement fixation positive in any titer, and enzyme immunoassay); culture or direct visualization (i.e., smear or microscopy) of *Coccidioides* in any body fluid; PCR; and coccidioidin skin test (any reaction at any dilution). We considered infiltrates or abnormalities on chest radiography radiographic evidence of infection, whereas we defined radiograph findings reported as equivocal or nonspecific as equivocal. Symptomatic patients were those with clinical symptoms consistent with coccidioidomycosis, regardless of diagnostic studies.

We classified outbreaks as environmental or nonenvironmental (i.e., resulting from healthcare or laboratory exposure) in origin. We further subclassified environmental outbreaks by whether a probable exposure source (e.g., soil disruption, dust storm) was identified. Environmental outbreaks without a probable exposure source were limited to those clearly bound by beginning and end time points; we excluded generalized increases in incidence among specific populations. We further characterized outbreaks by their association with military activity, incarceration, residential areas, laboratory activities, archaeology and field studies, or travel. Incarceration included military and civilian incarceration, as well as any group of persons detained against their will (e.g., in Japanese internment camps). Outbreaks were classified as residential if the report described the outbreak at or near a residential area, including the residences of the persons exposed. Outbreaks were determined to be travel-associated if travel was explicitly mentioned, and all military outbreaks were also considered to be travel-associated.

Outbreak location was defined as the geographic location of the probable exposure source or the patients' location when no source was identifiable. Occupational exposures were those clearly related to employment, as well as activities associated with archaeology, field studies, and military imprisonment. We also assessed reports for statements about whether outbreaks occurred in areas where, according to the original authors, coccidioidomycosis was not previously known to be endemic.

We determined epidemiologic metrics on the basis of the original authors' definitions and defined the number of persons possibly exposed as those exposed to the probable source, if present. We also recorded the number of cases resulting in hospitalization, dissemination, meningitis, or death in outbreak reports that described any of these clinical outcomes. We considered patients with meningitis or sepsis to have disseminated disease. We documented use of antifungal drugs when available and recorded or estimated minimum, maximum, median, and mean symptom durations from figures and individual case reports when available. We further documented data pertaining to incubation periods for outbreaks that had total exposure periods (i.e., date of last exposure minus date of first exposure) lasting <2 weeks.

We directly documented all data in our analysis from the literature or estimated data using conservative assumptions (e.g., majority of cases was defined as 50% + 1). In the absence of explicit data or the above conservative estimates, we noted information as unknown and omitted it from our analysis.

Results

A total of 47 coccidioidomycosis outbreaks met our study criteria (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-0623-Techapp1.pdf). These outbreaks involved 1,464 cases (1,451 symptomatic and 13 asymptomatic) (Table 1). An additional 128 symptomatic persons were reportedly affected by these outbreaks, but their illnesses did not meet the original authors' case criteria. Outbreaks ranged in size from 2 to 379 cases (mean 31, median 10). Five (11%) outbreaks had 2 cases each, 8 (17%) had 3–5 cases, 8 (17%) had 6–9 cases, 12 (26%) had 10–14 cases, 9 (19%) had 15–99 cases, and 5 (11%) had ≥100 cases.

More than 60% of cases occurred during 1940–1949 (32%) and 1970–1979 (29%) (Figure 1, panel A). Among the 33 (70%) environment-associated outbreaks in the Northern Hemisphere for which onset month or season were reported, 10 (30%) started in summer (June–August).

Table 1. Characteristics of published coccidioidomycosis outbreaks and outbreak-associated cases, United States and worldwide, 1940–2015

Outbreak characteristic	Outbreaks, no. (%), N = 47	Cases, N = 1,464	
		Total no. (%)	Median (min–max)*
Environmental†	40 (85)	1,425 (97)	10 (2–379)
Probable source of exposure reported‡	35 (88)	1,218 (85)	10 (2–379)
Associated with large natural phenomena	2 (4)	582 (40)	291 (203–379)
Revealed new, or confirmed suspected, endemic area§	16 (43)		
Occupational	25 (53)	616 (42)	10 (2–150)
Military	11 (23)	442 (30)	14 (8–150)
Archaeology/field studies	7 (15)	82 (6)	10 (5–27)
Laboratory	4 (9)	28 (2)	5.5 (2–5)
Construction¶	7 (15)	247 (17)	21 (8–119)
Other activity			
Armadillo hunting in northern Brazil	5 (11)	14 (1)	3 (2–4)
Native American site disruption	2 (4)	6 (<1)	3 (2–4)
Location			
Travel-associated	21 (45)	566 (39)	12 (5–150)
Residential	8 (17)	625 (43)	9 (2–379)
Incarceration	5 (11)	316 (22)	30 (7–150)

*Max, maximum; min, minimum.
†That is, not laboratory- or healthcare-associated.
‡Of 40 environmental outbreaks with 1,425 outbreak-associated cases.
§Of 37 environmental outbreaks for which this information was available.
¶Three outbreaks associated with construction were not considered occupational outbreaks, including 2 associated with construction by volunteers in Mexico and 1 associated with construction adjacent to a prison in California's Central Valley.

Thirty-one (66%) outbreaks reported total numbers of persons possibly exposed, ranging from 2 to 676,667 (median 27). Clinical attack rates ranged from 0.03% to 100% (mean 44%, median 43%).

Exposure Characteristics

Forty (85%) outbreaks were associated with environmental exposure, 33 (83%) of which occurred in the United States. Among the US environment-associated outbreaks, 25 (76%) occurred in California, 4 (12%) in Arizona, and 2 (6%) each in Utah and Texas (Figure 1, panel B). Of the remaining 7 (18%) environment-associated outbreaks, 5 (71%) occurred in Brazil and 2 (29%) in Mexico (Figure 2). Thirty-five (88%) environment-associated outbreaks involved a documented probable exposure source.

We found 7 (15%) nonenvironmental outbreaks. Four of these were related to laboratory exposures, 2 involved transmission through organ transplantation, and 1 was a nosocomial outbreak.

Two (4%) outbreaks comprising 582 (40%) cases resulted from separate natural phenomena in California: the Northridge earthquake in 1994 in Ventura County and the "Tempest from Tehachapi" dust storm in 1977. Twenty-one (45%) outbreaks comprising 566 (39%) cases were travel-associated. There were 316 (22%) cases in the 5 (11%) outbreaks among incarcerated populations, including 160 cases from 2 outbreaks in prisoner-of-war camps, 30 cases from an outbreak in a Japanese internment camp, 119 cases from an outbreak in a civilian prison, and 7 cases from an outbreak in a juvenile work camp.

Thirty-seven (79%) outbreaks reported whether the given outbreak revealed previously unknown information about endemicity. Of these, 12 (32%) revealed a new area (in Arizona, California, Texas, Utah, and Brazil) to which coccidioidomycosis was endemic, and 4 (11%) confirmed the endemicity of a suspected endemic area (in Arizona and California).

Occupational Exposures

Twenty-five (53%) outbreaks were associated with occupational exposures. Eleven (23%) outbreaks were associated with the military. Seven (15%) outbreaks were associated with construction, and another 7 (15%) were associated with archaeology or other field studies. Laboratory activities were associated with 4 (9%) outbreaks.

Incubation Period and Symptom Duration

Fifteen (32%) outbreaks included incubation period data meeting predefined inclusion criteria. Fourteen of these outbreaks stated lower and upper incubation period limits; the lower limit ranged from 1 to 14 days (mean 7, median 8) and the upper limit from 4 to 70 days (mean 21, median 17). The median incubation period, reported for 12 outbreaks, ranged from 4 to 31 days (mean 12, median 13), and the mean incubation period, reported for 11 outbreaks, ranged from 4 to 33 days (mean 14, median 12).

Of the 13 (28%) outbreak reports that included symptom duration, the lower limit ranged from 1 day to 17 weeks (mean 3.4 weeks, median 2 weeks), and the upper limit ranged from 3 to 52 weeks (mean 16.2, median 10). The median symptom duration ranged from 1 to 18 weeks (mean 7.1, median 4.5), and the mean symptom duration ranged from 3 to 19.6 weeks (mean 9.3, median 6.3).

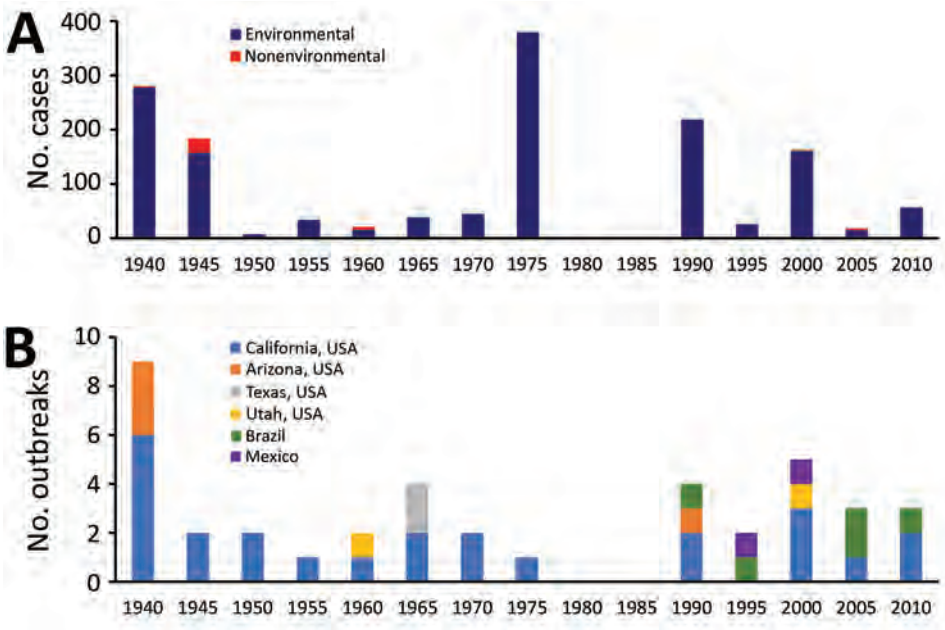

Figure 1. Coccidioidomycosis cases, United States and worldwide, 1940–2015. A) Outbreak-related cases, by onset year (in 5-year periods) and environmental association (N = 1,464 cases). B) Environment-associated outbreaks, by onset year and outbreak location (N = 40 outbreaks).

Laboratory and Radiologic Evidence

Thirty-nine (83%) outbreaks had cases confirmed by laboratory evidence. Radiographic evidence was reported for 153 (10%) cases in 22 (47%) outbreaks (Table 2). Among the 1,464 cases, at least 1,001 (68%) reported diagnostic evidence of coccidioidomycosis, including 334 with positive serologic results, 30 with positive culture or histopathologic visualization results, 378 with positive skin test results, and 153 with positive chest radiograph results.

Treatment and Outcomes

Health outcome data were available for 35 (74%) outbreaks in which 544 (43%) patients were hospitalized (Table 2). Thirty-two (3%) patients had disseminated coccidioidomycosis and 20 (2%) had meningitis; 18 (1%) died. Of the 20 (43%) outbreaks that described antifungal treatment, 128 (18%) patients in 13 outbreaks were treated with antifungal drugs, including amphotericin B, fluconazole, and other azole antifungals.

Discussion

We reviewed 47 coccidioidomycosis outbreaks comprising 1,464 cases, for an average of 19 outbreak-related cases annually during 1940–2015. Although reported outbreak-related cases are relatively uncommon compared with the thousands of annual reported cases in the United States alone, coccidioidomycosis outbreaks have helped inform our understanding of geographic risk, high-risk populations and activities, and clinical features of the infection.

Outbreaks have further defined the geographic distribution of *Coccidioides*, initially identified in part by large-scale coccidioidin skin test surveys (*13*). In fact, 7 of the first 9 environmental coccidioidomycosis outbreaks in the early 1940s either confirmed suspicion of or revealed previously unknown areas in southern California and western Arizona to which the fungus is endemic. Over time, outbreak investigations uncovered additional coccidioidomycosis-endemic areas throughout California's Central Valley, Texas, Utah, and areas of Brazil. Of outbreak reports that described endemicity, nearly half (43%) involved outbreaks that occurred in locations where the infection was not known to be endemic. Outbreak data also suggest that some geographic regions seem particularly well suited for *Coccidioides* growth and human exposure. For example, the arid hills bordering the southwestern portion of California's Central Valley were the setting for 5 outbreaks within 150 miles of each other.

Although outbreaks can help identify areas with geographic risk for coccidioidomycosis, few outbreaks have been reported from some regions to which coccidioidomycosis is known to be highly endemic. Specifically, only 4 outbreaks were reported from Arizona (3 of which occurred in the early 1940s), even though several Arizona counties report some of the highest coccidioidomycosis incidence rates nationwide. In contrast, 25 outbreaks occurred in California, a state with localized areas of similarly high incidence. A possible explanation for this discrepancy is that most of Arizona's population resides in areas where coccidioidomycosis is highly endemic, and exposure probably is common during daily activities, resulting in many sporadic cases and making outbreak detection challenging in this setting of high baseline incidence. In contrast, much of California's population resides outside of its most highly coccidioidomycosis-endemic areas, possibly enabling easier outbreak detection against lower baseline rates. Another possible contributing explanation for this discrepancy is that cases of coccidioidomycosis have been determined to be a compensable work-related condition in

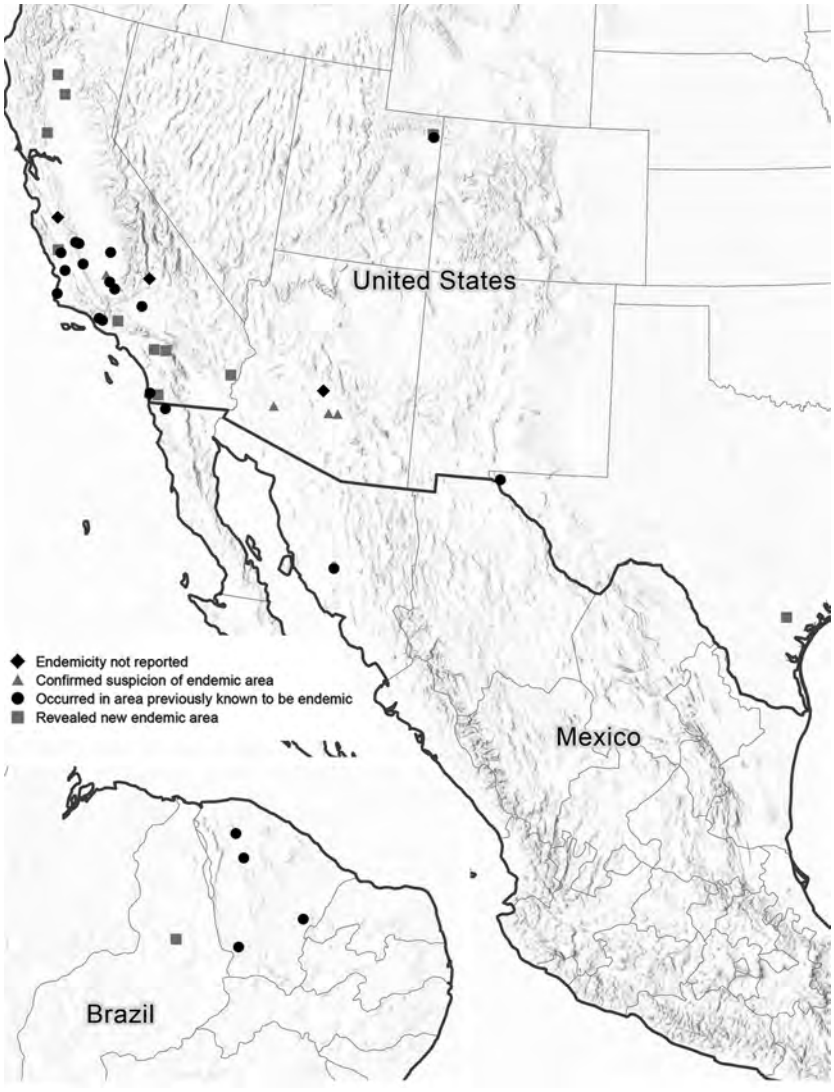

Figure 2. Locations of environment-associated coccidioidomycosis outbreaks, by state or territory and whether the outbreak revealed new or confirmed suspected endemicity (n = 40), United States, Mexico, and Brazil, 1940–2015.

California, whereas cases in Arizona have not, which might influence outbreak recognition (*14*). However, in a recent work-related outbreak in California, public health investigators initially identified few cases by reviewing workers' compensation claims, highlighting the need for data integration from multiple sources to identify and describe some outbreaks (*15*). Systematic collection of information on occupation, industry, and workplace as part of coccidioidomycosis surveillance might facilitate identifying future workplace-associated outbreaks. Outbreak detection is challenging in general because Arizona and many counties in California currently use laboratory-based coccidioidomycosis surveillance and patients are not routinely interviewed to detect possible common exposures.

In addition to the challenges associated with outbreak detection, defining whether a cluster of coccidioidomycosis cases or a period of elevated incidence truly represents an outbreak can be difficult, particularly when a specific exposure source is not apparent. Coccidioidomycosis incidence fluctuates with season and weather (*16*), and increased reports resulting from seasonal sporadic infection could be interpreted as an outbreak. For this review, we excluded several reports noting increased incidence over a prolonged period, despite specific identification with the terms "outbreak" or "epidemic" (*16–18*), to focus on outbreaks with more clearly defined sources. Outbreaks without a probable exposure source included in this review had a clear association among a group of persons, frequently those who traveled from areas to which coccidioidomycosis is not known to be endemic. Periods of widespread, elevated coccidioidomycosis incidence associated with seasonal or weather changes contrast with outbreaks clearly resulting from defined natural phenomena. The 2 largest outbreaks in this review, resulting from the 1994 Northridge Earthquake (*19*) and a 1977 dust storm originating near the Tehachapi mountains in Southern California (*20*), together comprised

Table 2. Characteristics of patients with coccidioidomycosis outbreak–associated cases, United States and worldwide, 1940–2015

Patient characteristic	Time period, no. (%) cases			
	1940–1959	1960–1979	1980–2015	Total 1940–2015
Presentation and diagnosis				
Total	503 (100)	482 (100)	479 (100)	1,464 (100)
Symptomatic	493 (98)	481 (100)	477 (100)	1,451 (99)
Any positive studies	290 (58)	466 (97)	242 (51)	998 (68)
Positive serology	156 (31)	68 (14)	110 (23)	334 (23)
Positive skin test result	270 (54)	82 (17)	26 (5)	378 (26)
Positive culture	15 (3)	2 (<1)	13 (3)	30 (2)
Positive chest radiograph	50 (10)	67 (14)	36 (8)	153 (10)
Clinical outcome				
Total*	475 (100)	472 (100)	320 (100)	1,267 (100)
Hospitalized	430 (91)	20 (4)	94 (29)	544 (43)
Dissemination	7 (1)	16 (3)	9 (3)	32 (3)
Meningitis	3 (1)	15 (3)	2 (1)	20 (2)
Deaths	5 (1)	8 (2)	5 (2)	18 (1)
Treated with antifungal drugs†	0	5 (1)	123 (44)	128 (18)

*Cases among outbreaks reporting health outcomes.
†Of 717 cases among 20 outbreaks documenting antifungal drug treatment.

40% of all outbreak-related cases, illustrating the potential for these events to affect many persons across large geographic areas.

This review highlights the well-recognized risk for coccidioidomycosis among several specific populations, including military personnel, incarcerated persons, and outdoor workers. Many of the earliest outbreaks occurred among service members stationed in semiarid desert areas of Arizona and California during and shortly after World War II (*21*,*22*). These early outbreaks were studied extensively by C.E. Smith, who advocated for dust control measures, such as planting grass, paving, and applying oil to soil in athletic areas, to reduce risk for infection (*21*–*23*). Three exclusively military-associated outbreaks have been described since then (1958, 1992, and 2001), resulting in many fewer cases than those in the early 1940s (51 vs. 391 cases). Coccidioidomycosis continues to be reported among service members (*24*,*25*), most likely as a consequence of immune-naive persons entering areas to which it is endemic, although evidently not to the same magnitude as during the World War II era.

This review also highlights the ongoing challenge of coccidioidomycosis in California prisons. The 5 outbreaks among incarcerated persons were similar to those among the military in that affected persons often did not have prior exposure to coccidioidomycosis-endemic areas (*22*,*26*–*29*). In an effort to minimize illness, inmates who are immunosuppressed, are African-American or Filipino, or have diabetes mellitus are no longer housed in several prisons in California's Central Valley (*30*). Additionally, inmates are offered coccidioidal skin testing to further reduce the risk for contracting coccidioidomycosis in prisons located in areas to which it is highly endemic (*31*).

More than half of the coccidioidomycosis outbreaks we reviewed were associated with occupational exposure, often related to soil-disrupting activities. The association between coccidioidomycosis and these activities, specifically construction, is often described, although evidence from a nonoutbreak setting in Arizona suggests that exposure working near a construction site does not always appear to be associated with increased risk for coccidioidomycosis (*32*). In general, workers who disrupt soil in areas to which the fungus is endemic are believed to be at higher risk than the general population. Recommendations from the California Department of Public Health (*33*) and the United States Geological Survey (*34*) to prevent work-related coccidioidomycosis focus on strategies such as education about coccidioidomycosis for workers and supervisors, dust-control methods such as wetting soil before disrupting it, cleaning potentially contaminated materials to prevent *Coccidioides* from being transported away from the worksite, and using respiratory protection; however, the efficacy of these interventions is difficult to measure. Furthermore, these types of control measures can be difficult to implement or enforce. For example, during a 2011 outbreak among construction workers building a solar power farm in San Luis Obispo County, California, 88% of interviewed patients reported receiving safety training on Valley fever, but their descriptions of the training varied widely (*15*). Clearly, further research on preventing work-related coccidioidomycosis is needed.

Nonenvironmental coccidioidomycosis outbreaks were uncommon, and most were caused by laboratory exposure. *Coccidioides* cultures can be highly infectious; 1 incident caused 15 coccidioidomycosis cases, affecting persons in several rooms on the same floor (*35*). No laboratory-associated outbreaks have been published since 1949, consistent with notably improved prevention and laboratory safety measures (*36*).

With the advent of transplant medicine, at least 2 coccidioidomycosis outbreaks have been associated with organ transplantation, 1 of which was confirmed by whole-genome sequencing of isolates from 3 organ recipients (*37*). Coccidioidomycosis poses a serious risk for transplant recipients,

and further studies are needed to determine whether serologic screening on donors from coccidioidomycosis-endemic areas minimizes transmission (*38*).

Clinical features of coccidioidomycosis cases in this review were similar to those in previous studies. The median shortest incubation period was 8 days, median longest 17 days, and overall median 13 days, consistent with the commonly reported incubation period range of 1 to 3 weeks (*39*). The median shortest symptom duration was 2 weeks, the median longest was 10 weeks, and the overall median was 4.5 weeks. By comparison, among patients with reported coccidioidomycosis in Arizona, median symptom duration was ≈6 weeks among patients who had recovered at the time of the interview and ≈22 weeks among patients who had not recovered (*40*). A possible explanation for this difference is that cases reported to public health might be more severe than those that go unreported, whereas cases detected as part of outbreaks might reflect a wider spectrum of illness; in addition, many cases detected by surveillance occur in older adults who might have comorbidities and slower recovery times, whereas many outbreak-associated cases occurred in occupational settings and most likely affected younger adults. However, the median clinical attack rate (43%) and proportions of patients who were hospitalized (43%), had disseminated disease (3%), and died (1%) were generally similar to those described elsewhere (*5–7*,*40*,*41*), suggesting that other factors might explain the differences in symptom duration observed. Notably, hospitalization varied substantially by time period; 91% of patients during 1940–1959 were hospitalized but only 29% during 1980–2015. This trend follows an increase in antifungal drug use from 0% to 44% during the same period. These patterns probably reflect changes in medical practice.

This review is limited by the fact that many outbreaks probably are not recognized, not reported to public health, not investigated, or not published, although we also attempted to capture reports from the gray literature. Other limitations include the heterogeneity of available data and our restriction to English-language reports.

Nevertheless, outbreaks are a key data source regarding modes and locations of exposure. Thus, increased attention to outbreak identification and tracking is worthwhile given the continued population growth in coccidioidomycosis-endemic areas, increased settlement at the wildland–urban interface, and the incompletely understood effects of intensifying climate change on *Coccidioides*. Monitoring outbreaks could be critical in identifying new areas of endemicity and high-risk activities. Increased awareness of coccidioidomycosis among employers of persons in potentially high-risk occupations, the public, and healthcare providers is needed to reduce both the risk and severity of future outbreaks.

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About the Author

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Multistate Epidemiology of Histoplasmosis, United States, 2011–2014[1]

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Histoplasmosis is one of the most common mycoses endemic to the United States, but it was reportable in only 10 states during 2016, when a national case definition was approved. To better characterize the epidemiologic features of histoplasmosis, we analyzed deidentified surveillance data for 2011–2014 from the following 12 states: Alabama, Arkansas, Delaware, Illinois, Indiana, Kentucky, Michigan, Minnesota, Mississippi, Nebraska, Pennsylvania, and Wisconsin. We examined epidemiologic and laboratory features and calculated state-specific annual and county-specific mean annual incidence rates. A total of 3,409 cases were reported. Median patient age was 49 (interquartile range 33–61) years, 2,079 (61%) patients were male, 1,273 (57%) patients were hospitalized, and 76 (7%) patients died. Incidence rates varied markedly between and within states. The high hospitalization rate suggests that histoplasmosis surveillance underestimates the true number of cases. Improved surveillance standardization and surveillance by additional states would provide more comprehensive knowledge of histoplasmosis in the United States.

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Histoplasmosis is an infection caused by the soil-dwelling thermally dimorphic fungus *Histoplasma capsulatum* (*1*). Infection typically results from inhalation of aerosolized spores. Only 1% of sporadic infections are estimated to be symptomatic, although attack rates during outbreaks have been as high as 50%–100%, possibly from high-dose exposure (*2*). Most symptomatic infections involve primary pulmonary disease; however, extrapulmonary and severe disseminated disease can occur, especially in immunosuppressed persons.

Histoplasmosis is often described as the most common mycosis endemic to North America (*1*), although data to support this statement are limited, given a lack of national public health surveillance. Although once thought to be endemic to a relatively narrow geographic area, histoplasmosis has been increasingly detected in many parts of the world (*3*,*4*). According to histoplasmin skin test surveys performed in the 1950s and 1960s in the United States, areas surrounding the Ohio and Mississippi River Valleys are recognized as the regions of predominant histoplasmosis endemicity (*5*). However, locally acquired infections have been described outside these areas, suggesting that the geographic range of *Histoplasma* in the United States is wider than is often appreciated (*6*). This incomplete knowledge about geographic areas of risk could deter clinicians from considering histoplasmosis as a cause of illness, leading to misdiagnosis and inappropriate treatment.

A key data source for information about the current geographic distribution and epidemiology of histoplasmosis is disease surveillance. As of 2016, histoplasmosis was reportable in 10 states but not notifiable nationally. Reportable diseases are those that healthcare providers and laboratories are required to report to state, territorial, or local public health authorities. Each jurisdiction's regulation or law determines

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which diseases are reportable. For diseases selected as nationally notifiable by the Council of State and Territorial Epidemiologists (CSTE), jurisdictions voluntarily notify the Centers for Disease Control and Prevention (CDC) of cases, and data are summarized for national surveillance (*7*). Until 2016, no national case definition existed for histoplasmosis surveillance. Given this void, each state implemented different case definitions, which generally included similar clinical and laboratory criteria (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1258-Techapp1.pdf).

Current knowledge about this disease and its geographic risk is limited. To improve understanding of the burden of histoplasmosis in the United States, we collected and summarized surveillance data from 12 states.

Methods

To create a multistate dataset, we combined deidentified data on histoplasmosis cases reported during 2011–2014 from the 10 states where histoplasmosis was reportable in 2016 (Arkansas, Delaware, Illinois, Indiana, Kentucky, Michigan, Minnesota, Nebraska, Pennsylvania, and Wisconsin) and from 2 states where it previously had been reportable (Alabama and Mississippi, which both removed histoplasmosis from their list of reportable diseases in 2015). For states that classified cases as confirmed, probable, or suspected, we included only confirmed and probable cases; from states that did not use such a classification system, we included all cases. Although histoplasmosis is not reportable in Ohio, a comparable convenience sample of data received from reference laboratories was available for 2012–2015 and was analyzed separately.

Because data from each state were collected in different formats, we implemented the following rules to standardize data for analysis. We included variables collected by at least 3 states. Because of inconsistent availability of detailed data, we considered all histoplasmosis laboratory test results recorded as positive to be positive even without an explicitly stated qualitative or quantitative result. Immunodiffusion test results indicating H band, M band, or both were considered positive. Complement fixation titers to the yeast-phase or mycelial-phase antigen ≥1:8 were considered positive, and, for patients for whom >1 complement fixation titer was available, we retained the highest titer for analysis. We created dichotomous variables to indicate whether positive test results for histoplasmosis were obtained by enzyme immunoassay (of serum, urine, or another or unspecified specimen type), immunodiffusion, complement fixation, PCR, culture, microscopy, or other or unspecified histoplasmosis test. Data for negative histoplasmosis test results were not routinely available and were therefore not included.

We calculated state-specific annual incidence and county-level mean annual incidence per 100,000 persons by using yearly population estimates from the US Census Bureau (https://www.census.gov). County-level incidence estimates represent patients' county of residence (or, in Ohio, the county of the facility that ordered the laboratory test). To identify factors significantly associated with hospitalization or death, we performed bivariable analyses using χ^2, Fisher exact, and *t*-tests at $p \leq 0.05$. We calculated 95% CIs for relative risks.

Results

Descriptive Analysis Results

During 2011–2014, a total of 3,409 histoplasmosis cases were reported from 12 states (Table 1). Median patient age was 49 (range 0–94, interquartile range [IQR] 33–61) years, and most (2,079 [61%]) patients were male. Of the 1,729 patients in 8 states that contributed race data, 1,079 (62%) were white, 446 (26%) were of unknown race, and 166 (10%) were black. Of the 1,620 patients in these 8 states for whom ethnicity data were available 1,072 (66%) were non-Hispanic or Latino, 503 (31%) were of unknown ethnicity, and 45 (3%) were Hispanic or Latino. Of the 2,542 patients in 10 states for whom case status was assigned, 1,465 (58%) had confirmed and 1,077 (42%) had probable cases.

Symptom data were available from 4 states. The most common symptoms were cough (67% [range by state 56%–81%]), shortness of breath (64% [range 50%–77%]), and fever (56% [range 46%–66%]). Data on immune status were available for 1,154 patients from 3 states; of these, 649 (56%) patients were not immunocompromised, 344 (30%) were immunocompromised, and immune status was unknown for 161 (14%). Hospitalization data were available for 2,218 patients. More than half (1,273 [57%]) of patients were hospitalized; median hospitalization duration for 548 patients for whom hospitalization duration was known was 7 (range 1–126, IQR 4–138) days. Mortality data were available for 1,142 patients; 76 (7%) died.

Three states reported whether cases were associated with an outbreak (816 patients); association for 511 (63%) was unknown, 195 (24%) were not associated, and 110 (14%) were associated (range by state 3%–45%). Exposure data were collected by 3 other states. In Michigan, 29% of patients reported exposure to bird or bat droppings in the 6 weeks before symptom onset; in Illinois, 24% of patients had exposure to "large quantities of bird/bat droppings"; and in Pennsylvania, 8% of interviewed patients noted "contact with bird/bat droppings."

Nine states contributed laboratory data (Table 2). Of 1,929 patients with any positive histoplasmosis test result, antigen test results were positive for 644 (33%), antibody test results were positive for 1,052 (55%), and culture results were positive for 257 (13%). Of the 644 patients with a positive antigen test result, 536 (83%) had tests performed on urine specimens, 146 (23%) had tests performed on serum, and 42 (7%) had tests performed on a specimen of unspecified type. Of the 1,052 patients with a positive antibody test result, antibodies were detected in 618 (59%)

patients by an immunodiffusion test and in 849 (81%) by a complement fixation test. The median highest complement fixation titer was 1:64 (range 1:8–1:4,096). Positive results for other positive histoplasmosis tests not mentioned above or that could not be classified as a specific test type were reported for 248 (13%) patients.

Ohio contributed data on 303 histoplasmosis cases. Median patient age was 53 (range 6–92, IQR 40–67) years. Most (183 [61%]) patients were male. Positive antigen test results were reported for 128 (42%) patients (87 urine and 41 unspecified specimen type), positive antibody test results for 129 (43%) (127 complement fixation and 2 immunodiffusion), and positive other or unspecified test types for 46 (15%).

Bivariable Analysis Results

Factors significantly associated with hospitalization were age >50 years (relative risk 1.23, 95% CI 1.14–1.32); male sex (1.08, 95% CI 1.01–1.45); nonwhite race (1.26, 95% CI 1.13–1.41); immunocompromised status (1.78, 95% CI 1.62–1.96); and positive antigen test result (1.75, 95% CI 1.62–1.89) or confirmatory test result (1.21, 95% CI 1.09–1.34) (Table 3). Patients with a positive antibody test result were less likely to be hospitalized than those without a positive antibody test result (0.58, 95% CI 0.53–0.63). Factors significantly associated with death were age >50 years (6.28, 95% CI 3.43–11.49), immunocompromised status (6.07, 95% CI 2.61–14.11), positive antigen test result (1.73, 95% CI 1.04–2.87), or positive confirmatory test result (2.13, 95% CI 1.28–3.54). Patients with a positive antibody test result were less likely to die than those without a positive antibody test result (0.41, 95% CI 0.24–0.71).

Incidence

Annual incidence rates were highest for Arkansas, Illinois, Indiana, Michigan, and Minnesota (Figure 1). State-specific annual incidence rates ranged from 0 to 4.3 cases/100,000 population, and no consistent increases or decreases occurred over the 4-year period. Mean county-level incidence ranged from 0 to 39 cases/100,000 population by county (Figure 2).

Discussion

This summary of 2011–2014 state-based public health surveillance data on 3,409 histoplasmosis patients in 12 US states provides a broad, population-level epidemiologic description of this underrecognized disease. Key findings include granular data about geographic distribution of the

Table 1. Patient characteristics for 3,409 histoplasmosis cases reported to public health departments, 12 US states, 2011–2014*

Characteristic, no. patients, no. states contributing information	No. (%)
Sex, 3,405 patients, 12 states	
M	2,079 (61.1)
F	1,323 (38.9)
Unknown	3 (0.1)
Race, 1,729 patients, 8 states	
White	1,079 (62.4)
Black	166 (9.6)
Other	24 (1.4)
Asian or Pacific Islander	9 (0.5)
American Indian or Alaska Native	5 (0.3)
Unknown	446 (25.8)
Ethnicity, 1,620 patients, 8 states	
Non-Hispanic or Latino	1,072 (66.2)
Hispanic or Latino	45 (2.8)
Unknown	503 (31.1)
Case status, 2,542 patients, 10 states	
Confirmed	1,465 (57.6)
Probable	1,077 (42.4)
Outbreak-associated illness, 816 patients, 3 states	
Yes	110 (13.5)
No	195 (23.9)
Unknown	511 (62.6)
Immunocompromised, 1,154 patients, 3 states	
Yes	344 (29.8)
No	649 (56.2)
Unknown	161 (14.0)
Hospitalized, 2,218 patients, 9 states	
Yes†	1,273 (57.4)
No	851 (38.4)
Unknown	94 (4.2)
Died, 1,142 patients, 8 states	
Yes	76 (6.7)
No	906 (79.3)
Unknown	160 (14.0)

*Median (range) age for 3,401 patients from 12 states was 49 (0–94) y.
†Median (range) duration for 548 patients from 9 states was 7 (1–126) d.

disease, patient demographic features, and common methods of laboratory diagnosis. These data suggest substantial underdetection of histoplasmosis and a need for more standardized histoplasmosis surveillance. By characterizing populations at greater risk, these data can help increase public awareness and help healthcare providers better target diagnostics and early antifungal treatment.

Given that most infected persons are asymptomatic, the high proportions of patients who were hospitalized (57%) and died (7%) suggest relatively severe illness for these patients. In comparison, a review of 105 US histoplasmosis outbreaks during 1938–2013 found that 25% of all outbreak-associated patients were hospitalized and 1% died; hospitalization and death have become less common in recent years (*8*). National hospitalization data, which indicate that >5,000 histoplasmosis admissions (including multiple admissions per patient) occurred during 2012 (*9*), also suggest underdetection and underreporting in our surveillance data. We saw an annual mean of 852 cases from 12 states. Extrapolating from the hospitalization rate of 57% of patients for whom data were available, an estimated ≈485 patients (852 × 0.57) with reported disease would be hospitalized annually. To suggest that all remaining >4,500 estimated national hospitalizations occur in states without surveillance seems unlikely. Rather, the more likely explanation is that substantial underreporting occurs in states in which histoplasmosis is reportable, even among hospitalized patients. Furthermore, a retrospective cohort study in a tertiary care center found that the 6-month all-cause mortality rate among symptomatic histoplasmosis patients was 4% and was associated with older patient age (*10*). Our analysis, which included inpatients and outpatients, showed a higher mortality rate than the 5% in-hospital mortality rate found by an analysis of nationwide histoplasmosis-associated hospitalizations during 2001–2012 (*9*), further supporting the conclusion that current histoplasmosis surveillance captures only a small subset of more severely ill persons. To provide more information about the clinical spectrum, burden, and outcomes of each of the different forms of histoplasmosis, future surveillance could be improved by more consistent collection of data on severity and disease forms (e.g., acute pulmonary, chronic pulmonary, disseminated).

Table 2. Positive histoplasmosis laboratory test results among 1,929 histoplasmosis cases reported to public health departments, 9 US states, 2011–2014*

Test type	No. (%)
Antigen	644 (33.4)
Urine	536 (27.8)
Serum	146 (7.6)
Unspecified specimen type	248 (12.9)
Antibody	1,052 (54.5)
Immunodiffusion	618 (32.0)
Complement fixation	849 (44.0)
Confirmatory and histopathology	262 (13.5)
PCR	5 (0.3)
Culture	257 (13.3)
Microscopy	24 (1.3)
Other or unspecified type	248 (12.9)

*For some patients, >1 type of test was performed.

Current maps showing the presumed geographic distribution of histoplasmosis are still primarily informed by large-scale skin testing performed in the 1950s and 1960s, which identified the Ohio and Mississippi River Valleys as having the highest proportion of positive skin test reactions (*5*). In our analysis, we included much of the traditionally defined population at greatest risk; we did not include 2 (Missouri and Tennessee) of the 5 states with areas for which skin test positivity rates were >85%. Again, considering the apparent reporting bias of our data toward more severe cases, underdiagnosis and underreporting were likely. These results also support existing evidence that cases occur outside of the traditionally defined regions (*6*,*11*). Knowledge of histoplasmosis-endemic regions is particularly helpful for clinicians when risk-stratifying their patients and deciding whether to test for this disease. Because of the variability in state-specific case definitions, incidence rates might not be directly comparable between states but are useful for identifying trends within states. For example, Minnesota (only a small portion of which is traditionally considered histoplasmosis endemic) reported an incidence rate nearly double that of the 4 states with the next highest rates, and that rate has remained consistently high over the same period, possibly as a result of a broader case definition and strong surveillance system. Despite these limitations in

Table 3. Patient factors associated with hospitalization or death among histoplasmosis cases reported to public health, 12 US states, 2011–2014*

	Hospitalization		Death	
Characteristic	RR (95% CI)	p value	RR (95% CI)	p value
Age >50 y	1.23 (1.14–1.32)	<0.001	6.28 (3.43–11.49)	<0.001
Male sex	1.08 (1.01–1.45)	0.033	1.02 (0.65–1.58)	0.944
Nonwhite race†	1.26 (1.13–1.41)	<0.001	0.82 (0.36–1.86)	0.627
Immunocompromised‡	1.78 (1.62–1.96)	<0.001	6.07 (2.61–14.11)	<0.001
Positive laboratory test result§				
Antigen	1.75 (1.62–1.89)	0.001	1.73 (1.04–2.87)	0.033
Antibody	0.58 (0.53–0.63)	<0.001	0.41 (0.24–0.71)	0.001
Confirmatory	1.21 (1.09–1.34)	0.001	2.13 (1.28–3.54)	0.003

*RR, relative risk.
†Data collected by 8 states.
‡Data collected data by 3 states.
§Data collected by 9 states.

interstate comparability, data from Minnesota, Wisconsin, and Michigan suggest that histoplasmosis routinely occurs in areas where histoplasmosis was not previously considered endemic. Although interstate travel could account for some cases, higher incidence in the central and northern areas of these states, farther from known disease-endemic areas, suggests that local acquisition is likely. County-level incidence rates are also useful for demonstrating the distribution of cases within states. For instance, in Illinois, incidence was highest in counties clustered in the central region; in Arkansas and Mississippi, incidence was higher in counties along the Mississippi River.

These surveillance data provide a valuable window into the descriptive epidemiology of histoplasmosis in the United States. Although the large proportion of patients for whom race data were missing precluded a comparison of incidence by race, nonwhite patients were more likely than white patients to have been hospitalized, a finding that warrants future study. Black race has been associated with more severe histoplasmosis in patients with AIDS (*12*), but in general, no racial disposition has been documented for histoplasmosis (*3*,*13*) as it has been for coccidioidomycosis. In contrast, a sex disparity was readily evident. Male patients accounted for nearly two thirds of cases and were more likely than female patients to be hospitalized. A similar male predominance was seen in other studies of histoplasmosis and resulting hospitalizations (*3*,*8*–*11*) and for patients with other fungal infections, including coccidioidomycosis and blastomycosis (*11*,*14*,*15*). The reasons for this disparity are not entirely known, although different outdoor recreational and occupational exposures have been suggested (*3*,*14*,*15*). Of note, we did not observe an increased risk for death among male patients. Other studies of death from histoplasmosis in the United States also have not found male sex to be a risk factor for death (*10*,*16*).

In the 3 states with available data, nearly one third of histoplasmosis patients were reported to have been immunocompromised, providing additional evidence that current histoplasmosis surveillance, and perhaps clinical diagnosis, tends to detect more severe cases. We were unable to parse information about specific immunocompromising conditions from surveillance data. However, according to a study of histoplasmosis-associated hospitalizations, HIV infection was the most common concurrent immunocompromising condition listed on histoplasmosis-associated discharge records in the early 2000s; by 2012, diabetes mellitus (21%) had eclipsed HIV infection (17%) (*9*). The proportion of hospitalizations for immune-mediated inflammatory disease (rheumatoid arthritis, inflammatory bowel disease, and psoriasis) listed on discharge records also increased from 4% in 2001 to 10% in 2012, as did the proportion with solid organ or stem cell transplant (from 1% to 6%) (*9*). Standardized surveillance data would provide additional insight into the populations at highest risk for histoplasmosis and could help identify possible prevention opportunities.

Although state case definitions used different laboratory criteria for case classification, the laboratory data reported by 9 states provide a window into the most commonly positive test types and associations with disease outcomes. The fact that culture results were positive for 13% of patients for whom data were available again underscores the bias of detection and reporting toward severe cases because cultures are more often positive for patients with disseminated or chronic pulmonary disease than for those with milder disease (*17*). Accordingly, a positive confirmatory test result was associated with higher risk for hospitalization and death. Of note, a positive antigen test result, reported for one third of patients, was even more strongly associated with hospitalization than was a positive confirmatory test result, although the associations were similar for death. Antigen testing is particularly useful for immunocompromised patients and patients with severe disease, who might not mount an immune response, and is less sensitive for patients with subacute pulmonary disease (*18*). More than half of patients had a positive antibody test result, and these patients were less likely to have been hospitalized or die than those without a positive result, probably because these tests are more sensitive than others for patients with milder disease and might be used more routinely in outpatient settings. Serologic cross-reactions, particularly with antigen testing, and misclassification of blastomycosis cases

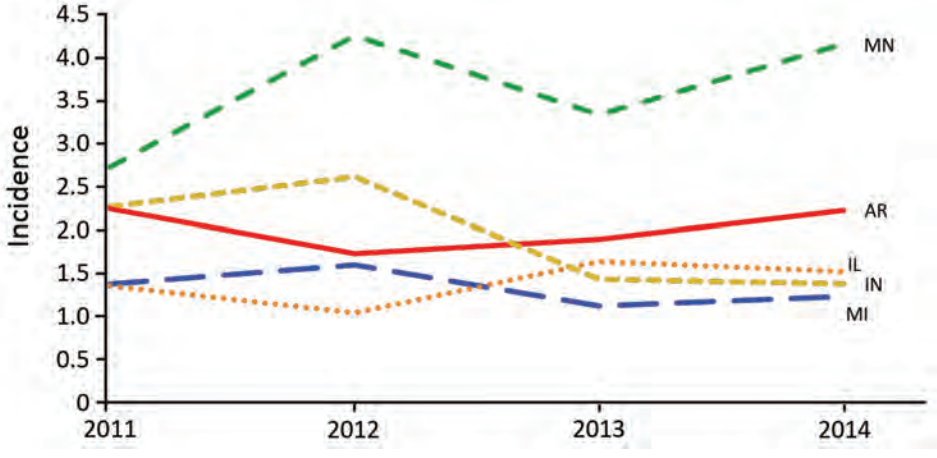

Figure 1. Annual state-specific histoplasmosis incidence (no. cases/100,000 population) for the 5 US states in which incidence was highest, 2011–2014.

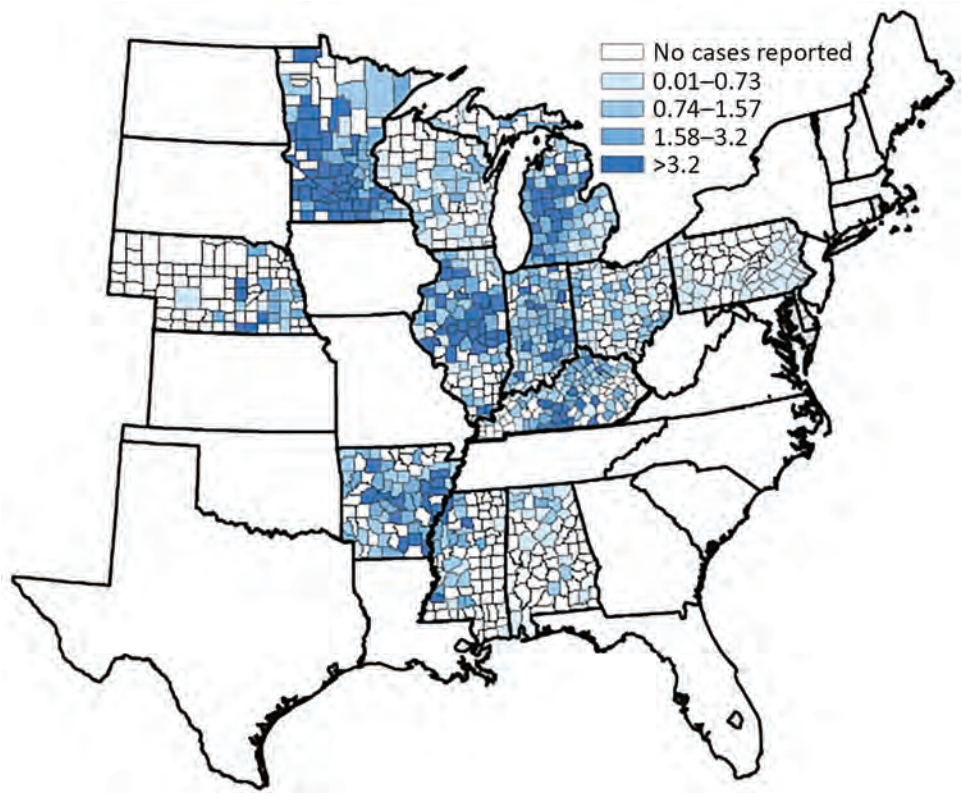

Figure 2. County-specific histoplasmosis incidence (no. cases/100,000 population) for the 12 US states from which surveillance data were available, 2011–2014.

as histoplasmosis might have occurred in areas in which both diseases are endemic; however, because extensive follow-up to differentiate between the 2 diseases occurred in some areas, the overall contribution of such false-positive histoplasmosis cases in our analysis is probably small.

As with other mycoses endemic to certain areas, assessing risk associated with recreational and occupational exposures is useful for identifying clusters of cases and developing recommendations for subsequent prevention of additional cluster-associated cases. A review of US histoplasmosis outbreaks described the presence of either birds or bats for 77% of outbreaks (*8*). However, limited published data are available about the proportion of sporadic (nonoutbreak) cases resulting from these types of exposures. For our analysis, only 3 states had collected similar data; reports of exposure to birds, bats, and their droppings were reported much less frequently (<30%) for sporadic cases than for outbreaks. Overall, exposure data were assessed for a relatively small portion of cases, and given the differences in how these data were collected, these numbers might not be directly comparable. The frequency of these exposures among the general population is also not known. Although histoplasmosis surveillance might not be able to detect broad exposures that lead to prevention messaging, tracking cases can enable detection of spatial and temporal hotspots and clusters that can lead to more intensive exposure investigations.

Our analysis has limitations inherent in summarizing disparate public health surveillance data. Primarily, differing state case definitions limit most direct comparisons between states. In addition, states collected different data, so denominators differed for many epidemiologic characteristics. Another limitation is the large proportion of missing and unknown data (either because surveillance investigators did not intend to collect specific data or because they did attempt to collect the data but were unable to do so). For this reason, we were not able to perform multivariable analyses. We were also not able to determine whether deaths were associated with histoplasmosis. Ultimately, more standardized histoplasmosis surveillance data would enable a better understanding of this disease by facilitating comparisons across states. The standardized surveillance case definition approved by CSTE in June 2016 will probably enable more consistent comparisons of incidence and trends in states that use this case definition (*19*).

This multistate comparison of histoplasmosis surveillance data is a first step toward an updated understanding of the burden of this disease in the United States. Other actions that would improve our understanding of histoplasmosis include expanding the number of states in which it is reportable and making it nationally notifiable. Although underreporting of milder cases might explain the high severity of disease among reported cases, the fact that laboratory-based reporting is common suggests that at least some of the skewed disease spectrum results from underdiagnosis.

Increased awareness about histoplasmosis among the public, the public health community, and healthcare providers could improve diagnosis, leading to appropriate treatment and better patient outcomes and reducing harm from administering multiple courses of antibacterial drugs ineffective against fungi, as commonly occurs for coccidioidomycosis (*20*,*21*). To identify populations at highest risk and opportunities for prevention, additional study of the incidence and epidemiologic, clinical, and laboratory features of histoplasmosis cases nationwide is needed.

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Epidemiology of Recurrent Hand, Foot and Mouth Disease, China, 2008–2015

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Release date: February 16, 2018; Expiration date: February 16, 2019

Learning Objectives

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

- Describe the epidemiologic features of recurrent hand-foot-and-mouth disease (HFMD), using national surveillance data during 2008–2015 in mainland China
- Identify the virologic features of recurrent HFMD
- Determine the clinical implications of the epidemiologic and virologic features of recurrent HFMD

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Using China's national surveillance data on hand, foot and mouth disease (HFMD) for 2008–2015, we described the epidemiologic and virologic features of recurrent HFMD. A total of 398,010 patients had HFMD recurrence; 1,767 patients had 1,814 cases of recurrent laboratory-confirmed HFMD: 99 reinfections of enterovirus A71 (EV-A71) with EV-A71, 45 of coxsackievirus A16 (CV-A16) with CV-A16, 364 of other enteroviruses with other enteroviruses, 383 of

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EV-A71 with CV-A16 and CV-A16 with EV-A71, and 923 of EV-A71 or CV-A16 with other enteroviruses and other enteroviruses with EV-A71 or CV-A16. The probability of HFMD recurrence was 1.9% at 12 months, 3.3% at 24 months, 3.9% at 36 months, and 4.0% at 38.8 months after the primary episode. HFMD severity was not associated with recurrent episodes or time interval between episodes. Elucidation of the mechanism underlying HFMD recurrence with the same enterovirus serotype and confirmation that HFMD recurrence is not associated with disease severity is needed.

Hand, foot and mouth disease (HFMD) is a common childhood infectious disease that is mainly caused by enterovirus A71 (EV-A71), coxsackievirus A16 (CV-A16), and CV-A6 (*1*). Most HFMD patients exhibit a benign, self-limiting illness characterized by skin eruptions on the hands, feet, or buttocks and ulcers or blisters in the mouth with or without fever (*2*). However, some patients develop neurologic or cardiopulmonary complications or die (*3*,*4*). In the past 2 decades, outbreaks of HFMD have been documented in countries of the Western Pacific, including Malaysia, Japan, Singapore, Vietnam, and Cambodia (*5*–*9*). In China, HFMD has been prevalent since 2007. During 2008–2015, ≈13 million HFMD cases were reported, including 123,261 severe cases and 3,322 deaths in 31 provinces of mainland China.

Three inactivated monovalent EV-A71 vaccines have been licensed in China. Phase 3 clinical trials proved these vaccines had high efficacy (90.0%–97.4%) against EV-A71–associated HFMD (*10*,*11*) but did not confer cross-protection for HFMD caused by non–EV-A71 enteroviruses (*11*). A natural infection with EV-A71 also confers no or only short-term (<2 months duration) cross-protection against CV-A16–associated illness (*12*,*13*). Because of this limited cross-protection from infections of different enterovirus serotypes, multiple HFMD episodes can occur in a single person. Although observational studies indicate that the antibody response induced by the EV-A71 vaccine could last >2 years, reinfection with an enterovirus of the same serotype is still possible because the immunity induced by a natural enterovirus infection might not be lifelong (*14*). We accessed the national surveillance data for HFMD diagnosed during 2008–2015 in China, in an attempt to describe the epidemiologic features of patients with recurrent HFMD and examine the relationship between disease severity and HFMD recurrence.

Materials and Methods

Data Sources

As described previously (*1*), HFMD cases were reported voluntarily to the Chinese Center for Disease Control and Prevention (China CDC) during January 1, 2008–May 1, 2008, and starting May 2, 2008, cases were mandatorily reported online to China CDC within 24 hours after diagnosis. We collected information on basic demographics (name, sex, national identification number, date of birth, home address, name of either of the patient's parents, contact telephone number); case classification (probable or confirmed); disease severity (severe or mild); date of illness onset, diagnosis, and death (if applicable); and enterovirus serotype (for confirmed cases). For virologic surveillance, clinical specimens were collected from a subsample of cases from each province and tested by PCR with primers and probes for panenterovirus, EV-A71, and CV-A16. We assumed that the enterovirus identified in HFMD patient samples was the causative enterovirus of the HFMD episode.

We included the HFMD surveillance data of 29 provinces of mainland China collected during January 1, 2008–December 31, 2015. We excluded data from Hunan and Hubei Provinces from this study because (since 2010 for Hunan Province and 2012 for Hubei Province) most of the hospitals in these provinces reported EV-A71 infection on the basis of EV-A71 IgM antibody detection assays, which are unreliable tests (*15*–*17*).

Case Definitions

We defined a probable HFMD patient as a patient who had rashes on the hands, feet, mouth, or buttocks and ulcers or vesicles in the mouth with or without fever. We defined a laboratory-confirmed patient as a probable patient with laboratory evidence of infection with EV-A71, CV-A16, or other enteroviruses. The diagnostic tests used for enterovirus detection were reverse transcription PCR and real-time reverse transcription PCR. Patients were classified as having severe HFMD if they had any complications (i.e., aseptic meningitis, brainstem encephalitis, encephalitis, encephalomyelitis, acute flaccid paralysis or autonomic nervous system dysregulation, pulmonary edema, pulmonary hemorrhage, or cardiorespiratory failure). Otherwise, patients were classified as having mild HFMD.

We identified patients with ≥2 episodes of HFMD reported in the national HFMD surveillance system by matching records using any of the following 3 screening criteria: 1) having identical identification number and identical or highly similar patient name; 2) having identical patient's parent name, home address, and birth date and identical or highly similar patient name; and 3) having identical contact telephone number, home address, and birth date and identical or highly similar patient name (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1303-Techapp1.pdf). We considered patients to have recurrent HFMD if they experienced ≥2 independent episodes of HFMD. We defined independent episodes as consecutive episodes separated by

an interval of ≥14 days if the previous episode was mild and ≥23 days if the previous episode was severe. We had estimated the time intervals defining 2 independent episodes by adding the longest duration of HFMD reported (7 days for mild illness and 16 days for severe illness) (*18*–*21*) plus the longest incubation period reported (7 days) (*4*,*22*–*26*). We classified patients with ≥2 independent episodes of laboratory-confirmed HFMD as having recurrent laboratory-confirmed HFMD. Otherwise, we classified patients as having recurrent probable HFMD. When counting the number of cases of reinfection with EV-A71, CV-A16, or other enteroviruses (Figure 1; Table 1), we considered any 2 laboratory-confirmed episodes as 1 case of reinfection; therefore, we classified patients with 3 laboratory-confirmed HFMD episodes as having 3 cases of reinfection (i.e., we grouped episodes 1 and 2, 1 and 3, and 2 and 3 together) and 4 laboratory-confirmed HFMD episodes as having 6 cases of reinfection (i.e., we grouped episodes 1 and 2, 1 and 3, 1 and 4, 2 and 3, 2 and 4, and 3 and 4 together).

Data Analysis

We used medians and interquartile ranges (IQRs) to describe continuous variables and numbers and percentages to summarize categorical variables. We used logistic regression with the forward stepwise selection approach to explore the association between HFMD recurrence and severe disease. We denoted the results as odds ratios (ORs) with 95% CIs. All statistical tests were 2-sided, and we considered an α of 0.05 statistically significant.

We defined the probability of HFMD recurrence as the probability of occurrence of HFMD among children who previously had HFMD and estimated recurrence using survival analysis (the Kaplan-Meier method). To calculate the probability of HFMD recurrence, we took only the event of interest into account and censored other events at the end of observation. When estimating the probability of HFMD recurrence, we enrolled all patients with recurrent HFMD (whether probable or laboratory-confirmed) in the analysis. We censored patients with only 1 HFMD episode. When estimating the probability of reinfection after EV-A71 with EV-A71, we included only the case-patients with a primary episode of EV-A71 infection who were later reinfected with EV-A71. We censored case-patients who had just 1 infection with EV-A71 (i.e., case-patients who were infected with EV-A71 then infected with CV-A16 or other enteroviruses, case-patients who were infected with EV-A71 then had probable HFMD, and case-patients with a single episode of EV-A71 infection). We used similar analyses to estimate the probability of reinfection with the same serotype for other serotypes of enterovirus.

We also conducted a sensitivity analysis to account for the uncertainty of the intervals used to define 2 independent HFMD episodes; in this analysis, we used 16 days (for previous mild episodes) and 61 days (for previous severe episodes) as cutoff values, which were derived from another investigation conducted in China that also investigated HFMD recurrence (*27*). We conducted data cleaning and analyses using R Project version 3.2.5 (http://cran.r-project.org) and ArcGIS 10.2 (http://www.esri.com/arcgis/about-arcgis). This study was approved by the ethics review committees of China CDC (Beijing, China).

Results

During 2008–2015, a total of 12,256,102 HFMD episodes occurring in 29 provinces of China were reported to the China CDC surveillance system. When using ≥14-day and ≥23-day intervals for defining independent infections, 398,010 patients (having 820,355 [7%] episodes) were identified as having recurrent HFMD, of which 1,767 (0.4%) patients (having 3,717 episodes) had recurrent laboratory-confirmed HFMD (Figure 1). The number of patients with recurrent HFMD was similar when we repeated this analysis using the 16-day and 61-day cutoff values in the sensitivity analysis, indicating that our estimation of HFMD recurrence was robust (online Technical Appendix Figure). Compared with patients with only 1 laboratory-confirmed HFMD episode, patients with recurrent laboratory-confirmed HFMD had a similar seasonal pattern, presenting semiannual peaks of activity with a ma-jor peak in the spring and early summer (April–June) followed by a smaller peak in autumn (September–October) (Figure 2, panels A, B). Similar seasonality was also observed for patients with a single episode of and recurrent probable HFMD (Figure 2, panels C, D).

We next focused on analyzing the 1,767 patients with recurrent laboratory-confirmed HFMD. During the study period, 90.3% (1,595) of these patients had 2 episodes and 9.7% (172) had >2 episodes: 161 (9.1%) patients had 3 episodes and 11 (0.6%) patients had 4 episodes. In total, 9% (154/1,767) of the patients with recurrent laboratory-confirmed HFMD still had ≥1 episode of probable HFMD, and 1,613 patients had only episodes of laboratory-confirmed HFMD. Of the 157 (8.9%) patients with ≥1 severe HFMD episode (183 total severe episodes), 26 patients (20 with 2 episodes, 3 with 3 episodes, and 3 with 4 episodes) experienced 2 severe HFMD episodes. A total of 1,814 cases of recurrence occurred among the 1,767 patients with recurrent HFMD. Only 144 (8%) of these 1,814 cases involved reinfection with an enterovirus of the same serotype: 99 (5.5%) with EV-A71 and 45 (2.5%) with CV-A16 (Figure 1). Most recurrent HFMD cases were caused by enteroviruses of different serotypes. Of the 1,767 patients, 5 (0.3%) were found to have an interval of ≤20 days between consecutive HFMD episodes: 2 patients who were reinfected with enteroviruses of different serotypes and 3 patients who were reinfected with enteroviruses of the same serotype.

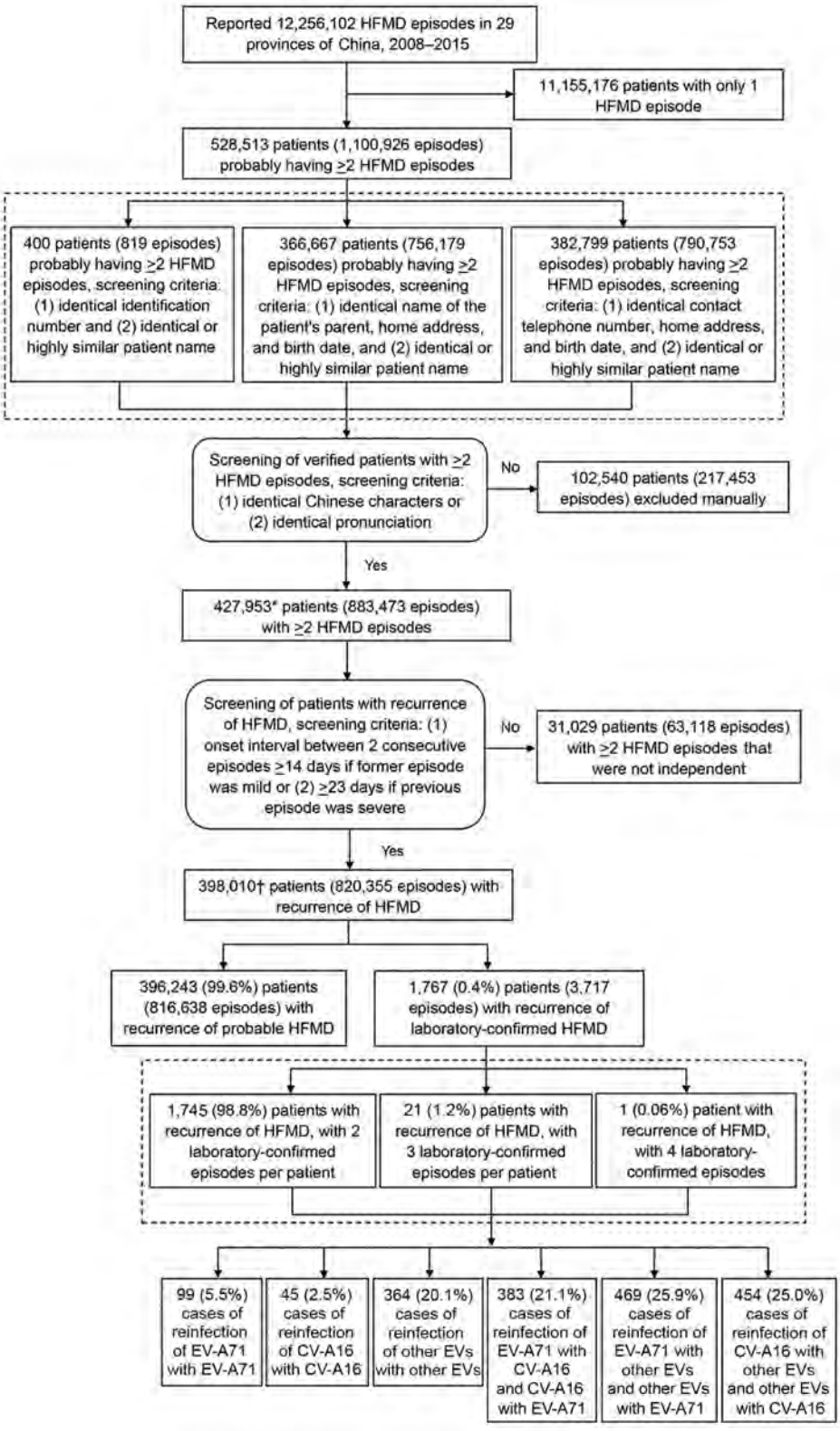

Figure 1. Flowchart showing screening for and analysis of patients with recurrent HFMD from the national HFMD surveillance database, 29 provinces of China, 2008–2015. Percentages do not equal 100% because of rounding. *The number of patients (427,953) with ≥2 HFMD episodes is higher than expected (528,513 – 102,540 = 425,973) because of improved patient matching. In some situations, the number of patients with ≥2 episodes did not change; for example, a patient initially identified with 3 episodes might have been determined to have only 2 episodes, with the third episode being attributed to a different patient. In other situations, the number of patients with ≥2 episodes decreased; for example, a patient initially identified as having 3 episodes might have been determined to be 3 different patients with 3 different episodes. Therefore, the reduced number of patients (528,513 – 427,953 = 100,560) with ≥2 HFMD episodes is smaller than the number of patients (102,540) excluded manually. †The number of patients (398,010) with recurrence of HFMD is higher than expected (427,953 – 31,029 = 396,924) because some patients needed to be excluded and included. In some situations, patients were completely included or excluded from the recurrent HFMD patient population sample; for example, all 3 episodes of a patient could have been determined to not be independent from each other. In other situations, patients were included and excluded from the recurrent HFMD patient population sample; for example, a patient with 3 episodes might have had 2 episodes that were not independent from each other. In these cases, the patient had 2 episodes included and 1 episode excluded; therefore, the number of included patients plus excluded patients (398,010 + 31,029 = 429,039) exceeded the starting population number (427,953). CV-A16, coxsackievirus A16; EV-A71, enterovirus A71; HFMD, hand, foot and mouth disease; other EVs, other non–EV-A71 and non–CV-A16 enteroviruses.

Table 1. Demographic characteristics of patients with recurrent probable and laboratory-confirmed HFMD in 29 provinces of China, 2008–2015*

Characteristic	Patients with recurrent probable HFMD, N = 396,243	Patients with recurrent laboratory-confirmed HFMD, N = 1,814†						Patients with recurrent laboratory-confirmed HFMD, N = 1,767
		Reinfection after EV-A71 with EV-A71, n = 99	Reinfection after CV-A16 with CV-A16, n = 45	Reinfection after other EVs with other EVs, n = 364	Reinfection after EV-A71 with CV-A16 or after CV-A61 with EV-A71, n = 383	Reinfection after EV-A71 with other EVs or after other EVs with EV-A71, n = 469	Reinfection after CV-A16 with other EVs or after other EVs with CV-A16, n = 454	
Age at first episode								
Age, mo, median (IQR)	20.8 (12.2–31.4)	24.2 (15.6–36.5)	27.1 (20.9–39.4)	18.8 (12.2–31.4)	26.3 (17.7–36.8)	22.6 (14.5–34.4)	22.8 (14.2–32.8)	22.6 (14.2–34.0)
Age group								
<6 mo	7,279 (2)	1 (1)	0	10 (3)	1 (0.3)	4 (1)	7 (2)	23 (1)
6–11 mo	80,982 (20)	10 (12)	7 (16)	77 (21)	39 (10)	76 (16)	72 (16)	283 (16)
12–23 mo	155,973 (39)	46 (46)	14 (31)	144 (40)	132 (35)	181 (39)	176 (38)	696 (39)
24–59 mo	145,289 (37)	39 (39)	22 (49)	129 (35)	203 (53)	204 (43)	192 (42)	738 (42)
5–9 y	6,526 (2)	2 (2)	2 (4)	4 (1)	8 (2)	4 (1)	7 (2)	26 (2)
10–14 y	158 (0.04)	1 (1)	0	0	0	0	0	1 (0.05)
≥15 y	36 (0.01)	0	0	0	0	0	0	0
Age at second episode								
Age, mo, median (IQR)	36.4 (24.3–48.5)	40.0 (27.4–50.2)	40.7 (25.7–55.8)	34.7 (24.2–45.9)	42.3 (32.5–53.2)	37.1 (26.8–49.4)	36.8 (26.8–49.2)	36.5 (25.7–48.7)
Age group								
<6 mo	236 (0.06)	0	0	1 (0.3)	0	0	0	1 (0.05)
6–11 mo	14,239 (4)	2 (2)	0	12 (3)	3 (1)	14 (3)	13 (3)	47 (3)
12–23 mo	83,568 (21)	17 (17)	8 (18)	89 (25)	35 (9)	73 (16)	73 (16)	309 (17)
24–59 mo	257,729 (65)	69 (70)	29 (64)	232 (64)	298 (78)	335 (71)	315 (69)	1,234 (70)
5–9 y	39,786 (10)	10 (10)	7 (16)	29 (8)	47 (12)	45 (10)	52 (12)	170 (10)
10–14 y	640 (0.16)	1 (1)	1 (2)	1 (0.3)	0	2 (0.4)	1 (0.2)	6 (0.33)
≥15 y	45 (0.01)	0	0	0	0	0	0	0
Male sex	259,028 (65)	74 (75)	31 (69)	247 (68)	270 (70)	326 (70)	291 (64)	1,208 (68)
Rural residence	186,700 (47)	49 (49)	19 (42)	115 (32)	187 (49)	190 (41)	167 (37)	716 (41)
Frequency of episodes								
2	373,745 (95)	91 (92)	41 (91)	303 (83)	356 (93)	404 (86)	400 (88)	1,595 (90)
3	21,023 (5)	7 (7)	4 (9)	54 (15)	22 (6)	59 (13)	49 (11)	161 (9)
≥4‡	1,475 (0.4)	1 (1)	0	7 (2)	5 (1)	6 (1)	5 (1)	11 (1)
Death	20 (0.005)	0	0	0	0	0	0	0

*Data are no. (%) patients unless otherwise indicated. CV-A16, coxsackievirus A16; EV-A71, enterovirus A71; HFMD, hand, foot and mouth disease; IQR, interquartile range; other EVs, other non–EV-A71 and non–CV-A16 enteroviruses.
†For patients with laboratory-confirmed recurrence of 3 or 4 HFMD episodes, any 2 laboratory-confirmed HFMD episodes were combined to form a case of recurrence of laboratory-confirmed HFMD. For example, patients with 3 episodes were categorized as having 3 reinfections, with the first and second, first and third, and second and third infections being grouped together.
‡For patients with recurrence of laboratory-confirmed HFMD, the highest number of episodes was 4. For patients with recurrence of probable HFMD, the highest number of episodes was 8.

Demographic Characteristics

The median ages of patients with recurrent laboratory-confirmed HFMD were 22.6 (IQR 14.2–34.0) months for the first episode and 36.5 (IQR 25.7–48.7) months for the second episode. Younger children had more episodes of recurrent laboratory-confirmed HFMD ($p<0.001$) and recurrent probable HFMD ($p = 0.001$) (online Technical Appendix Table 1). Few patients (1.5%) had their first episode of HFMD after 5 years of age. Approximately two thirds (68% or 1,208) of the patients affected were boys, and 41% were residents of rural areas. The demographic characteristics age, sex, and rural residence and the frequency of episodes were similar among patients with recurrent laboratory-confirmed HFMD, regardless of the enterovirus serotypes of reinfections (Table 1).

Geographic Distribution

Patients with recurrent laboratory-confirmed HFMD were observed in all of the 29 provinces of China we examined except Tibet. The number of recurrent laboratory-confirmed HFMD cases varied substantially, ranging from 7 cases in Qinghai Province to 658 cases in Guangdong Province. Half of the cases with recurrent laboratory-confirmed HFMD were reported in 3 provinces: Guangdong (658 cases), Yunnan (153 cases), and Sichuan (107 cases) (Figure 3).

Probability of HFMD Recurrence

Patients in this cohort were under observation for a median of 38.0 (range 0–97.4) months after their first HFMD diagnosis (online Technical Appendix Table 2). The recurrent episode occurred 0.5–93.4 (median 11.7) months after the primary HFMD episode in patients with recurrence of probable HFMD and 0.5–62.1 (median 12.0) months in patients with recurrence of laboratory-confirmed HFMD. The probability of HFMD recurrence was 1.9% at 12 months, 3.3% at 24 months, and 3.9% at 36 months; however, recurrence remained at 4.0% at 38.8 months after the primary episode of HFMD (Figure 4, panel A). For patients with primary EV-A71 infections, the probability of reinfection with CV-A16 (0.11% [95% CI 0.10%–0.13%]) or other enteroviruses (0.14% [95% CI 0.13%–0.16%]) was higher than that of reinfection with EV-A71 (0.05% [95% CI 0.04%–0.07%]; p<0.001) (Figure 4, panel B). For patients with primary CV-A16 infections, the probability of reinfection with EV-A71 (0.18% [95% CI 0.15%–0.20%]) or other enteroviruses (0.21% [95% CI 0.18%–0.23%]) was higher than that of reinfection with CV-A16 (0.04% [95% CI 0.03%–0.05%]; p<0.001) (Figure 4, panel C). These findings suggest that risk for reinfection with different enterovirus serotypes might be higher than that for reinfection with identical enterovirus serotypes.

Relationship between HFMD Recurrence and Severe Illness

Unsurprisingly, we found that illness severity was inversely associated with age and onset-to-diagnosis interval. EV-A71 infections (OR 7.2, 95% CI 4.0–13.0) and other enterovirus infections (OR 2.7, 95% CI 1.5–5.0) were more severe than CV-A16 infections. Patients who lived in urban areas also had increased risk for severe illness (OR 1.8, 95% CI 1.3–2.5). After adjusting for these risk factors, recurrent HFMD episodes were not found to be associated with illness severity, which means the second and third HFMD episodes did not appear to be more or less severe

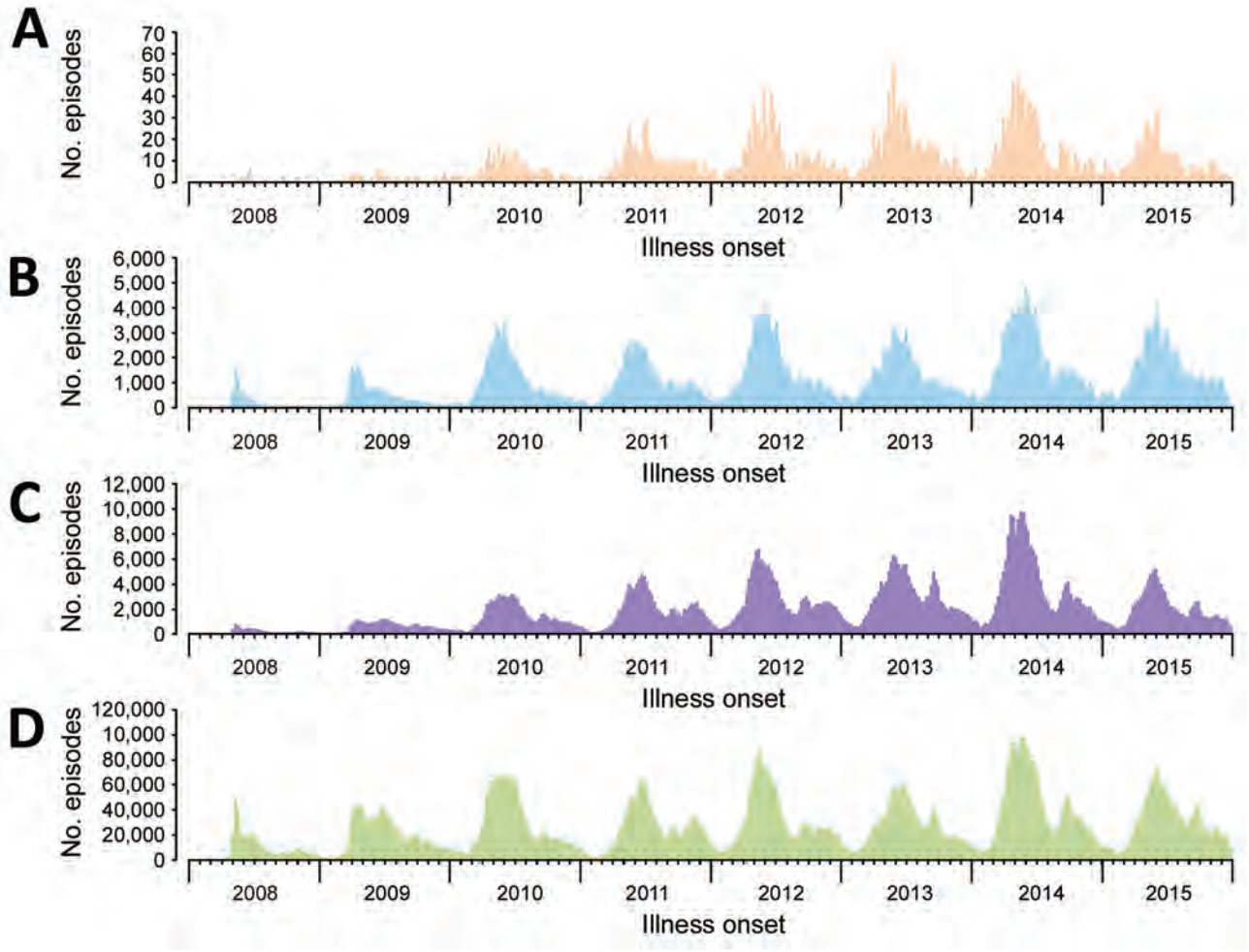

Figure 2. Hand, foot and mouth disease (HFMD) episodes in 29 provinces of China, 2008–2015. A) Patients with recurrent laboratory-confirmed HFMD. B) Patients with single episode of laboratory-confirmed HFMD. C) Patients with recurrent probable HFMD. D) Patients with single episode of probable HFMD.

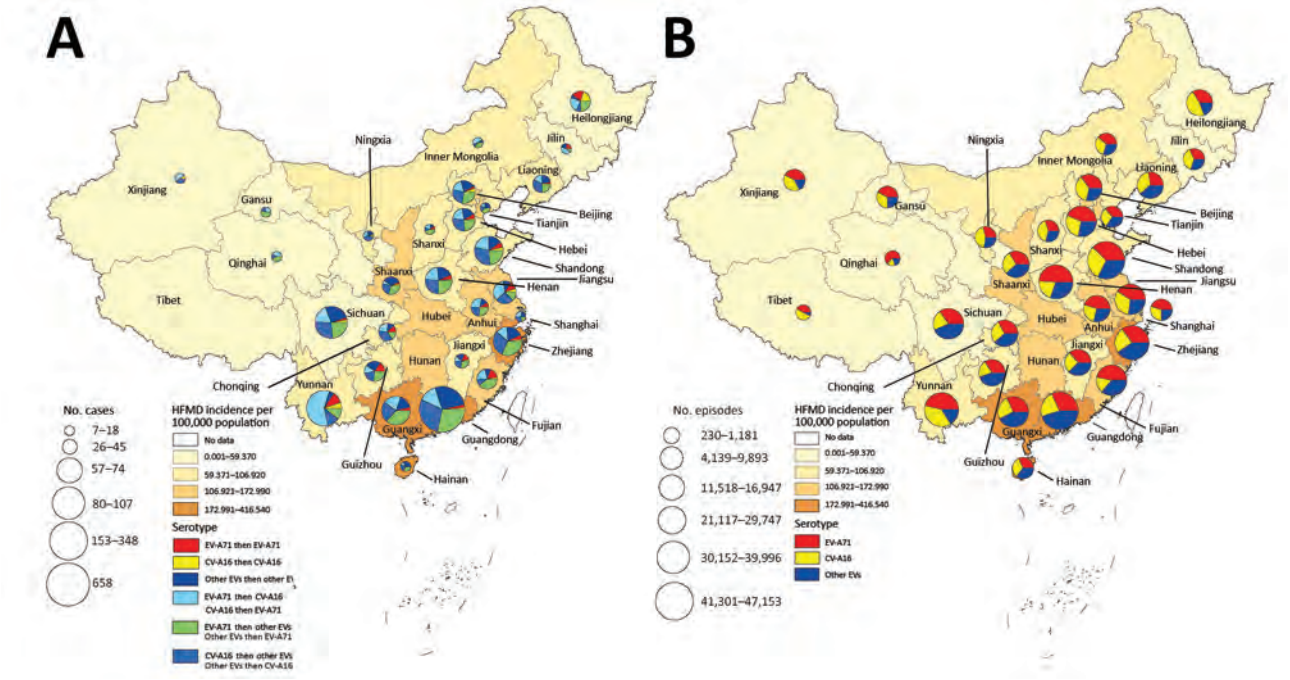

Figure 3. Geographic distribution of patients with recurrent HFMD (A) and episodes of enterovirus infection (B) in 29 provinces of China, 2008–2015. A) Pie charts correspond to the number of recurrent laboratory-confirmed HFMD cases. B) Pie charts correspond to the number of laboratory-confirmed HFMD episodes. CV-A16, coxsackievirus A16; EV-A71, enterovirus A71; HFMD, hand, foot and mouth disease; other EVs, non–EV-A71 and non–CV-A16 enteroviruses.

than the first episode. In addition, the interval between the 2 episodes was not related to disease severity of the latter episode (OR 0.97, 95% CI 0.95–1.01) (Table 2).

Discussion

During 2008–2015, we found that 398,010 HFMD patients with ≥2 episodes (a total of ≈820,000 episodes) were reported among children in China; 1,767 of these recurrences were laboratory-confirmed. The patients who were reinfected with different enterovirus serotypes had similar age, sex, residence, and frequency of episodes. Recurrence of HFMD mainly occurred 0–38.8 months after the primary episode, with a recurrence probability of 4% at 38.8 months. Reinfection with a different enterovirus serotype was more likely than reinfection with an identical enterovirus serotype. The severity of HFMD

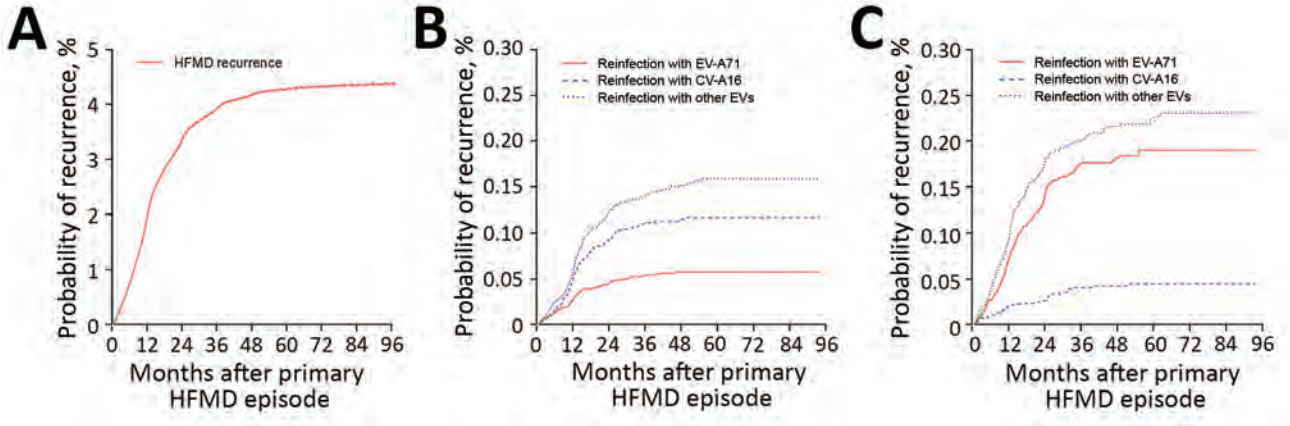

Figure 4. Kaplan-Meier analysis of survival from HFMD recurrence after primary HFMD diagnosis, 29 provinces of China, 2008–2015. A) Probability of HFMD recurrence among all patients who had probable and laboratory-confirmed HFMD. B) Probability of HFMD recurrence among case-patients whose primary episode was an infection with EV-A71. C) Probability of HFMD recurrence among case-patients whose primary episode was an infection with CV-A16. CV-A16, coxsackievirus A16; EV-A71, enterovirus A71; other EVs, non–EV-A71 and non–CV-A16 enteroviruses; HFMD, hand, foot and mouth disease.

Table 2. Risk factors associated with severe illness in cases of recurrent laboratory-confirmed HFMD, China, 2008–2015*

Risk factor	Mild, N = 3,187, no. (%)	Severe, N = 172, no. (%)	Adjusted OR (95% CI)
Age at HFMD onset, mo			
>23	2,054 (96.9)	66 (3.1)	Reference
12–23	836 (91.5)	78 (8.5)	2.4 (1.7–3.6)
<12	297 (91.4)	28 (8.6)	2.6 (1.6–4.4)
Sex			
F	1,010 (95.5)	48 (4.5)	Reference
M	2,177 (94.6)	124 (5.4)	0.9 (0.6–1.2)
Enterovirus serotype			
CV-A16	857 (98.5)	13 (1.5)	Reference
Other EVs	1,452 (96.0)	61 (4.0)	2.7 (1.5–5.0)
EV-A71	878 (90.0)	98 (10.0)	7.2 (4.0–13.0)
Residence			
Rural	1,342 (92.8)	104 (7.2)	Reference
Urban	1,845 (96.4)	68 (3.6)	1.8 (1.3–2.5)
Order episode occurred			
First	1,523 (93.9)	99 (6.1)	Reference
Second or after	1,664 (95.8)	73 (4.2)	0.8 (0.5–1.2)
Onset-to-diagnosis interval, d			
≤1	1,443 (96.3)	55 (3.7)	Reference
2–3	896 (94.0)	57 (6.0)	1.6 (1.1–2.4)
≥4	848 (93.4)	60 (6.6)	1.8 (1.2–2.7)
Interval between successive episodes			0.97 (0.95–1.01)

*CV-A16, coxsackievirus A16; EV-A71, enterovirus A71; HFMD, hand, foot and mouth disease; OR, odds ratio; other EVs, other non–EV-A71 and non–CV-A16 enteroviruses.

was not associated with recurrent infections or the time interval between HFMD episodes.

In a report on a phase 3 clinical trial, an EV-A71 neutralizing antibody titer of 1:16 was associated with protection against EV-A71–associated HFMD (*28*). In addition, EV-A71 was observed to confer cross-neutralization activity against major global EV-A71 genotypes (A, B1, B3–B5, C1–C5), although the degree of cross-neutralization varied (*29*–*31*). In an EV-A71 vaccine study, participants were observed for only 2 years, but results suggested that the vaccine can provide protection against EV-A71–associated HFMD for ≥2 years (*14*). Infection with enteroviruses seems to confer lifelong immunity to HFMD, given that adult cases are rare (*1*). The reasons underlying the cases of HFMD recurrence caused by reinfections with the same serotype, which have a 12-month median interval to reinfection, are not clear. Measles has been deemed to provide long-lasting protection against reinfection. However, measles reinfections have occurred in vaccinated and presumptively immune persons, either because of insufficient primary antigenic stimulation or inadequate duration of antibody response (*32*). Study results have suggested the involvement of multiple cellular deficiencies, including low memory B-cell count, reduced polyclonal naive and memory T-cell responses, and suboptimal antigen-presenting cell responses, in children with low vaccine responses (*33*,*34*). Agammaglobulinemia is another condition of immunodeficiency associated with recurrent infections (*35*). In patients with influenza, suboptimal immune responses after the primary infection led to the failure to generate a protective immune response that could have prevented reinfection (*36*). Children with immature immunity or immunodeficiency (*37*) probably are not able to induce sufficient immune responses (or might induce low-level serologic responses that wane rapidly) after infection with EV-A71 or CV-A16; thus, these children are likely susceptible to reinfection with an enterovirus of the same serotype as their primary episode. Another possibility (though less likely) is that high neutralizing antibody titers might not protect some persons from illnesses induced by enteroviruses. Further investigations are needed to provide a scientific explanation.

Even though the genotypes of EV-A71 and CV-A16 were not available in this study, previous studies have shown that the predominant EV-A71 and CV-A16 genotypes circulating in China have been consistent. Phylogenetic analysis of viral protein 1 gene sequences revealed that the EV-A71 genotype circulating in China since 2008 has been C4 (*38*–*42*); hence, the monovalent EV-A71 vaccines licensed in China were designed to target the C4 genotype. B1 has been reported to be the predominant genotype of CV-A16 circulating in China (*40*–*43*). Therefore, we reasonably conclude that in our cohort HFMD recurrences involving reinfections with enteroviruses of the same serotype were also highly likely reinfections with the same genotype.

Recurrent laboratory-confirmed HFMD was largely (at least 72% of cases) attributable to different serotypes of enterovirus; thus, undoubtedly hundreds of thousands of patients with HFMD recurrence with different serotypes should have occurred, given clinical samples were collected from only 4.2% of the patients with probable HFMD episodes for virologic diagnosis. Our results support

the notion that limited or no cross-protection against different serotypes of enterovirus occurs after natural infection, which is consistent with observations from the EV-A71 vaccine study (*12*,*14*) and a modeling study of natural infections (*13*). Antibody-dependent enhancement, which has been commonly seen in dengue, was also observed in EV-A71 and CV-B3 infections in mouse models; the severity of the subsequent episode of infection was enhanced by a subneutralizing level of antibody after primary infection (*44*,*45*). However, we did not observe that HFMD recurrence or the interval between successive episodes had any effect on disease severity in humans. It seems that antibody-dependent enhancement does not happen in human infections with enterovirus, further implying that no or short-term cross-reactivity develops for different serotypes of enterovirus.

Three monovalent EV-A71 vaccines are administered in China (*46*). Our study indicates that children who receive an EV-A71 vaccine can still develop HFMD after vaccination, which is a challenge for monovalent EV-A71 vaccines. Even though the EV-A71 vaccine protects against >90% of the EV-A71 infections that occur in children, children still face the risk for infection with other enterovirus serotypes after vaccination. Hence, public health authorities should inform parents and caregivers about the risk for the development of HFMD after EV-A71 vaccination, and multivalent vaccines for HFMD (e.g., EV-A71 combined with other prevalent circulating serotypes CV-A16 and CV-A6) are needed for the HFMD epidemic.

This study has several limitations. First, the burden of HFMD recurrence was underestimated because the disease is substantially underreported in the surveillance system (16%–36% estimated) (*47*) and the observation period for assessing recurrence was insufficient, especially among patients identified in more recent years. Although the recurrence of HFMD is rarely reported in other countries (*48*,*49*), our study suggests that it is not uncommon. Second, because clinical samples were not collected from all patients during each HFMD episode and tested, we could not determine the real number of recurrent HFMD cases; thus, the probabilities of reinfection with an identical enterovirus serotype (i.e., EV-A71 and CV-A16) we calculated might be underestimated. It is not favorable to estimate the number of patients with reinfections of the same serotype because only a small proportion (4.2%) of HFMD episodes have been tested for enterovirus diagnosis, although mathematical modeling methods could be used to solve this problem. This topic requires further exploration. Third, we were unable to describe the features of patients with reinfection of non–EV-A71 and non–CV-A16 serotypes. Fourth, the short interval between consecutive episodes in some patients suggests the potential for co-infections rather than reinfections; thus, co-infections might have occurred and caused a slight overestimation of the recurrence rate for HFMD. However, patients with short intervals between consecutive episodes accounted for a small proportion of the patients with recurrent HFMD, so the effect that co-infections played is relatively limited.

In conclusion, our 8-year surveillance study indicates a high burden of HFMD recurrence among children in China and shows that each episode of recurrent HFMD is more likely caused by a different enterovirus serotype than that of the primary episode (both for patients with EV-A71 and CV-A16 primary infections). Further studies in which virologic diagnosis is performed for all HFMD episodes are needed to better quantify the probability of HFMD recurrence and probability of reinfection by enterovirus serotype, including non–EV-A71 and non–CV-A16 serotypes. Further investigations are also warranted to elucidate the mechanism underlying HFMD recurrences resulting from reinfections with enteroviruses of the same serotype; the protective antibody levels for EV-A71, CV-A16, and other enterovirus serotypes; and the duration of immunity and cross-immunity between serotypes. Finally, more work is needed to study the effect of HFMD recurrence on disease severity, even though no association was observed in this patient cohort.

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Capsule Typing of *Haemophilus influenzae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry[1]

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Encapsulated *Haemophilus influenzae* strains belong to type-specific genetic lineages. Reliable capsule typing requires PCR, but a more efficient method would be useful. We evaluated capsule typing by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Isolates of all capsule types (a–f and nontypeable; n = 258) and isogenic capsule transformants (types a-d) were investigated. Principal component and biomarker analyses of mass spectra showed clustering, and mass peaks correlated with capsule type-specific genetic lineages. We used 31 selected isolates to construct a capsule typing database. Validation with the remaining isolates (n = 227) showed 100% sensitivity and 92.2% specificity for encapsulated strains (a–f; n = 61). Blinded validation of a supplemented database (n = 50) using clinical isolates (n = 126) showed 100% sensitivity and 100% specificity for encapsulated strains (b, e, and f; n = 28). MALDI-TOF mass spectrometry is an accurate method for capsule typing of *H. influenzae*.

Haemophilus influenzae is subdivided into encapsulated strains, which express different serotypes of capsular polysaccharide (designated types a–f), and nonencapsulated strains, which are designated nontypeable *H. influenzae* (NTHi) (*1*). Since the introduction of conjugate vaccines against *H. influenzae* type b (Hib), a common cause of meningitis, epiglottitis, and sepsis in small children, the epidemiology of invasive *H. influenzae* disease has changed dramatically, with an increase in the diversity of serotypes responsible for illness.

Although the incidence of Hib disease has decreased in countries implementing childhood vaccination (*2*), invasive disease caused by NTHi has become more prominent during the same period, especially among newborns and the elderly (*3–6*). In the postvaccination era, increasing incidences and outbreaks of invasive *H. influenzae* type a (Hia) infections have been reported in South and North America (*7–10*), particularly among the indigenous populations in Canada and the United States (*7,8,10*). Studies have also suggested an increase in cases of invasive *H. influenzae* type e (Hie) and type f (Hif) disease (*4,11,12*). Hib vaccine failures have been described (*13*), and omission of booster dose(s) appears to result in a rapidly increased incidence of invasive Hib disease (*14,15*), suggesting continued circulation of Hib isolates in the community. Globally, one third of eligible children still do not receive adequate vaccination (*16*).

Encapsulated *H. influenzae* strains are generally genetically clonal. This finding was first demonstrated by multilocus enzyme electrophoresis, by which encapsulated isolates could be separated into different genetic lineages that correspond to different capsule types (*17*). This clonal population structure has been confirmed by multilocus sequence typing (MLST), which assigns isolates to different sequence types (STs), although some differences have been observed in the organization of different lineages (*18*). There are 3 known major genetic groups of Hia and Hib (*18,19*). Two genetic groups of Hia (related to ST21 and ST23) account for most Hia isolates in the MLST database (*20*). For Hib, ST6-related isolates account for most cases (*17,18*), whereas the second most common genetic group is related to ST222 (*18,21*). There is 1 known lineage each for serotypes c through f (*18,19*). In contrast, NTHi are genetically heterogenous (*19*).

Capsule typing of *H. influenzae* has traditionally been performed by using slide agglutination with antisera (conventional serotyping), but incorrect results are common,

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[2]These senior authors contributed equally to this article.

and specificity for encapsulated isolates is low (*22*,*23*). Determination of presence of the capsule gene complex (*bexA* or *bexB*) by PCR, followed by type-specific *cap* a–f PCRs has excellent sensitivity and specificity but is laborious and time-consuming (*24*–*27*). Because of limitations of current typing methods, typing might be delayed or not performed in clinical practice. However, rapidly obtained information on capsule type is still of interest for the treating clinician (*5*) and, in particular, for monitoring of capsule type distribution and effectiveness of Hib vaccination programs, especially with respect to invasive disease.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is commonly used to identify bacterial and fungal species, including *H. influenza*e, by analyzing the composition of ribosomal proteins in a sample. It is a rapid and convenient method and has a low cost per sample (*28*). Recently, we have shown that MALDI-TOF mass spectrometry can separate Hib from non-b *H. influenzae* (*29*). In this study, we examined the capacity of MALDI-TOF mass spectrometry to perform full capsule typing of *H. influenzae*. This method would be valuable for first-line diagnostics of *H. influenzae* to identify patients at risk for immunodeficiency or anatomic cerebrospinal fluid space defect, and to detect rapidly outbreaks caused by specific capsule types. It would also increase time and cost effectiveness of surveillance of *H. influenzae* epidemiology and Hib vaccination efficacy.

Materials and Methods

Bacterial Isolates

We used 2 culture collections in this study (Figure 1). The first collection was an evaluation set of isolates used to construct a coherent reference database and was composed of 258 *H. influenzae* strains. It included isolates from 3 major clinical laboratories in Sweden (Malmö/Lund, Gothenburg, and Stockholm) obtained in 1997–2011 but also a wide range of international strains from different countries, continents, and time periods (n = 41; online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/3/17-0459-Techapp1.pdf). In addition, we included 4 isogenic capsule-transformed strains of types a (Rb^-/a^+:02), b (Rb^+:02), c (Rb^-/c^+:02), and d (Rb^-/d^+:02) (*30*) in the study. These strains originate from strain Rd, a capsule-deficient type d strain (*31*). For validation of the new MALDI-TOF mass spectrometry typing method, we used a second collection composed of 126 bloodstream and cerebrospinal fluid *H. influenzae* isolates obtained in Sweden during 2010 and 2013–2016. All isolates were identified as *H. influenzae* by using standard laboratory taxonomy techniques and were grown on chocolate agar plates overnight (18–24 h) in a humid atmosphere at 37°C containing 5% CO_2 before any experiments were conducted.

PCR for Capsule Typing and MLST

We prepared DNA by adding a few colonies of bacteria to distilled water. After heating at 98°C for 10 min, we centrifuged each sample at 16,000 × *g* for 5–10 min and collected the supernatant. In a few instances, we extracted DNA by using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. We performed capsule typing by PCR using *bexB* and type-specific *cap* primers for all isolates as described (*24*–*26*).

We performed PCR for MLST genes as described (*18*) and sequenced the resulting PCR products by using the forward primer and, if necessary for adequate sequence quality, the reverse primer. We trimmed and

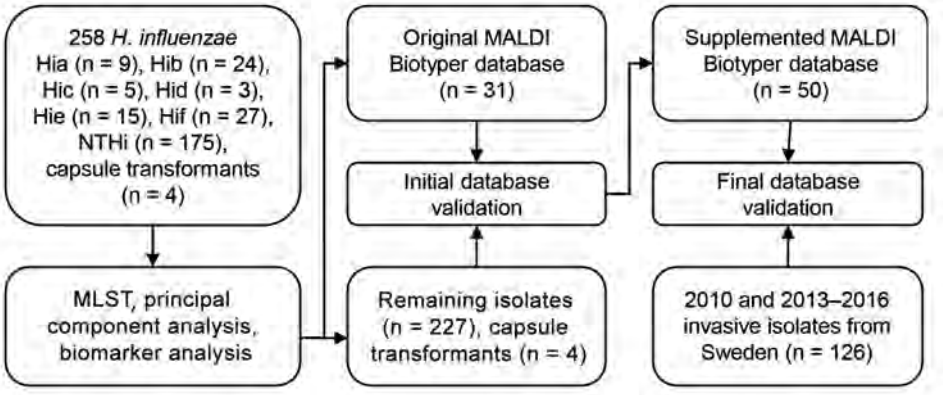

Figure 1. Culture collections and methods used to investigate capsule typing of *Haemophilus influenzae* by MALDI-TOF mass spectrometry. An evaluation set of *H. influenzae* isolates of all capsule types from diverse geographic origins and time periods and isogenic capsule transformants (*30*) were used to investigate capsule type-specific differences in MALDI-TOF mass spectra. MLST was used to ensure adequate coverage of different genetic lineages of encapsulated *H. influenzae*. Reference isolates from the evaluation set (encapsulated and nonencapsulated) were selected to construct a new typing database in MALDI Biotyper. This database was tested with the remaining isolates in the set, and misclassified isolates were added to the database. The final supplemented database was blindly validated with a second culture collection that consisted of clinical invasive isolates. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MLST, multilocus sequence typing; NTHi, nontypeable *H. influenzae*.

edited sequences before concatenation (total length 3,057 bp). We deposited MLST nucleotide sequences in GenBank (accession nos. MG550316–MG550889). Some isolates had been previously typed by MLST. In these instances, we retrieved MLST data from the MLST database (*20*).

Analysis of MLST Data

We determined sequence types by using the MLST database. We aligned concatenated sequences in Geneious 9.1.8 (Biomatters, Auckland, New Zealand) and used the PAUP* 4.0a158 plug-in (http://phylosolutions.com/paup-test/) to construct a maximum-likelihood phylogenetic tree. The best fitting model was estimated to be the generalized time-reversible model including invariant sites and gamma distribution by using the Akaike information criterion in jModelTest 2.1.10 (*32*,*33*). We visualized the resulting tree by using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). All isolate and ST information has been submitted to the MLST database.

Acquisition of MALDI-TOF Mass Spectrometry Data

We acquired mass spectra by using a Microflex LT MALDI-TOF mass spectrometry system (Bruker Daltonics, Bremen, Germany), with default settings as described (*29*). We prepared all isolates for acquisition of spectra by using the ethanol–formic acid procedure described by the instrument manufacturer. We spotted isolates on 2 spots and analyzed each spot 3 times, resulting in 6 spectra/isolate. Isolates in the reference database were spotted on 8 spots, resulting in 24 spectra, before being added to the database.

Analysis of MALDI-TOF Mass Spectrometry Data

In Mass-Up 1.0.13 (*34*), we preprocessed and analyzed raw spectra of all isolates in the evaluation set (n = 258) and capsule transformants (n = 4) by using the integrated MALDIquant analysis package for R (http://strimmerlab.org/software/maldiquant/). We performed preprocessing with intensity transformation (square root), smoothing (Savitzky–Golay), baseline correction (Top-Hat), and intensity standardization (total ion current). We performed peak detection with a signal-to-noise ratio of 2, a half window size of 50, and no minimum peak intensity. We calculated a consensus spectrum for each isolate with a peak tolerance of 0.002 and percentage of presence of 60%. For principal component analysis (PCA) (*35*) and biomarker analysis, we performed intersample matching with a peak tolerance of 0.002. PCA was performed with default settings (maximum number of components = –1 and 0.95 of the total variance covered). In the biomarker analysis, we calculated a p value for each peak by using the randomization test of independence.

Construction and Validation of a MALDI Biotyper Database for Capsule Typing

We used selected isolates from the evaluation set to create main spectra (MSPs) for a new MALDI Biotyper 4.1 database (Bruker Daltonics) (Figure 1). These reference isolates were selected to represent all capsule types and genetic lineages. NTHi strains were selected with the aim of including isolates of all known genetic clades (*19*). Spectra of reference isolates were controlled by using FlexAnalysis (Bruker Daltonics). We performed smoothing (Savitzky-Golay) and baseline correction (Top-Hat) and excluded spectra with outlier appearance (lacking or having an extra peak) and low quality (peaks outside a 500 ppm range). If <20 spectra remained after the control, new spectra for that specific isolate were obtained. We used default settings for spectra preprocessing, MSP creation, and identification as described (*29*).

We used isolates in the evaluation set not selected as reference isolates for initial validation of the database (Figure 1). Because all isolates were *H. influenzae*, high score values (≥2.0) were expected. Thus, we classified each spectrum according to the top matching MSP in the new database. For isolate classification, ≥5/6 spectra classified to the same type (a–f or NTHi) were required. If ≤4/6 spectra were classified to the same type, the isolate was classified as inconclusive. To improve the specificity of the typing method, considering the known heterogeneity of NTHi, we supplemented the capsule typing database with NTHi isolates not correctly classified in the initial validation until all isolates in the evaluation set not included in the database were correctly classified on every single spectrum. Finally, we blindly validated the supplemented database by using MALDI-TOF mass spectrometry classification of invasive isolates (n = 126) obtained during 2010 and 2013–2016 and calculated sensitivity and specificity by using PCR typing as the standard (Figure 1).

Results

Genetic Lineages of Encapsulated *H. influenzae* in the Evaluation Set

To construct a clinically useful reference database for capsule typing by MALDI-TOF mass spectrometry, we aimed to identify and collect isolates from all known lineages of encapsulated *H. influenzae* (Figure 2; online Technical Appendix Table). We performed MLST for all Hia (n = 9) and Hib isolates (n = 24) in the evaluation set, in addition to a subset of isolates of other capsule types (c–f), capsule transformants (n = 4), and NTHi. Phylogenetic analysis confirmed that the collection contained isolates from different genetic lineages of encapsulated *H. influenzae*, including the 2 major genetic groups of type a and all 3 lineages of type b (Figure 2). Capsule transformants belonged

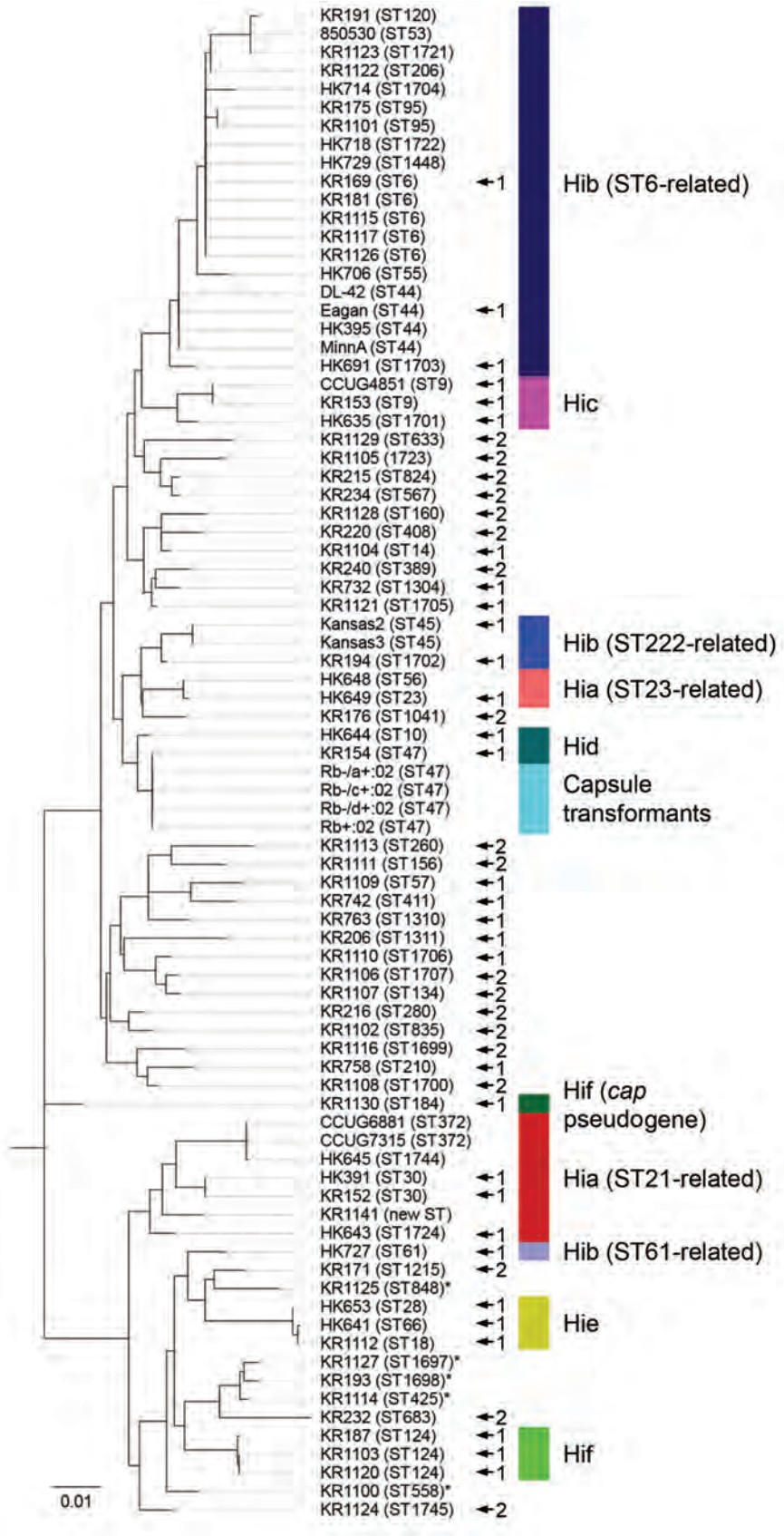

Figure 2. Multilocus sequencing typing (MLST) of encapsulated and nonencapsulated *Haemophilus influenzae* isolates. MLST was performed on a subset of encapsulated isolates (n = 44) from the evaluation set, including all type a and type b isolates (n = 33). All major genetic lineages (indicated by colors), except the least common lineage of Hia (ST4-related), of encapsulated *H. influenzae* were represented in the collection, including the 2 more common lineages of Hia and all 3 lineages of Hib. An isolate typed by PCR as Hif (KR1130) with a nonexpressed pseudogene *cap* locus was also included. This isolate was not part of the established Hif lineage, and was initially suspected to be an outlier on the basis of differences from other Hif in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra. All capsule transformants (n = 4) were ST47 (same as the parental strain Rd) (*30,31*) and were part of the Hid lineage. Nontypeable *H. influenzae* (NTHi; no color) included as reference isolates in the capsule typing databases (n = 28) were included in the analysis. NTHi in the evaluation set misclassified as type e (n = 5, indicated by asterisks) were also included. These isolates belonged to 3 separate genetic lineages, all related to the Hie lineage. Isolates included as references in MALDI-TOF mass spectrometry databases are indicated by arrows and numbers (1 for isolates in the original database and 2 for isolates added during supplementation of the database). Scale bar indicates nucleotide substitutions per site. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

to the known genetic lineage of Hid isolates (Figure 2) and were the same ST as the parental strain Rd (*18*). One isolate (KR1130) was typed by PCR as Hif (*bexB*- and *cap f*-positive) but phylogenetically belonged to a lineage separate from all other Hif isolates. Thus, this isolate was not part of the established, ST124-related Hif lineage (Figure 2). The *cap* locus of this isolate was sequenced and found to be a nonexpressed pseudogene (data not shown).

MALDI-TOF Mass Spectrometry of Genetic Lineages of Encapsulated *H. influenzae*

We performed PCA for all isolates in the evaluation set (n = 258) and the capsule transformants (n = 4). As expected, NTHi formed a large heterogeneous group, but clustering of encapsulated isolates of the same capsule types was found (Figure 3, panel A). When PCA was performed on encapsulated isolates (n = 83) and capsule transformants (n = 4) only, the clustering became clearer and was particularly evident for Hib, Hie, and Hif isolates (Figure 3, panel B). Encapsulated isolates segregated in groups according to capsule type and, for Hia and Hib isolates, by genetic lineage according to MLST (Figure 2; 3, panel B). Capsule transformants were found as a separate group in close proximity of Hid isolates and not distributed according to their respective capsule type (Figure 3, panel B). Isolate KR1130 did not cluster with Hif isolates of the ST124-related lineage (Figure 3, panel B).

Biomarker analysis of encapsulated isolates and capsule transformants identified several peaks conserved within the different genetic lineages of capsule types, indicating the possibility of separating them on the basis of MALDI-TOF mass spectra (Figure 4). Capsule transformants expressed similar peak patterns relative to each other but differed in many peaks when compared with wild-type strains of the same capsule types.

Sensitivity and Specificity of Automated Capsule Typing by MALDI-TOF Mass Spectrometry

An initial capsule typing reference database was constructed in MALDI Biotyper. Encapsulated isolates (n = 22) representing all major genetic lineages of encapsulated *H. influenzae* were included (Figure 2). To ensure adequate coverage of potential variation within each lineage, multiple reference isolates were chosen for each lineage (when possible) on the basis of geographic origin and variations in mass spectra. In addition, NTHi (n = 9) representing 8 of 10 known genetic clades of NTHi (*19*) were included in the database (Figure 2).

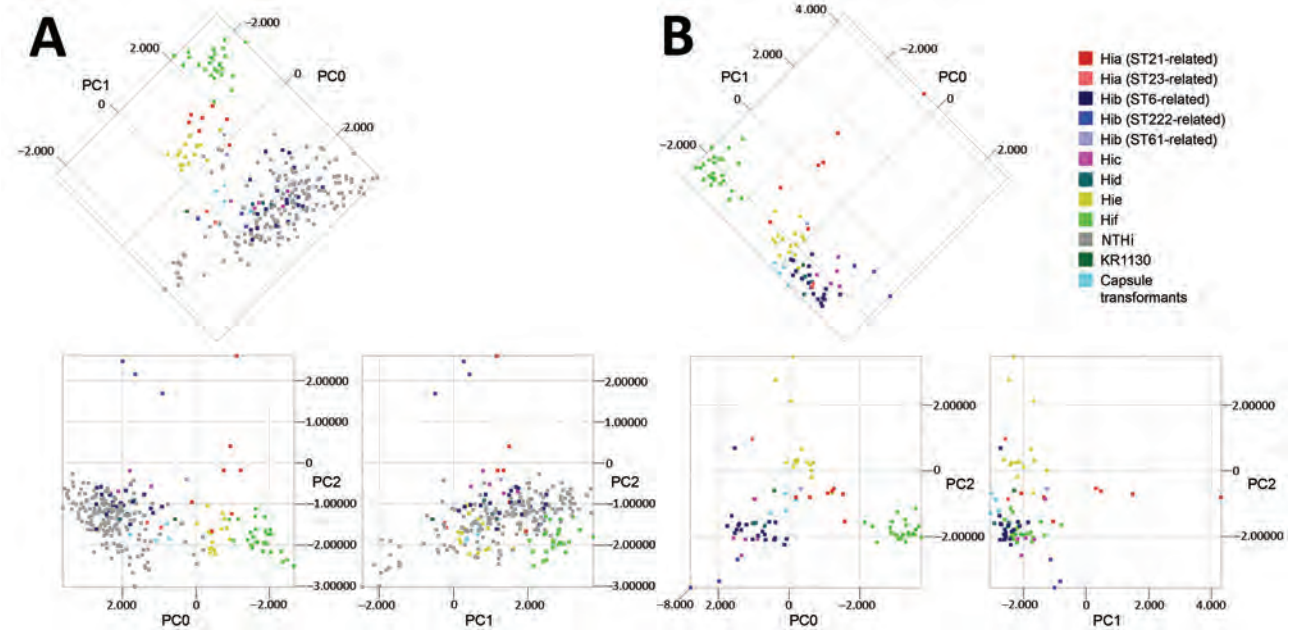

Figure 3. Principal component analysis (PCA) of matrix-assisted laser desorption/ionization time-of-flight mass spectra of encapsulated and nonencapsulated *Haemophilus influenzae*. A) PCA of all isolates (n = 258) of *H. influenzae* in the evaluation set representing all capsule types, which are color-coded according to capsule type and for Hia and Hib isolates by genetic lineage as shown by multilocus sequence typing (MLST) and capsule transformants (n = 4). The first 3 principal components (PC0, PC1, and PC2) are shown in 2-dimensional plots. Analysis showed the diversity of nontypeable *H. influenzae* (NTHi). Encapsulated isolates showed discrete clustering, which was further evaluated by PCA of encapsulated isolates separately. B) PCA of encapsulated isolates in the evaluation set (n = 83) and capsule transformants (n = 4) presented and color-coded as in panel A. Clustering of isolates on the basis of capsule type was evident, particularly for Hib, Hie, and Hif isolates. Different genetic lineages of the same capsule type (Hia and Hib) clustered separately. KR1130 (with a pseudogene type f *cap* locus) did not cluster with the other Hif isolates. Capsule transformants clustered together in proximity of Hid isolates, and not with their respective capsule type. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

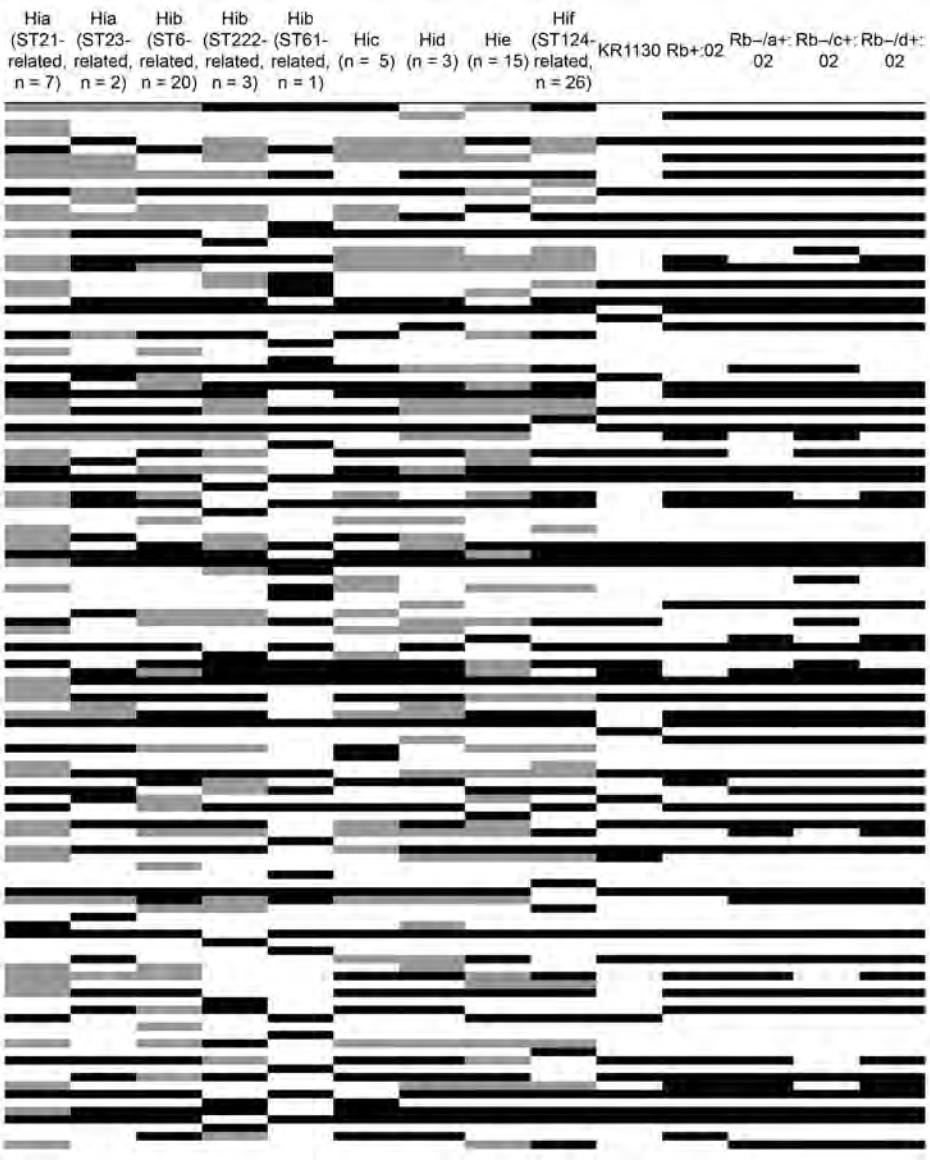

Figure 4. Biomarker analysis of matrix-assisted laser desorption/ionization time-of-flight mass spectra of encapsulated *Haemophilus influenzae*. Analysis was performed on all encapsulated isolates in the evaluation set (n = 83) and capsule transformants (n = 4). Rows represent peaks (2,000–20,000 m/z in descending order), and columns represent groups of encapsulated *H. influenzae*. A total of 124 peaks with discriminatory power ($p<0.05$) between different capsule types and genetic lineages were identified. Peak expression is indicated by shades of black (black >75%, gray ≥25% but ≤75%, and white <25% of isolates in the group express the peak). Several peaks conserved within capsule types and genetic lineages with the possibility for separation were observed, as indicated by the mosaic of peak patterns. Capsule transformants showed similar peak patterns, and lacked many of the capsule type-specific peaks for their respective phenotypic capsule types. KR1130 expressed different peaks than Hif of the ST124-related lineage. Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; ST, sequence type.

Validation of the original database (n = 31) using the remaining isolates in the evaluation set (n = 227) showed 100% sensitivity for encapsulated isolates (Table 1), and every isolate was correctly classified on every spectrum. All capsule transformants were classified as type d, the original serotype of the parental strain Rd (*31*). No isolate matched KR1130, the isolate typed by PCR as Hif with a pseudogene *cap* locus.

A few NTHi were either inconclusively typed or misclassified as encapsulated, resulting in reduced specificity for encapsulated isolates (Table 1). For this reason, we supplemented the capsule typing database with misclassified NTHi from the evaluation set until the database correctly classified all the remaining isolates in the evaluation set on every single spectrum. This modification resulted in an additional 19 NTHi being added to the MALDI Biotyper database (Figure 2). When PCA was performed separately for NTHi in the evaluation set, it was evident that the supplemented database covered the heterogeneity of NTHi better than the original database (Figure 5). The same finding was evident from phylogenetic analysis (Figure 2). Five NTHi were misclassified as Hie and could not be added to the database because they interfered with classification of true Hie isolates and would decrease sensitivity for Hie (Figure 2).

As a final performance test, we blindly validated the supplemented database (n = 50) by using a separate culture collection consisting of clinical invasive isolates from Sweden (n = 126) obtained during 2010 and 2013–2016. When we compared MALDI-TOF mass spectrometry capsule typing results with PCR capsule typing results, all encapsulated isolates (types b, e, and f; n = 28)

Table 1. Validation of the original MALDI-TOF mass spectrometry capsule typing database (n = 31) by classification of the remaining 227 isolates in the evaluation set and 4 capsule transformants of *Haemophilus influenzae**

Capsule type	No.	No. correct†	No. inconclusive‡	No. incorrect§	Sensitivity, %	Specificity, %
Hia, ST21-related	4	4	0	0	100	99.1
Hia, ST23-related	1	1	0	0	100	100
Hib, ST6-related	17	17	0	0	100	100
Hib, ST222-related	1	1	0	0	100	100
Hic	2	2	0	0	100	100
Hid	1	1	0	0	100	98.2
Hie	12	12	0	0	100	97.7
Hif	23	23¶	0	0	100	99.0
All encapsulated isolates, a–f	61	61	0	0	100	92.2
NTHi	166	122	31	13#	73.5	100
Rb-negative capsule transformants	4	1**	0	3**	NA	NA

*Hia, *H. influenzae* type a; Hib, *H. influenzae* type b; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NA, not applicable; NTHi, nontypeable *H. Influenzae*; ST, sequence type.
†≥5/6 spectra classified to the same correct type.
‡≤4/6 spectra classified to the same type.
§≥5/6 spectra classified to the same incorrect type.
¶All classified to the established ST124-related Hif lineage.
#Two isolates classified as Hia (ST21-related), 4 as Hid, 5 as Hie, and 2 as Hif (ST124-related).
**All classified as type d (i.e., the same type as the parental strain Rd) (*30,31*), resulting in correct classification of isolate Rb–/d+:02 and incorrect classification of isolates Rb+:02, Rb–/a+:02, and Rb–/c+:02.

were correctly classified on every single spectrum (Table 2). Of 98 NTHi, only 5 were not correctly classified. These isolates were all classified as inconclusive. Thus, no NTHi was incorrectly classified as encapsulated, and the resulting sensitivity and specificity of capsule typing was 100% in the final validation (Table 2).

Discussion

In this study, we have shown that encapsulated *H. influenzae* have different MALDI-TOF mass spectra that correlate with genetic lineages representing different capsule types. We have demonstrated that, after construction of a comprehensive reference database, routine MALDI-TOF mass

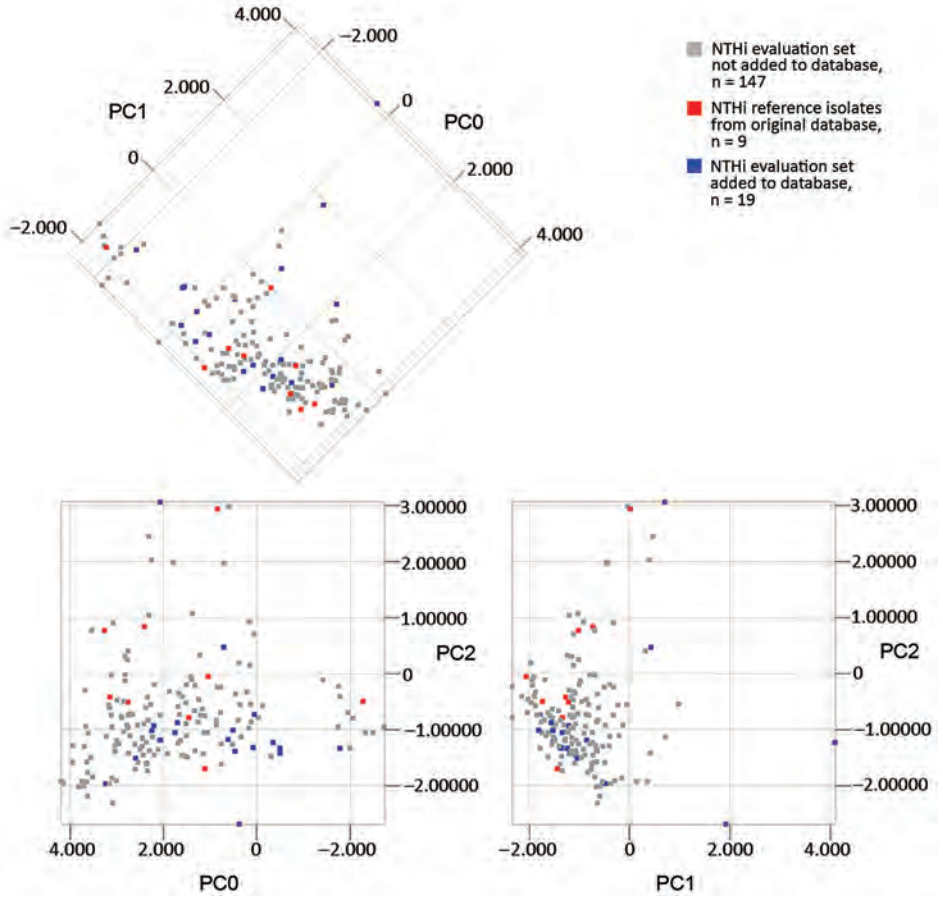

Figure 5. Principal component analysis of matrix-assisted laser desorption/ionization time-of-flight mass spectra of NTHi in the original and supplemented databases. Isolates are color-coded according to database affiliation, and the first 3 principal components (PC0, PC1, and PC2) are shown in 2-dimensional plots. No clustering similar to that for encapsulated isolates was observed. NTHi reference isolates in the original capsule typing database (n = 9), representing different genetic clades, were evenly distributed in the group. Supplementing the reference database with another 19 isolates improved coverage of the heterogeneity of NTHi. NTHi, nontypeable *H. influenzae*; PCA, principal component analysis.

Table 2. Validation of the supplemented MALDI-TOF mass spectrometry capsule typing database (n = 50) by classification of 126 invasive isolates of *Haemophilus influenzae* from Sweden*

Capsule type	No. tested	No. correct†	No inconclusive‡	No. incorrect§	Sensitivity, %	Specificity, %
Hib	8	8¶	0	0	100	100
Hie	5	5	0	0	100	100
Hif	15	15#	0	0	100	100
All encapsulated isolates (b, e, and f)	28	28	0	0	100	100
NTHi	98	93	5	0	94.9	100

*Hib, *H. influenzae* type b; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NTHi, nontypeable *H. influenzae*; ST, sequence type.
†≥5/6 spectra classified to the same correct type.
‡≤4/6 spectra classified to the same type.
§≥5/6 spectra classified to the same incorrect type.
¶All classified to the ST6-related Hib lineage.
#All classified to the established ST124-related Hif lineage.

spectrometry analysis has excellent capacity to identify type-specific genetic lineages associated with encapsulated *H. influenzae* and thereby can be used for capsule typing of *H. influenzae*.

Our study had several strengths. We analyzed a large collection of well-characterized strains collected at different times from various geographic regions to ensure the robustness of our findings. Using MLST, we ensured adequate coverage of the major genetic lineages of encapsulated *H. influenzae* in the MALDI-TOF mass spectrometry reference database. Moreover, the database was carefully evaluated and supplemented to ensure adequate coverage of the heterogeneity of NTHi. We blindly validated the supplemented database to mimic an authentic clinical or epidemiologic situation and demonstrated excellent sensitivity and specificity compared with conventional PCR-based typing. During construction of the capsule typing database, we identified several isolates previously typed by PCR or agglutination (by us or others) in which the MALDI-TOF mass spectrometry results did not match the suggested capsule type. When we retyped these isolates by PCR, the capsule type suggested by MALDI-TOF mass spectrometry proved to be correct in all instances (except for the NTHi typed as Hie) (Figure 2), and isolates were reassigned to a new capsule type, further supporting the capacity of MALDI-TOF mass spectrometry for capsule typing.

Our study had some limitations. The first limitation reflects the limited availability of some rare variants. Our collection contained no Hia isolates belonging to the uncommon ST4-related genetic group. For the ST61-related lineage of Hib, we had access to only 1 isolate, which was included in the reference database and thus not represented in the test collection. However, our ST61 isolate was separable when mass spectra were analyzed by PCA and biomarker analysis, and no isolate was misclassified to this lineage in the initial or final validation of the typing databases. Furthermore, we have demonstrated that identification of the ST222-related Hib lineage by MALDI-TOF mass spectrometry is possible (Table 1), which was not the case previously (*29*). The second potential limitation arises through the genetic heterogeneity of NTHi, making adequate representation in the reference database a challenge (*19*). This limitation was apparent during the initial evaluation of the typing method, when some NTHi were misclassified. To address this issue, we supplemented the database with 19 additional reference NTHi strains. The final validation of our typing method demonstrated excellent specificity for NTHi, but the sensitivity for identifying encapsulated isolates remained unchanged. Because most invasive infections in countries implementing Hib vaccination are caused by NTHi, a high specificity is desirable (*3*–*5*).

MALDI-TOF mass spectrometry has proved valuable in subtyping several clinically relevant bacteria, including *Clostridium difficile* (*36*), methicillin-resistant *Staphylococcus aureus* (*37*,*38*), and enterohemorrhagic *Escherichia coli* (*39*). Subtyping generally relies on common genetic differences between isolates, reflected in the composition of the proteins measured. In our study, wild-type isolates of different capsule types could be separated, but isogenic capsule transformants could not. These isolates were classified as type d, the original capsule type of the parental strain Rd. This finding confirms that capsule type identification is based on a proxy identification of genetic lineage, rather than identification of capsule biosynthesis-associated proteins. Thus, our method is an indirect typing method, as opposed to serotyping, which identifies the capsule polysaccharide, and PCR, which identifies the capsule gene complex directly.

Although there is little evidence that new lineages of encapsulated *H. influenzae* have appeared historically, novel lineages of encapsulated strains might appear and be missed by the method. Isolate KR1130 used in this study was initially suspected to represent such a lineage. However, its *cap* locus was shown to be on a nonexpressed pseudogene. Only 1 other isolate of the same ST (ST184) is currently registered in the MLST database, and it is a nontypeable isolate. No other Hif strain in this study or the MLST database belongs to this genetic lineage (*20*).

One advantage of indirect capsule type identification by MALDI-TOF mass spectrometry is that determination of genetic lineage of encapsulated isolates can be made without further analysis. The method can also identify

previously encapsulated capsule-deficient strains, which have lost parts or all of the *cap* locus, either during infection or laboratory handling (*40*–*42*).

A concern regarding subtyping by MALDI-TOF mass spectrometry (*43*) is the potential need for special sample preparations, such as growth conditions and type of matrix. In several studies, differences in mass spectra between subtypes of various species were observed but no automated classification methods were was reported (*43*), which might limit general applicability. In this study, we used standard growth conditions, as well as routine ethanol–formic acid extraction and mass spectra acquisition protocols. The software used (MALDI Biotyper) also has the advantage of being a standard software used in clinical settings. These factors greatly increased the chance of clinical implementation of our findings.

In conclusion, our study demonstrated that rapid capsule typing of *H. influenzae* by identification of capsule type-specific genetic lineages using routine MALDI-TOF mass spectrometry is possible and highly accurate. After further large-scale validation, this method has the potential for clinical and research use. With the increasing heterogeneity in capsule types of disease-causing *H. influenzae* observed since Hib conjugate vaccines were introduced, the method can become a valuable tool in clinical diagnostic laboratories.

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Emergence of *Streptococcus pneumoniae* Serotype 12F after Sequential Introduction of 7- and 13-Valent Vaccines, Israel

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Israel implemented use of 7- and 13-valent pneumococcal vaccine in 2009 and 2010, respectively. We describe results of prospective, population-based, nationwide active surveillance of Streptococcus pneumoniae serotype 12F (Sp12F) invasive pneumococcal disease (IPD) dynamics in the 7 years after vaccine introduction. Of 4,573 IPD episodes during July 2009–June 2016, a total of 434 (9.5%) were caused by Sp12F. Sp12F IPD rates (cases/100,000 population) increased in children <5 years of age, from 1.44 in 2009–2010 to >3.9 since 2011–2012, followed by an increase in all ages. During 2011–2016, Sp12F was the most prevalent IPD serotype. Sp12F isolates were mostly penicillin nonsusceptible (MIC >0.06 μg/mL; MIC_{50} = 0.12) and predominantly of sequence type 3774), a clone exclusively found in Israel (constituting ≈90% of isolates in 2000–2009). The sharp increase, long duration, and predominance of Sp12F IPD after vaccine implementation reflect a single clone expansion and may represent more than a transient outbreak.

Streptococcus pneumoniae is a leading cause of illness and death worldwide; the highest incidence occurs in children <2 years of age and in the elderly (*1*–*5*). For this reason, pneumococcal diseases are a target for global immunization programs in children and adults (*1*,*6*,*7*). Most reported cases of invasive pneumococcal disease (IPD) are sporadic, and outbreaks occur infrequently (*5*). Reported outbreaks have been of limited epidemiologic significance and associated with specific serotypes, including *Streptococcus pneumoniae* serotype 12F (Sp12F) (*5*,*8*,*9*).

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Since the introduction of pneumococcal conjugated vaccine (PCV), a substantial increase in nasopharyngeal carriage of nonvaccine serotypes (NVT) has been observed in surveillance studies (*10*–*14*) and randomized trials assessing the effect of PCV on NVT carriage, comparing vaccinated children and unvaccinated controls (*10*). This increase in NVT carriage, commonly referred to as the replacement phenomenon, was accompanied by an increase in rates of IPD caused by NVT, diminishing the overall impact of PCVs on IPD rates (*10*,*11*).

In Israel, the 7-valent PCV (PCV7) was introduced to the national immunization plan (NIP) in July 2009, with a catch-up plan for children <2 years of age, and was gradually replaced with the 13-valent PCV (PCV13) in November 2010, without further catch-up. Uptake rates were high (>90%) (*14*). PCV7/PCV13 sequential introduction was followed by a substantial decline in overall IPD incidence among all age groups, driven mainly by the near-elimination of vaccine serotype disease, but accompanied by a substantial increase in IPD caused by non-PCV13 serotypes (*15*,*16*).

Sp12F is a nonvaccine serotype that is uncommonly carried in healthy persons (*12*,*13*,*17*). Sp12F was relatively rare in Israel among IPD cases in the pre-PCV era but has increased noticeably following PCV introduction (*15*). This serotype has been associated with outbreaks of community-acquired pneumonia and IPD in the United States (*17*–*20*) and Canada (*8*,*21*). Globally, most reported Sp12F isolates were of sequence type (ST) lineages ST989 and ST218 (*17*,*18*,*22*,*23*). An outbreak clone in Canada has been reported to have acquired resistance to macrolides and fluoroquinolones, replacing susceptible clones (*21*), but in general Sp12F is penicillin susceptible.

We assessed the dynamics of Sp12F IPD rates after sequential introduction of PCV7/PCV13 in Israel. Additionally, we investigated the molecular and antimicrobial drug susceptibility characteristics of these serotype isolates before and after vaccine introduction.

Materials and Methods

Study Design

Our data derive from ongoing nationwide, prospective, population-based, active surveillance on IPD in children and adults in Israel (*15*,*16*). This report concentrates on data collected during the 7 years that followed PCV introduction in Israel (July 2009–June 2016). The study was conducted in all 27 medical health centers routinely obtaining cerebrospinal fluid (CSF) and blood cultures from children and adults: 26 hospitals and 1 major outpatient health maintenance organization. Less than 1% of blood cultures and no CSF cultures were obtained outside these centers. This setting enabled us to cover all culture-confirmed IPD cases among the population of Israel (*15*).

IPD isolates are sent regularly to the national reference center at the Central Laboratories of the Ministry of Health in Jerusalem for confirmation and serotyping (*15*,*16*). Since 2009, active surveillance on IPD in all ages has been conducted in all 27 laboratories performing blood cultures in Israel. The capture–recapture method ensured the reporting of >95% of cases. Using these data, we completed the isolates missing from the passive surveillance system, so that all *S. pneumoniae* isolates from blood, CSF, or both collected during the relevant period in Israel were included in this study. Eventually, *S. pneumoniae* strains from 4,573 IPD cases, isolated from blood or CSF, were included in the study. The population breakdown during the study period is given in Table 1. During this period, the Jewish population was ≈79% of the total population (*24*).

Case Definition

We defined an IPD episode by isolation of *S. pneumoniae* from blood or CSF. We excluded positive cultures from sterile sites other than blood or CSF (i.e., joint/pleural fluid or peritoneal fluid) (*15*,*16*), as well as diagnoses based solely on nonculture methods (PCR, antigen testing, Gram stain, or clinical diagnosis only).

Vaccine Uptake

The method of evaluating vaccine uptake initiated in July 2009 has been described elsewhere (*14*,*15*). In June 2009, the proportion of children 12–23 months of age who received ≥2 of any PCV doses was 20%; this proportion increased to 71% in June 2010 and has increased to ≈95% since 2011.

Bacteriology

We inoculated *S. pneumoniae* cultures onto tryptic soy agar plates supplemented with 5% defibrinated sheep blood (Hy-labs, Rehovot, Israel) and incubated them at 37°C for 24 hours in a 5% enriched CO_2 atmosphere. Identification of *S. pneumoniae* was done as previously described (*15*).

Serotypes

All strains were serotyped by the Quellung test; since 2013, the serotype has been determined by a validated combination of the capsular sequence typing molecular typing protocol and serotyping using the antisera of Statens Serum Institute (Copenhagen, Denmark). Serotype 6A was differentiated from serotype 6C by PCR (*10*).

Antimicrobial Drug Susceptibility Testing

We determined susceptibility to penicillin for Sp12F strains by the oxacillin disk diffusion screening method of Bauer and Kirby and Etest (BioMérieux, Marcy l'Étoile, France) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (*25*). Because Sp12F often causes meningitis, we set the susceptibility cutoff values according to the CLSI cutoff for meningitis. Thus, we defined isolates with penicillin MICs of ≤0.06 μg/mL as susceptible to penicillin and considered those with MICs >0.06 μg/mL to be nonsusceptible.

Pulsed-Field Gel Electrophoresis

We determined the genetic relatedness for all IPD strains isolated since 2009 and all Sp12F strains isolated during 2000–2008 available in our strains bank. We prepared and analyzed chromosomal DNA fragments generated by *Sma*I digestion as described previously (*11*,*26*,*27*), with modifications.

We performed genotype analysis and clustering using the Bionumerics version 7.6 software package (Applied-Maths, Sint-Martens-Latem, Belgium) with dice

Table 1. Age distribution of case-patients with Sp12F infection, Israel, July 2009–June 2016*

	Age <5 y		Age 5–17 y		Age 17–64 y		Age ≥65 y	
Year	No. cases	Total population	No. cases	Total population	No. cases	Total population	No. cases	Total population
Jul 2009–Jun 2010	11	763,700	1	1,706,200	3	4,341,250	5	743,250
Jul 2010–Jun 2011	17	784,000	2	1,737,250	13	4,405,100	9	768,100
Jul 2011–Jun 2012	38	804,750	1	1,768,700	16	4,465,500	14	799,000
Jul 2012–Jun 2013	36	824,300	2	1,802,100	17	4,526,750	13	831,750
Jul 2013–Jun 2014	48	841,400	2	1,841,100	15	4,589,000	12	866,050
Jul 2014–Jun 2015	34	864,000	4	1,904,700	32	4,692,000	18	919,600
Jul 2015–Jun 2016	42	885,600	2	1,947,100	13	4,765,500	14	959,650
Total no. cases	226		14		109		85	

*Sp12F, *Streptococcus pneumoniae* serotype 12F.

coefficients, a 1% position tolerance, and optimization values. We performed cluster analysis by the unweighted pair-group mean analysis. All isolates in the same cluster, defined as clonal, were assigned a letter, A to T, for analysis purposes.

Multilocus Sequence Typing

We characterized selected isolates representing pulsed-field gel electrolysis (PFGE) cluster and isolate years by multilocus sequence typing (MLST). The 7 housekeeping loci (*aro*E, *ddl*, *gdh*, *gki*, *rec*P, *spi*, and *xpt*) were amplified according to the *S. pneumoniae* PubMLST (*28*). We crudely extracted bacterial cells by boiling and performed PCR and sequencing by using a revised protocol provided by Bruno Pichon (Antimicrobial Resistance and Healthcare Associated Infection Unit, Public Health England, pers. comm., 2016 Sept 16).

We designed new primers for allele amplification and sequencing. These primers do not contain degenerative bases; an M13 tag on each 5′ primer sequence was added to ease sequencing setup (Table 2). Sequencing of the forward and reverse amplicons was performed at the Center for Genomic Technologies, Institute of Life Sciences, The Hebrew University of Jerusalem (Jerusalem, Israel), using BigDye Terminator v1.1 chemistry (Applied Biosystems, Foster City, CA, USA).

Genomic Data Analysis

We performed genomic data analysis for capsular sequence typing, PFGE, and MLST using Bionumerics version 7.6 software (Applied Maths). We retrieved MLST allelic profiles and STs from the *S. pneumoniae* MLST database (*29*) and submitted the new alleles and STs to the database curator for ST assignment. We compared MLST data with all Sp12F strains submitted to PubMLST.

Statistical Analysis

We performed statistical analysis using SPSS version 14.0 software for Windows (IBM SPSS, Chicago, IL, USA). A p value <0.05 was considered statistically significant.

The data from the active surveillance during 2009–2016 are presented according to epidemiologic years, July through June. Each isolate was counted only once per episode; episodes were separated by ≥30 days for the same serotype or by any interval for different serotypes. Rate reductions and ratios were calculated. The following age groups were defined: <5 years, 5–17 years, 18–64 years, and ≥65 years of age.

Results

During the study, we identified 4,573 IPD episodes (93% bacteremia and 7% meningitis). Of those, 434 (9.5%) were caused by serotype 12F (Figure 1).

Study Population Characteristics

Patients <5 years of age accounted for 27% of the overall study population; those 5–17 years of age, 8%; those 18–64 years of age, 31%; and those ≥65 years of age, 34%. Overall, 56.3% of all episodes occurred in male patients, and 86.4% of all episodes occurred among the Jewish population.

Overall IPD

We compared the first (2009–2010) and the last (2015–2016) study years and found that overall IPD rates (cases/100,000 population) declined by 34% (incidence rate ratio [IRR] 0.66, 95% CI 0.59–0.74), from 10.2 to 6.7 (Figure 1). IPD rates substantially declined in all age groups but most notably in children <5 years of age (48% rate reduction, from 30.9 to 16.1 cases/100,000 population). The results for the first study year (2009–2010)

Table 2. Multilocus sequence typing PCR amplification and sequencing primers for study of *Streptococcus pneumoniae* serotypes, Israel, July 2009–June 2016

Primers	Sequence, 5′ → 3′
PCR	
aroE/M13F	TGTAAAACGACGGCCAGTcgtttagctgcagttgttgc
aroE/M13R	CAGGAAACAGCTATGACCcccacactggtggcattaac
ddl/M13F	TGTAAAACGACGGCCAGTttgccatggataaaacacgac
ddl/M13R	CAGGAAACAGCTATGACCcgcgcttgtcaaaactttcc
gdh/M13F	TGTAAAACGACGGCCAGTgtgctgaaaagattaaggtct
gdh/M13R	CAGGAAACAGCTATGACCtgcttccagctttatagtcatg
gki/M13F	TGTAAAACGACGGCCAGTggcattggaatgggatcacc
gki/M13R	CAGGAAACAGCTATGACCtctcccgcagctgacac
recP/M13F	TGTAAAACGACGGCCAGTgccaactcaggtcatccagg
recP/M13R	CAGGAAACAGCTATGACCttcgatagcagcatggatgg
spi/M13F	TGTAAAACGACGGCCAGTcgcttagaaaggtaagttatg
spi/M13R	CAGGAAACAGCTATGACCaggctgagattggtgattctc
xpt/M13F	TGTAAAACGACGGCCAGTggaggtcttatgaaattattag
xpt/M13R	CAGGAAACAGCTATGACCagatctgcctccttaaatac
Sequencing	
M13 F	TGTAAAACGACGGCCAGT
M13 R	CAGGAAACAGCTATGACC

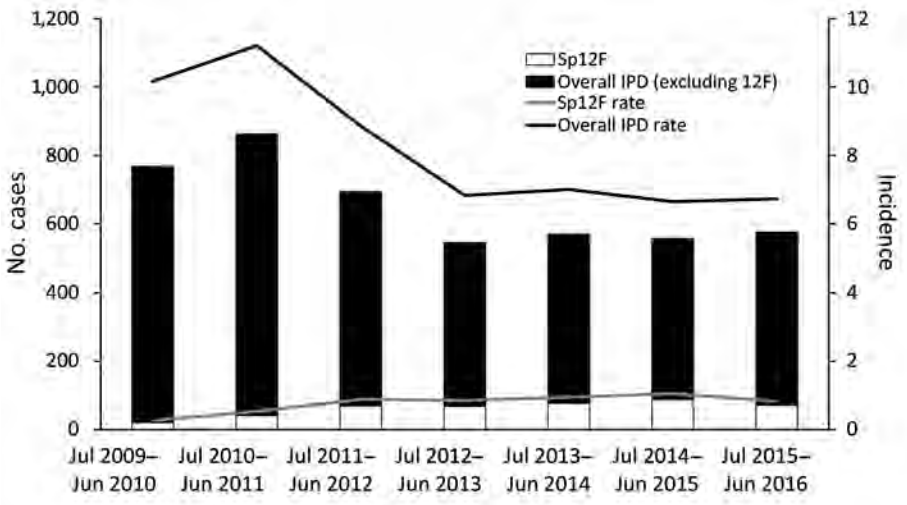

Figure 1. Overall invasive pneumococcal disease (IPD) and *Streptococcus pneumoniae* serotype 12F (Sp12F) cases and incidence (cases/100,000 population), Israel, July 2009–June 2016.

already showed a substantial reduction compared with the pre-PCV period (*15*,*16*) (Figure 1).

IPD Caused by PCV13 Serotypes

Rates of IPD caused by PCV13 serotypes and proportions of all IPD episodes declined substantially throughout the study period. Rates (cases/100,000 population) of IPD caused by PCV7 serotypes declined by 90%, from 2.6 to 0.5, from the first to the last study years. Similarly, rates of IPD caused by PCV13 serotypes declined by 80%, from 7.4 to 1.5, during the study period.

Proportions of all IPD episodes caused by PCV7 serotypes substantially declined by 74%, from 24.8% in 2009–2010 to 6.4% in 2015–2016. Proportions of all IPD episodes caused by PCV13 serotypes decreased by 68%, from 69.4% to 22.4%.

IPD Caused by Non-PCV13 Serotypes

IPD caused by non-PCV13 serotypes increased by 93%. Rates (cases/100,000 population) increased from 2.8 in 2009–2010 to 5.4 in 2015–2016.

Sp12F

We observed a steady increase (except for the last study year) in the proportion of Sp12F out of all IPD episodes throughout the study: 2.7% in 2009–2010, 4.9% in 2010–2011, 10.1% in 2011–2012, 12.6% in 2012–2013, 13.7% in 2013–2014, 16.1% in 2014–2015, and 12.7% in 2015–2016 (Figure 2; Table 1). The incidence of IPD (in all ages) caused by Sp12F increased over the same period except for 1 slight decrease: 0.26 in 2009–2010, 0.53 in 2010–2011, 0.88 in 2011–2012, 0.85 in 2012–2013, 0.95 in 2013–2014, 1.05 in 2014–2015, and 0.83 in 2015–2016. Similar trends were observed in all age groups, with the sharpest increase observed in children <5 years of age, for which the respective figures by year were 1.44, 2.17, 4.72, 4.37, 5.70, 3.94, and 4.74 (Figure 3). Of note, 91.7% of all Sp12F episodes occurred in the Jewish population.

Analysis of Sp12F Isolates

To assess the association between PCV7/PCV13 introduction and the dynamics of Sp12F rates, we analyzed strains isolated during 2000–2015. Overall, we analyzed 445 Sp12F strains by PFGE (Figure 4). The isolate population consists of a predominant pulsotype (A; 90.1% of isolates), and several other diverse pulsotypes (Figure 5). The predominant pulsotype was observed throughout the study period and was consistently prevalent among the Sp12F population; thus, the observed more general serotype Sp12F increase following PCV7/PCV13 introduction can be attributed to an expansion of this single clone during the study period.

MLST of Sp12F

We further analyzed representative Sp12F isolates by MLST. Among 25 isolates with the predominant PFGE pulsotype, 24 were typed as ST3774 and 1 as the closely related ST3524. Four isolates from other PFGE pulsotypes were typed as ST989 and 1 isolate from another PFGE pulsotype was typed as ST3377.

We compared the MLST type with isolates reported as Sp12F in the global PubMLST database (*29*). This database contains 283 isolates serotyped as Sp12F and 105 sequence types. Among these isolates, the most common STs are ST218 and ST989, as previously published (*17*,*18*,*22*,*23*) (Figure 6). ST3774 is rare among isolates submitted to this global database, with only 1 isolate reported from Israel in 2006.

Antimicrobial Drug Resistance of Sp12F

Among all Sp12F isolates, 89% were penicillin nonsusceptible (MIC >0.06); nonsusceptibility rates varied by

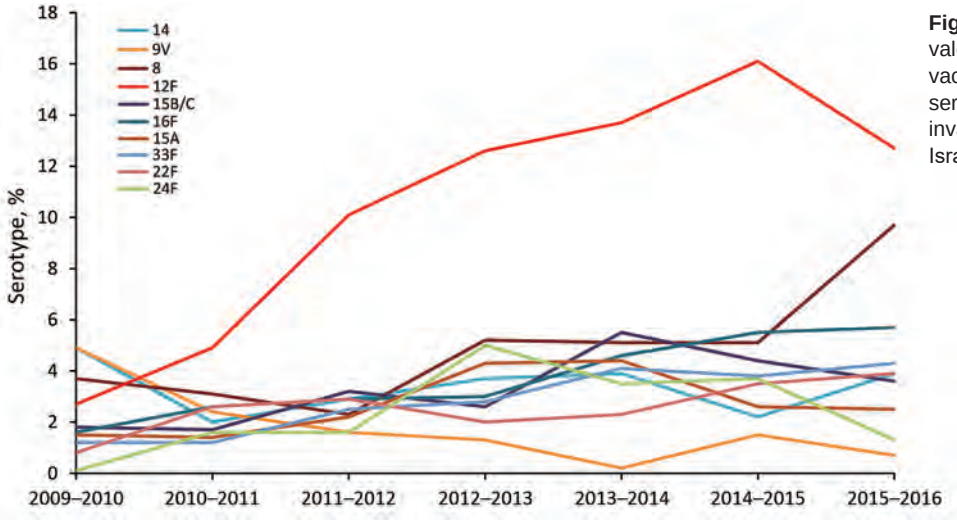

Figure 2. Predominant non–13-valent pneumococcal conjugate vaccine *Streptococcus pneumoniae* serotypes as proportions of overall invasive pneumococcal disease, Israel, July 2009–June 2016.

year from 70% to 99% (MIC_{50} 0.12 μg/mL, range 0.02–1.2 μg/mL). However, most strains were fully susceptible to ceftriaxone (99%; MIC_{50} 0.06 μg/mL, range 0.02–1.0 μg/mL), clindamycin (95%), rifampin (100%), and vancomycin (100%).

Discussion

S. pneumoniae serotype 12F, a non-PCV13 serotype, is currently the most prevalent pneumococcal serotype causing IPD in Israel. This previously rare serotype caused 434 IPD cases during July 2009–June 2016 and became prevalent in all age groups, most distinctly in young children. The population of Sp12F during 2000–2016 was >90% of the same clonal complex according to PFGE and MLST analyses, indicating the expansion of the ST3774 sequence type, which is currently unique to Israel. The predominant global Sp12F outbreak clones ST218 and ST989 (*21*) are very rare in IPD cases in Israel.

Sp12F was previously reported to have high invasive disease potential and is sometimes called hyperinvasive (*8*,*30*–*32*). It was reported as the most common serotype causing IPD in Japan (*31*,*32*), and it has been the cause of an IPD outbreak in Canada (*8*). Furthermore, several sites reported high disease potential for meningitis with Sp12F (*30*,*33*), and several Sp12F outbreaks have been reported in the literature among closed or crowded populations (*18*,*34*), including daycare centers (*35*), military groups (*34*), a jail (*20*), and a homeless shelter (*36*).

In a recent study from France that evaluated the invasive potential of specific serotypes and compared

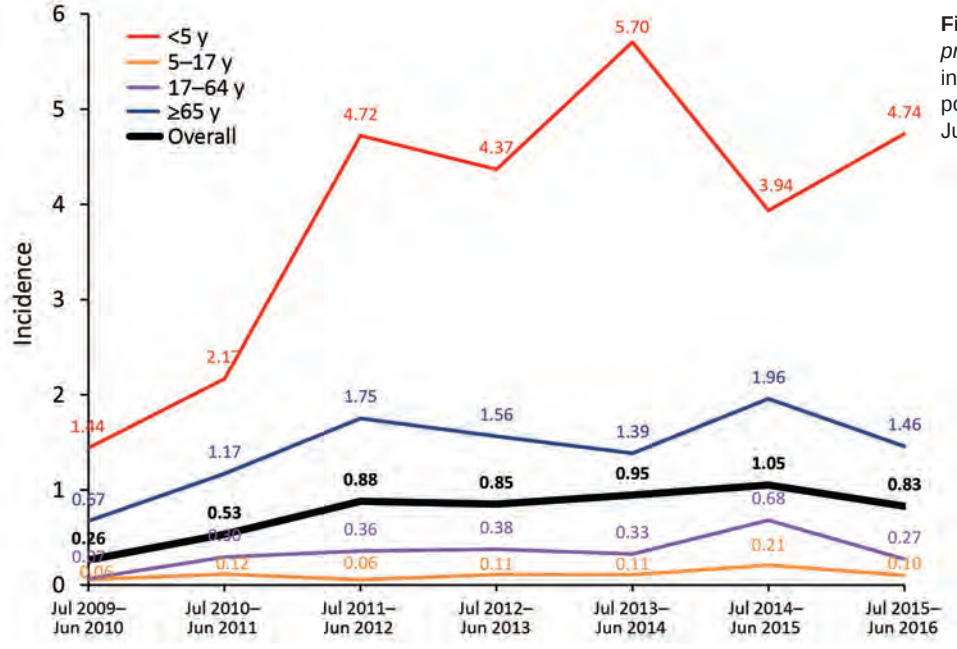

Figure 3. *Streptococcus pneumoniae* serotype 12F incidence (cases/100,000 population) by age group, Israel, July 2009–June 2016.

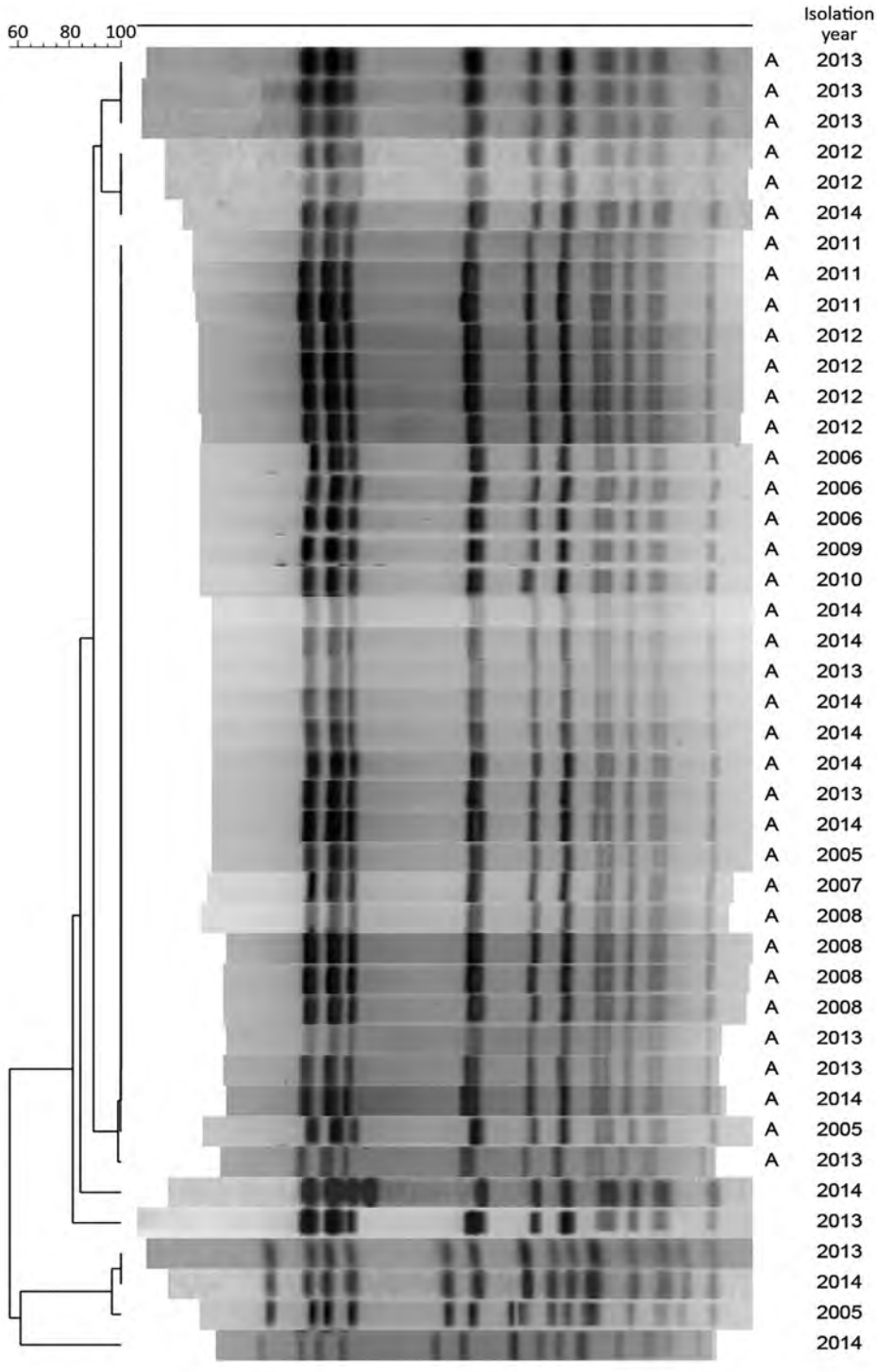

Figure 4. Pulsed-field gel electrophoresis analysis of a sample of *Streptococcus pneumoniae* serotype 12F isolates from Israel, 2000–2015. A indicates predominant pulsotypes.

nasopharyngeal carriage in healthy children to carriage in children infected with IPD, Sp12F was shown to be a major cause of IPD but was not found in nasopharyngeal cultures (*30*). Furthermore, in Israel, Sp12F was rarely found in carriage studies before and after vaccination trials (*14*,*37*,*38*). These findings emphasize both the invasive nature of Sp12F and its failure to become a successful colonizer.

The emergence of IPD caused by Sp12F in Israel, mainly by clonal expansion, may be attributed, at least in part, to the observed more general serotype replacement after PCV implementation. The emergence of this serotype in the youngest age groups after PCV7/PCV13 introduction supports this hypothesis. However, the previously reported low carriage rate of Sp12F may counter this possibility, and

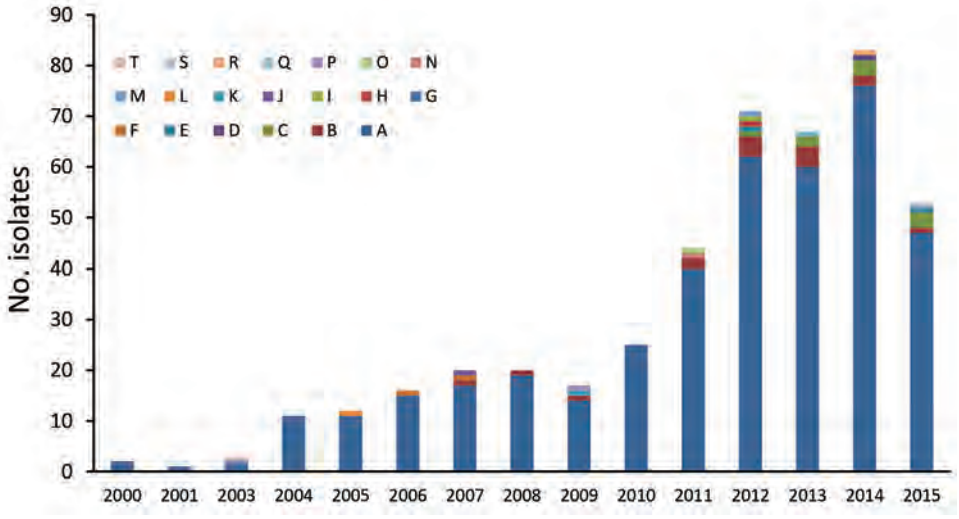

Figure 5. Pulsotype distribution of 445 *Streptococcus pneumoniae* serotype 12F isolates from Israel, by year, 2000–2015.

current data on Sp12F carriage in Israel are needed to support this theory. Alternatively, a large-scale clonal outbreak, of long duration and high invasiveness rather than true replacement, may be at the origin of this serotype emergence.

Our results emphasize the role of Sp12F as a serious current public health challenge. Continued monitoring of IPD and specific serotype distribution are needed to enable the development of future vaccination strategies. The

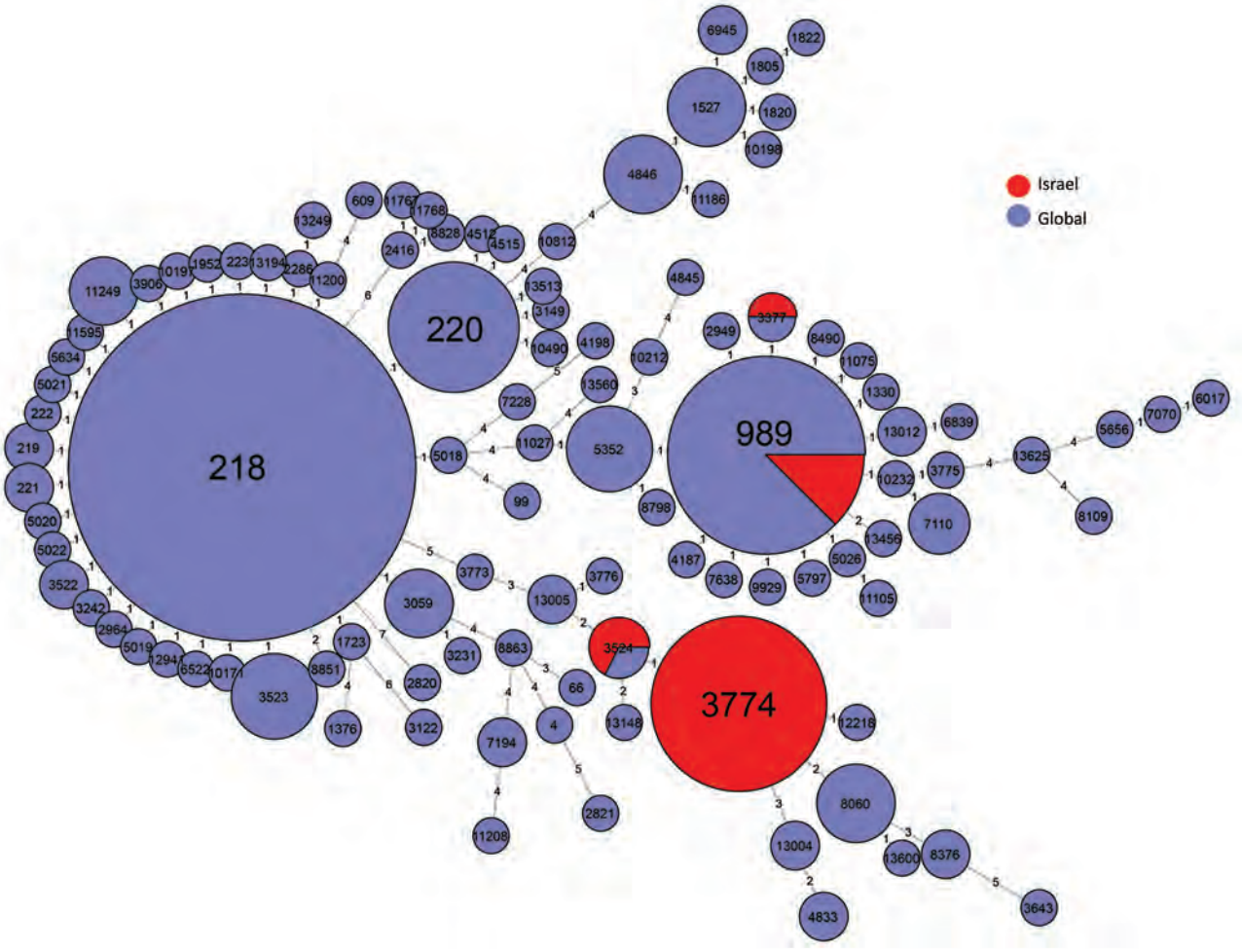

Figure 6. Multilocus sequence typing comparison of *Streptococcus pneumoniae* serotype 12F serotype isolates from Israel and globally.

evolutionary origin of this emerging clone will be further studied by whole genome sequencing, which may offer insights into the virulence of Sp12F strains.

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Major Threat to Malaria Control Programs by *Plasmodium falciparum* Lacking Histidine-Rich Protein 2, Eritrea

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False-negative results for *Plasmodium falciparum* histidine-rich protein (HRP) 2–based rapid diagnostic tests (RDTs) are increasing in Eritrea. We investigated HRP gene 2/3 (*pfhrp2*/*pfhrp3*) status in 50 infected patients at 2 hospitals. We showed that 80.8% (21/26) of patients at Ghindae Hospital and 41.7% (10/24) at Massawa Hospital were infected with *pfhrp2*-negative parasites and 92.3% (24/26) of patients at Ghindae Hospital and 70.8% (17/24) at Massawa Hospital were infected with *pfhrp3*-negative parasites. Parasite densities between *pfhrp2*-positive and *pfhrp2*-negative patients were comparable. All *pfhrp2*-negative samples had no detectable HRP2/3 antigen and showed negative results for HRP2-based RDTs. *pfhrp2*-negative parasites were genetically less diverse and formed 2 clusters with no close relationships to parasites from Peru. These parasites probably emerged independently by selection in Eritrea. High prevalence of *pfhrp2*-negative parasites caused a high rate of false-negative results for RDTs. Determining prevalence of *pfhrp2*-negative parasites is urgently needed in neighboring countries to assist case management policies.

Eritrea, which is located in the Horn of Africa, has reduced malaria mortality and incidence rates extensively over the past decade (*1*,*2*). This reduction is attributed largely to integrated vector management, early diagnosis, and effective treatment implemented by the national malaria control program (NMCP). However, malaria remains a public health concern because there were ≈65,000 cases and 5 million persons living in malaria-prone areas in 2015 (*3*). Malaria is unstable in Eritrea, and seasonal and transmission patterns vary across 3 ecologic zones. *Plasmodium falciparum* infection accounts for ≈70% of confirmed malaria cases and *P. vivax* for the remaining ≈30% (*4*).

Microscopy remains the mainstay of malaria diagnosis at hospitals. Rapid diagnostic tests (RDTs) were introduced at the community level and primary health facilities in 2006, which paved the way for implementation of artemisinin-based combinations as first-line treatment in 2007. RDTs that detect *P. falciparum* and *P. vivax* simultaneously by targeting histidine-rich protein 2 (HRP2) and *P. vivax* plasmodium lactate dehydrogenase (pLDH), and met the World Health Organization (WHO)–recommended procurement criteria (*5*) were implemented in Eritrea with a quality assurance program that included training operators regularly and testing RDT lots before distribution.

In 2014, the NMCP received reports of false-negative RDT results in microscopically confirmed cases of *P. falciparum* and *P. vivax* malaria at several health facilities. The RDTs involved were from 10 lots that had passed testing at a WHO–Foundation for Innovative New Diagnostics lot-testing laboratory. To address this issue, the Ministry of Health (MOH) conducted exploratory investigations at 12 health facilities located in 4 regions of Eritrea and used different brands of RDTs. Results showed an overall false-negative result rate of 80% (41/50) for microscopically confirmed cases of *P. falciparum* malaria (*6*).

Deficiencies in RDT storage and operational issues were ruled out as causes of the false-negative results. Furthermore, samples of deployed RDTs were retrieved from the field and retested at a WHO–Foundation for Innovative New Diagnostics lot-testing laboratory against well-characterized reference samples. All RDTs passed testing,

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further suggesting that RDT quality issues did not cause false-negative results for *P. falciparum* (*6*). The cause of false-negative results for *P. vivax* was believed to be low parasite density. On the basis of these findings, the MOH recalled the RDTs and investigated *P. falciparum*–specific parasite factors, such as presence of *P. falciparum* lacking HRP2, as a primary cause.

HRP2-based RDTs target parasite HRP2, and some cross-react with HRP3 because of sequence similarities between the 2 proteins. *P. falciparum* lacking HRP2 and HRP3 caused by deletions of genes encoding these antigens (*pfhrp2* and *pfhrp3*) in clinical cases were first reported in the Amazon region of Peru (*7*) and subsequently in Colombia (*8*), Suriname (*9*), and Brazil and Bolivia (*10*) (prevalence 4%–41%). The high prevalence of *pfhrp2*-negative parasites in Peru correlated with poor performance of HRP2-based RDTs (*11*) and led WHO to recommend using non–HRP2-based RDTs for case management in affected areas.

Outside South America, sporadic and low prevalence of *pfhrp2*-negative parasites has been reported in India (*12*); along the China–Myanmar border (*13*); and in countries in Africa, including Mali (*14*), Senegal (*15*), and the Democratic Republic of the Congo (*16*). A high prevalence (22%–41%) of *pfhrp2*-negative parasites was reported in 2 areas of Ghana (*17*). However, *pfhrp2*-negative parasites have not been detected in East Africa.

Unlike South America and Asia, where *P. vivax* infections dominate, most countries in Africa have predominantly *P. falciparum* infections. Thus, many of these countries have adopted HRP2-based RDTs only for *P. falciparum*. These RDTs do not detect *pfhrp2*-negative parasites, which results in *P. falciparum* infections not being detected and treated. Use of only HRP2-based RDTs in areas where *pfhrp2*-negative parasites are present will lead to increases in disease burden and transmission (*18*). Therefore, emergence of *pfhrp2*-negative parasites poses a serious threat to malaria control programs in Africa where malaria disease burden is high and RDT is the preferred diagnostic test. We investigated *P. falciparum* lacking *pfhrp2* and *pfhrp3* in Eritrea to determine whether *pfhrp2/pfhrp3* deletions were a cause of false-negative results for HRP2-based RDTs and to describe origins of parasites.

Methods

Study Site

Eritrea is divided into 6 administrative regions. This study was conducted at Ghindae Hospital and Massawa Hospital in the Northern Red Sea Region (Figure 1) in March 2016. These locations were selected because of the timing of the malaria transmission season, easy access to infrastructure, and a high incidence of false-negative RDT results reported in this region (*6*).

Patient Enrollment and Sample Collection

Consecutive nonpregnant persons >5 years of age who came to the 2 hospitals and confirmed as being infected with *P. falciparum* by microscopy and a pan-pLDH single test line RDT (Carestart Malaria pLDH(PAN) G0111; Access

Figure 1. Location of study sites at Ghindae and Massawa Hospitals, Eritrea, for analysis of a major threat to malaria control programs by *Plasmodium falciparum* lacking histidine-rich protein 2. Inset shows the location of the study sites in Eritrea.

Bio, Inc., Somerset, NJ, USA) were invited to participate. Venous blood (50 µL) was obtained from each consenting patient, spotted onto Whatman (Brentford, UK) 3M filter paper, dried, stored in a zip-lock bag containing desiccant, and used to prepare thin and thick blood smears. Smears and dried blood spots were shipped to different laboratories for further analyses.

Microscopy and HRP2-Based RDT

Blood smears were stained and examined at both hospitals and the Eritrean National Reference Laboratory (NRL; Asmara, Eritrea) according to the WHO microscopy manual (*19*). Final parasite species and density counts were determined by NRL microscopists who participated in the WHO/National Institute for Communicable Diseases malaria microscopy proficiency testing program. Blood samples were tested by using a combination HRP2/*P. falciparum* pLDH/*P. vivax* pLDH RDT (Bioline Malaria Ag Pf/Pf/Pv 05FK120; Standard Diagnostics, Gyeonhhi-do, South Korea) according to the manufacturer's instructions.

DNA Extraction and *Plasmodium* Speciation

Each dried blood spot was processed into 3 discs from which genomic DNA was extracted by using QIAamp DNA Mini Kits and a QIAcube Robot (QIAGEN, Crawley, UK) according to the manufacturer's instructions. DNA was eluted into a volume of 100 µL, and 10 µL was used in each PCR. An 18S rRNA gene-based multiplex PCR (*20*) was used to determine whether 4 human *Plasmodium* spp. were present in each sample.

Characterization of *pfhrp2* and *pfhrp3* Gene Deletions and Flanking Genes

Presence or absence of *pfhrp2* (Pf3D7_0831800) and *pfhrp3* (Pf3D7_1372200) genes was characterized by amplifying across exon1–exon2 and exon2 (*7*). Outcomes were classified as *pfhrp2*-positive or *pfhrp3*-positive (PCR result positive for exon1 and exon2 of the *pfhrp2* or *pfhrp3* gene) or *pfhrp2*-negative or *pfhrp3*-negative (PCR result negative for exon1 and exon2 of the *pfhrp2* or *pfhrp3* gene, but PCR positive result for 3 single-copy *P. falciparum*–specific genes [*P. falciparum* merozoite surface protein 1, *P. falciparum* merozoite surface protein 2, and *P. falciparum* glutmate-rich protein]) (*21*). Presence or absence of genes flanking *pfhrp2* (Pf3D7_0831900/MAL7P1_230 and Pf3D7_0831700/MAL7P1_228) and *pfhrp3* (Pf3D7_1372400/MAL12P1_485 and Pf3D7_1372100/MAL13P1_475) were characterized by PCR amplification of a fragment in each gene (*7*).

HRP2 Levels

HRP2 levels were measured for each sample by using Luminex mulitplex bead-based immunoassay described elsewhere (*22*). Dried blood spots were incubated overnight in elution buffer (0.05% phosphate-buffered saline, Tween 20, 0.05% NaN_3) to give a 1:20 dilution of whole blood. A 50-µL elution was used in each assay. The threshold mean fluorescence intensity minus background (MFI – bg) signal that indicated true negativity for HRP2 was derived by testing 86 blood samples from a setting to which malaria was not endemic. The MFI – bg positivity cutoff value was the lognormal mean ± 3 SD for this malaria-negative population.

Microsatellite Analysis

We amplified 7 neutral microsatellite markers (TA1, PolyA, PfPK2, TA109, 2490, 313, and 383) from each sample (*23*) and assayed for size by using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We scored alleles manually by using Peak Scanner Software version 1.0 (Applied Biosystems), using a height of 300 relative fluorescence units as the minimal peak threshold.

Genetic Diversity and Population Genetic Analyses

We determined haplotypes for each parasite isolate from 7 microsatellite markers and used for genetic diversity and genetic relatedness analysis among parasites from Eritrea and between parasites from Eritrea and Peru (*23*) by using PHYLOViZ software (*24*). We calibrated sizes of 7 microsatellite markers against those of *P. falciparum* strain 3D7 before conducting genetic relatedness analysis, using a cutoff value of 2 (minimum differences for 2 loci) to compare parasites. We calculated expected heterozygosity (H_E) values for each of the 7 microsatellite markers by using FSTAT software (https://www2.unil.ch/popgen/softwares/fstat.htm) and derived the mean H_E for *pfhrp2*-positive and *pfhrp2*-negative parasite populations in Eritrea.

Statistical Analysis

We conducted graphics and statistical analysis by using GraphPad Prism 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). Parasite densities were log-transformed for analysis and the geometric mean is reported. We used a Mann-Whitney test to compare log parasite densities between hospitals and between *pfhrp2*-positive and *pfhrp2*-negative parasites.

Ethics Considerations

The study was approved by the Eritrean MOH Research and Ethical Committees. The Northern Red Sea branch of the MOH and authorities of the 2 hospitals were informed, through an official letter, on the scope, coverage, and objectives of the study.

Patients were enrolled after providing consent following a detailed explanation about the investigation. Data from patients were recorded on structured forms.

All patient specimens were given a unique identification number after collection, and only this number was used for data linkage.

Laboratory analyses of *pfhrp2/pfhrp3* status of parasites and their genetic diversity and relatedness was conducted at the Australian Defence Force Malaria and Infectious Disease Institute. Analyses were approved by the Australian Defence Joint Health Command Low Risk Ethics Panel (LREP 15–004).

Results

Patient Characteristics

A total of 51 patients recruited from the 2 hospitals participated in the study. We obtained characteristics for all of these patients (Table 1).

Microscopy and *Plasmodium* Speciation PCR

Microscopy at the NRL confirmed 50 infections only with *P. falciparum* and 1 infection only with *P. vivax*. These results were confirmed by PCR; no mixed infections or infections with other *Plasmodium* species were identified. The 1 infection with *P. vivax* was excluded from further analysis. The number of *P. falciparum*–infected patients was approximately equal between the hospitals, and patients from the 2 hospitals had comparable parasite densities (p = 0.47) (Table 1).

RDT

All 50 confirmed *P. falciparum* samples showed positive results for the pan-pLDH RDT, but only 19 showed positive results for the HRP2-based RDT. These samples showed an overall false-negative rate of 62.0%; a total of 21 (80.8%) of 26 at Ghindae Hospital and 10 (41.7%) of 24 at Massawa Hospital were false negative (Table 1).

Presence or Absence of *pfhrp2*- and *pfhrp3*-Negative Genes

At Ghindae Hospital, 80.8% (21/26) of samples contained *pfhrp2*-negative parasites and 92.3% (24/26) *pfhrp3*-negative parasites. At Massawa Hospital, 41.7% (10/24) of samples contained *pfhrp2*-negative parasites and 70.8% (17/24) *pfhrp3*-negative parasites (Table 2). When we combined sample data, 62.0% (31/50) of samples contained *pfhrp2*-negative parasites and 82.0% (41/50) *pfhrp3*-negative parasites. All *pfhrp2*-negative samples (31/50, 62.0%) were also *pfhrp3* negative, but only 9 (18.0%) of 50 *pfhrp3*-negative samples were also *pfhrp2*-negative (Table 2; Figure 2).

Presence or Absence of Genes Flanking *pfhrp2* and *pfhrp3*

All 31 *pfhrp2*-negative isolates had a deletion of the upstream gene but retained the downstream gene of *pfhrp2*. A total of 8 *pfhrp2*-positive samples had a deletion of the upstream gene. In contrast, of 41 *pfhrp3*-negative samples, 30 had deletions of both flanking genes, 2 had deletions of only the upstream gene, and 9 had deletions of only the downstream gene. We obtained different patterns of *pfhrp2*, *pfhrp3*, and their flanking gene status in samples collected from both hospitals (Table 2).

RDT Results and *pfhrp2/pfhrp3*

All 31 samples positive for *P. falciparum* by microscopy and PCR but negative by HRP2-based RDTs had deletions of *pfhrp2* and *pfhrp3*. Conversely, all 19 *pfhrp2*-positive samples showed positive results by HRP2-based RDTs, including 12 *pfhrp3*-negative samples (Figure 2).

Parasite Density and *pfhrp2*/*pfhrp3*

We determined the relationship between parasite density and *pfhrp2/pfhrp3* status (Figure 2). The geometric mean parasite density for the 5 *pfhrp2*-positive patients at Ghindae

Table 1. Characteristics of patients infected with *Plasmodium falciparum* at 2 hospitals, Eritrea*

Characteristic	Ghindae	Massawa	Overall
No. enrolled	26	25	51
Mean age, y (range)	29.15 (10–60)	29.08 (7–68)	29.12 (7–68)
No. males:no. females (ratio)	16:10 (1.6:1)	13:12 (1.08:1)	29: 22 (1.32:1)
Travel history outside area of residence, no. positive/no. tested	2/26	3/25	5/51
Clinical history in previous 2 weeks, no. positive/no. tested			
Any malaria symptoms	25/26	24/25	49/51
Fever	10/26	24/25	34/51
Antimalarial treatment	0	0	0
Microscopy results			
No. *P. falciparum* positive	26	25	50
GM parasite density, parasites/μL (95% CI), [range]	6,851 (3,490–13,448) [16–71,000]	5,304 (2,620–10,734) [55–100,000]	6,059 (3,782–9,705) [16–100,000]
PCR results			
No. *P. falciparum* positive	26	24	50
No. *P. vivax* positive	0	1	1
RDT results			
No. pan-pLDH positive	26	25	51
No. HRP-based *P. falciparum* positive	5	14	19
False-negative result for *P. falciparum*, %	80.8	41.7	62.0

*GM, geometric mean; HRP2, histidine-rich protein 2; pLDH, *Plasmodium* lactate dehydrogenase; RDT, rapid diagnostic test.

Table 2. Frequency of *pfhrp2*, *pfhrp3,* and flanking genes in patients infected with *Plasmodium falciparum* at 2 hospitals, Eritrea*

Hospital	Upstream	*pfhrp2*	Downstream	No. (%)	Upstream	*pfhrp3*	Downstream	No. (%)
Ghindae	+	+	+	4 (15.4)	+	+	+	2 (7.7)
					+	–	–	2 (7.7)
	–	+	+	1 (3.9)	–	–	–	1 (3.9)
	–	–	+	21 (80.8)	–	–	–	21 (80.8)
Subtotal				26				26
Massawa	+	+	+	7 (29.2)	+	+	+	6 (25.0)
					+	–	–	1 (4.2)
	–	+	+	7 (29.2)	+	+	+	1 (4.2)
					+	–	–	6 (25.0)
	–	–	+	10 (41.7)	–	–	+	2 (8.3)
					–	–	–	8 (33.3)
Subtotal				24				24
Combined	+	+	+	11 (22.0)	+	+	+	8 (16.0)
					+	–	–	3 (6.0)
	–	+	+	8 (16.0)	+	+	+	1 (2.0)
					+	–	–	6 (12.0)
					–	–	–	1 (2.0)
	–	–	+	31 (62.0)	–	–	+	2 (4.0)
					–	–	–	29 (58.0)
Total				50				50

*Parasites with same presence or absence pattern for *pfhrp2* and flanking genes but different presence or absence pattern for *pfhrp3* and flanking genes are separated into subgroups. *pfhrp, P. falciparum* histidine-rich protein +, positive; –, negative.

Hospital was 11,069 parasites/μL (95% CI 4,886–25,074 parasites/μL), which is comparable with that for the 21 *pfhrp2*-negative patients (6,111 parasites/μL, 95% CI 2,664–14,020 parasites/μL; p = 0.86). The geometric mean parasite density for the 14 *pfhrp2*-positive patients at Massawa Hospital was 2,999 parasites/μL (95% CI 1,113–8,080 parasites/μL), which was significantly lower than that for the 10 *pfhrp2*-negative patients (11,783 parasites/μL, 95% CI 4,695–29,568 parasites/μL; p = 0.021). When data for patients from the 2 hospitals were combined, geometric mean parasite densities in *pfhrp2*-positive and *pfhrp2*-negative patients were not significantly different (p = 0.085).

HRP2 Levels and *pfhrp2/pfhrp3*

HRP2 levels, as indicated by mean MFI – bg values, were 18,885 (95% CI 16,061–21,710) for 19 *pfhrp2*-positive samples and 15.2 (95% CI 9.9–20.4) for 31 *pfhrp2*-negative samples. We found no significant difference between hospitals (p>0.05). When MFI – bg values were converted to HRP2 concentrations, *pfhrp2*-negative samples had an undetectable amount of HRP2, and *pfhrp2*-positive samples had an HRP level >9.5 ng/mL (Figure 3).

Genetic Diversity of Parasites

We determined 14 unique haplotypes for 50 samples on the basis of 7-loci microsatellite genotyping (Figure 4, panel A). All samples contained only 1 dominant haplotype. Nine haplotypes were detected for 19 *pfhrp2*-positive samples, and the maximum number of isolates sharing 1 haplotype (H10) was 7. Six haplotypes were detected for 31 *pfhrp2*-negative samples. Different haplotypes were observed for *pfhrp2*-negative and *pfhrp2*-positive populations except for haplotype H5, which was present in 20 *pfhrp2*-negative/*pfhrp3*-negative isolates and 1 *pfhrp2*-positive/*pfhrp3*-negative isolate (Figure 4, panel A). The 3 dominant haplotypes (H3, H5, and H10) were detected at both hospitals (Figure 4, panel B).

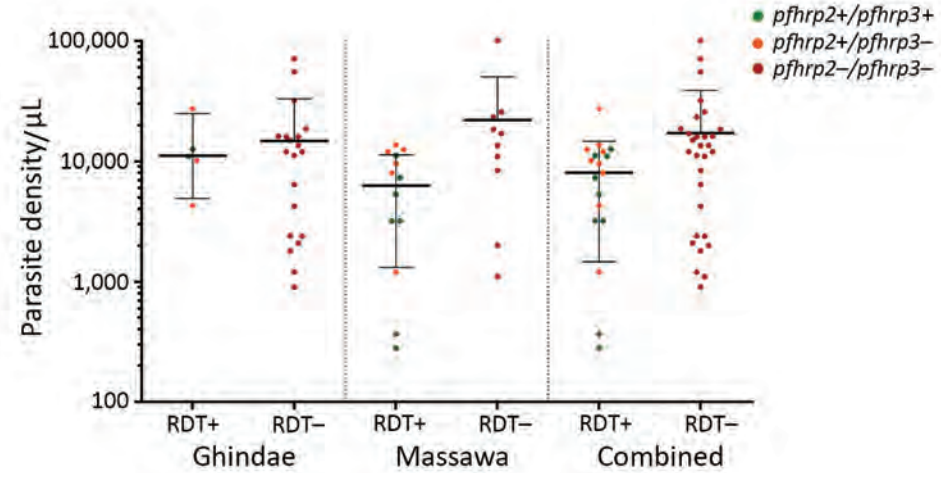

Figure 2. *Plasmodium falciparum* histidine-rich protein 2–based malaria RDT results and presence or absence of the *pfhrp2/pfhrp3* genes, in relation to parasite density, Eritrea. Horizontal lines indicate geometric means, and error bars indicate 95% CIs. *pfhrp, P. falciparum* histidine-rich protein; RDT, rapid diagnostic test; – negative; +, positive.

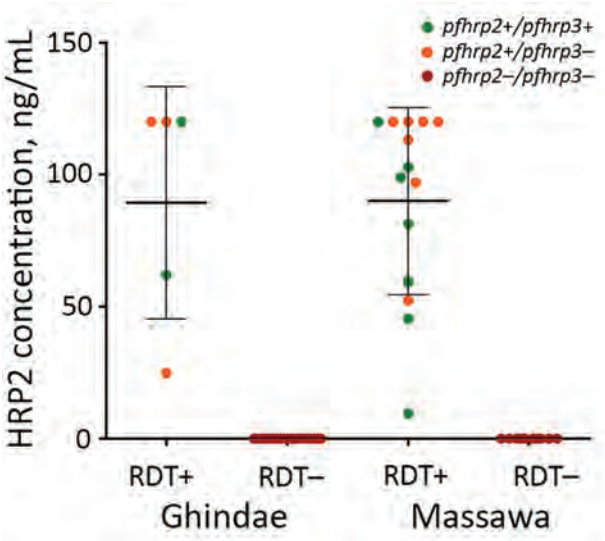

Figure 3. *Plasmodium falciparum* HRP2 antigen levels in relation to presence or absence of *pfhrp2/pfhrp3* genes and HRP2-based malaria RDT results, Eritrea. Horizontal lines indicate means, and error bars indicate SDs. HRP2, histidine-rich protein 2; *pfhrp, P. falciparum* histidine-rich protein; RDT, rapid diagnostic test; – negative; +, positive.

H_E values were 0.44 for *pfhrp2*-positive parasite populations and 0.11 for *pfhrp2*-negative parasite populations. These values indicated an overall lower level of genetic diversity in the *pfhrp2*-negative parasite population. Three of the 7 markers in *pfhrp2*-negative parasites had H_E values of 0, indicating no diversity, but only 1 marker in *pfhrp2*-positive parasites had an H_E value of 0.

Genetic Relatedness of Parasites

Of 9 haplotypes for *pfhrp2*-positive parasites from Eritrea, 4 (H9, H10, H11, and H12) were scattered and 2 (H7 and H8) were genetically closely related (Figure 5, panel A). In contrast, for *pfhrp2*-negative parasites, 5 of the 6 haplotypes (H1, H2, H3, H5, and H13) were genetically closely related and formed a cluster. Also linked to this cluster were haplotypes H6 and H14, which were *pfhrp2* positive/*pfhrp3* negative. We found 1 unrelated *pfhrp2*-negative haplotype (H4) outside the major cluster, which indicated different genetic lineages (Figure 5, panel A). There were 2 clusters for *pfhrp3*-negative parasites.

The 14 haplotypes observed in Eritrea were unrelated to any of the 5 haplotypes observed in Peru. This finding suggested distant genetic lineages between isolates from these 2 countries (Figure 5, panel B).

Discussion

Since malaria RDTs became available in the 1990s, growth in the number of tests, manufacturers, and volumes sold has been exponential (*25*,*26*). In parallel, the proportion of fever case-patients having access to diagnostic services before treatment has also expanded, particularly in Africa, largely attributed to the implementation of HRP2-based malaria RDTs (*3*). To continue reducing malaria transmission, use of RDTs must be expanded further, and tests must remain affordable, accurate, and user friendly.

Many factors can affect the accuracy of RDTs, and each factor should be investigated as a potential cause of false-negative results (*27*). In this instance, the Eritrea MOH had already investigated reports of false-negative results for RDTs and ruled out several possible causes (*6*). In our report, molecular and protein characterizations of prospectively collected specimens provided unambiguous evidence that confirmed that incidences of false-negative results in Eritrea were caused by a high prevalence of parasites having deletions of the *pfhrp2* and *pfhrp3* genes.

Our data showed that, in March 2016, a total of 80.8% (21/26) patients at Ghindae Hospital and 41.7% (10/24) at Massawa Hospital were infected with *pfhrp2*-negative parasites. All of these patients had undetectable levels of HRP2 in blood samples, and all showed negative results by HRP2-based RDTs and positive results by pan-pLDH–based RDTs. All samples were confirmed by microscopy and PCR as having only *P. falciparum* infections. Conversely, the remaining patient samples were *pfhrp2* positive, and all showed positive results for HRP2-based and pan-pLDH–based RDTs, and had HRP2 concentrations >9.5 ng/mL. These data confirmed that false-negative RDT results were caused by parasites lacking *pfhrp2*.

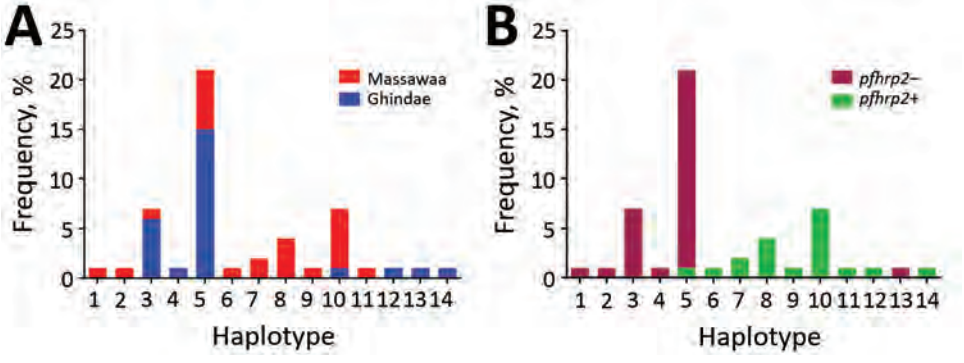

Figure 4. Number and frequency of *Plasmodium falciparum* haplotypes detected in patients at 2 hospitals, Eritrea, by hospital (A) and by *pfhrp2*-positive versus *pfhrp2*-negative parasite populations (B). *pfhrp, P. falciparum* histidine-rich protein; – negative; +, positive.

Although *pfhrp2*-negative parasites have been increasingly reported in several countries in South America, Asia, and Africa, they were mostly sporadic and showed low prevalences, except in the Amazon region of South America. Our study showed a high prevalence of parasites lacking *pfhrp2* that caused false-negative RDT results outside South America and a 100% correlation between parasites lacking the *pfhrp2* gene, the HRP2 antigen, and false-negative RDT results. These *pfhrp2* gene deletions were detected after investigations triggered by field reports of false-negative RDT results for symptomatic patients and categorically confirmed the role of this issue in malaria case management.

We showed correlations between patient demographics, parasite densities, and parasite *pfhrp2* gene status at the individual level. Our data showed that *P. falciparum* infections occurred across a range of parasite densities and age groups. All but 2 patients were symptomatic over the 2 weeks before testing, regardless of being infected with *pfhrp2*-positive or *pfhrp2*-negative parasites; the remaining 2 patients were infected with *pfhrp2*-negative parasites. Geometric mean parasite densities for *pfhrp2*-negative patients were comparable to those for *pfhrp2*-positive patients in this sample set. Further studies with larger sample sizes are required to confirm this finding.

Another useful finding of our study was the high prevalence of *pfhrp3* gene deletions in samples from Eritrea, which resulted in a high prevalence of parasites with concomitant *pfhrp2* and *pfhrp3* gene deletions. Every sample that was *pfhrp2*-negative was also *pfhrp3*-negative, which showed that 2 genes on different chromosomes were deleted in parallel. It appears that the dual-deleted parasites (*pfhrp2* negative/*pfhrp3*-negative) form clusters, and *pfhrp2*-positive/*pfhrp3*-negative parasites are scattered outside these clusters, suggesting that these parasites might have evolved from different genetic backgrounds. Although *pfhrp3* status did not affect results of RDTs used in this study, this status could affect results of other RDTs because *pfhrp3* shares sequence similarity with *pfhrp2*, and cross-reactivity between HRP2 and HRP3 has been reported for some RDTs (*28*). It is clear that regardless of the brand of HRP2-based RDTs used, parasites with dual *pfhrp2* and *pfhrp3* deletions will cause failure of RDTs because there is no possibility for cross-reactivity between HRP3 and HRP2 to occur.

A major issue is whether *pfhrp2*-negative parasites emerged locally and what is the main driving force behind their prevalence. In Peru, *pfhrp2*-negative parasites were detected in 4 of 5 major parasite populations during the late 1990s, and their prevalence increased in the absence of HRP2-based RDT pressure because microscopy was the primary diagnostic test used in that country (*23*). Spread of these parasites in Peru might have been driven by an undefined biological advantage associated with deletion of the *pfhrp2* gene.

In Eritrea, low genetic diversity of *pfhrp2*-negative parasites compared with that of *pfhrp2*-positive parasites and presence of a closely related cluster consisting of all but 1 *pfhrp2*-negative parasite suggest that clonal expansion of *pfhrp2*-negative parasites was probably caused by selection by use of HRP2-based RDTs. Because HRP2-based RDTs have been widely used in Eritrea since 2006, compliance with the recommended test before treatment is universally applied, and treatment adherence is high, conditions are ideal for selection of *pfhrp2*-negative parasites. This selection is predicted by recent mathematical modeling, which showed that exclusive use of HRP2-based RDTs exerts strong selection pressure for *pfhrp2*-negative parasites in communities and that HRP2/*P. falciparum* pan-LDH combination RDTs do not provide selection pressure for *pfhrp2*-negative parasites (*18*). Furthermore, low malaria prevalence in Eritrea might

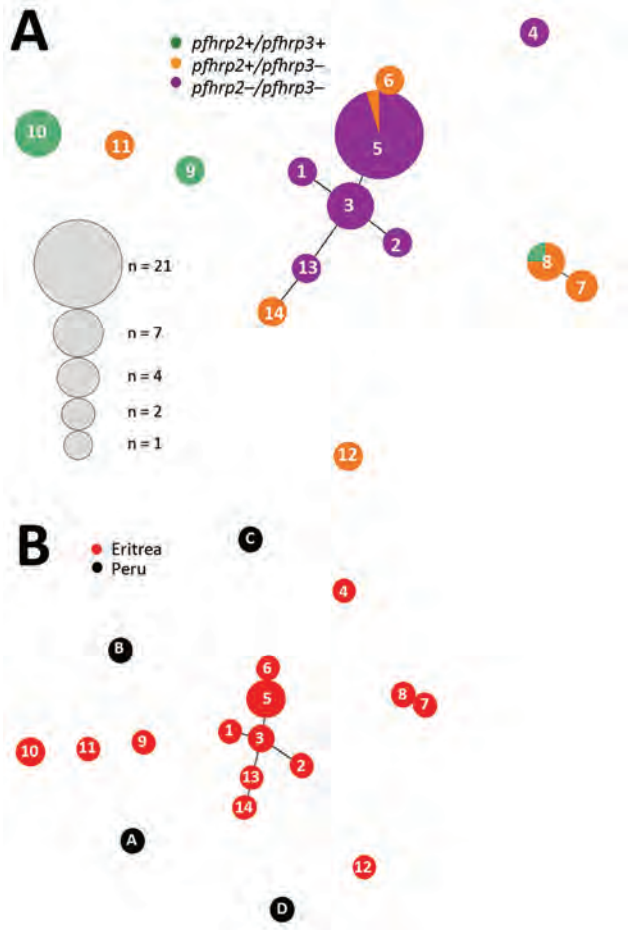

Figure 5. Genetic relatedness among *Plasmodium falciparum* parasite populations in Eritrea differing in *pfhrp2* and *pfhrp3* gene status (A) and comparison of parasite populations from Eritrea and Peru (B). Plots were produced by using Phyloviz software (*24*) at a cutoff value of 2 (minimum differences for 2 loci). Numbered circles indicate specific haplotypes. Circle sizes indicate number of samples with a particular haplotype. *pfhrp, P. falciparum* histidine-rich protein; – negative; +, positive.

have also contributed to selection of *pfhrp2*-negative parasites once they emerge. However, historical samples were not available for a retrospective analysis of the dynamics and evolution of the *pfhrp2*-negative parasites in Eritrea.

The presence of a unique *pfhrp2*-negative parasite outside the main cluster suggests that the *pfhrp2* deletion has occurred at least twice in parasites in Eritrea. The *pfhrp2*-negative parasites in Eritrea and Peru showed distinct haplotypes, strongly suggesting de novo development of these parasites in both locations. This suggestion would imply that all malaria-endemic areas are at risk and that there is an urgent need to map the prevalence of *pfhrp2*-negative parasites to inform case management policy. The immediate mapping priority should be for countries/areas near Eritrea. It is also critical for research and development and improvements in alternative biomarkers of *P. falciparum*. Currently, there is only 1 *P. falciparum* and 1 *P. falciparum/P. vivax*–detecting RDT specific for pLDH that meet WHO procurement criteria. Most manufacturers have difficulties in producing RDTs that can consistently detect *P. falciparum* at a concentration of 200 parasites/μL by using antibodies against *P. falciparum*–pLDH (*29*).

Emergence of *pfhrp2*-negative parasites poses a major threat to malaria control programs because patients infected with these parasites are not given a correct diagnosis and treatment. Because of broad use of HRP2-based RDTs, particularly in Africa, the magnitude of selection pressure and potential scope of the problem are large. Confirming *pfhrp2/3* deletions as the cause of false-negative RDT results requires multiple PCRs performed by experienced technicians. Guidance on investigating *pfhrp2* deletions and accurate reporting is available (*21*). In addition, WHO has recently released a standard survey tool to determine whether *pfhrp2/3* gene deletions in *P. falciparum* isolated from patients with confirmed symptomatic malaria have reached a threshold triggering a change in diagnostic strategy (*30*).

In conclusion, we confirmed that frequently reported false-negative results for *P. falciparum* in specimens tested by using HRP2-based RDTs in Eritrea were caused by high proportions of parasites with deletions of *pfhrp2/pfhrp3* genes. It is clear that HRP2-based RDTs are no longer appropriate diagnostic tools for malaria case management in this country. RDTs detecting other parasite antigens, such as *P. falciparum*–pLDH, have been implemented. Our results show the need to seriously investigate reports of false-negative RDT results and conduct surveys to determine the prevalence of *pfhrp2/3* deletions in neighboring regions. International technical guidance is also needed to assist countries in planning *pfhrp2/3* surveillance activities and laboratory investigations and adapting procurement and case management strategies when *pfhrp2/3* gene deletions are confirmed.

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A.B., S. Mihreteab, S. Mohamed, F.H., G.E., A.C., and A.Z. conducted hospital surveys and performed training and sample collection; K.A. performed molecular characterization of *pfhrp2/pfhrp3* and flanking genes; K.G. and S.D. performed microsatellite typing and genetic analyses; N.C.W. and Q.C. performed molecular supervision; E.R. and V.U. performed antigen detection; K.G., Q.C., and M.L.G. analyzed data; A.B., S. Mihreteab, E.R., Q.C., and J.C. wrote the manuscript; and J.C. coordinated the study.

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Use of Influenza Risk Assessment Tool for Prepandemic Preparedness

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In 2010, the Centers for Disease Control and Prevention began to develop an Influenza Risk Assessment Tool (IRAT) to methodically capture and assess information relating to influenza A viruses not currently circulating among humans. The IRAT uses a multiattribute, additive model to generate a summary risk score for each virus. Although the IRAT is not intended to predict the next pandemic influenza A virus, it has provided input into prepandemic preparedness decisions.

Planning and preparation for influenza pandemics are major challenges to public health authorities for many reasons, not the least of which is the inherent variability and unpredictability of the influenza virus (*1*). Just in the past decade, infections from multiple new influenza viruses have occurred in humans, representing influenza A subtypes such as H1N2, H3N2v, H5N1, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N8. In response to these findings, prepandemic vaccines were developed for some of these viruses (*2*–*5*). In 2009, a new virus, subsequently designated influenza A(H1N1)pdm09, emerged in humans in North America and quickly spread, causing the first influenza pandemic of the 21st century (*6*). Although only 3 hemagglutinin (HA) subtypes of influenza (H1, H2, and H3) are known to have caused human pandemics (*7*), the emergence and spread of influenza A(H5N1) and, more recently, influenza A(H7N9), with associated high death rates in humans, are of great concern. If these or other influenza A viruses not currently circulating among humans develop the capability to transmit efficiently among humans, they pose a risk for causing a pandemic that could be associated with high rates of illness and death (*8*,*9*).

The task of risk mitigation planning and preparedness for pandemic influenza is difficult, and a tool is needed that systematically evaluates different influenza viruses to inform decisions related to the prioritization and allocation of resources for vaccine development, influenza surveillance strategies, and research initiatives. In this context, the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) developed the Influenza Risk Assessment Tool (IRAT) with the goal to systematically evaluate influenza A viruses that are not circulating in humans but potentially pose a pandemic risk (*10*).

The IRAT uses a common decision analysis approach that incorporates input from multiple elements or attributes, applies a weighting scheme, and generates a score to compare various options or decisions (*11*). In regard to the evaluation of animal-origin influenza viruses for their potential human pandemic risk, 2 specific questions were developed related to the potential risk for emergence and consequent potential impact: 1) What is the risk that a virus not currently circulating in humans has the potential for sustained human-to-human transmission? (emergence question); and 2) If a virus were to achieve sustained human-to-human transmission, what is the risk that a virus not currently circulating among humans has the potential for substantial impact on public health? (impact question).

In developing the IRAT, a working group of international influenza experts in influenza virology, animal health, human health, and epidemiology identified 10 risk elements and definitions. These elements were described previously (*10*); in brief, they include virus properties (genomic variation, receptor-binding properties, transmissibility in animal models, and antiviral treatment susceptibility) and host properties (population immunity, disease severity, and antigenic relationship to vaccines). The final 3 elements are based on the epidemiologic and ecologic evidence: infection in humans, infections in animals, and global distribution in animals. These elements are used to answer the 2 risk questions to evaluate an influenza virus of interest. The 10 elements are ranked and weighted on the basis of their perceived importance to answering the specific risk questions and an aggregate risk score is generated.

Since its inception, the IRAT has facilitated the evaluation of multiple viruses and contributed information to decisions related to US pandemic planning, such as selection of candidate vaccine viruses (CVVs) and vaccines for the Strategic National Stockpile of prepandemic influenza vaccines (*12*). We summarize the evaluation of 14 animal viruses and discuss the strengths and limitations of the IRAT as a tool supporting CDC's Preparedness and Response Framework for Influenza Pandemics (*13*), a document that outlines key public health decisions and actions to be taken at specific times during an influenza pandemic.

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Methods

Scoring Procedure

The ranking and weighting of risk elements used to answer the 2 standard IRAT risk questions (emergence, impact) was predetermined by the working group of international influenza experts (*10*). Discussion and debate about the importance of each risk element to answer the 2 questions resulted in a consensus ranking of risk elements for each question after 2 rounds of ranking all elements. Subsequently, when viruses were evaluated by subject-matter experts (SMEs), their task was to consider a virus solely within the definition of the individual risk element they were scoring. SMEs scored a specifically selected virus of interest; only data related to this strain were considered to avoid confusion over potentially significant strain differences. Each evaluation was conducted in the context of data available at the time of the evaluation. Multiple SMEs scored each risk element, but the maximum number of risk elements scored by any one SME evaluating a virus was set at 3 in an attempt to maintain a high level of expertise, assuming that most SMEs are not experts across all the technical areas represented by the various risk elements. Limiting the number of elements any SME scores also reduced the burden on any one SME, which can potentially shorten the time to evaluate a given virus and removed the possibility of potential bias introduced by an SME scoring most or all elements.

The SMEs provided a point estimate based on a standardized scale of 1–10 using the definition and criteria of the risk element. For each element, the lowest risk score possible is 1, and the highest risk score possible is 10. The SMEs also provided an upper and lower bound of acceptability in scores they would consider reasonable from other experts, assuming the same knowledge base regarding the risk element. In addition, a justification was solicited that summarized the critical basis contributing to the SME's point estimate, as well as his or her judgment of confidence in the quality of the data. Scoring was collected in 2 phases. In the initial phase, preliminary data were reviewed, and variation in the individual risk element scores was noted. A wide range of point estimate scores for a particular element potentially indicated that SMEs operated from a different knowledge base, such as when unpublished data were available to only a select few or alternatively indicated that few data were available to use in generating scores. In the second phase, SMEs were presented with a summary of the preliminary data for their element(s). In instances with a wide range of scores for a given element, the justification information was anonymized and redistributed to all the SMEs who scored that particular element, with the invitation to reconsider or confirm their initial score (Figure 1). Results for the IRAT average point scores were used to calculate an overall virus risk score for each of the 2 risk questions. Generally, virus scores of 1–3 were considered a low potential risk by the IRAT; scores of 4–7 were moderate; and scores of 8–10 were potential high risk. Scores at the

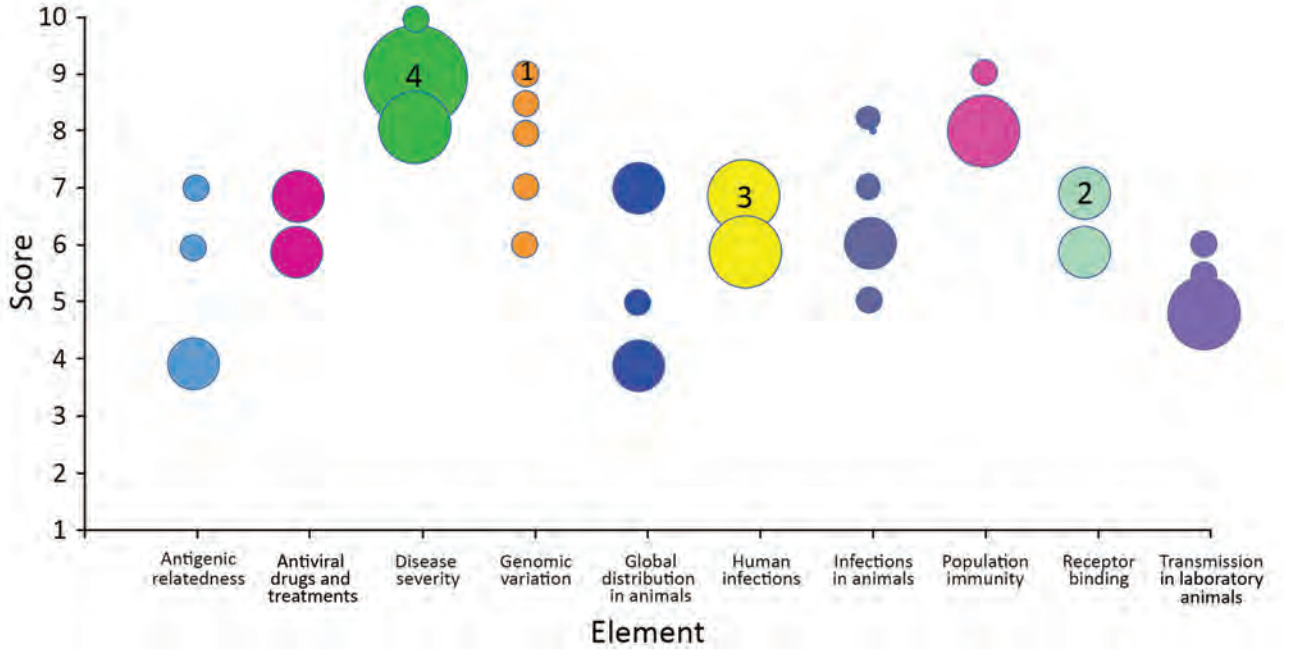

Figure 1. Individual subject-matter expert point scores by element for the May 2017 scoring of influenza A(H7N9) virus, A/Hong Kong/125/2017, based on risk element definitions. Circles indicate individual point scores; circle sizes (examples indicated by a number inside) correspond to the frequency of each point score.

boundaries of those ranges are described by a combination term such as moderate-high for a score of 7.5.

Establishment of a Point of Reference

As a point of reference for low-risk viruses with respect to both IRAT questions, potential risk for emergence and risk for potential impact, SMEs evaluated the North America avian influenza A(H1N1) virus, A/duck/New York/1996. As expected, this virus received low risk scores from the SMEs; the summary average risk score was 2.3 (i.e., low risk) to achieve sustained human-to-human transmission. Similarly, the average risk score for the virus to substantially impact public health if it were to achieve sustained human-to-human transmission was 2.4 (low risk).

Results

During 2011–2017, SMEs evaluated 14 animal-origin influenza viruses using the IRAT. The emergence and impact scores are plotted for each virus (Figure 2). Of the viruses scored thus far by IRAT, avian influenza A(H7N9) A/Hong Kong/125/2017 ranked highest for potential risk. Other viruses, such as A/Indiana/08/2011, an influenza A(H3N2) variant (H3N2v), had a similar score for risk for emergence similar to that of A/Hong Kong/125/2017 but a much lower estimated risk for potential impact.

Influenza A(H7N9) and the IRAT

On March 31, 2013, the China Health and Family Planning Commission notified the World Health Organization (WHO) of 3 cases of human infection with influenza A(H7N9) (*14*). Three viruses were isolated and analyzed at the China WHO Collaborating Center and the complete viral genome sequences deposited in a publicly accessible influenza database. After these reports, CDC used the IRAT to assist the US Department of Health and Human Services' Biomedical Advanced Research and Development Authority with the overall prepandemic risk assessment of these viruses.

Although laboratories had begun the animal transmission challenge work, study results were not available. Hence, the IRAT risk element of transmissibility in animal models lacked data. This element is ranked as the second most important and thus carries a high weight in the computation of a final score for the IRAT emergence question. Therefore, it was necessary to assign a score for this element without data. Point scores for the other 9 elements were gathered and used to populate the IRAT to generate a partial risk score.

Although information about the outcome of laboratory animal transmission studies was scarce, previous observations showed significant correlation between other IRAT risk elements (receptor-binding properties, genomic variation, and human infections) and this element. A moderate score for this element extrapolated from other elements would greatly improve the ability to compare this new virus with other viruses evaluated previously with the IRAT. Based on the evidence of increased α2,6 receptor binding, the presence of L226 in the HA receptor binding pocket and the ability to infect humans, this element was assigned a score of 7 in the moderate risk category (range 4–7). Uncertainty was captured by assigning the risk element transmissibility in animal models a score of 1, 7, or 10 (Table 1). Using these 3 possible scores for this risk element, the summary risk score for the emergence question would be 5.2, 6.4, or 7.0, respectively. Assigning these same scores to this risk element to calculate the impact score, the summary risk scores would be 7.1, 7.4, or 7.5, respectively (Table 2). The much greater range in emergence (1.7) than impact (0.3) score is understandable

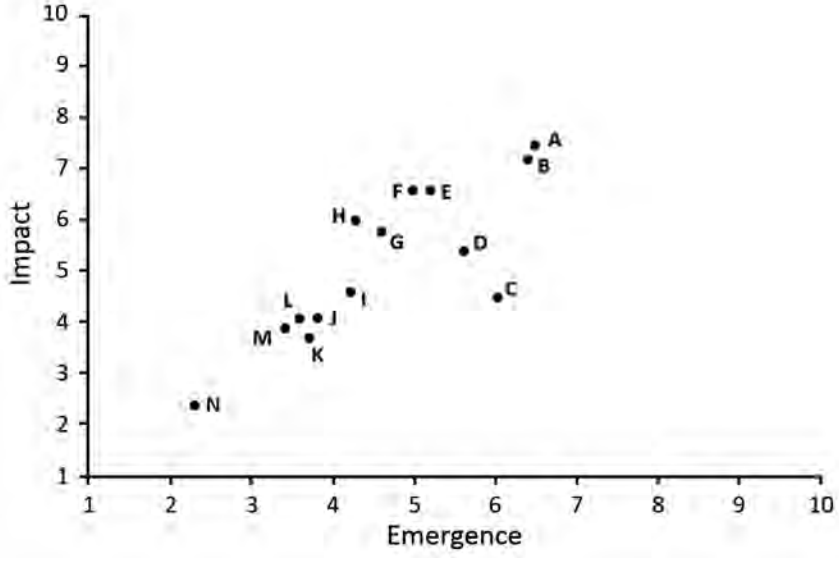

Figure 2. Comparison of average emergence and impact scores for 14 animal-origin influenza viruses using the Influenza Risk Assessment Tool. Circle represents each virus: A, H7N9 A/Hong Kong/125/2017; B, H7N9 A/Shanghai/02/2013; C, H3N2 variant A/Indiana/08/2011; D, H9N2 G1 lineage A/Bangladesh/0994/2011; E, H5N1 clade 1 A/Vietnam/1203/2004; F, H5N6 A/Yunnan/14564/2015-like; G, H7N7 A/Netherlands/2019/2003; H, H10N8 A/Jiangxi-Donghu/346/2013; I, H5N8 A/gyrfalcon/Washington/41088/2014; J, H5N2 A/Northern pintail/Washington/40964/2014; K, H3N2 A/canine/Illinois/12191/2015; L, H5N1 A/American green-winged teal/Washington/1957050/2014; M, H7N8 A/turkey/Indiana/1573-2/2016; N, H1N1 A/duck/New York/1996. Additional information about virus scores and individual viruses is available at https://www.cdc.gov/flu/pandemic-resources/monitoring/irat-virus-summaries.htm.

Table 1. Emergence question IRAT score calculation for 2 similar influenza A(H7N9) viruses, A/Shanghai/2/2013 and A/Anhui/1/2013, with data missing on the risk element "transmission in laboratory animals" during scoring of 2013 outbreak in China*

		Transmission in laboratory animals					
		R = 1†		R = 7‡		R = 10§	
Element	W	R	W × R	R	W × R	R	W × R
Human infections	0.2929	5.00	1.46		1.46		1.46
Transmission in lab animals	0.1929	1.00	0.19	7.00	1.35	10.00	1.93
Receptor binding	0.1429	6.70	0.96		0.96		0.96
Population immunity	0.1096	9.00	0.99		0.99		0.99
Infections in animals	0.0846	6.00	0.51		0.51		0.51
Genomic variation	0.0646	8.60	0.56		0.56		0.56
Antigenic relatedness	0.0479	6.00	0.29		0.29		0.29
Global distribution in animals	0.0336	1.00	0.03		0.03		0.03
Disease severity and pathogenesis	0.0211	9.00	0.19		0.19		0.19
Antiviral drug and treatment options	0.0100	5.40	0.05		0.05		0.05
Total	1.0001		5.24		6.40		6.98

*IRAT, Influenza Risk Assessment Tool; R, average risk point score; W, weight.
†Substituting the lowest possible risk score (1) to calculate summary IRAT score.
‡Substituting a moderate risk score (7) to calculate summary IRAT score.
§Substituting the highest possible risk score (10) to calculate summary IRAT score.

when the relative weight assigned to this risk element is considered in the 2 different scenarios.

Only minimal data were available for 2 other elements (global distribution in animals and infections in animals) in April 2013. For the purposes of the risk scoring, we gave the global distribution in animals element a score of 1 because this virus had been identified in only a few live-bird markets in China. Because of the lack of information, confidence scores were low for this element. SMEs gave infection in animals a higher risk (mean 6, moderate risk) and confidence scores probably because of other H7N9 viruses associated with avian species. Because these elements carry less weight in risk scoring for both questions, they did not heavily affect the final score. In general, the SMEs agreed about risk scores for these elements.

The uncertainty and the data gaps, particularly for the transmissibility in the animal models element, were presented to decision makers and discussed. Particular attention was given to explain the range of scoring generated about the emergence question. However, the SMEs agreed that the impact score was less influenced by the missing information and the risk score did not significantly affect the final summary score.

In May 2013, 1 month after the initial assessment, information became available to inform the risk element transmissibility in animal models. More information was available for all other elements as well. The viruses were rescored in May 2013. The resulting average summary risk score for the 2 similar influenza A(H7N9) viruses (A/Anhui/1/2013 and A/Shanghai/2/2013) was 6.4 for the emergence risk and 7.2 for impact on public health if this virus gains the ability to transmit from person to person. SMEs reported a higher level of confidence in their risk scores at this time, although most element risk scores did not change appreciably. Since May 2013, these viruses have been scored annually in 2014, 2015, 2016, and again in early 2017, with little to no change in scoring, but with higher levels of confidence in individual scores each year.

Adaptation of the IRAT to Assess Influenza A(H5N1) Viruses

In 2014, the IRAT was used to compare several influenza A(H5N1) viruses that circulated during 2011–2014. Use of the IRAT is predicated on the assumption that each risk element will independently assess some aspect inherent in or associated with the various viruses included in the assessment. Based on available information, 5 of the risk elements would have had virtually the same score for all the H5N1 viruses. Although these 5 risk elements are useful for discriminating among other viruses, when comparing H5N1 viruses, sufficient information is lacking to enable the distinctions among the viruses necessary for the IRAT. These 5 elements (disease severity, population immunity, antiviral treatment susceptibility, receptor-binding properties, and transmissibility in animal models) were therefore removed from the IRAT scoring.

To use the IRAT to compare these viruses with each other, we tailored specific questions for this effort. Two questions were generated that related specifically to prepandemic mitigation of the impact these viruses could have on public health. The risk assessment focused on 2 questions about the risk element of antigenic relationship and availability of vaccines:

1. Considering the vaccine antigens that are in the US Strategic National Stockpile or are currently being generated for this purpose, what H5N1 viruses pose the greatest potential risk to public health?
2. Considering what CVVs are available or in development, what H5N1 viruses pose the greatest potential risk to public health?

Table 2. Impact question IRAT score calculation for 2 similar influenza A(H7N9) viruses, A/Shanghai/2/2013 and A/Anhui/1/2013, with data missing on the risk element "transmission in laboratory animals" during scoring of 2013 outbreak in China*

		Transmission in laboratory animals					
		R = 1†		R = 7‡		R = 10§	
Element	W	R	W × R	R	W × R	R	W × R
Disease severity and pathogenesis	0.2929	9.00	2.64		2.64		2.64
Population immunity	0.1929	9.00	1.74		1.74		1.74
Human infections	0.1429	5.00	0.71		0.71		0.71
Antiviral drug and treatment options	0.1096	5.40	0.59		0.59		0.59
Antigenic relatedness	0.0846	6.00	0.51		0.51		0.51
Receptor binding	0.0646	6.70	0.43		0.43		0.43
Genomic variation	0.0479	8.60	0.41		0.41		0.41
Transmission in lab animals	0.0336	1.00	0.03	7.00	0.24	10.00	0.34
Global distribution in animals	0.0211	1.00	0.02		0.02		0.02
Infection in animals	0.0100	6.00	0.06		0.06		0.06
Total	1.0001		7.14		7.35		7.45

*IRAT, Influenza Risk Assessment Tool; R, average risk point score; W, weight.
†Substituting the lowest possible risk score (1) to calculate summary IRAT score.
‡Substituting a moderate risk score (7) to calculate summary IRAT score.
§Substituting the highest possible risk score (10) to calculate summary IRAT score.

To answer these 2 questions, SMEs scored the element of antigenic relatedness twice, in relationship to 1) a currently available WHO CVV or 2) antigens already prepared and stockpiled. Stockpiled antigen would be more quickly available for use than antigen in early development as a CVV.

Ten H5N1 clades were considered to be circulating during 2011–2014. SMEs scored these 10 using the IRAT based on information available for 5 elements that could be used to distinguish between these related viruses. SMEs were asked to provide a risk score for 1) human infections, 2) antigenic relationship of the viruses, 3) global distribution in animals, 4) infections in animals, and 5) genomic variation. The elements are listed in order of importance (i.e., the most heavily weighted element is human infections, and the next most important is the antigenic relatedness).

Initial SME scores for the 5 elements were averaged and presented to the same SMEs, and consensus on the final scores was reached through discussion. These risk scores were then multiplied by the appropriate weighting factor to generate summary risk scores. Because this risk assessment comprises only 5 elements, weights were apportioned on the basis of 5 elements rather than on the standard 10 elements. The IRAT definitions for the elements remained the same.

When scoring the 10 H5N1 viruses for antigenic relatedness, the SMEs based their first risk score on knowledge of currently available WHO CVVs and applied the IRAT definition of antigenic relatedness. The same SMEs then provided a second risk score for antigenic relatedness to US stockpiled antigens when considering the same 10 clades. In some instances, the average risk scores for this element differed. In each case, the average risk score for this element was multiplied by 0.2567, providing 2 possible summary risk scores for each virus (Tables 3, 4).

When SMEs considered risk scores associated with antigenic relatedness to CVVs, clade 1.1.2 was the only virus clade that scored >5.0. When considering summary risk scores when the antigenic relatedness element was based on already stockpiled antigens, SMEs scored 4 virus clades >5.0: clade 1.1.2, clade 2.1.3.2a, clade 2.3.2.1a, and clade 2.3.2.1c (Table 5).

Ultimately, the US Department of Health and Human Services' Biomedical Advanced Research and Development Authority decided to base the antigen to add to the US stockpile on influenza A(H5N1) clade 2.3.2.1a. This clade did not score the highest through the IRAT, but additional information, such as production deadlines, availability of the CVVs, and contractual obligations, also was considered before a final decision was reached, reinforcing that the IRAT is just 1 input for decision makers.

Table 3. Product of average risk point scores multiplied by weight for each of the 10 influenza A(H5N1) clades for the IRAT when the antigenic relatedness score is based on the virus' relatedness to a CVV*

	Clade									
Element	1.1.1	1.1.2	2.1.3.2a	2.2.1	2.2.1.1	2.3.2.1a	2.3.2.1b	2.3.2.1c	2.3.4.2	7.2
Human infections	1.96	3.06	2.28	2.28	0.91	1.96	1.05	1.83	1.83	0.59
Antigenic relatedness to CVV	0.59	0.69	0.59	0.59	1.54	0.59	0.59	0.59	0.69	2.05
Global distribution in animals	0.63	0.78	0.78	0.47	0.47	0.94	0.89	1.21	0.74	0.67
Infection in animals	0.45	0.63	0.63	0.66	0.27	0.57	0.48	0.72	0.51	0.42
Genomic variation	0.12	0.25	0.13	0.13	0.18	0.16	0.12	0.25	0.11	0.19
Total	3.75	5.42	4.42	4.13	3.27	4.22	3.13	4.60	3.87	3.93

*Weight for human infections = 0.4567; weight for antigenic relatedness to CVV = 0.2567; weight for global distribution in animals = 0.1567; weight for infections in animals = 0.09; weight for genomic variation = 0.04. CVV, candidate vaccine virus; IRAT, Influenza Risk Assessment Tool.

Table 4. Product of average risk point scores multiplied by weight for each of the 10 influenza A(H5N1) clades for the IRAT when the antigenic relatedness score is based on the virus' relatedness to a US stockpiled antigen*

	Clade									
Element	1.1.1	1.1.2	2.1.3.2a	2.2.1	2.2.1.1	2.3.2.1a	2.3.2.1b	2.3.2.1c	2.3.4.2	7.2
Human infections	1.96	3.06	2.28	2.28	0.91	1.96	1.05	1.83	1.83	0.59
Antigenic relatedness to stockpiled antigen	0.77	1.21	1.28	0.69	2.05	1.98	1.98	1.98	1.62	2.05
Global distribution in animals	0.63	0.78	0.78	0.47	0.47	0.94	0.89	1.21	0.74	0.67
Infection in animals	0.45	0.63	0.63	0.66	0.27	0.57	0.48	0.72	0.51	0.42
Genomic variation	0.12	0.25	0.13	0.13	0.18	0.16	0.12	0.25	0.11	0.19
Total	3.93	5.94	5.11	4.23	3.78	5.61	4.52	5.99	4.80	3.93

*Weight for human infections = 0.4567; weight for antigenic relatedness to candidate vaccine virus = 0.2567; weight for global distribution in animals = 0.1567; weight for infections in animals = 0.09; weight for genomic variation = 0.04. IRAT, Influenza Risk Assessment Tool.

Discussion

The objective of the IRAT development was to assist decision makers in pandemic planning by creating a tool that facilitates the assessment of influenza A viruses not circulating in humans but potentially posing a pandemic risk. A common misconception is that the IRAT is a prediction tool to identify the next likely pandemic virus; however, that is neither the intent nor within the capability of the IRAT. Without a complete understanding of all the mechanisms and factors associated with the emergence of a pandemic virus, let alone the plausibility of detecting and characterizing the immediate precursor of the next pandemic influenza virus, prediction is not possible at this time. However, on the basis of 10 individual risk elements weighted specifically in relationship to their importance in answering specific risk questions, the IRAT process evaluates viruses systematically. This assessment enables comparison of different viruses when prioritization decisions must be made.

The IRAT development objective was fulfilled in 3 important ways. First, the IRAT provides a systematic procedure and framework for acquiring, analyzing, and combining information on multiple attributes of influenza A viruses deemed important to the consideration and communication of pandemic risk by influenza SMEs. The IRAT simplifies interpretation of multiple complex data elements but requires the interpretation of complex data by SMEs within their respective areas of expertise to generate an overall assessment of the perceived pandemic risk. Second, the IRAT has shown the requisite flexibility required to deal with practical issues of characterizing newly emerging influenza viruses, such as lack of data within specific risk elements. As in the initial evaluations of influenza A(H7N9), to address missing data regarding a critical risk element, a range of scores for that element was used to generate a range of possible summary risk scores that was easily communicated to decision makers. Third, the IRAT supports CDC's Pandemic Preparedness and Response Framework by summarizing information to assist in prepandemic decisions (*13*).

Other initiatives addressing pandemic influenza risk assessment have taken an approach similar to IRAT or used a modification of epidemiologic risk modeling. WHO's Global Influenza Program has recently introduced the Tool for Influenza Pandemic Risk Assessment (TIPRA) (*15*) to supplement its Pandemic Influenza Risk Management guideline (*16*). Although TIPRA uses the same decision analysis approach as IRAT, some subtle and some more major differences make the TIPRA unique. Risk questions similar to the IRAT are used, but fewer individual risk elements are used along with different definitions. In addition, a gateway question of evidence for population immunity dictates whether use of the TIPRA is indicated. An alternative approach has been taken by the FLURISK project (*17*), an activity funded by the European Food Safety Authority. By combining an estimate of human–livestock contact intensity with influenza strain–specific outbreak information and the virus' estimated capability to cause human infection, a quantitative risk for ≥1 human infections is calculated. Pandemic risk is therefore not specifically addressed in this model, but rather the risk for an influenza A virus to make the species jump into humans, a prerequisite of a pandemic, is estimated (*18*).

As research progresses into influenza virus mechanisms of transmission and adaptation to mammalian hosts, particularly in relation to humans, more risk elements for use in IRAT may be identified or existing risk elements may be modified and redefined. In a recent review of pandemic influenza risk assessment, the review authors contended that assessment of influenza pandemic risk should include 3 specific viral factors: HA receptor binding specificity, HA pH of activation, and polymerase complex efficiency (*19*). IRAT addresses receptor binding specificity directly but does not specifically incorporate the other 2

Table 5. Summary of scoring of the 7 highest-scoring influenza A(H5N1) virus clades related to antigen in the US SNS and to nearest related CVV*

	Antigenic relatedness*	
Clade	To SNS	To CVV
2.3.2.1c	5.99	4.60
1.1.2	5.94	5.42
2.3.2.1a	5.61	4.22
2.1.3.2a	5.11	4.42
2.3.4.2	4.80	3.87
2.3.2.1b	4.52	3.13
2.2.1	4.23	4.13

*Relatedness based on hemagglutinin inhibition testing. CVV, candidate virus vaccine; SNS, Strategic National Stockpile.

factors. Consideration of these, as well as other sources of data related to answering the IRAT risk questions, will be investigated for potential inclusion in the IRAT. Thus, the IRAT is a carefully defined tool that provides standardized risk assessment scores and a flexible framework that can be modified for special cases and as additional information becomes available.

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Use of Verbal Autopsy to Determine Underlying Cause of Death during Treatment of Multidrug-Resistant Tuberculosis, India

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Of patients with multidrug-resistant tuberculosis (MDR TB), <50% complete treatment. Most treatment failures for patients with MDR TB are due to death during TB treatment. We sought to determine the proportion of deaths during MDR TB treatment attributable to TB itself. We used a structured verbal autopsy tool to interview family members of patients who died during MDR TB treatment in India during January–December 2016. A committee triangulated information from verbal autopsy, death certificate, or other medical records available with the family members to ascertain the underlying cause of death. For 66% of patient deaths (47/71), TB was the underlying cause of death. We assigned TB as the underlying cause of death for an additonal 6 patients who died of suicide and 2 of pulmonary embolism. Deaths during TB treatment signify program failure; accurately determining the cause of death is the first step to designing appropriate, timely interventions to prevent premature deaths.

Mycobacterium tuberculosis resistant to ≥2 of the most potent TB drugs, isoniazid and rifampin, is classified as multidrug-resistant tuberculosis (MDR TB). Worldwide, an estimated 580,000 MDR TB cases emerge annually (*1*). Unfortunately, there are substantial gaps in MDR TB detection and treatment. Approximately 1 of 5 persons needing MDR TB treatment actually receive it, and among those who do receive treatment, less than half (48%) who start treatment finish successfully (*1*,*2*). These rates are driven by treatment failure, loss to follow-up, and premature death. In 2016, the proportion of deaths during MDR TB treatment in India was higher than the global average (20% vs. 14%) (*3*).

India follows the routine surveillance and reporting guidelines recommended by the World Health Organization (WHO) and considers any death that occurs during TB treatment as a TB-related death. Several studies have used all-cause mortality as a surrogate marker of mortality attributable to TB (*4*–*6*). This method of attributing all-cause mortality can overestimate TB case-fatality rates. Accurately determining the cause of death is the first step to designing appropriate and timely interventions to prevent premature deaths.

In settings with no or poorly documented vital registration and medical certification of the cause of death, verbal autopsy can be an essential public health tool for obtaining a reasonable estimation of the cause structure of mortality (*7*). Verbal autopsy uses systematic retrospective inquiry of family members about the symptoms and signs of illness before death to help determine the putative medical cause of death (*8*). The demand for and use of verbal autopsy data has rapidly gained importance and has been used to set global health priorities (*9*,*10*). Verbal autopsy data may improve surveillance and program monitoring and evaluation and could stimulate change in public health policy (*11*–*13*). In our study, we used the verbal autopsy method to determine the underlying causes of death for persons who died during MDR TB treatment (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1718-Techapp1.pdf).

Methods

Study Design and Population

We conducted a cross-sectional study of patients who died during MDR TB treatment during January–December

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2016 at 3 drug-resistant TB (DR-TB) treatment centers in southwestern Karnataka state in India (Figure). The DR-TB centers serve 3 major populations of Karnataka (Bangalore, 17.9 million persons from 7 districts; Mangalore, 4.6 million persons from 3 districts; and Mysore, 10.1 million persons from 6 districts) (*3*). DR-TB centers, established with the support of Revised National Tuberculosis Control Program of India (RNTCP), are located within tertiary care centers, public hospitals, and teaching hospitals and follow national guidelines for the treatment of drug-resistant TB (*2*). The study participants included family proxies of deceased patients. For the purpose of data collection, family proxies included spouses, parents, adult children, siblings, or relatives involved in providing care to the deceased person.

MDR TB patients were started on a standardized second-line treatment regimen at DR-TB centers followed by ambulatory care in the community. Pretreatment clinical evaluations were required; these included chest radiograph; complete hemogram; liver, renal and thyroid function tests; HIV serology; screening for diabetes mellitus; calculation of body mass index; and for women, pregnancy tests. All patients were required to complete ≥7 days of inpatient treatment at DR-TB centers, where they began treatment for MDR TB in accordance with national guidelines (i.e., 6 months of kanamycin, levofloxacin, ethionamide, pyrazinamide, ethambutol, and cycloserine followed by 18 months of levofloxacin, ethionamide, ethambutol, and cycloserine). Patients who did not achieve culture conversion at 6 months, or who experienced culture reversion at any time during treatment, were considered to have poor treatment response and required reevaluation and second-line drug susceptibility testing. Throughout treatment, community-based directly observed treatment (DOT) providers monitored patients for adverse drug effects. DOT providers referred patients experiencing any major adverse drug reactions to appropriate health facilities. The management of existing comorbidities such as diabetes, liver or renal disease, and neurological or psychiatric disorders is also recommended by the RNTCP (*2*).

In accordance with national policy, district TB officers should conduct an audit for all deaths occurring among MDR TB patients to better understand potential programmatic gaps in TB diagnosis and treatment (*2*). However, TB death audits do not look into the underlying cause of death.

We developed a semistructured questionnaire as a verbal autopsy tool to elicit information about behavioral, demographic, socioeconomic, and comorbid conditions; detailed symptoms; and chronology of events preceding death, with the most recent condition first and the earliest (e.g., the condition that started the sequence of events between normal health and death) last. We also abstracted relevant clinical information from medical records and, when available from the family, the cause of death as documented on a death certificate. We abstracted the information on date of death and the date of initiation of treatment from programmatic management of drug-resistant TB treatment cards to calculate the median duration of MDR

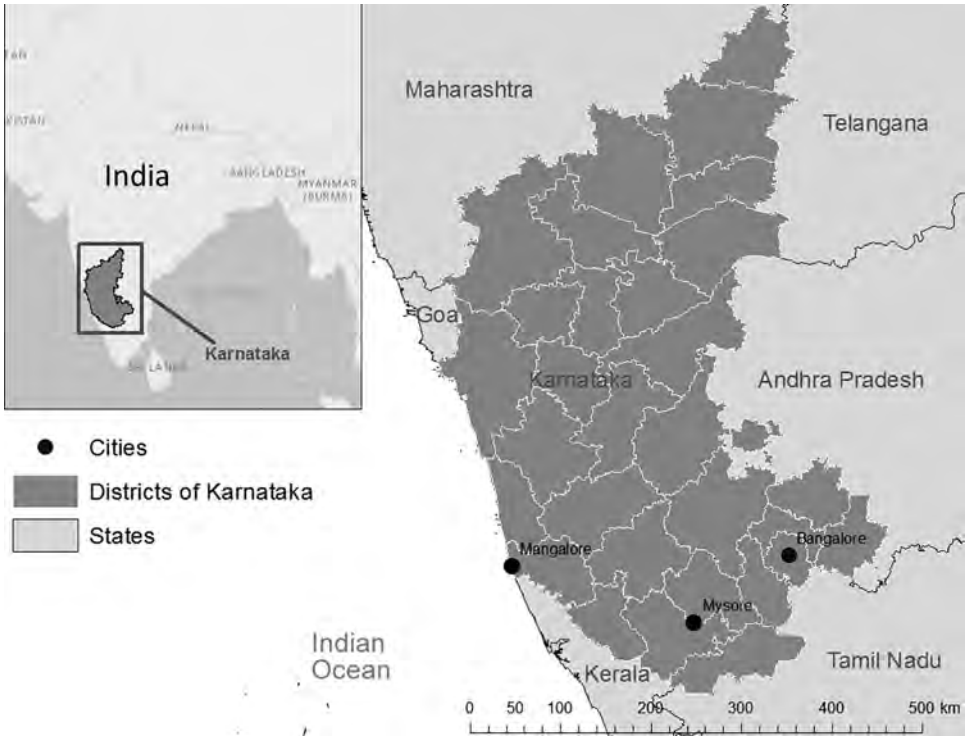

Figure. Locations of the 3 drug-resistant TB treatment centers in the state of Karnataka, India. Inset shows location of Karnataka in India.

TB treatment. A committee of 3 clinicians independently reviewed all available evidence to ascertain the underlying cause of death following national guidelines (*14*). A final assignment of the cause of death required the concurrence of ≥2 committee members. If 2 reviewers did not concur on cause of death, then they would meet in person to discuss their disagreement. The committee assigned the underlying cause of death as TB if it had initiated the sequence of illness events leading directly to death (*14*). If the underlying cause of death was not TB, then the committee also recorded what was believed to be the specific cause of death using codes from the International Classifications of Diseases, 10th Revision, as recommended by WHO (*15*). For patients with a cause of death not directly attributed to TB but that may have resulted from TB or TB treatment (e.g., drug-induced psychosis leading to suicide), we listed TB as an underlying cause of death.

Data Collection

We conducted proxy interviews during December 2016–March 2017. The principal investigator, who had prior experience in conducting verbal autopsy, trained a team of investigators to obtain clinical histories and document the sequence of events preceding death using the verbal autopsy tool. The field investigators prepared participants psychologically for the verbal autopsy process and provided appropriate grief counseling when needed. The principal investigator supervised the field investigators to ensure correct protocol implementation, made home visits to identify potential challenges, provided feedback, and recommended corrective action to ensure quality data collection.

Families with an annual income <10,000 Indian rupees are eligible for government supplemental support with Below Poverty Line (BPL) ration cards (*16*). We used BPL cards as a proxy for socioeconomic status of the families.

We included history of smoking and alcohol consumption of the deceased person in the proxy questionnaire. We did not quantify the number of cigarettes smoked, nor the amount of alcohol consumed, because these details were deemed not accurate through proxy.

Data Analysis

We used EpiData (EpiData Association, Odense, Denmark) to double enter, validate, and analyze quantitative data. We calculated simple proportions of selected demographic, behavioral, and clinical characteristics among cases with and without TB as the underlying cause. We used the χ^2 test to detect differences in proportions among cases with and without TB as the underlying cause of death and the Fisher exact test when individual cell counts were <5 (α<0.05). We calculated the duration of MDR TB treatment as the number of days from date of initiation of treatment to date of death. We abstracted missed doses of TB drugs from treatment cards and used medians and interquartile ranges (IQRs) to describe the number of doses missed and duration of MDR TB treatment. We compared medians between cases with and without TB as the underlying cause of death using a Mann-Whitney test.

Ethics Considerations

We obtained approval for the study protocol from the Yenepoya University Ethics Committee (Mangalore, India); Ethics Advisory Group of International Union Against Tuberculosis and Lung Disease (Paris, France); National Tuberculosis Institute (Bangalore, India); and US Centers for Disease Control and Prevention (Atlanta, GA, USA). We conducted proxy consent and verbal autopsies in the local language, Kannada. We ensured strict privacy and confidentiality during all participant encounters. All interviews occurred at participants' homes at a time convenient to them. Institutional-, district-, and state-level authorities granted appropriate permission before initiating the study.

Results

A total of 75 TB-related deaths occurred during the study period. For these, 72 family proxies (96%) consented to verbal autopsy. Most of the proxies were the spouse of the deceased (37%), followed by parents (16%), adult children (16%), siblings (16%), in-laws (10%), and other relatives (5%). In addition to verbal autopsy data available for 72 of the deceased patients, medical records for 8 patients were available from the family, and 4 patients had death certificates that their proxies provided during interviews. The death audit report was available for 2 patients.

The committee reviewed all available data and independently and unanimously assigned TB as the underlying cause of death for 49 (68%) patients. For the remaining 23 patients, the committee reached consensus after face-to-face deliberation (Table 1). The committee members assigned the underlying cause of death for 71 out of 72 patients. For 1 patient, there was insufficient evidence to assign a specific cause of death; we excluded this patient from further analysis. TB was the underlying cause for 77% (55/71) of the deaths (Table 2) (*14*). After extensive deliberations and discussions, the committee deemed TB or TB treatment the underlying cause of death for 6 patients who died of suicide. Suicidal ideation may be associated with TB because of psychological stress, social stigma, hopelessness, and depression caused by MDR TB diagnosis or treatment; in addition, cycloserine used to treat MDR TB may have caused psychosis or depression resulting in suicidal ideations (*17*,*18*). For 2 patients who died of pulmonary embolism, the committee decided that immobilization related to severe MDR TB disease predisposed them to pulmonary embolism and increased their risk of death.

Table 1. Medical conditions related to underlying cause of death for tuberculosis patients, Karnataka, India*

Consensus reached from verbal autopsy	Consensus reached after discussion
Breathlessness	HIV-related opportunistic infections
Chest pain	Acute myocardial infarction
Hemoptysis	Hypertensive heart disease
Cardiac arrest	History of cardiac enlargement
Productive cough	Bilateral foot edema suggestive of congestive cardiac failure
Loss of appetite	Hemorrhagic shock and anemia
History of stroke	Alcoholic gastritis
Mental confusion	Alcoholic liver disease
Death by hanging	Hepatic failure
Drowning	Sepsis due to tuberculosis
Acid consumption	Unilateral feet edema suggestive of pulmonary embolism
HIV/TB co-infection	Oral cancer

*A 3-member commission determined underlying cause of death after verbal autopsy (interviews and record review), or after in-person discussion if the members could not reach consensus.

We described the characteristics of the 71 MDR TB patients who died during treatment by underlying cause of death (Table 3). The mean age of the patients was 44 years (SD ± 14 years), and more than two thirds (69%) were male. Deaths with TB as the underlying cause were significantly less common (p = 0.011) among male patients (Table 3). The median (IQR) duration of treatment for patients with TB as underlying cause of death was 192 (101–365) days and for those without was 158 (76–459) days. The median (IQR) number of missed TB doses for patients with TB as underlying cause of death was 24 (6–60) and for those without was 20 (9–46). We saw no significant differences in the duration of treatment (p = 0.50) or the number of missed doses (p = 0.16) between patients with TB as underlying cause of death and those without.

Discussion

Our methods were consistent with the current national policy in India of conducting death audits by public health authorities to ascertain the cause of death and identify gaps in program implementation (*19*). We were not aware of previous reports of using verbal autopsy to ascertain the underlying cause of death among patients treated for MDR TB in India. Death rates among MDR TB patients treated in Maharashtra and Western India ranged 21%–30% (*20*,*21*). These reports defined TB-related deaths in accordance with the RNTCP treatment outcome variable (i.e., all-cause mortality) and may not reflect the true estimate of deaths attributable to TB. A recent study among a smaller cohort of HIV-infected MDR TB patients treated in Mumbai reported 4 (31%) of 13 deaths were attributed to causes not related to TB or treatment (*22*). These data suggest that the RNTCP may be overestimating TB-related deaths among MDR TB patients. In our study, we found that 23% (16/71) of patients who died during MDR TB treatment did not have TB as an underlying cause of death.

Our study had several strengths. We identified all TB patients in the national control program who died during the study period and had a high proxy response rate (96%). Therefore, selection bias was unlikely. In contrast to using the death audit method, which is not well-defined (*19*), we systematically gathered the medical history of

Table 2. Underlying cause of death for patients who died during treatment for multidrug-resistant tuberculosis, Karnataka, India*

Underlying cause of death (ICD-10 code)	No. patients, n = 72
Infectious diseases (A00–B99)	
Tuberculosis (A15)*	55
Diarrhea and gastroenteritis of presumed infectious origin (A09)	2
HIV disease resulting in infectious and parasitic infection (B20)	3
Neoplasms (C00–D48)	
Malignant neoplasm of other and unspecified parts of tongue (C02)	1
Disease of the nervous system (G00–G99)	
Hemiplegia (G81)	1
Diseases of the circulatory system (I00–I99)	
Hypertensive heart failure (I11)	1
Acute myocardial infarction (I21)	1
Hypotension (I95)	1
Diseases of the digestive system (K00–K93)	
Alcoholic gastritis (K29.2)	1
Peptic ulcer, site unspecified (K27)	1
Alcoholic liver disease (K70)	3
Acute hepatic failure, not otherwise specified (K72)	1
Symptoms, signs, and abnormal clinical and laboratory finding, not elsewhere classified (R00–R99)	
Other ill-defined and unspecified causes of mortality (R99)	1

*Underlying cause of death was assigned as TB if it had initiated the sequence of morbid events leading directly to death. ICD-10, International Classification of Diseases, 10th Revision.

Table 3. Characteristics associated with death in patients who died during treatment for MDR TB, Karnataka, India*

Characteristic	No. (%) for whom TB is the underlying cause of death	No. (%) for whom TB is not the underlying cause of death	χ^2 test result (p value)†
Total, n = 71	55 (77)	16 (23)	
Age, y			
15–30	9 (90)	1 (10)	3.06 (0.383)
31–45	22 (82)	5 (18)	
46–60	16 (76)	5 (24)	
61–75	8 (62)	5 (38)	
Sex			
M	33 (69)	15 (31)	6.44 **(0.011)**
F	22 (96)	1 (4)	
Socioeconomic status			
Above poverty line	5 (63)	3 (37)	1.49 (0.474)
Below poverty line	42 (81)	10 (19)	
Not available	8 (73)	3 (27)	
Smoking history			
Yes	20 (67)	10 (33)	3.73 (0.155)
No	33 (85)	6 (15)	
Don't know	2 (100)	0	
Alcohol consumption history			
Yes	18 (64)	10 (36)	4.71 (0.095)
No	36 (86)	6 (14)	
Don't know	1 (100)	0	
Diabetes			
Yes	11 (79)	3 (21)	0.012 (0.912)
No	44 (77)	13 (23)	
Hypertension			
Yes	11 (79)	3 (21)	0.012 (0.912)
No	44 (77)	13 (23)	

*The data do not include 1 patient whose underlying cause of death could not be ascertained. MDR, multidrug-resistant; TB, tuberculosis.
†Bold indicates statistically significant p values (p<0.05).

patients who had died by interviewing key informants and reviewing medical records and death certificates. These additional sources of information increased the total data available for each case that might otherwise be missed by reviewing medical records alone. Furthermore, we constituted an independent committee of medical specialists not affiliated with RNTCP to review all the available evidence and code the underlying causes of death, which reduced bias that may occur when TB program personnel conduct a death audit. Consensus among committee members increased the validity of our results. In addition, causes of death secondary to TB, such as treatment-related suicide and pulmonary embolism (*23*), enriched the qualitative aspects of TB-related deaths that could otherwise be missed.

Our study had some limitations. In India, as many as 2 million TB patients seek care in the largely unregulated private sector (*24*). We included only patients who received treatment from the national TB control program; thus, our study findings do not reflect all MDR TB patients in India. Unlike the RNTCP death audit, which focuses on identifying potential gaps in program implementation (*19*), our study focused only on ascertaining the medical cause of death. Therefore, we are unable to comment on the potential gaps in program implementation that may have precipitated death. Furthermore, as with all verbal autopsy studies, the robustness of the results depends on the accuracy of the information provided by key informants. We worked to minimize errors by following standard procedures, but it is possible that some errors may have occurred. We did not collect information on whether the deceased patients had undergone drug susceptibility testing for the second-line TB drugs and so are unable to comment if persons with more severe forms of TB disease were more likely to die of TB than those with less severe forms of TB.

Despite these limitations, the study has several implications for policy and practice. First, a substantial proportion of deaths during MDR TB treatment were not TB related. Our results suggest the national TB program may be overestimating TB case-fatality rates. Incorporating verbal autopsies as part of the death audit may help improve the accuracy of defining TB-related deaths. Second, suicide was common (n = 6) and attributed as secondary to TB disease or treatment of TB. These deaths were potentially preventable. Cycloserine is known to lead to psychiatric adverse reactions, including suicidal tendencies (*2*,*17*). WHO recently recommended that cycloserine be replaced with other oral second-line drugs (*25*). In addition, 4 patients had alcohol-related deaths. While these deaths were not considered secondary to TB, it is unclear if these patients also had depression. A population-based study in Kerala reported higher risk for poor treatment outcome, including death, among MDR

TB patients consuming alcohol (*26*). These data reinforce the need for professional counseling and psychiatric care integration for MDR TB care (*2*). A recent study assessed the feasibility of integrated psychiatric and medical TB care and treatment and suggested the immediate need in India (*27*). Third, our finding that male sex was associated with deaths due to causes other than TB may be helpful in generating hypotheses for further research, such as risk-factor analysis in a larger, more representative cohort of patients throughout India. Finally, we acknowledge that many TB-related deaths may occur before the start of MDR treatment, after completion of MDR treatment, and among persons lost to follow-up. We are hopeful our findings will stimulate further research to document all potential TB-related deaths in the community and aid in monitoring India's progress toward reducing TB deaths by 95% by 2035 (*28*).

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Increasing Prevalence of Nontuberculous Mycobacteria in Respiratory Specimens from US-Affiliated Pacific Island Jurisdictions[1]

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Nontuberculous mycobacteria (NTM) respiratory infections represent a growing public health problem in many countries. However, there are limited published epidemiologic studies for the Western Pacific region. We reviewed respiratory specimens submitted to Diagnostic Laboratory Services in Hawaii, USA, for culture of *Mycobacterium tuberculosis* during August 2007–December 2011 to determine the NTM isolation rate. We observed a statistically significant increase in the rate of specimens with NTM isolated in respiratory culture (adjusted rate ratio per year 1.65, 95% CI 1.54–1.77; p<0.01). In contrast, the number of patients with respiratory cultures positive for *M. tuberculosis* showed no increase (adjusted rate ratio per year 0.98, 95% CI 0.94–1.01; p = 0.19). A 6-month subset of NTM isolates was identified by using a nucleic acid probe or 16S rRNA sequencing. *M. avium* complex and *M. fortuitum* were the most common NTM identified.

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and have been identified repeatedly from the soil and municipal and other water supplies by using both molecular and traditional methods (*1*–*3*). NTM might vary in their pathogenicity, but are most commonly associated with pulmonary infections (*4*). The incidence of NTM pulmonary infections is increasing and is an emerging public health concern (*5*–*8*). It has been proposed that the increased use of piped, chlorinated water might serve as a common thread between the increasing incidence of NTM pulmonary disease in the industrialized and developing worlds, especially those infections caused by the *Mycobacterium avium* complex (MAC) (*9*). The resistance of NTM to chlorine might enable NTM to have a selective advantage in their microenvironment (*2*). NTM have also been isolated from shower heads, which provide a moist and conducive environment for NTM to grow and form biofilms. Consequently, NTM might be aerosolized and then inhaled (*10*), thereby causing pulmonary infection and disease in susceptible persons. Use of more accurate detection methods has also contributed to a documented increase in NTM-associated disease (*6*).

The incidence and prevalence of NTM and associated respiratory disease is unknown for US-affiliated Pacific Islands (USAPI). The specific islands or atoll chains involved in this study are composed of 6 jurisdictions that have formal relationships with the United States: the territories of American Samoa and Guam, the Commonwealth of the Northern Mariana Islands, and the Freely Associated States. These states are the Republic of Palau, the Republic of the Marshall Islands, and the Federated States of Micronesia (the island states of Chuuk, Kosrae, Pohnpei, and Yap) (*11*). The United States and each of the Freely Associated States have a Compact of Free Association that provides citizens of these nations the rights of entry, residence, and employment without being subjected to the usual health screening required for new immigrants from other countries (*12*).

The Centers for Disease Control and Prevention (CDC) has been actively involved in the prevention and control of

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several infectious diseases, including tuberculosis, in the USAPI. In 2010, the USAPI regional rate of infection with *M. tuberculosis* (MTB) (106.0 cases/100,000 persons) was >12 times the rate in Hawaii (8.8 cases/100,000 persons) and almost 30 times the US national rate (3.6 cases/100,000 persons) (*13*). In contrast, there is no systematic monitoring of NTM-related respiratory infections, and thus there is no information on the epidemiology and potential role of NTM in respiratory disease in the USAPI.

Reports from other regions indicate that the epidemiology of NTM can be highly variable (*6*). Patients with previous pulmonary MTB infections are also at an increased risk for development of pulmonary NTM infections. This finding might be caused by structural lung damage arising from pulmonary MTB or because of host defense or immune defects (*4*).

We conducted this study to investigate the prevalence of NTM isolation in respiratory specimens from the USAPI. Description of the prevalence of NTM pulmonary isolates and disease in the USAPI can have major implications in screening MTB disease and NTM disease in this region and in regions to which patients immigrate.

Methods

Specimen Collection

Diagnostic Laboratory Services (DLS) (Aiea, HI, USA) performed specimen testing for acid-fast bacilli (AFB) under the scope of a federal contract; Guam was not included in this contract during the years studied and is not included in this analysis. The primary focus of the contract was on enabling effective MTB control through the delivery of laboratory and diagnostic services to the USAPI. Respiratory specimens consisting of either 2 or 3 expectorated sputum samples were collected within a 24-hour period according to recommendations of the World Health Organization or CDC. Only MTB complex was definitively identified; all other *Mycobacterium* spp. were reported to the respective USAPI as NTM without further identification. Samples were obtained primarily from patients undergoing evaluation for possible MTB disease either because of tuberculosis symptoms or from close contact with patients with infectious MTB disease.

Laboratory Testing

Specimens were digested by using the standard NALC-NaOH method, followed by centrifugation (*14*). A portion of the sediment was then used to prepare an AFB smear. The remaining sediment was resuspended and used to inoculate 7H9 liquid medium (BACTEC MGIT 960; Becton Dickinson, Franklin Lakes, NJ, USA), broth medium, and solid medium (Middlebrook 7H10 agar slant; Becton Dickinson). The broth medium was incubated in a MGIT 960 semiautomated system (Becton Dickinson) and the 7H10 agar slant was incubated at 35°C in an incubator containing 5% CO_2. The broth was monitored for mycobacterial growth for ≤6 weeks, and the 7H10 agar slant was monitored for 8 weeks.

The prepared smear was heat-fixed at 70°C–80°C for 2 h. The smear was then stained by using the fluorochrome stain method, which used auramine O, a decolorizing solution, and a potassium permanganate counterstain and read by using a fluorescent microscope. All AFB smear-positive specimens were analyzed by using the Gen-Probe Amplified Mycobacterium Direct Test (Hologic, Marlborough, MA, USA) for nucleic acid amplification testing specific for the MTB complex.

All AFB-positive cultures were also tested for MTB by using the MTB AccuProbe System (Hologic), which is used to directly identify the mycobacterial species in MTB-positive cultures (i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*). If the AFB-positive culture was negative for MTB by MTB AccuProbe, the isolate was routinely reported only as an NTM without speciation according to the CDC contract.

Subset Analysis

All consecutive NTM-positive respiratory specimens collected during May–November 2010 were further analyzed to identify the species of NTM from the region. A total of 35 respiratory specimens, all from different patients, were positive for NTM during that period. Samples exhibiting features of MAC by light microscopy were initially tested by using the AccuProbe *Mycobacterium avium* Complex Test (Hologic). This nucleic acid test identifies mycobacterial species in the MAC (e.g., *M. avium*, *M. intracellulare*, and others). Specimens with negative results for MAC by AccuProbe were then further identified by using 16S rRNA sequencing (MicroSeq 500-bp 16S rDNA and MicroSeq ID Analysis software; Thermo Fisher Scientific, Waltham, MA, USA) as described by Hall et al. (*15*). NTM identification by 16S rRNA sequencing was analyzed by using RipSeq software (Pathogenomix, Santa Cruz, CA, USA) and a corresponding database (*16*).

Epidemiologic and clinical data were obtained on the described subset of patients by paper chart review at respective MTB treatment centers. Data were successfully obtained for 29 (82.9%) of 35 patients. This information was relayed back to DLS in a secure manner and deidentified before analysis. This study was approved by the institutional review board of the Queen's Medical Center and each of the involved USAPI jurisdictions.

Statistical Analysis

Descriptive statistics included frequencies and prevalence percentages calculated by participant characteristics and

calendar year. The outcome variable was a positive mycobacteria culture. Mycobacteria cultures were further defined as NTM or MTB. Patients with multiple positive cultures of the same mycobacterial species (i.e., MTB or NTM) were counted only once. Patients who were subsequently positive for a different species of mycobacteria (e.g., positive for MTB in 2008 and NTM in 2010) were counted as having 2 separate cases. Variables of interest were calendar year, age, sex, and geographic site. We calculated annual and overall period prevalence for each case group by using population data obtained from the US Census Bureau. We used Poisson regression models to estimate rate ratios of positive mycobacteria cultures per year for NTM and MTB. We further adjusted all estimates by age group, sex, and geographic site to determine the relative prevalence of positive cultures. Results of the regression models are expressed as relative rates with 95% CIs. Analyses included unadjusted models and models adjusted for age or for age, sex, and geographic sites. A p value <0.05 was considered statistically significant. We performed analyses by using SPSS software (IBM Corporation, Armonk, NY, USA) and SAS software (SAS Institute, Cary, NC, USA).

Results

A total of 15,811 respiratory specimens collected from 5,807 patients were sent to DLS for AFB smear and culture during August 2007–December 2011. A total of 998 patients had ≥1 AFB-positive respiratory culture: 675 (67.6%) were MTB positive and 323 (32.4%) were NTM positive. Five patients were classified as being co-infected.

We obtained baseline characteristics for patients from the various regions of the USAPI (Table 1). Patients with NTM respiratory isolates were older than the overall USAPI population (median age 34.8 years vs. 24.0 years), but a similar proportion were female (49.2% vs. 49.1%). MTB-positive patients were also older than NTM-positive patients (median age 42.6 years vs. 34.8 years); however, sex was similar (49.3% female vs. 49.2% male).

The rate of NTM isolation increased from 0.5% of patients screened in 2007 to 11.3% of patients screened in 2011. Conversely, the rate of MTB detection in the population screened remained relatively stable but showed a statistically significant change from 11.5% in 2007 to 13.7% in 2008 (p = 0.001) (Table 2). However, there was no major change thereafter.

We observed an increasing trend in annual prevalence of patients who were NTM culture positive from August 2007 (i.e., extrapolated for 2007) through December 2011 (Figure). This prevalence increased from 2 cases/100,000 persons in 2007 to 48 cases/100,000 persons in 2011 (adjusted rate ratio 1.65, 95% CI 1.54–1.77; p<0.01). In contrast, annual prevalence of MTB remained relatively stable over the same period and fluctuated from 43 cases/100,000 persons in 2008 to 58 cases/100,000 population in 2010 (adjusted rate ratio 0.98, 95% CI 0.94–1.01; p = 0.19) (Table 3).

The overall period prevalence of NTM isolation for our study period (August 2007–December 2011) was 106 cases/100,000 persons. Age data were available for 244 (75.5%) of 323 patients with NTM-positive specimens. Prevalence for persons 50–64 years of age (242 cases/100,000 persons) and for persons >64 years of age (278 cases/100,000 persons) was more than double the overall prevalence. Prevalence of NTM isolation varied greatly for USAPI regions; the lowest rate was observed in American Samoa (22 cases/100,000 persons) and the highest rate was observed in the Federated States of Micronesia (164 cases/100,000 persons).

The cumulative rate for MTB isolation over the same period was 221 cases/100,000 persons. This rate was highest for persons 50–64 years of age (330 cases/100,000 persons) and similar for persons 15–49 years of age (229 cases/100,000 persons) and persons >64 years of age (211

Table 1. Baseline characteristics of patients from various regions in the US-Affiliated Pacific Islands with respiratory tract culture specimens tested for AFB, August 2007–December 2011*

Characteristic	Total population†	AFB positive		AFB negative
		NTM	MTB	
Total	304,542 (100)	323 (0.1)	675 (0.2)	4,804 (2)
Median age, y	24.0	34.8	42.6	39.9
Sex, %				
M	50.9	50.8	50.7	54.4
F	49.1	49.2	49.3	45.6
Island nations				
American Samoa	55,529 (18.2)	12 (3.7)	10 (1.4)	120 (2.5)
Commonwealth of the Northern Mariana Islands	55,121 (18.2)	29 (9.0)	87 (12.8)	522 (11.5)
Federated States of Micronesia	107,154 (35.1)	176 (54.5)	266 (39.4)	2,140 (44.5)
Palau	20,879 (6.8)	32 (9.9)	51 (7.5)	882 (18.4)
Republic of the Marshall Islands	65,859 (21.6)	74 (22.9)	261 (38.6)	1,140 (23.7)

*Values are no. (%) patients unless otherwise indicated. American Samoa, Federated States of Micronesia, and the Commonwealth of the Northern Mariana Islands reported 2, 2, and 1 patients with co-infections, respectively. Specimens from Guam were not included in specimens provided to Diagnostic Laboratory Services during this period. AFB, acid-fast bacilli; MTB, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria.
†Data were obtained from https://www.census.gov/population/international/data/idb/informationGateway.php.

Table 2. Age- and site-adjusted relative rates of positive cultures by year, US-affiliated Pacific Islands, August 2007–December 2011

Bacteria and year	No. patients screened	Positive cultures, %	Adjusted relative rates (95% CI)*	p value
Nontuberculous mycobacteria				
2007†	595	0.53	1 (referent)	
2008	996	3.31	7.53 (3.20–17.71)	<0.001
2009	1,224	5.99	14.22 (6.24–32.36)	<0.001
2010	1,556	6.37	19.48 (8.62–44.05)	<0.001
2011	1,436	11.32	29.04 (12.89–65.41)	<0.001
Mycobacterium tuberculosis				
2007†	595	11.54	1 (referent)	
2008	996	13.71	1.40 (1.15–1.69)	0.001
2009	1,224	12.41	1.14 (0.95–1.36)	0.17
2010	1,556	12.36	1.05 (0.88–1.25)	0.60
2011	1,436	11.67	1.07 (0.89–1.28)	0.45

*Adjusted for age and geographic sites.
†August–December 2007.

cases/100,000 persons). The highest rate was observed in the Republic of the Marshall Islands (396 cases/100,000 persons), more than 20-fold higher than the rate in American Samoa (18 cases/100,000 persons). We also determined period prevalence for the rest of the island nations and stratified prevalence by age groups (Table 4).

Subset Analysis with NTM Identification

A total of 35 consecutive patients who had respiratory specimens were positive for NTM during May 31–November 12, 2010. We further identified specimens to the species level by using AccuProbe MAC or 16S rRNA sequencing. We conducted database comparison by using GenBank or RipSeq (Pathogenomix). The mycobacterial species identified in these respiratory specimens were 11 (31.4%) MAC (including *M. intracellulare* and *M. chimaera*), 7 (20.0%) *M. fortuitum*, 5 (14.3%) *M. gordonae*, 2 (5.7%) *M. abscessus/chelonae*, 2 (5.7%) *M. parascrofulaceum/M. fortuitum*, 1 (2.9%) *M. kansasii*, 1 (2.9%) *M. florentinum*, 1 (2.9%) *M. mucogenicum*, 1 (2.9%) *M. paraffinicum*, 1 (2.9%) *M. simiae*, and 1 (2.9%) *M. terrae*. Two specimens were positive for >1 NTM species: 1 (2.9%) *M. fortuitum* and *M. triplex*, and 1 (2.9%) *M. fortuitum* and *M. gordonae*.

By paper chart review, we obtained clinical data for 29 of 35 patients with sputum samples positive for NTM during May 12–November 12, 2010. Median age for these patients was 51 years (range 8–86 years). Nineteen (65.5%) of 29 responders were male, median height was 163 cm, and median weight was 59 kg. Most patients were unemployed, retired, or did not provide any information on their occupation. Self-reported ethnicity was 86.2% (25/29) Pacific Islanders, 6.9% (2/29) Filipinos, 3.4% (1/29) Chinese, and 3.4% (1/29) unknown.

Chronic cough, sputum production, weight loss, and fatigue/malaise were the most commonly reported signs or symptoms. The most common concurrent diagnosis involving the lung was chronic obstructive pulmonary disease, which affected 8 (27.6%) of 29 patients for whom clinical data were collected. A total of 6 (31.0%) of 29 patients were current smokers, and 3 (10.3%) of 29 had

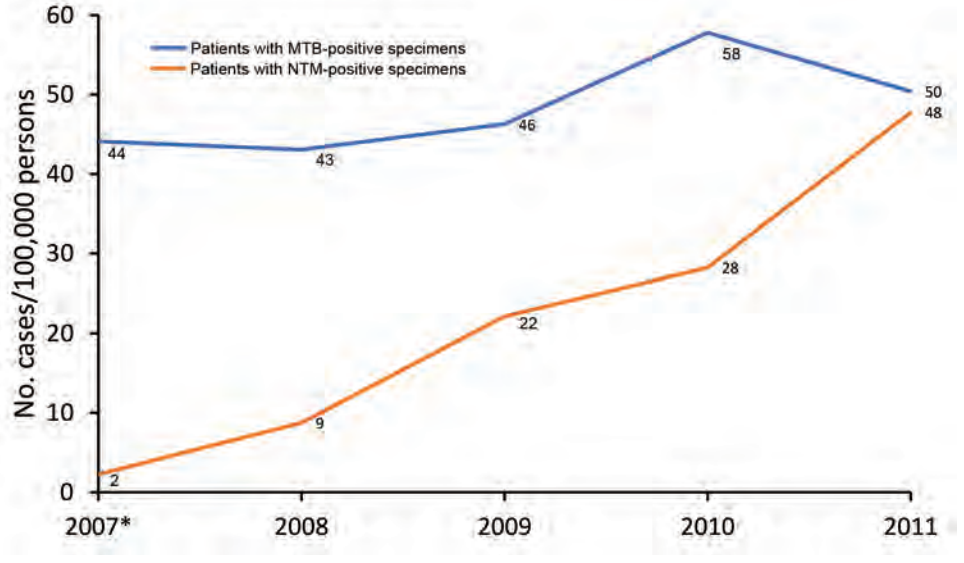

Figure. Prevalence of positive test results for NTM and MTB in respiratory specimens from patients in US-affiliated Pacific Island jurisdictions, 2007–2011. *Data for 2007 were extrapolated from data for August–December 2007. MTB, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria.

Table 3. Rate ratios for positive mycobacteria cultures per year in US-affiliated Pacific Islands, August 2007–December 2011

	Unadjusted		Adjusted*	
Variable	Rate ratio per year (95% CI)	p value	Rate ratio per year (95% CI)	p value
Negative culture	1 (referent)		1 (referent)	
Mycobacterium tuberculosis	0.98 (0.93–1.04)	0.55	0.98 (0.94–1.01)	0.19
Nontuberculous mycobacteria	1.57 (1.43–1.74)	<0.001	1.65 (1.54–1.77)	<0.01

*Adjusted for age, sex, and site.

been given a diagnosis of diabetes mellitus. There was a paucity of radiologic investigations available for USAPI patients; only 2 patients had chest radiographs performed. Radiographic findings showed nodular lesions and 1 cavitary lesion (*1*) and multiple cavitary lesions with bronchiectasis (*1*). Thirteen of the 29 patients had received empiric therapy for MTB.

Discussion

Data for this study identified major epidemiologic trends and differences in the prevalence of NTM isolation in the USAPI. We found a major increase in annual prevalence of patients with NTM respiratory isolates in the USAPI during August 2007–December 2011. The annual prevalence of NTM isolation in 2011 (48 cases/100,000 persons) is similar to recently published data for Hawaii (*8*).

Increased testing, as shown by gradually increased number of patients screened, probably contributed to the upward trend in NTM isolation. However, the rate of increase of NTM isolation seems out of proportion to the modest increase in the number of patients screened. It is possible that actual prevalence of NTM respiratory diseases is increasing in this region in a manner similar to that of neighboring Hawaii (*8*) because most patients had respiratory signs and symptoms and were suspected to have MTB infections. Our findings are also consistent with those reported for the continental United States, where the prevalence of NTM pulmonary isolates has been shown to be increasing (*6*,*7*).

The high prevalence of NTM infections in the USAPI might also be attributable to environmental conditions in the area. Adjemian et al. postulated that soil composition and greater persistence of moisture droplets in the air contributed to the increased prevalence of NTM infection in Hawaii (*8*). It is likely that similar environmental conditions are present in the USAPI, although specific and systematic environmental sampling studies will need to be conducted to confirm this possibility.

The increase in NTM infections in other countries has been associated with urbanization and increased availability of piped water. The urban population growth rate during 2010–2015 of the various study regions was –0.1% for American Samoa, 0.3% for the Federated States of Micronesia, 0.4% for the Commonwealth of the Northern Mariana Islands, 0.6% for the Republic of the Marshall Islands, and 1.7% for Palau (*17*). Although information about access to piped water for our patients is not available, the stable urban/rural makeup of the population in these regions makes access to piped water an unlikely cause for the increased prevalence of NTM.

The prevalence of NTM isolation showed major variations for different island nations. Although the urbanization rate did not change much over our collection period, the degree of urbanization within the different nations might be contributing to differences in prevalence for the nations. The Federated States of Micronesia, which had the highest period prevalence of NTM isolation, also had the lowest percentage of its population living in urban areas (22.4%) (*17*). Conversely, American Samoa, which had the lowest prevalence of NTM isolation, also had a relatively high urban population (87.2%). Variations in the percentage of urban dwellers in the other regions were

Table 4. Period prevalence of persons with ≥1 AFB-positive culture in US-affiliated Pacific Islands, August 2007–December 2011*

Characteristic	Total population	NTM	MTB
Territory			
American Samoa	55,529	12 (22)	10 (18)
Commonwealth of the Northern Mariana Islands	55,121	29 (53)	87 (158)
Federated States of Micronesia	107,154	176 (164)	248 (266)
Palau	20,879	32 (153)	51 (244)
Republic of the Marshall Islands	65,859	74 (112)	261 (396)
Total no. cases	304,542	323 (106)	675 (221)
Cases with age data, no. (%)	NA	244 (75.5)	541 (80.1)
Age, y			
0–14	101,812	31 (30)	44 (43)
15–49	158,365	102 (64)	363 (229)
50–64	33,932	82 (242)	112 (330)
>64	10,433	29 (278)	22 (211)

*Values are no. with positive specimens (rate/100,000 persons) except as indicated. Population data were obtained from the US Census Bureau (http://www.census.gov/population/international/index.html) by using the midyear population in 2010. MTB, *Mycobacterium tuberculosis*; NA, not applicable; NTM, nontuberculous mycobacteria.

less drastic: 72.7% for the Republic of the Marshall Islands, 87.1% for Palau, and 89.2% for the Commonwealth of the Northern Mariana Islands. More studies are needed to evaluate the relative contribution of each factor to the rate of NTM isolation and disease.

The highest NTM isolate rates were observed for older persons (those 50–64 years of age and those >64 years of age). This finding is consistent with results from another study (*6*).

NTM identification in the limited subset analysis showed numerous NTM with variable pathogenicity. Although MAC was the most commonly isolated NTM species in our study, its isolation was not as common in the USAPI as it was in North America and Europe (*4*,*18*). Rapidly growing mycobacteria, in particular *M. fortuitum*, appear to be comparatively more prevalent in the subset. This finding is supported by epidemiologic studies in Asia, which suggested that Pacific Islanders, similar to Asians, might have an ethnic susceptibility to rapidly growing mycobacteria. Subset analysis in our study suggests that the predominant NTM species in the USAPI might be similar to the NTM species reported from Asia (*18*).

Almost half of the subset patients surveyed had evidence of treatment for infection with MTB. It is common to start effective MTB therapy in patients with a high pretest probability of MTB. However, only 2 of these patients had chest radiographs performed. This finding is a reflection of limited resources available in clinics in this region. In these patients, routine use of direct nucleic acid amplification testing (e.g., GeneXpert MTB/RIF; Cepheid Inc., Sunnyvale, CA, USA) for AFB-positive smears might provide more support for final diagnosis and treatment options.

In contrast to findings for NTM, the prevalence of patients with MTB-positive respiratory cultures remained relatively stable over the study period. The Marshall Islands have been identified as having the highest rate of tuberculosis infections in the Western Pacific islands (and one of the highest rates worldwide); this rate has been linked to high rates of diabetes in the region (*19*). The epidemiology of MTB in the region has been extensively discussed (*13*,*20*,*21*).

This study had several limitations. First, the study used microbiological data for patients who were suspected to have MTB infection and thus might not be representative of the entire population. However, the most common signs and symptoms of NTM infection (chronic or recurring cough, fatigue, malaise, dyspnea, fever, hemoptysis, chest pain, and weight loss) are not dissimilar from those with MTB infection (*4*). Furthermore, because testing was not performed primarily to detect NTM infection, it is more likely that our study underestimated the prevalence of NTM isolation, and that the actual prevalence is probably higher. Second, although the number of patients from whom respiratory samples were collected remained relatively stable, the total number of respiratory specimens submitted increased over time. This limitation might have contributed to the increasing trend of NTM-positive respiratory specimens in our data. Third, data collection systems in the region were rudimentary, and only sex and age data were provided to DLS during specimen collection. Fourth, identification of NTM-positive respiratory specimens was not routinely performed, and nonpathogenic species, such as *M. gordonae*, were not excluded, potentially overestimating the prevalence of NTM respiratory infections in the region.

Identification data were obtained for a limited subset of 35 patients, and further studies should be conducted to identify the composition of subspecies from the region. Clinical and epidemiologic data for this group are incomplete and cannot be used to make meaningful conclusions about the rest of the study population.

In conclusion, the prevalence of patients with NTM respiratory isolates from the USAPI is higher than that reported in data for the continental United States; this rate steadily increased during 2007–2011. Although these data were obtained from a cohort suspected to have MTB infection and might not be representative of the general population, the high proportion of patients with suspected MTB that cultured NTM has major clinical and public health implications. A limited subset analysis in this study suggested that there might be relatively fewer MAC isolates and a greater number of *M. fortuitum* isolates in the USAPI than in North America and Europe. Further studies and more data are required to increase our understanding of NTM infection in this region.

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Use of Genome Sequencing to Define Institutional Influenza Outbreaks, Toronto, Ontario, Canada, 2014–15

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Adequacy of the current clinical definition of institutional influenza outbreaks is unclear. We performed a retrospective genome sequencing and epidemiologic analysis of institutional influenza outbreaks that occurred during the 2014–15 influenza season in Toronto, Canada. We sequenced the 2 earliest submitted samples positive for influenza A(H3N2) from each of 38 reported institutional outbreaks in long-term care facilities. Genome sequencing showed most outbreak pairs identified by using the current clinical definition were highly related. Inclusion of surveillance samples demonstrated that outbreak sources were likely introductions from broader circulating lineages. Pairwise distance analysis using majority genome and hemagglutinin-specific genes enabled identification of thresholds for discrimination of within and between outbreak pairs; the area under the curve ranged 0.93–0.95. Routine genome sequencing for defining influenza outbreaks in long-term care facilities is unlikely to add significantly to the current clinical definition. Sequencing may prove most useful for investigating sources of outbreak introductions.

Current definitions for influenza outbreaks in hospitals or chronic/long-term care facilities (LTCFs) are ill-defined, being typically based on ≥2 symptomatic patients in a 48–72-hour period and ≥1 microbiologic sample documented as positive for influenza (*1*,*2*). However, this definition does not conclusively determine whether transmission events have occurred within the institution or if a linked outbreak is emerging. Influenza outbreaks in hospitals and LTCFs are associated with significant rates of illness and death (*3*). Annually, 1% of adults >65 years of age in North America are hospitalized because of influenza symptoms, and case-fatality rates have been reported at 50% in some groups (*4*). Outbreak prevention measures, including chemoprophylaxis and traditional infection control approaches, have demonstrated benefits in confirmed outbreaks (*5*). However, these benefits are balanced by resource expenditures, use of chemoprophylaxis in uninfected persons, and potentially detrimental interruptions in care introduced by infection prevention and control measures (*6*). The 2014–15 influenza season in North America was dominated by influenza A(H3N2) but had a poor vaccine match and efficacy to the circulating H3N2 strain, and high case numbers were counted (*7*). The US Centers for Disease Control and Prevention categorized the season as moderately severe and among the longest seasons in the previous decade (*8*).

By using currently employed direct fluorescent antibody or PCR diagnostic techniques, it is often impossible to determine whether detected influenza strains in an outbreak are related. It is probable that some outbreaks represent independently introduced cases, without direct transmission. Even in large institutional outbreaks of influenza, multiple strain types can be introduced (*9*). Although the purpose of identifying outbreaks and enacting infection control measures in facilities is to limit linked transmission (*1*), the identification of an outbreak may be more of a reflection of the force of infection in the population in general at that time: 25%–50% of LTCFs report ≥1 influenza outbreak annually (*4*). If facility outbreak rates mirror those of outbreaks in the general population, measures to limit transmission in the facility are expected to have limited benefit because cases are actually introduced from outside. Despite the aim of outbreak infection control measures to reduce institutional transmission, we have a limited understanding of the adequacy of the broadly applied clinical definition of influenza outbreaks created for identification of transmission. Genome sequencing has the potential to discern differences in influenza strains and clarify related and unrelated strains (*10*).

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Genome sequencing is increasingly being incorporated into clinical care, including outbreak investigations and infection control (*11*–*13*). Recent technological advancements offer increasingly portable and rapid tools that have the potential to revolutionize clinical microbial diagnostics (*14*). However, to date, the role of genome sequencing in defining institutional influenza outbreaks has not been systematically evaluated. Here, we evaluate whether influenza genome sequencing could improve understanding of the utility of current influenza outbreak definitions and whether it could play a role in routine outbreak identification.

Materials and Methods

We conducted a retrospective analysis of influenza outbreaks in LTCFs comprising chronic care hospitals, long-term care institutions, and retirement homes across the city of Toronto, Ontario, Canada, during the 2014–15 influenza season. We performed genome sequencing on 38 pairs of influenza-positive outbreak samples that had been collected and sampled prospectively throughout the outbreak season. Pairs of samples for each evaluated outbreak constituted the 2 earliest positive samples for influenza A(H3N2) from each outbreak, provided the outbreak had ≥2 adequate influenza-positive samples. Samples submitted from suspected outbreaks were initially screened by real-time reverse transcription PCR (RT-PCR). The Public Health Ontario research ethics board in the province of Ontario, Canada, approved this study.

Influenza Season Epidemiology

We used prospectively collected data on influenza A(H3N2) outbreaks occurring in Toronto during the 2014–15 influenza season, which included the following variables: outbreak number, outbreak size, date of outbreak onset, and date of submitted samples. Publicly available information on weekly totals of PCR-confirmed influenza A–positive samples tested at Public Health Ontario Laboratory (PHOL), the reference microbiology laboratory for Ontario, were also included (*15*).

Sequencing

We sequenced primary clinical specimens. For each sample, total nucleic acid was extracted on the easyMag extraction system (bioMérieux, Saint-Laurent, Quebec, Canada) by using 250 μL of sample with 25 μL eluate. We used universal primers (MBTuni-12/13) (*16*) to amplify influenza A–specific RNA (Superscript III One-Step RT PCR; Thermo Fisher Scientific Inc., Waltham, MA, USA). We fragmented amplicons and tagged them with sequencing adapters by using the Nextera XT DNA Library Kit (Illumina, San Diego, CA, USA) and run on an Illumina MiSeq sequencing system. Due to insufficient coverage of the polymerase basic (PB) 1 (segment 2) gene across many samples (mean read depth in sample with lowest coverage was 0.42×), we excluded this segment from the genome analysis. Mean coverage depth for all segments (excluding segment 2) was 2,800×. We generated consensus sequences on the basis of the most common nucleotide for a given position. In this study, we define majority genome as sequences containing all influenza genome segments except segment 2. Sequences were aligned to an influenza A(H3N2) isolate from Switzerland (GISAID accession nos. EPI614438–EPI1614444 and EPI680123; http://www.gisaid.org) by using Bowtie2 (*17*). We used Samtools and mpileup (*18*) to call single-nucleotide polymorphisms to the reference, and we used bcftools (*18*) and vcfutils (*19*) for further assembly.

Additional Surveillance Samples

In addition to the outbreak samples, we also evaluated the hemagglutinin (HA) sequences of 13 influenza A(H3N2)–positive samples available from the same season that were not associated with these LTCF outbreaks. To eludicate the changes occurring in circulating community influenza lineages throughout the influenza season, the PHOL performs prospective surveillance by randomly selecting submitted samples for sequencing, originating from all possible sources including outpatient and inpatient care. The 13 samples selected for this analysis represent a subset of these samples that were analyzed directly from clinical specimens and had adequate HA sequence data. For these surveillance samples, we sequenced HA by using Sanger sequencing (*20*) by using the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3730XL genetic analyzer (both from Applied Biosystems). We aligned consensus surveillance HA sequences to the H3N2 isolate from Switzerland by using MAFFT (*21*). All positions containing missing data were eliminated. We submitted all 76 majority genome outbreak sequences and surveillance HA sequences to GenBank (accession nos. MF806611–MF807155).

Phylogenetic Analysis

We inferred evolutionary history by maximum likelihood analysis. We used PhyML (SeaView version 4.6.1, http://doua.prabi.fr/software/seaview) with a general time-reversible model and approximate likelihood ratio test branch support, where the tree with the greatest log likelihood was retained. We generated initial trees by using BioNJ (*22*) with optimized tree topology. We used FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/) to manipulate the phylogenetic trees and to root to the outgroup influenza A(H3N2) isolate from Switzerland. The trees were drawn to scale (with scale bars noted) and branch length as a function of substitutions per site. We generated phylogenetic trees for both majority genome (including all outbreak pairs), as well as specifically the HA gene (including all outbreak pairs and the surveillance samples).

Pairwise Distance Analysis

For all genes (majority genome and HA gene, including surveillance samples), we calculated pairwise distances within-outbreak sample pairs and between-outbreak sample pairs (random sample from each pair) by using MEGA-CC version 7.0.18 (*23*). We calculated pairwise distances as total number of single-nucleotide polymorphisms per base pair over the entire alignment. For between-outbreak pairs, we matched outbreaks to the most temporally coincident outbreak. The median and interquartile range of time between processing times of temporally coincident outbreak samples were 0 and 1 day, respectively.

Results

During the 2014–15 influenza season (October 15, 2014–March 23, 2015), a total of 108 influenza A(H3N2) outbreaks in Toronto healthcare institutions were laboratory-confirmed at PHOL. Of those, ≥2 positive H3N2 samples were confirmed in 87 outbreaks, and samples from 38 outbreaks had adequate volumes and primer amplification to be suitable for genome sequencing, and were selected for analysis. Of these analyzed outbreaks, 2 occurred in chronic care hospitals, 31 in long-term care institutions, and 5 in retirement homes. Total outbreak case counts in the analyzed outbreaks were 5–67 persons. The first outbreak analyzed occurred on November 24, 2014, and the last occurred on March 23, 2015. The temporal distribution of the 108 outbreaks, along with the 38 analyzed outbreaks and citywide positive influenza sample counts, are shown in Figure 1. The median time from recognition of a potential outbreak to the first sample processing was 1 day. The median time between the first and second samples for each outbreak pair was 0 days.

We constructed phylogenetic trees for the majority influenza genome (Figure 2, panel A, https://wwwnc.cdc.gov/EID/article/24/3/17-1499-F2.htm) and the HA gene alone (Figure 2, panel B). Surveillance samples with previously sequenced HA genes were included in the HA phylogenetic analysis (Figure 2, panel B).

Histograms of pairwise distances for within-outbreak pairs and between contemporaneous outbreaks are shown in Figure 3. Pairwise distances for majority genomes ranged 0–0.003 for pairs within outbreaks and 0.0002–0.016 for pairs between outbreaks. For the HA gene, pairwise distances ranged 0–0.0018 for pairs within outbreaks, and 0–0.011 for pairs between outbreaks. A receiver operator characteristic curve (ROC) analysis (Figure 4) for differentiating within- and between-outbreak pairs identified an optimal cutoff for pairwise distances in majority genome analysis of 0.0005, giving an area under the curve (AUC) of 0.95 (95% CI 0.89–1.00) and sensitivity and specificity of 0.89 and 0.95, respectively. Of 38 outbreak pairs, 2 demonstrated between-outbreak relatedness that was equal to or greater than within-outbreak relatedness, and both of these pairs demonstrated within-outbreak pairwise distances greater than the ROC defined threshold value (Figure 2, pairs

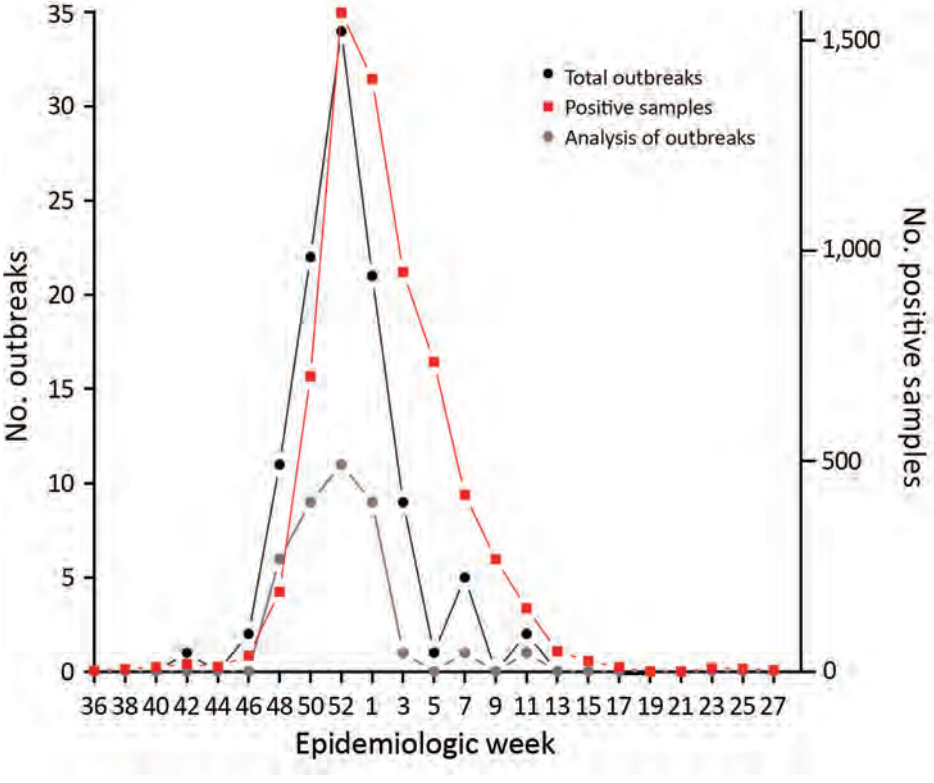

Figure 1. Epidemiologic curves of influenza A cases and outbreaks in long-term care facilities, by epidemiologic week, Toronto, Ontario, Canada, 2014–15. Shown are the total number (n=6,573) of influenza A–positive cases reported during the season for the province (red line), the 108 influenza A(H3N2) outbreaks in long-term care facilities analyzed at the provincial public health laboratory (black line), and the 38 outbreaks evaluated by genome sequencing in this study (gray line).

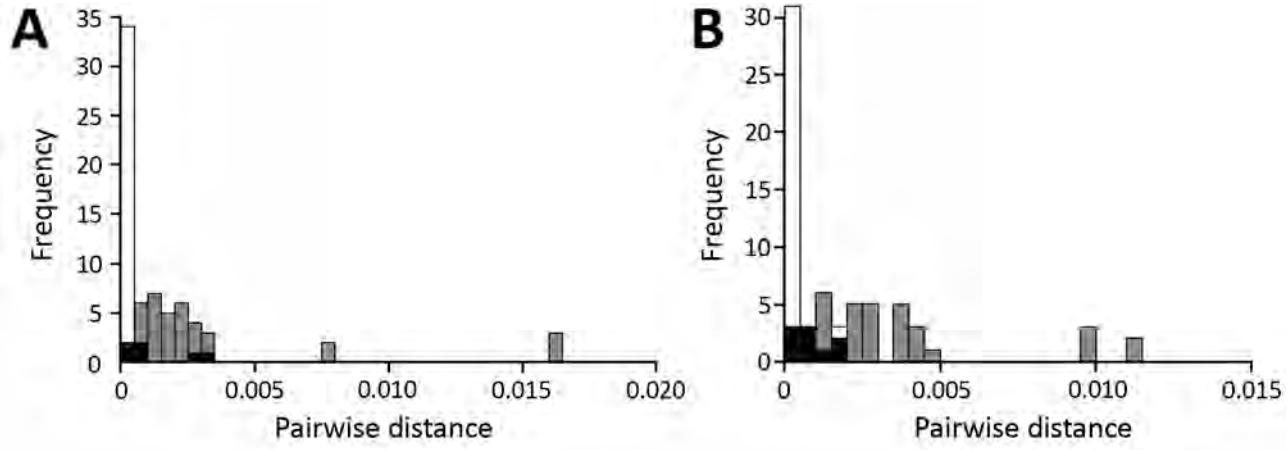

Figure 3. Histograms of pairwise distances for within-outbreak pairs (white) and between contemporaneous outbreak pairs (light gray) for influenza A(H3N2) samples from patients in long-term care facilities, Toronto, Ontario, Canada, 2014–15. A) Majority genome; B) hemagglutinin gene. Black indicates overlap between categories.

25 and 37). Two optimal cutoffs for pairwise distances in HA gene-specific analysis were identified, and we selected the threshold (0.0009) with the most balanced sensitivity and specificity, which provided an AUC of 0.93 (95% CI 0.87–0.98) (Figure 4) and sensitivity and specificity of 0.89 and 0.84, respectively. For HA gene analysis, 2 of 38 outbreak pairs demonstrated between-outbreak relatedness equal to or greater than within-outbreak relatedness, and both of these pairs demonstrated within-outbreak pairwise distances greater than the ROC-defined threshold value (Figure 2, pairs 25 and 37). We used a Wilcoxon rank-sum test to compare the outbreak case numbers when majority genome pairwise distances were below the optimal ROC threshold (34/38 outbreaks) and when pairwise distances were higher than the optimal ROC threshold (4/38). We found no statistically significant difference in distribution of outbreak sizes between the 2 groups (p = 0.94).

Discussion

In this retrospective genomic study of influenza A(H3N2) outbreaks in LTCFs during the 2014–15 influenza season in Toronto, we evaluated the potential role of genome sequencing in clinically defined outbreaks with ≥2 available pairs of H3N2-positive respiratory specimens. Our analysis indicates that, in ≈90% of cases, the initial sample pairs were sufficiently closely related, suggesting that direct transmission had

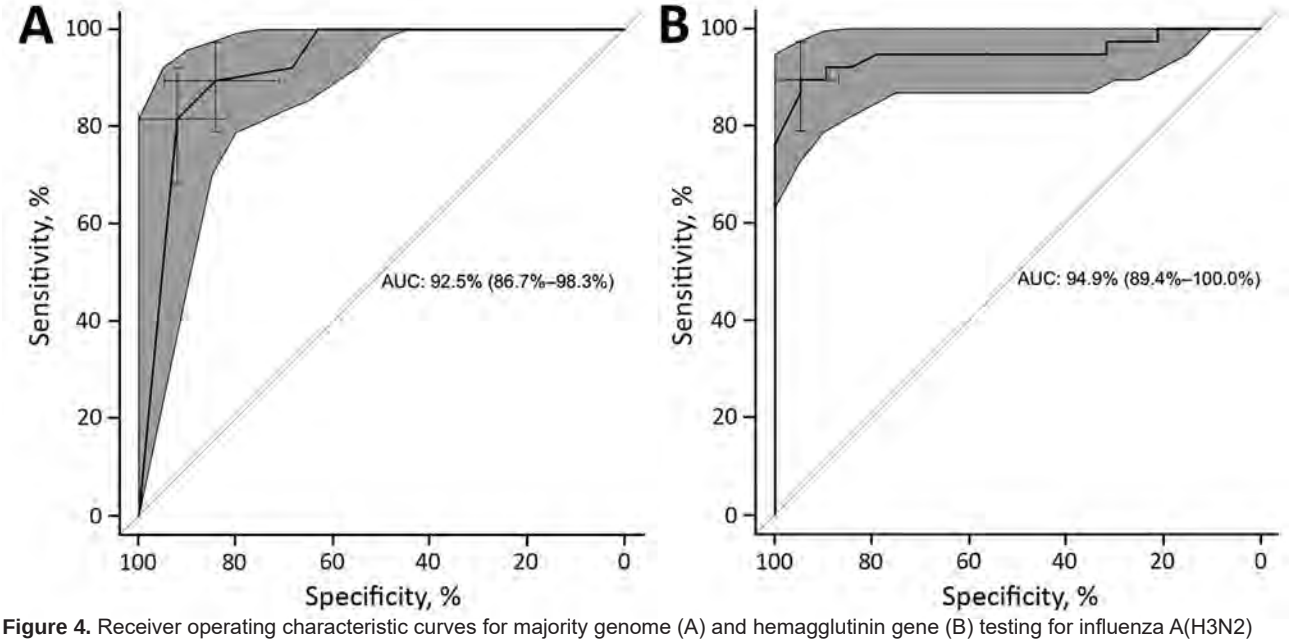

Figure 4. Receiver operating characteristic curves for majority genome (A) and hemagglutinin gene (B) testing for influenza A(H3N2) samples from patients in long-term care facilities, Toronto, Ontario, Canada, 2014–15. AUC values and 95% CIs are shown. The predicted binary outcome is within versus between (contemporaneous) outbreaks. AUC, area under the curve.

occurred. Thus, routine genome sequencing for supporting influenza outbreak definitions is unlikely to add statistically significant improvements over current clinical definitions.

Epidemiologic curves for the influenza season evaluated in this study show that activity (based on submitted samples to the provincial public health laboratory) peaked at week 52, and the number of analyzed outbreaks paralleled these trends. Outbreaks ranged widely in sizes and reflect a large patient population affected by influenza effects and outbreak mitigating measures.

To determine whether these influenza outbreaks were consistent with LTCF person-to-person spread, we constructed phylogenetic trees; the majority genome and HA gene trees show that most outbreak pairs are closely related. Including HA gene analysis of surveillance samples from the same season confirmed that the specific outbreak pairs generally appear to be sampled from the same population as the surveillance cases, which we inferred to be representative of circulating influenza lineages. This finding supports the idea that outbreak strains are being introduced from broadly circulating virus lineages as opposed to circulating preferentially within an LTCF reservoir (*9*). It is likely that the remaining 10% of cases that appeared unrelated represent transmission from occult sources. However, further study of the nature and epidemiology of these seemingly unlinked outbreaks is needed.

Although most outbreak pairs were closely related, some were not. To quantify this, we calculated a pairwise distance matrix and compared distance within pairs from the same LTCF with outbreaks occurring in a different location but at the same time. When we used the majority genome analysis, the pairwise distances within outbreaks formed a Poisson-shaped distribution abutting zero genetic distance. Distribution of pairwise distances between outbreaks that overlapped was normal but was centered to the right of the within-outbreak pairwise distances. We generated an ROC curve to assist with applying a threshold to classify those outbreak pairs that were or were not caused by direct transmission. The AUC for this curve was high, indicating a strong ability to discriminate among, within, and between outbreak pairs. Analysis of the HA pairs shows similar relationships but with less clearly differentiated pairwise distance distributions. We found no obvious classification benefit of majority genome sequencing versus HA-specific gene sequencing. This finding is supported by a report of a multisite outbreak in which strain differentiation with whole-genome sequencing offered no obvious benefit over HA/neuraminidase gene sequencing for infection control purposes (*24*).

From these analyses, we see that nearly all pairs within outbreaks appeared to be highly related, likely representing linked transmission occurring within individual LTCFs. These findings suggest that current clinically defined outbreak definitions for identifying within-LTCF transmission when ≥2 samples are positive for influenza are highly specific. Moreover, the high proportion of related strains reinforces the importance of measures for mitigating transmission within facilities through established and routine approaches (e.g., hand and respiratory hygiene, management of ill healthcare workers, adherence to infection control practices, vaccination) (*25*). Although we demonstrate reasonable approaches to differentiate among, within, and between outbreak strains, it is unlikely that these would be useful for prospective classification of outbreaks in the facilities evaluated in this report, considering the high incidence of presumed related strain outbreaks. This conclusion is supported by the fact that outbreak size appeared to have no obvious relationship to strain relatedness. There may, however, be utility for gene sequencing in acute-care environments to better ascertain transmission sources and support infection prevention and control investigations (*26*). Moreover, it is reasonable to expect that this approach could be applied to other subtypes of influenza A.

This study has several limitations. First, we were only able to assess outbreaks with ≥2 PCR-positive influenza A(H3N2) samples and cannot comment on the validity of outbreak definitions in circumstances under which only 1 patient is positive for influenza. One may expect the specificity of the clinical definition in this context to be lower, although it is challenging to validate this in the absence of microbiologic sample availability (which may be compounded by limitations of influenza tests, specifically false negatives) (*27*). Similarly, additional microbiologic samples are often not obtained after the outbreak has been initially identified, which prevents us from assessing ongoing strain relatedness throughout the evolving outbreak. Our surveillance sample was also limited, in both number of sequences and length of the alignment, impairing our ability to thoroughly characterize and compare the features of influenza circulating in the 2 settings. Because of limitations of data linkage with epidemiologic data, we cannot assess other features of outbreaks, including LTCF size and geographic location, as well as individual patient factors and epidemiologic links. However, the relative consistency of the results suggests these factors are likely less relevant. Last, we did not seek to evaluate the role of deep sequencing in ascertaining linked transmission, although this particular approach warrants additional study (*28*).

In summary, current clinical definitions of influenza A outbreaks with ≥2 positive influenza samples appear reasonably specific for identifying presumed within-facility transmission. As a result, routine gene sequencing as part of outbreak identification does not offer clear additional benefit. However, whole/majority genome or HA-specific sequencing may prove useful to identify sources of influenza introductions where it is clinically indicated.

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Influenza Vaccination and Incident Tuberculosis among Elderly Persons, Taiwan[1]

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Experimental studies have demonstrated that influenza vaccination may protect against tuberculosis (TB) through a Th17 response. This nationwide cohort study aimed to evaluate the association of influenza vaccination with incident TB among elderly persons in Taiwan. This 2005–2012 study included 99,982 elderly persons (64,290 vaccinated and 35,692 unvaccinated) from the Taiwan National Health Insurance Research Database. During the 738,367 person-years of follow-up, 1,141 (1.14%) persons had incident TB. The cumulative incidences of TB were 145.2 cases/100,000 person-years among vaccinated elderly persons and 175.5 cases/100,000 person-years among unvaccinated elderly persons (p = 0.002). The time-dependent Cox proportional hazards model revealed that influenza vaccination was an independent protective factor for incident TB. Our results suggest that influenza vaccination is associated with a lower risk of incident TB among elderly persons in Taiwan. Further investigation of biologic mechanisms is warranted.

Tuberculosis (TB) remains a common and deadly disease throughout the world (*1*). In 2014, there were 9.6 million cases of TB worldwide, and 1.5 million persons died of this disease (*2*). When *Mycobacterium tuberculosis* infects a host, T-cell–mediated immunity plays a major role in protecting against the development of TB. In this context, *M. tuberculosis*–induced T-cell–mediated immunity activates macrophage phagocytosis and kills intracellular *M. tuberculosis* (*3*). Activation of helper T cells also drives their secretion of tumor necrosis factor α, which attracts additional macrophages and lymphocytes and promotes granuloma formation to control the spread of *M. tuberculosis* (*3*). Previous studies have also demonstrated that the *M. bovis* bacillus Calmette-Guerin vaccine effectively protects infants from TB through the activation of conventional T-cell immunity (*4*,*5*). Although there is no effective vaccine to prevent TB in older adults, a recent report has demonstrated that a partially effective (e.g., 60% efficacy) vaccine targeted at adults can reduce the prevalence of TB (*6*).

Influenza vaccination is a safe and effective method of preventing influenza infections in elderly persons (*7*–*9*). A previous study demonstrated that the activation of CD4+ T cells (Th1) by influenza vaccination leads to the secretion of Th1-type cytokines (e.g., interferon-γ) (*10*), which activates macrophage phagocytosis and may subsequently kill intracellular *M. tuberculosis*. Recent animal studies have also demonstrated that influenza vaccination may provide protection against a range of pathogens (e.g., *M. tuberculosis*) by activating a Th17 response (*11*). Although several reports have indicated a possible interaction between influenza vaccination and TB, little is known regarding the association between influenza vaccine and subsequent TB development. Therefore, we conducted a nationwide, population-based cohort study during 2005–2012 to evaluate the association between the influenza vaccine and incident TB among elderly persons in Taiwan.

Methods

Background Information

The National Health Insurance (NHI) program in Taiwan is a universal and comprehensive program that was implemented on March 1, 1995 (*12*). The NHI program covers inpatient, outpatient, and emergency care, as well as alternative medicine, dental services, and prescription drugs. More than 99% of Taiwan's population is enrolled in the NHI program (*12*). Since 2001, Taiwan's government has implemented a free annual influenza vaccination program for elderly persons (≥65 years of age) (*13*).

Study Population

This nationwide cohort study used the National Health Insurance Research Database (NHIRD), which is managed by the Taiwan National Health Research Institutes. The

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Longitudinal Health Insurance Database, a subset of the NHIRD, is a representative database of 1 million persons randomly sampled by the National Health Research Institutes from the registry of all NHI enrollees. The NHIRD can be accessed at http://nhird.nhri.org.tw/en/index.htm, and the data are offered to scientists for research purposes. Personally identifiable information is encrypted. The NHIRD contains comprehensive medical information for insured persons, which includes drug prescriptions (e.g., influenza vaccinations) and diagnostic codes using the format of the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM). The accuracy of major disease diagnoses (e.g., diabetes mellitus and cerebrovascular disease [CVD]) in the NHIRD has been well validated (*14*,*15*).

We selected persons who were ≥65 years of age in 2005. Those who had received a TB diagnosis (ICD-9-CM codes 010–018) before enrollment were excluded. All persons were followed until a TB diagnosis, death, or December 31, 2013. This study was approved by the institutional review board of Taipei Veterans General Hospital (IRB 2015-04-004AC).

Outcome Variable

The outcome variable was new-onset TB during the follow-up period. Incident TB was defined using the presence of the appropriate ICD-9-CM codes (010–018) (*16*) and the prescription of ≥2 anti-TB drugs (isoniazid, ethambutol, rifampin, or pyrazinamide) for 4 weeks.

Main Explanatory Variable

The main explanatory variable was influenza vaccination, which was identified using the drug codes for influenza vaccination (*17*). The free annual influenza vaccination program for elderly persons in Taiwan usually begins at the beginning of October and continues through the end of April in the following year. The influenza season in Taiwan was typically defined as the period from October through the following March (*17*). The vaccines in the influenza immunization program were Fluvirin (Novartis Vaccines, Basel, Switzerland); Vaxigrip (Pasteur Merieux Connaught, Lyon, France); and Fluarix (GlaxoSmithKline, Research Triangle Park, NC, USA). The status of influenza vaccination was recorded in each influenza vaccination program for all elderly persons in Taiwan; time-dependent Cox proportional hazard models were used to evaluate the association between influenza vaccination and incident TB. For descriptive analysis, we defined 2 groups on the basis of whether or not they had ever received an influenza vaccination.

Controlling Variables

The controlling variables in this study were the persons' sociodemographic characteristics (income level and urbanization) and concurrent conditions. Income level was calculated using the average monthly income of the insured person; persons were categorized into 3 levels: low (≤20,000 new Taiwan dollars [NTD]), intermediate (20,000 NTD to <40,000 NTD), or high (≥40,000 NTD). Urbanization was categorized as whether the person lived in an urban, suburban, or rural area. Concurrent conditions were diabetes (ICD-9 code 250), congestive heart failure (CHF; ICD-9 code 428.0), CVD (ICD-9 codes 430–437, excluding 432), hypertension (ICD-9 codes 401–405), chronic kidney disease (CKD; ICD-9 codes 580–587), cancer (ICD-9 codes 140–208), chronic obstructive pulmonary disease (COPD; ICD-9 codes 491, 492, and 496), and asthma (ICD-9 code 493). A person was considered to have a concurrent condition only if the condition occurred in an inpatient setting or at ≥2 outpatient visits (*18*).

Validation of TB Diagnosis

Identification of patients with an ICD-9-CM code for TB was validated by analysis of selected samples from the claims database of Taipei Veterans General Hospital (a 2,800-bed tertiary referral hospital in Taiwan) for 2014. The content of this database is used for reimbursement and is similar to that of the NHIRD. Two pulmonologists (V.Y.-F.S. and S.-W.P.) independently reviewed the clinical and laboratory data from the selected samples. We used a positive TB culture as the standard for the diagnosis of TB.

Statistical Analysis

Continuous sociodemographic data were presented as mean (SD); we used the 2-sample *t*-test for intergroup comparisons. We analyzed categorical data using the Pearson χ^2 test, as appropriate. We calculated the incidence of TB per 100,000 person-years for vaccinated and unvaccinated elderly persons and estimated the hazard ratios (HRs) of incident TB among vaccinated elderly persons (compared with unvaccinated elderly patients) using Cox proportional hazard models. We defined the exposed preventive fraction as the theoretically preventable proportion of the incidence rate of the outcome of interest (incident TB) in the exposed population if the influenza vaccination had been implemented for all persons. We calculated the preventive fraction using the equation (*19*) preventive fraction among exposed persons = $[(CI_u) - (CI_e)] / (CI_u) = 1 -$ relative risk, where CI_u is the cumulative incidence in the unexposed group (no influenza vaccination) and CI_e is the cumulative incidence in the exposed group (influenza vaccination).

We defined the population preventive fraction as the theoretically preventable proportion of the incidence rate of the outcome of interest (incident TB) in the entire population if the influenza vaccination had been implemented

for all persons. We calculated the population preventive fraction using the equation population preventive fraction = (proportion of exposed cases) × (preventive fraction in the exposed cases), where the proportion of exposed cases is the prevalence of influenza vaccination.

We analyzed all data regarding influenza vaccination that were recorded in each influenza vaccination program for all persons. In addition, we analyzed the status of concurrent conditions as collected annually among elderly patients. Thus, information regarding the change in vaccination and status of concurrent conditions was available for each person throughout the study period. Therefore, we used Cox proportional hazards models with time-dependent covariates (*20*) to evaluate the associations of influenza vaccination with incident TB. In these models, influenza vaccination and concurrent conditions were considered the time-dependent variables (*20*), whereas potential confounders (e.g., age, sex, and income level) that were collected at baseline were considered fixed covariates. Because a previous report showed that influenza vaccine–induced immunity could last for 4 months in the elderly population (*21*), we hypothesized the duration of influenza vaccine–induced immunity as 4 months in the time-dependent Cox proportional hazards model. The formula for the Cox proportional hazards model is $\log h_i(t) = \log h_0(t) + (\beta_1 x_{i1} + \beta_2 x_{i2} + \ldots + \beta_k x_{ik}) + [\lambda_1 x_{j1}(t) + \lambda_2 x_{j2}(t) + \ldots \lambda_k x_{jk}(t)]$, where x_{i1} = (age, sex, and income level) are the fixed variables and x_{j1} = (influenza vaccination and concurrent conditions) are the time-dependent variables.

We examined the proportional hazard assumption by plotting the log–log plots according to influenza vaccination status; the plot showed lines for elderly persons in Taiwan with and without influenza vaccination that were straight and parallel. Adjusted HRs with 95% CIs were reported to indicate the strength and direction of the associations. To examine the interaction between influenza vaccination and other covariates in the multivariate analysis, we conducted subgroup analyses after stratifying study subjects by sex, age, and concurrent conditions. We conducted all data management and analyses using SAS software version 9.4 (SAS Institute, Cary, NC, USA).

Results

Patient Selection

This study identified 102,123 elderly persons in Taiwan who were ≥65 years of age in 2005. After excluding persons with antecedent TB (n = 568) and those with incomplete data (n = 1,573), we included the remaining 99,982 elderly persons in our analyses (Figure). During the follow-up period, 64.3% (64,290) of the elderly persons received influenza vaccination; 14.2% (14,167) were vaccinated once, 10.3% (10,277) twice, 8.6% (8,615) 3 times, 7.8% (7,800) 4 times, 6.9% (6,815) 5 times, 6.2% (6,188) 6 times, 5.6% (5,591) 7 times, and 4.8% (4,801) 8 times. The mean (SD) age was 73.7 (7.0) years and 49.5% were men. During the 738,367

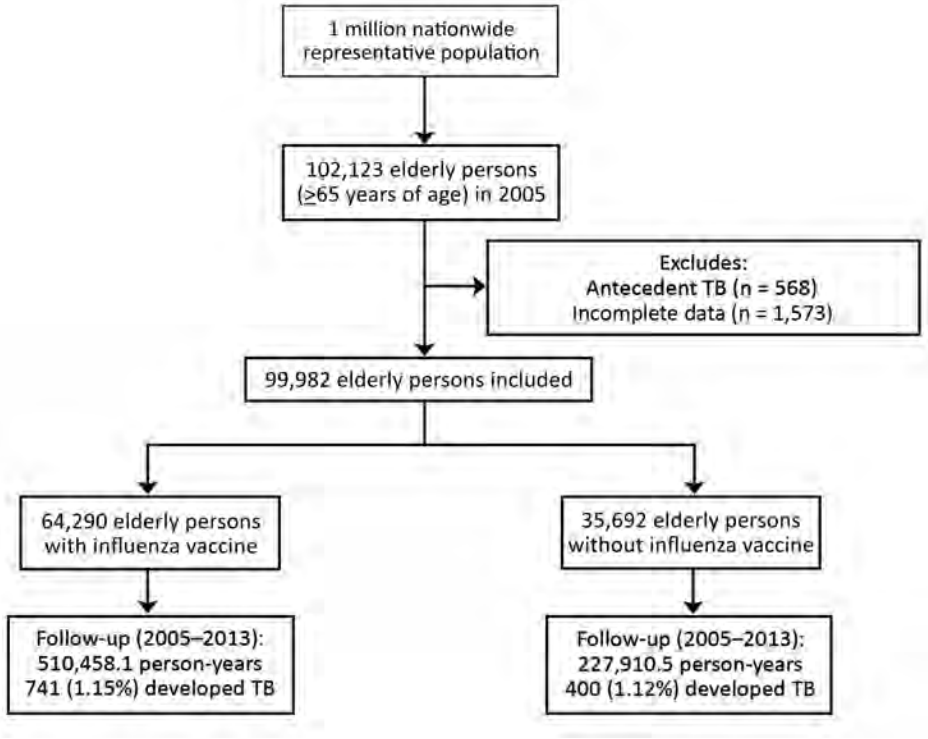

Figure. Flowchart showing enrollment and follow-up for elderly persons with and without influenza vaccination, Taiwan, 2005–2012. TB, tuberculosis.

person-years of follow-up, we observed 1,141 (1.14%) incident TB cases.

Demographic Characteristics and Concurrent Conditions among Elderly Persons

We compiled demographic characteristics and concurrent conditions among elderly persons in Taiwan with and without vaccination (Table 1). Elderly persons with vaccination were slightly younger than those without vaccination (73.2 vs. 74.6 years of age). In addition, compared with unvaccinated elderly persons, vaccinated elderly persons had a higher proportion of some concurrent conditions (e.g., diabetes, COPD, asthma, and hypertension) and a lower proportion of other concurrent conditions (e.g., CHF, CKD, and cancer). We found no significant difference in the proportion of incident TB cases between vaccinated and unvaccinated elderly persons (1.15% vs. 1.12%).

Incidence Rate of TB

During the follow-up period, new-onset TB was diagnosed in 1,141 elderly patients. The cumulative incidence of TB was 145.2 cases/100,000 person-years among vaccinated elderly patients and 175.5 cases/100,000 person-years among unvaccinated elderly patients (Table 2). The HR of incident TB was 0.83 (95% CI 0.73–0.93) between vaccinated and unvaccinated elderly patients. The exposed fraction for incident TB was 17.3%, and the population preventive fraction for incident TB was 11.1%.

Protective and Risk Factors for Incident TB

We used a time-dependent Cox proportional hazards model to identify the independent protective and risk factors for incident TB (Table 2). After adjusting for the sociodemographic characteristics and concurrent conditions, we found that the risk of incident TB was 18% lower (95% CI 0.73–0.93) among elderly persons with influenza vaccination compared with unvaccinated persons. Another protective factor for incident TB was HTN. The risk factors for incident TB were age 70–74 years or ≥75 years, male sex, living in a rural area, diabetes, CHF, COPD, CKD, and cancer.

Sensitivity Analyses

We conducted sensitivity analyses regarding the associations of influenza vaccination with incident TB and stratified results according to age group, sex, and concurrent conditions (Table 3). Time-dependent Cox regression analyses revealed that influenza vaccination significantly reduced the risk of incident TB in men; persons with low or high income; persons living in urban or suburban areas; persons with COPD or CVD; and persons without diabetes, CHF, asthma, HTN, CVD, cancer, or CKD.

Table 1. Characteristics of elderly persons with and without influenza vaccination, Taiwan, 2005–2012*

Characteristic	With vaccination, n = 64,290	Without vaccination, n = 35,692	p value
Age, y, mean ± SD	73.2 ± 6.3	74.6 ± 8.2	<0.001
65–69	23,339 (36.3)	12,321 (34.5)	<0.001
70–74	17,848 (27.8)	7,842 (22.0)	
>75	23,103 (35.9)	15,529 (43.5)	
Sex			
F	32,426 (50.4)	18,042 (50.6)	0.734
M	31,864 (49.6)	17,650 (49.4)	
Income level			
Low	44,867 (69.8)	25,250 (70.7)	<0.001
Intermediate	11,353 (17.7)	5,692 (16.0)	
High	8,070 (12.5)	4,750 (13.3)	
Urbanization			
Urban	14,767 (23.0)	10,245 (28.7)	<0.001
Suburban	38,331 (59.6)	20,817 (58.3)	
Rural	11,192 (17.4)	4,630 (13.0)	
Concurrent conditions			
Diabetes	14,085 (21.9)	6,944 (19.5)	<0.001
CHF	3,200 (5.0)	2,257 (6.3)	<0.001
COPD	12,310 (19.2)	5,881 (16.5)	<0.001
Asthma	6,491 (10.1)	2,948 (8.3)	<0.001
CVD	10,824 (16.8)	5,938 (16.6)	0.419
HTN	36,444 (56.7)	16,998 (47.6)	<0.001
CKD	4,790 (7.5)	2,937 (8.2)	<0.001
Cancer	4,142 (6.4)	2,731 (7.7)	<0.001
Outcomes			
New-onset TB	741 (1.15)	400 (1.12)	0.649
Follow-up years, mean (SD)	7.9 (2.0)	6.4 (3.3)	<0.001
Total follow-up duration, person-years	510,458.1	227,910.5	<0.001

*Values are no. (%) persons unless otherwise indicated. CHF, congestive heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; HTN, hypertension; TB, tuberculosis.

Table 2. Univariate and multivariate analyses of the protective and risk factors for incident TB among elderly persons with and without influenza vaccination, Taiwan, 2005–2012*

Characteristic	Person-years	Incidence density†	Univariate analysis, HR (95% CI)	Multivariate analysis, adjusted HR (95% CI)
Influenza vaccination				
No	227,910.5	175.5	1	1
Yes	510,458.1	145.2	0.83 (0.73–0.93)‡	0.82 (0.73–0.93)‡
Age, y				
65–69	293,257.6	106.4	1	1
70–74	197,971.8	150.5	1.41 (1.21–1.66)§	1.18 (1.00–1.38)¶
≥75	247,139.3	214.9	2.02 (1.75–2.32)§	1.43 (1.24–1.66)§
Sex				
F	385,893.5	62.7	1	1
M	352,475.2	255.1	4.07 (3.53–4.69)§	3.32 (2.88–3.84)§
Income level				
Low	507,842.5	170.9	1	1
Intermediate	133,417.7	122.9	0.72 (0.61–0.85)§	0.88 (0.74–1.04)
High	97,108.5	112.2	0.66 (0.54–0.80)§	0.82 (0.67–1.00)
Urbanization				
Urban	187049.0	129.4	1	1
Suburban	436275.1	157.9	1.22 (1.05–1.41)‡	1.13 (0.98–1.31)
Rural	115044.5	182.5	1.41 (1.17–1.70)§	1.37 (1.13–1.65)¶
Concurrent conditions				
Diabetes				
No	596,098.4	153.5	1	1
Yes	142,270.2	158.9	1.03 (0.89–1.20)	1.17 (1.02–1.33)¶
CHF				
No	708,633.3	152.4	1	1
Yes	29,735.4	205.1	1.34 (1.04–1.74)¶	1.37 (1.16–1.62)§
COPD				
No	621,742.5	133.3	1	1
Yes	116,626.2	267.5	2.00 (1.76–2.28)§	2.77 (2.43–3.15)§
Asthma				
No	676,539.1	145.9	1	1
Yes	61,829.5	249.1	1.71 (1.44–2.02)§	1.12 (0.96–1.30)
CVD				
No	631659.3	152.0	1	1
Yes	106709.3	169.6	1.11 (0.95–1.31)	0.89 (0.78–1.02)
HTN				
No	360268.3	156.5	1	1
Yes	378100.3	152.6	0.97 (0.87–1.09)	0.86 (0.76–0.98)¶
CKD				
No	693223.9	149.2	1	1
Yes	45144.7	237.0	1.59 (1.30–1.94)§	1.31 (1.12–1.53)§
Cancer				
No	696681.8	150.9	1	1
Yes	41686.8	215.9	1.43 (1.15–1.77)‡	1.96 (1.71–2.26)§

*CHF, congestive heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; HR, hazard ratio; HTN, hypertension; TB, tuberculosis.
†Per 100,000 person-years.
‡p<0.01.
§p<0.001.
¶p<0.05.

Dose-Response Relationship between Influenza Vaccination and Incident TB

We evaluated the dose-response relationship between influenza vaccination and incident TB using the Cox proportional hazards models. The risk of developing TB (adjusted HR 0.81; 95% CI 0.79–0.83) decreased as the number of influenza vaccinations increased.

Validation

During January 1, 2014–December 31, 2014, a total of 433 patients had an ICD-9-CM code for TB. Of these patients, 326 had prescriptions for ≥2 anti-TB drugs for 4 weeks and were selected for validation. Among these 326 patients, the diagnosis of TB was confirmed in 314 and excluded in 12 (specificity 96.3%).

Discussion

Our longitudinal study aimed to evaluate the temporal association of influenza vaccination with incident TB during the 9-year follow-up period. The results indicate that, after adjusting for demographic data, concurrent conditions, and income level, elderly persons in Taiwan with influenza vaccination had a lower risk of incident TB than unvaccinated elderly persons. Our study also conducted a sensitivity

analysis of influenza vaccination with incident TB after stratifying persons according to age, sex, and concurrent conditions. Influenza vaccination significantly reduced the risk of incident TB in men; persons with low or high income; persons living in urban or suburban area; persons with COPD or CVD; and persons without diabetes, CHF, asthma, HTN, CVD, cancer, or CKD.

Activation of T-cell immunity by influenza vaccination may contribute to our observed lower risk of incident TB among vaccinated elderly Taiwanese patients. Activation of CD4+ Th1-type cells by influenza vaccination induces the secretion of Th1-type cytokines (e.g., interferon-γ) (*10*), which can activate macrophage phagocytosis and kill intracellular *M. tuberculosis*. The activation of Th1-type cells by influenza vaccination also accelerates granuloma formation and controls the spread of MTB (*3*). Furthermore, recent animal studies have demonstrated that influenza vaccination may provide protection against TB by initiating a Th17 response, which causes the recruitment of neutrophils, release of antimicrobial peptides, and interleukin-17–driven Th1 immunity (*11*). All these mechanisms indicate that the activation of helper T-cell immunity by influenza vaccination may contribute to the reduced risk of incident TB among vaccinated elderly persons.

Our study also revealed that the population preventive fraction of influenza vaccination for incident TB was 11.1%. Although influenza vaccination reduced the risk of incident TB by only 18% in an older population, a previous report showed that a relevant vaccine (when given to adults) would reduce the burden of TB, even when the vaccine is only partially effective in protecting against *M. tuberculosis* infection (*6*). Therefore, our findings suggest that influenza vaccination should be offered to elderly persons to prevent infections.

The aim of this cohort study was to determine the association between influenza vaccination and subsequent TB development. Our research design, which included unbiased patient selection and strict TB diagnostic criteria, supports the validity of these findings. In Taiwan, TB has been the most prevalent notified infectious disease for decades (*22*), and TB-related data are strengthened by the tight regulation of the TB reporting system in Taiwan. By law, clinics and hospitals in Taiwan must report TB cases to Taiwan's Centers for Disease Control within 7 days (*22*). Moreover, Taiwan's Centers for Disease Control convenes monthly expert committee meetings to discuss ambiguous reported TB cases (*22*). Our study also examined the internal validity of TB diagnosis classification using the same ICD-9-CM coding and confirmed that interobserver agreement and accuracy in identifying TB cases were excellent.

The lower risk of incident TB among vaccinated elderly patients is likely related to activation of T-cell–mediated immunity. A particular strength of this study is that we traced all elderly persons with minimal referral bias, because all medical care is covered by the Taiwan NHI program. Furthermore, the study's large sample size was sufficiently powered to detect the real, albeit subtle, difference between vaccinated and unvaccinated elderly persons. In addition, the annual influenza vaccination status for all persons was collected, and influenza vaccination was considered a time-dependent variable in the multivariate analysis. Longitudinal studies that do not account for changes in the annual vaccination will not be able to produce precise estimates of the vaccination's effect on incident TB (*23*).

This study has several limitations. First, some potential risk factors (e.g., smoking and obesity) were not available for our analysis. However, some smoking-related health effects would be partially reflected in the presentation of concurrent conditions (e.g., COPD and hypertension), which were included in our analysis. Second, influenza vaccination among elderly persons in Taiwan was voluntary, rather than randomly assigned. Therefore, unmeasured confounders (such as concurrent conditions) may exist for the association of influenza vaccination with incident TB and death from all causes. However, a previous study demonstrated that elderly persons in Taiwan with influenza vaccination have a

Table 3. Sensitivity analysis for the associations of influenza vaccination with incident TB after adjusting for demographic and medical characteristics, Taiwan, 2005–2012*

Study subgroup	Incident TB, adjusted HR (95% CI)
All persons, n = 99,982	0.82 (0.73–0.93)‡
Age 65–69 y, n = 35,660	0.81 (0.64–1.03)
Age ≥70 y, n = 64,322	0.85 (0.67–1.08)
Male patients, n = 49,514	0.81 (0.70–0.93)‡
Female patients, n = 50,468	0.89 (0.68–1.17)
Patients with low income, n = 70,117	0.82 (0.71–0.94)‡
Patients with intermediate income, n = 17,045	1.04 (0.75–1.45)
Patients with high income, n = 12,820	0.62 (0.40–0.97)†
Patients in urban area, n = 25,012	0.71 (0.53–0.95)†
Patients in suburban area, n = 59,148	0.82 (0.70–0.96)†
Patients in rural area, n = 15,822	0.97 (0.73–1.28)
Diabetes patients, n = 21,029	0.80 (0.61–1.06)
Patients without diabetes, n = 78,953	0.83 (0.72–0.95)‡
Patients with CHF, n = 5,457	0.75 (0.43–1.30)
Patients without CHF, n = 94,525	0.82 (0.72–0.93)‡
Patients with COPD, n = 18,191	0.74 (0.58–0.94)†
Patients without COPD, n = 81,791	0.88 (0.76–1.02)
Patients with asthma, n = 9,439	0.79 (0.57–1.10)
Patients without asthma, n = 90,543	0.83 (0.73–0.95)‡
Patients with HTN, n = 53,442	0.86 (0.72–1.02)
Patients without HTN, n = 46,540	0.80 (0.66–0.96)†
Patients with CVD, n = 16,762	0.70 (0.51–0.97)†
Patients without CVD, n = 83,220	0.85 (0.74–0.97)†
Patients with cancer, n = 6,873	0.85 (0.55–1.31)
Patients without cancer, n = 93,109	0.81 (0.71–0.92)‡
Patients with CKD, n = 7,727	0.80 (0.53–1.20)
Patients without CKD, n = 92,255	0.82 (0.72–0.94)‡

*CHF, congestive heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebral vascular disease; HR, hazard ratio; HTN, hypertension; TB, tuberculosis.
†p<0.05.
‡p<0.01.

greater number of concurrent conditions compared with their unvaccinated counterparts (*13*). Therefore, because persons with more chronic diseases have a higher risk of incident TB (*24*,*25*), this difference would lead to our underestimating the protective effect of influenza vaccination on TB development. Third, TB diagnoses that rely on administrative claims data (which are recorded by physicians or hospitals) may be less accurate than diagnoses that are made in a prospective clinical setting. Furthermore, the history of TB disease among elderly patients in Taiwan can be traced back only to 1995. However, there is no reason to suspect that the validity of claims data would differ with a patient's TB status. Moreover, diagnosis of TB by ICD-9-CM coding was validated in this study, indicating that the accuracy is excellent. Finally, because the Taiwan government provides free influenza vaccination only for persons who are ≥65 years of age (*13*), we could evaluate the association between influenza vaccination and incident TB only among elderly persons. Therefore, the generalizability of our findings to younger persons requires further verification.

In conclusion, this nationwide long-term cohort study determined the associations of influenza vaccination with incident TB among elderly persons in Taiwan. Influenza vaccination was associated with lower risks of incident TB, after adjusting for demographic data, concurrent conditions, and income level. Nevertheless, more comprehensive studies are needed to confirm our findings and identify the potential biologic mechanism(s) that explain these associations.

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About the Author

Dr. Yen is a clinical doctor at Taipei City Hospital, Taipei, Taiwan, specializing in infectious diseases. He is interested in epidemiology and infectious diseases and is actively engaged in TB control policy in Taiwan.

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Epidemiology and Molecular Identification and Characterization of *Mycoplasma pneumoniae*, South Africa, 2012–2015

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During 2012–2015, we tested respiratory specimens from patients with severe respiratory illness (SRI), patients with influenza-like illness (ILI), and controls in South Africa by real-time PCR for *Mycoplasma pneumoniae*, followed by culture and molecular characterization of positive samples. *M. pneumoniae* prevalence was 1.6% among SRI patients, 0.7% among ILI patients, and 0.2% among controls (p<0.001). Age <5 years (adjusted odd ratio 7.1; 95% CI 1.7–28.7) and HIV infection (adjusted odds ratio 23.8; 95% CI 4.1–138.2) among *M. pneumonia*–positive persons were associated with severe disease. The detection rate attributable to illness was 93.9% (95% CI 74.4%–98.5%) in SRI patients and 80.7% (95% CI 16.7%–95.6%) in ILI patients. The hospitalization rate was 28 cases/100,000 population. We observed the macrolide-susceptible *M. pneumoniae* genotype in all cases and found P1 types 1, 2, and a type 2 variant with multilocus variable number tandem repeat types 3/6/6/2, 3/5/6/2, and 4/5/7/2.

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In 1986, ≈4 million deaths were attributed to pneumonia in children <5 years old globally (*1*). This number declined to 1.2 million by 2011, largely because of interventions such as antimicrobial drugs and vaccination against leading pneumonia-causing pathogens (*1*). Despite this decline, pneumonia remains a major cause of illness and death globally, especially in children <5 years old (*2*).

Mycoplasma pneumoniae causes respiratory illness and pneumonia with estimated prevalence ranging 2%–35%, depending on the identification method, the study period, and the population under investigation (*3*–*5*). Data from 21 countries showed *M. pneumoniae* to be the most common atypical pneumonia-causing bacterium, responsible for ≈12% of community-acquired pneumonia during 1996–2004 (*6*). *M. pneumoniae* epidemics have been reported to occur in cycles of 3–5 years (*5*,*7*). Persons infected with *M. pneumoniae* are treated with macrolides or azalide antibiotics (*8*); however, because of excessive and inappropriate use of antibiotics, macrolide resistance is increasing (*9*–*11*).

Characterization of strains is important for outbreak investigations to understand disease epidemiology and to identify type switching that might occur because of population immune pressure. *M. pneumoniae* is typically characterized by typing the P1 adhesion molecule gene sequence, which distinguishes the 2 P1 types (*12*), or by using multilocus variable-number tandem-repeat analysis (MLVA), which is more discriminatory than P1 typing (*13*).

The prevalence of *M. pneumoniae* in South Africa is unknown because of the low availability of reliable tests and because clinicians rarely request testing. Here we describe the prevalence, epidemiology, molecular characteristics, and antimicrobial resistance properties of *M. pneumoniae* among patients with mild and severe respiratory illness in South Africa.

Materials and Methods

Study Design

We enrolled patients and asymptomatic persons during June 2012–May 2015 as part of 2 surveillance programs (1 for severe respiratory illness [SRI] and 1 for influenza-like illness [ILI]). SRI surveillance was conducted at 2 sentinel sites, Edendale Hospital in KwaZulu Natal Province and Klerksdorp-Tshepong Hospital Complex in North West Province. Patients enrolled in SRI surveillance were those hospitalized with clinical signs and symptoms of lower respiratory tract infection (LRTI), regardless of symptom duration. We included children 2 days to <3 months old who had physician-diagnosed sepsis or acute LRTI, children 3 months to <5 years old with physician-diagnosed LRTI, and persons >5 years old who met the World Health Organization case definition for LRTI (sudden onset of fever [temperature >38°C] or reported fever, cough or sore throat, and shortness of breath or difficulty breathing [*14*]).

ILI patients were outpatients who were seen at 2 primary health care clinics serving the 2 SRI sentinel sites. Patients were considered to have ILI if they had an acute fever of >38°C or a self-reported fever within the last 7 days and either a cough or sore throat. Asymptomatic persons included those who were seen at the same primary health care clinics and had no history of respiratory illness, diarrheal illness, or fever in the preceding 14 days. For asymptomatic persons, we aimed to enroll 1 HIV-infected and 1 HIV-uninfected person weekly in each clinic within the following age categories: 0–1, 2–4, 5–14, 15–54, and ≥55 years.

We obtained demographic and clinical information from all enrollees by using a standardized questionnaire. We reviewed hospital records of SRI patients to assess disease progression and outcome.

Specimen Collection

We collected combined nasopharyngeal and oropharyngeal swabs from ≥5 year-old persons and nasopharyngeal aspirates from <5 year-old persons (nasopharyngeal specimens) and placed the specimens in universal transport medium (Copan Italia, Brescia, Italy). We collected induced or expectorated sputum from SRI patients only. HIV status was determined as part of standard care or by using anonymized-linked dried blood spot testing for consenting enrollees (PCR for children <18 months old and ELISA for persons ≥18 months old [*15*]). We tested nasopharyngeal specimens for 10 respiratory viruses (influenza types A and B, adenovirus, enterovirus, rhinovirus, human metapneumovirus, respiratory syncytial virus, and parainfluenza virus types 1–3) by using an in-house multiplex real-time reverse transcription PCR (*16*).

Detection of *M. pneumoniae*

We extracted DNA from 200 μL of nasopharyngeal specimen and digested sputum by using the MagNA Pure 96 instrument (Roche Diagnostics, Mannheim, Germany) with the DNA and Viral NA SV kit (Roche Diagnostics). We performed an in-house multiplex real-time PCR for the detection of *M. pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, and *Legionella* spp., with human ribonuclease P gene serving as an internal control, as previously described (*17*). A positive *M. pneumoniae* patient was defined as a patient having a positive PCR result with a cycle threshold value ≤45 for *M. pneumoniae* on the nasopharyngeal specimen, sputum specimen, or both.

Culture and Molecular Characterization

We detected 82 cases of PCR-positive *M. pneumoniae* during study periods 1 (June 2012–May 2013) and 2 (June 2013–May 2014) and performed culture and molecular characterization retrospectively on 77 (94%) samples. Culture and further characterization could not be performed for 5 cases because of insufficient specimens.

We inoculated *M. pneumoniae*–positive specimens in SP4 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated them at 37°C in 5% CO_2 for up to 10 days. Growth was indicated by a color change from red to orange, without turbidity. We performed macrolide susceptibility analysis by using real-time PCR followed by high-resolution melt-curve (HRM) analysis by means of the Rotor-Gene Q6000 system (QIAGEN, Hilden, Germany), according to previously described methods (*18*).

We performed P1 genotyping by using real-time PCR targeting the 1900-bp region of the P1 gene, followed by HRM analysis using the Rotor-Gene Q6000 system according to previously described methods (*12*). We also performed MLVA typing on the same specimens by using 5 variable-number tandem-repeat loci (Mpn1, Mpn13, Mpn14, Mpn15, and Mpn16), as described by Dégrange et al. (*13*). However, for analysis, we used the 4-loci nomenclature as described by Sun et al. (*19*) because of the instability of the Mpn1 locus (*20*).

Statistical Analysis

We used the χ^2 or Fisher exact test for comparison of categorical variables. We used unconditional logistic regression to estimate the attributable fraction (AF) of *M. pneumoniae*–associated hospitalization and outpatient consultation by comparing the *M. pneumoniae* detection rate among SRI or ILI patients to that of controls. The AF was estimated from the odds ratio (OR) obtained from the regression models.

Among SRI patients, we estimated the AF for patients positive on nasopharyngeal specimens only as well as for patients positive on both nasopharyngeal and sputum

specimens. We adjusted all estimates for age, HIV status, underlying medical conditions other than HIV infection, and co-infections with the 10 respiratory viruses investigated in this study.

In addition, we used unconditional logistic regression to assess factors associated with *M. pneumoniae*–associated SRI hospitalization by comparing the characteristics of *M. pneumoniae*–positive SRI patients with those of *M. pneumoniae*–positive ILI patients. For the multivariable model, we assessed all variables that were significant at $p<0.2$ on univariate analysis and dropped nonsignificant factors ($p\geq0.05$) with manual backward elimination. We assessed pairwise interactions by inclusion of product terms for all variables remaining in the final multivariable additive model. We performed the analysis by using Stata 14 (StataCorp LLC, College Station, Texas, USA). Seasonality and periodicity were assessed over 3 periods (period 1, June 2012–May 2013; period 2, June 2013–May 2014; and period 3, June 2014–May 2015).

Calculation of Rates of *M. pneumoniae*–Associated SRI Hospitalization

We estimated the overall and age-specific rates of *M. pneumoniae*–associated SRI hospitalizations (per 100,000 population) by using the number of SRI hospitalizations and adjusting for nonenrollment (e.g., refusals to participate and no enrollment on weekends [*21*]) and healthcare-seeking behavior during 2013–2014. For all calculations, we assumed that the *M. pneumoniae* detection rate among persons tested and not tested was the same within age groups. We obtained age- and year-specific population denominators from projections of the 2011 census data (*21*), and we obtained age- and year-specific HIV prevalence in the study population from the projections of the Thembisa model (*22*).

We calculated 95% CIs for all estimated rates by using bootstrap resampling of all parameters included in the estimation over 1,000 replications. The upper and lower limits of the 95% CI were the 2.5th and 97.5th percentile of the estimated values from the bootstrapped datasets, respectively.

Results

Study Population

During June 2012–May 2015, we enrolled 11,391 persons, of whom 10,194 (89.5%) had specimens collected that were tested for *M. pneumoniae*. Of these specimens, 4,703 (46.1%) were from SRI patients, 3,721 (36.5%) were from ILI patients, and 1,770 (17.4%) were from controls. Among the SRI patients, 2,390 (50.8%) had a nasopharyngeal specimen tested only, 207 (4.4%) had sputum tested only, and 2,106 (46.8%) had both specimen types tested.

Among persons for whom age was known, children <5 years old accounted for 35.8% (1,678/4,687) of SRI patients, 30.7% (1,142/3,716) of ILI patients, and 35.2% (662/2,767) of controls. HIV status was known for 86.5% (8,815/10,194) of enrollees. HIV prevalence was 54.2% (2,117/3,902) among SRI patients, 28.8% (940/3,261) among ILI patients, and 42.7% (705/1,652) among controls (owing to enrollment criteria of controls) ($p<0.001$). Among SRI and ILI patients, HIV prevalence was lowest among infants <1 year old (SRI patients, 12.3% [99/805]; ILI patients, 2.5% [9/360]) and highest among persons 25–44 years old (SRI patients, 90.6% [1,180/1,303]; ILI patients, 59.0% [588/997]).

Detection Rate of *M. pneumoniae*

Overall, we detected *M. pneumoniae* in 1.0% (103/10,194) of persons tested; this rate was 1.6% (73/4,703) among SRI patients, 0.7% (27/3,721) among ILI patients, and 0.2% (3/1,770) among controls ($p<0.001$). We also compared detection rates by age and HIV status (Table 1). Among patients with SRI, the detection rate of *M. pneumoniae* differed by specimen type (1.1% [49/4,496] in nasopharyngeal specimens vs. 1.7% [39/2,313] in sputum; $p = 0.04$). Among the 2,106 SRI patients with *M. pneumoniae* results available for both specimen types, 15 (0.7%) patients tested

Table 1. *Mycoplasma pneumoniae* detection rate, by age and HIV status, among inpatients with SRI, outpatients with ILI, and controls, Klerksdorp and Pietermaritzburg, South Africa, June 2012–May 2015*

Characteristic	No. positive/no. tested (%)		
	SRI	ILI	Controls
Total	73/4,703 (1.6)	27/3,721 (0.7)	3/1,770 (0.2)
Age group, y			
<1	19/1,067 (1.8)	3/397 (0.8)	0/249 (0.0)
1–4	19/611 (3.1)	8/745 (1.1)	0/373 (0.0)
5–24	9/383 (2.3)	8/1,085 (0.7)	3/505 (0.6)
25–44	16/1,492 (1.1)	6/1,075 (0.6)	0/271 (0.0)
45–64	7/885 (0.8)	1/360 (0.3)	0/258 (0.0)
≥65	3/249 (1.2)	1/54 (1.9)	0/111 (0.0)
HIV status			
Negative	30/1,785 (1.7)	21/2,231 (0.9)	2/947 (0.2)
Positive	32/2,117 (1.5)	2/940 (0.2)	0/705 (0.0)

*ILI, influenza-like illness; SRI, severe respiratory illness.

positive on both specimens, 10 (0.5%) tested positive on nasopharyngeal specimen only, 20 (0.9%) tested positive on sputum only, and 2,061 (97.9%) tested negative on both specimen types. Of *M. pneumoniae*–positive patients with known outcome, 98% (97/99) survived and 2% (2/99) died. Both patients that died were adults admitted for SRI and were receiving treatment for tuberculosis with no other respiratory virus identified. One of these patients was HIV-positive, and HIV status for the other patient was unknown.

We observed no seasonality (Figure). However, we detected a significantly higher rate of *M. pneumoniae* in periods 1 and 2 compared with period 3 (period 1, 1.4% [50/3,651] vs. period 2, 1.2% [35/2,846] vs. period 3, 0.5% [18/3,594]; p<0.001).

Overall, we detected *M. pneumoniae* along with another virus or other atypical pneumonia-causing bacteria in 65% (62/103) of patients. *M. pneumoniae* was co-detected with rhinovirus most frequently (58% [36/62]), followed by adenovirus (43% [27/62]), respiratory syncytial virus (18% [11/62]), influenza virus (13% [8/62]), human metapneumovirus (8% [5/62]), enterovirus (6% [4/62]), parainfluenza virus (5% [3/62]), and *C. pneumoniae* (3% [2/62]).

AF of *M. pneumoniae*–Associated Hospitalization and Outpatient Consultation

The AF of *M. pneumoniae* detection to illness using nasopharyngeal specimens only for patients with ILI was 80.7% (95% CI 16.7%–95.6%) and for patients with SRI was 90.1% (95% CI 58.3%–97.7%). The AF of *M. pneumoniae* detection to illness for patients with SRI using both nasopharyngeal and sputum specimens was 93.9% (95% CI 74.4%–98.5%).

Factors Associated with Hospitalization among *M. pneumoniae* PCR-Positive Patients

On multivariable analysis, we compared *M. pneumoniae*–positive SRI patients to *M. pneumoniae*–positive ILI patients. Factors associated with increased risk of *M. pneumoniae*–associated hospitalization were age <5 years compared with ≥5 years (adjusted odds ratio [aOR] 7.1; 95% CI 1.7–28.7); HIV infection (aOR 23.8; 95% CI 4.1–138.2); and duration of symptoms ≥4 days (aOR 3.8; 95% CI 1.1–14.4) (Table 2).

Rates of *M. pneumoniae* SRI Hospitalization

The mean annual rate of hospitalization for *M. pneumoniae* patients during 2013–2014 was 27.9 cases/100,000 population (95% CI 18.9–37.4) (Table 3). HIV-infected persons had 19.5 (95% CI 14.4–26.4) times greater odds of *M. pneumoniae*–associated SRI hospitalization (102.2/100,000 [95% CI 64.9–136.4) than did HIV-uninfected persons (14.9/100,000 [95% CI 10.3–19.0]). The highest rate was in patients <5 years old (220.0/100,000 [95% CI 121.0–314.8]).

Culture and Molecular Characterization

We obtained cultures for 11/77 (14%) *M. pneumoniae*–positive specimens. HRM macrolide susceptibility profiles were available for 43% (33/77) of *M. pneumoniae*–positive specimens, and all were macrolide-susceptible.

We obtained HRM analysis results for P1 genotyping for 36% (28/77) of *M. pneumoniae* PCR-positive specimens (*12*). *M. pneumoniae* were P1 type 1 (29% [8/28]), P1 type 2 (61% [17/28]), and a variant of P1 type 2 (11% [3/28]).

MLVA types were available for 51% (39/77) of PCR-positive samples. On the basis of a combination of tandem repeats at the 4 loci, 3 distinct types were present: 3/5/6/2 (17/39 [44%]), 3/6/6/2 (15/39 [38%]), and 4/5/7/2 (7/39 [18%]). The remaining 49% (38/77) could not be assessed because of the inability to determine the fragment size of ≥1 of the 4 variable-number tandem-repeat loci.

Discussion

Overall, *M. pneumoniae* was detected in 1% of all patients (1.6% of SRI patients and 0.7% of ILI patients). Among

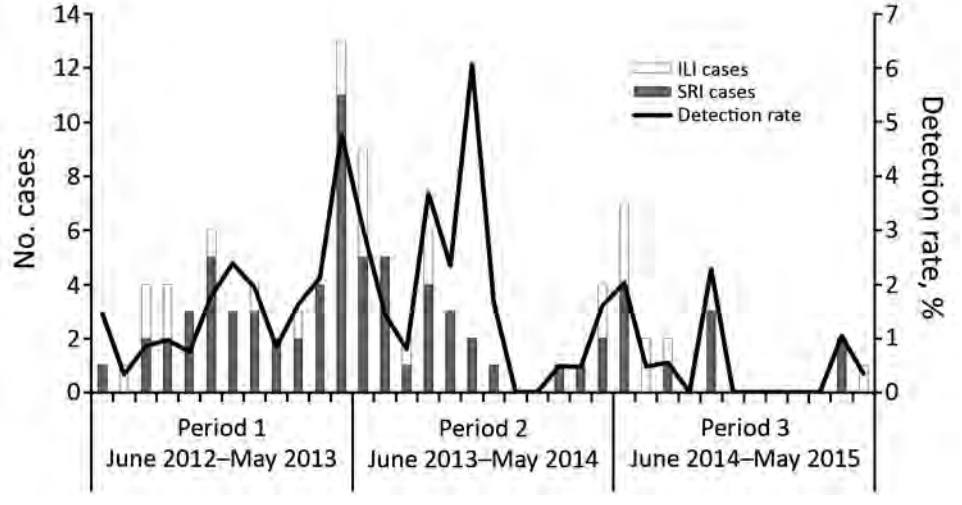

Figure. Number of *Mycoplasma pneumoniae*–positive cases and detection rate among inpatients with SRI and outpatients with ILI (N = 8,424), by month and period, Klerksdorp and Pietermaritzburg, South Africa, June 2012–May 2015. ILI, influenza-like illness; SRI, severe respiratory illness.

Table 2. Factors associated with patients being hospitalized for ILI and SRI associated with *Mycoplasma pneumoniae* infection, Klerksdorp and Pietermaritzburg, South Africa, June 2012–May 2015*

			Univariate analysis		Multivariable analysis†	
Factor	ILI, no. (%)	SRI, no. (%)	OR (95% CI)	p value	aOR (95%CI)	p value
Age <5 y	11/27 (40.7)	38/73 (52.1)	1.6 (0.6–3.9)	0.317	7.1 (1.7–28.7)	**0.006**
Female sex	16/17 (59.3)	38/72 (52.8)	0.8 (0.3–1.9)	0.565		
Crowding, no. persons/room						
≤2	18/27 (66.7)	49/72 (68.1)	Reference	NA		
3–4	7/27 (25.4)	19/72 (26.4)	1.0 (0.4–2.7)	0.996		
≥5	2/27 (7.4)	4/72 (5.6)	0.7 (0.1–4.4)	0.734		
Duration of symptoms ≥4 d	7/26 (26.9)	37/69 (53.6)	3.1 (1.2–8.4)	**0.023**	3.8 (1.1–14.1)	**0.046**
HIV infection	2/23 (8.7)	32/62 (51.6)	11.2 (2.4–51.9)	**0.002**	23.8 (4.1–138.2)	**<0.001**
Any underlying medical condition‡	3/27 (11.1)	10/72 (13.9)	1.3 (0.3–5.1)	0.716		
Any viral co-infection§	13/27 (48.1)	47/73 (64.4)	1.9 (0.8–4.7)	0.144		

*Bold indicates statistical significance. aOR, adjusted OR; ILI, influenza-like illness; NA, not applicable; OR, odds ratio; SRI, severe respiratory illness.
†Only variables found to be statistically significant (p<0.2) in univariate analysis were assessed in the multivariable model.
‡Underlying conditions defined as previously diagnosed chronic conditions including asthma, chronic lung diseases, cirrhosis or liver failure, chronic renal failure, heart failure, valvular heart disease, coronary heart disease, diabetes, burns, kwashiorkor or marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, emphysema, and cancer, or history of immunosuppressive therapy or splenectomy.
§Viruses tested were influenza types A and B, adenovirus, enterovirus, rhinovirus, human metapneumovirus, respiratory syncytial virus, and parainfluenza virus types 1–3.

M. pneumoniae patients, young age (<5 years) and HIV infection were associated with severe disease. *M. pneumoniae* strains were susceptible to macrolides and represented 3 P1 types. The higher detection rate of *M. pneumoniae* in periods 1 and 2 of the study suggests that there might be periodicity in *M. pneumoniae* infection in South Africa.

The prevalence of *M. pneumoniae* varies depending on whether a study was performed during an endemic or epidemic year, the laboratory detection method used, or the study participants (*3*). During 2010–2012, an epidemic of *M. pneumoniae* occurred in Denmark, England, Wales, Sweden, Finland, and Germany, with detection rates ranging from 12% to 17% (*23–26*). In France, detection rates of *M. pneumoniae* ranged from 2% to 10% during a 5-year period among outpatients with an acute respiratory illness (*5*). However, higher detection rates of 27%–30% among children with community-acquired pneumonia have been reported in the United States and Finland and up to 60% among hospitalized adults with pneumonia in Japan (*3*,*4*,*27*). Jain et al. reported that, among hospitalized children in the United States, *M. pneumoniae* was the most common bacterial cause of community-acquired pneumonia, accounting for 8% of cases (*28*), and among hospitalized adults in the United States, *M. pneumoniae* was identified in ≈2% of cases (*29*). The prevalence differences in our study compared with other studies might be attributable to a difference in enrollment criteria, the age group of participants, and HIV prevalence among the participants.

Despite having low detection rates, *M. pneumoniae* was significantly associated with illness. The fraction of illness attributable to *M. pneumoniae* in patients testing positive was 80.7% in ILI patients, 90.1% in SRI patients with *M. pneumoniae* detected on nasopharyngeal specimens only, and 93.9% in SRI patients with *M. pneumoniae* detected on both nasopharyngeal and sputum specimens. These results suggest that *M. pneumoniae* can be considered a likely pathogen when detected in patients with ILI or SRI, regardless of specimen type.

We did not observe a distinct seasonal pattern of *M. pneumoniae*. Several more years of surveillance of *M. pneumoniae* is essential to elucidate seasonality in our setting. However, a significant difference was noted in the detection rate over the 3 study periods. Layani-Milon et al. reported that, during a 5-year period (1993–1997), rates of *M. pneumoniae* disease varied monthly and yearly and *M. pneumoniae* occurred in epidemic cycles (*5*). Furthermore, in a serologic study in Johannesburg, South Africa, during

Table 3. Incidence of hospitalization for *Mycoplasma pneumoniae*, by age group and HIV status, Klerksdorp and Pietermaritzburg, South Africa, January 2013–December 2014*

	No. cases/100,000 population (95% CI)			Risk ratio (95% CI), HIV
Age group, y	All patients	HIV-infected patients	HIV-uninfected patients	infected vs. uninfected
<1	220.0 (121.0–314.8)	594.8 (316.4–873.2)	216.7 (128.3–303.8)	2.8 (0.4–18.6)
1–4	53.9 (30.1–75.6)	961.7 (518.4–1391.6)	35.9 (21.1–50.2)	26.8 (14.4–49.8)
5–24	14.1 (9.4–18.6)	130.0 (81.1–173.8)	5.5 (3.9–7.1)	23.8 (13.4–42.4)
25–44	21.4 (15.0–27.6)	70.1 (46.3–95.1)	0 (0–46.7)	NA
45–64	26.2 (17.6–34.9)	118.7 (81.2–159.3)	4.7 (3.4–6.2)	25.6 (9.9–65.9)
≥65	56.2 (37.5–74.2)	0 (0–234.1)	57.8 (41.3–74.9)	NA
<5	87.1 (60.2–114.0)	927.3 (621.3–1231.5)	72.3 (50.5–93.8)	16.1 (9.4–27.6)†
≥5	20.5 (14.0–26.9)	91.3 (59.0–122.4)	6.3 (4.5–8.4)	21.6 (14.8–31.7)†
All	27.9 (18.9–37.4)	102.2 (64.9–136.4)	14.9 (10.3–19.0)	19.5 (14.4–26.4)†

*NA, not applicable.
†Adjusted for age.

1969–1975, the periodicity of *M. pneumoniae* was shown to peak in 3-year intervals (*7*). During period 1 of our study, a cyclical epidemic of *M. pneumoniae* was probably occurring, and this epidemic reached its nadir in period 3 of the study. Longer study periods are required to evaluate the cyclical nature of *M. pneumoniae*.

In our study, a large proportion (65%) of *M. pneumoniae* patients were co-infected with a respiratory virus, of which rhinovirus and adenovirus were the most common. Similarly, Lieberman et al. reported that, in 65% of *M. pneumoniae* patients, ≥1 additional pathogens were detected, of which *Streptococcus pneumoniae* (43%) and *Legionella* spp. (15%) were most frequently detected (*30*).

We found that young age (<5 years) and HIV infection among *M. pneumoniae*–positive persons were independently associated with severe disease. HIV association with *M. pneumoniae* disease was reported in a study conducted in India during 2004–2007 (*31*). The incidence rate of *M. pneumoniae* in South Africa was 28 cases/100,000 population, with the highest incidence occurring in children <5 years old at a rate of 87 cases/100,000 population. We observed a greater disease prevalence among HIV-infected patients than HIV-uninfected patients. Other studies have reported incidence rates ranging from 180 to 1,290 cases/100,000 population/year (*3*,*5*,*32*). Studies have shown variability in detection rates among different age groups, especially in *M. pneumoniae*–endemic areas, where *M. pneumoniae* has occurred predominantly among children <5 years old (*33*,*34*).

A lack of consensus exists regarding the preferred specimen type for the identification of *M. pneumoniae* (*35*,*36*). We observed a significantly higher detection rate of *M. pneumoniae* in sputum compared to nasopharyngeal specimens, similar to results reported by Dorigo-Zetsma et al. (*37*) and Räty et al. (*38*). Although we detected a higher rate in sputum, nasopharyngeal specimens remain the preferred specimen type for surveillance because collecting a nasopharyngeal specimen is less invasive. In addition, a positive result on a nasopharyngeal specimen is a good indicator of disease as indicated by the AF.

During the *M. pneumoniae* epidemic that occurred in Europe during 2010–2012, P1 type 1 was dominant (*24*,*39*). In our population, P1 types 1 and 2 were circulating at equal frequencies. Likewise, in the United States, during an 8-year period (2006–2013) both P1 types were co-circulating (*40*). In China, during 2009–2011, P1 type 1, type 2, and variants of type 2 were identified; however, a higher frequency of type 1 compared with the other P1 types was observed (*41*). Continued surveillance is important to identify longer-term trends in *M. pneumoniae* strain prevalence in South Africa.

By using the 4-loci MLVA typing scheme and nomenclature, we identified 3 distinct MLVA types (3/6/6/2, 3/5/6/2, and 4/5/7/2), which are the same types circulating in the United States, Kenya, Guatemala, Egypt, Denmark, and Canada (*19*,*20*,*40*,*42*). However, the predominance of MLVA type 3/5/6/2 in our study was different to what has been previously described elsewhere in the world. The predominant MLVA type circulating in numerous countries during 1962–2013 was MLVA type 4/5/7/2 (*13*,*20*,*40*). Although no correlations of strain type with disease severity or outcomes have been established, these typing methods are useful for monitoring trends over time and during outbreak investigations.

Macrolide resistance of 17% was documented in *M. pneumoniae* in Japan during 2000–2003 (*43*), with even higher rates of up to 90% reported in China (*44*). In Germany, 1.2% and 3% of *M. pneumoniae* found in respiratory tract specimens were resistant to macrolides during 2003–2008 and 1991–2009, respectively (*45*). In a US study, macrolide resistance was reported for ≈3% of *M. pneumoniae* cases in patients hospitalized with community-acquired pneumonia (*46*). However, other studies have reported macrolide resistance of 10%–13% in sporadic and outbreak specimens in the United States (*40*,*47*). Resistance in Europe and the United States remains low relative to Asia, possibly because of the restricted availability of antimicrobial drugs. We did not identify macrolide resistance among the isolates in our study, and therefore macrolide treatment is probably effective against *M. pneumoniae* in our setting. However, excessive use of macrolides should be discouraged, given that in Japan inappropriate use of macrolides was shown to increase the likelihood of the organism developing mutations in the 23S rRNA gene (*11*). Therefore, identification of the etiologic cause of infection and its appropriate treatment are essential. In South Africa, first-line treatment for community-acquired pneumonia is penicillin (*48*). In severely ill persons or those in whom atypical pneumonia is suspected, macrolides are administered. A limitation of our study is that treatment data for patients with *M. pneumoniae* were limited.

We performed molecular characterization for samples collected during periods 1 and 2 of our study. Most of the positive specimens were obtained during these 2 periods, and results from these periods can be inferred for period 3 because no intervention was implemented. We obtained a low yield of isolates and were unable to determine the macrolide susceptibility trait and strain type for a proportion of specimens, most likely because of a low bacterial load in the specimen, which might have affected the ability to detect resistance particularly if a low prevalence of macrolide-resistant *M. pneumoniae* strains exists in South Africa.

We have shown that, although the *M. pneumoniae* detection rate was low, *M. pneumoniae* detection is probably associated with illness, underscoring the need for testing, especially among patients at higher risk for severe disease.

Such testing would result in an earlier diagnosis and improved management. Our study provides baseline data that can be used for future surveillance programs to better understand *M. pneumoniae* epidemiology in South Africa.

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All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Prospective Observational Study of Incidence and Preventable Burden of Childhood Tuberculosis, Kenya

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Prospective data on childhood tuberculosis (TB) incidence and case detection rates (CDRs) are scant, and the preventable burden of childhood TB has not been measured in prospective studies. We investigated 2,042 children (<15 years of age) with suspected TB by using enhanced surveillance and linked hospital, demographic, notification, and verbal autopsy data to estimate the incidence, CDR, risk factors, and preventable burden of TB among children in Kenya. Estimated TB incidence was 53 cases/100,000 children/year locally and 95 cases/100,000 children/year nationally. The estimated CDR was 0.20–0.35. Among children <5 years of age, 49% of cases were attributable to a known household contact with TB. This study provides much needed empiric data on TB CDRs in children to inform national and global incidence estimates. Moreover, our findings indicate that nearly half of TB cases in young children might be prevented by implementing existing guidelines for TB contact tracing and chemoprophylaxis.

Substantial progress has been made in the fight against tuberculosis (TB); however, new approaches are needed to achieve the current target set by the World Health Organization (WHO) to reduce TB incidence to 90% of 2016 levels by 2035 (*1*). A key element of WHO's End TB Strategy is the prioritization of preventive treatment (*2*). However, the preventable burden of childhood TB has not been quantified in prospective epidemiologic studies, and globally, only an estimated 7% of eligible children received isoniazid chemoprophylaxis in 2015 (*1*).

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Diagnosis of TB is more challenging in children than in adults (*3*). In low-resource settings, where TB burden is highest, diagnosis often relies on poorly validated clinical algorithms (*4*). As a result, adequate surveillance data are lacking, and published estimates of the global childhood TB burden vary widely (*1*,*5*–*11*). High-quality prospective data on the TB burden and case detection rate (CDR) in children are recognized priorities (*8*,*11*,*12*), and population-level data showing the preventable burden of childhood TB might reinforce the public health case for chemoprophylaxis in children. We designed the Kilifi Improving Diagnosis and Surveillance of Childhood TB (KIDS TB) Study to estimate the incidence, CDR, risk factors, and preventable burden of childhood TB in Kenya.

Methods

Study Sites

The study took place at Coast Provincial General Hospital (CPGH) and Kilifi County Hospital (KCH) in Coast Province, Kenya. CPGH provides primary and secondary care to the city of Mombasa and tertiary services for Coast Province. KCH is nested within the Kilifi Health and Demographic Surveillance System (KHDSS) (*13*), which covers a predominantly rural area of 891 km^2 that in March 2011 was home to 261,919 residents in 29,970 households; two thirds of pediatric admissions to KCH during the study period were derived from this system. Three other health facilities in the KHDSS provide TB smear microscopy; 12 clinics are designated TB treatment centers (Figure 1). Because of resource constraints, contact tracing was not routine and isoniazid chemoprophylaxis not available at the time of the study, despite the inclusion of these steps in national TB guidelines.

[1]The following members of the Kilifi Improving Diagnosis and Surveillance of Childhood TB (KIDS TB) Study Group also contributed to patient recruitment, investigation, and management: Victor Bandika, Jay Berkley, Kath Maitland, Susan Morpeth, Daisy Mugo, Robert Musyimi, Agnes Mutiso, John Paul Odhiambo, Monica Toroitich, and Hemed Twahir.

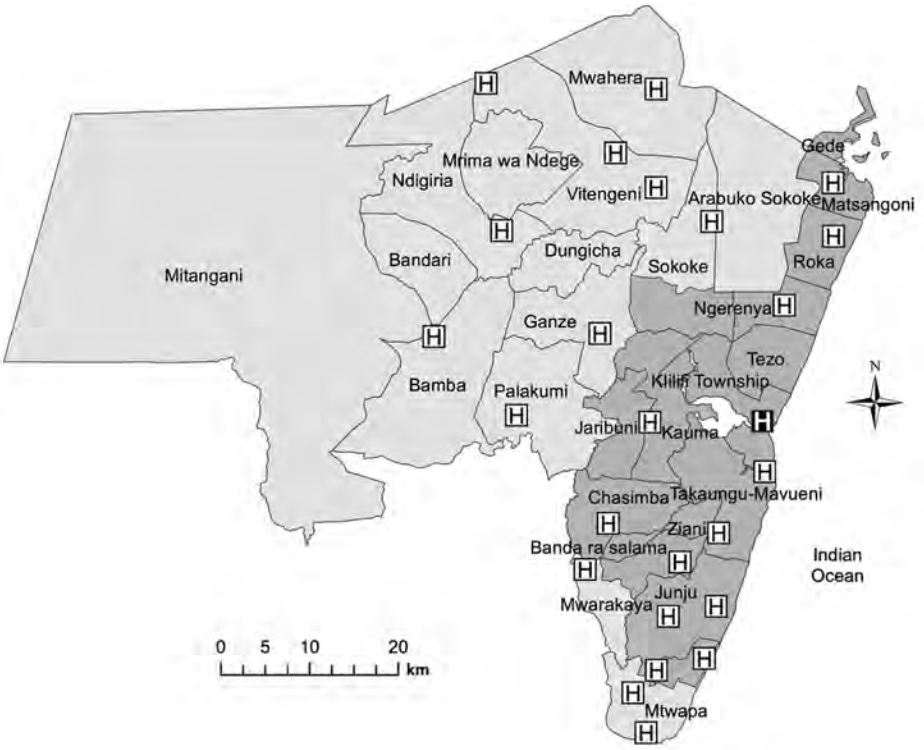

Figure 1. Kilifi District and the Kilifi Health and Demographic Surveillance Survey area (darker gray shading), showing administrative districts, Kilifi County Hospital (black square), and other tuberculosis treatment facilities (white squares), Kenya, 2010.

Participants

We established a system of enhanced passive and active childhood TB surveillance. In the passive case–detection arm, we prospectively recruited all children <15 years of age who were seen at KCH or CPGH during August 2009–July 2011 for an unexplained persistent cough for >2 weeks, pneumonia not responding to antibiotics, unexplained fever for >2 weeks, unexplained progressive weight loss or failure to thrive for >4 weeks, close contact with a person with TB, or clinical suspicion of TB for any other reason. Study clinicians and clinicians from the hospital and surrounding clinics were trained in the symptoms and signs of a range of TB presentations (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/3/17-0785-Techapp1.pdf). We excluded children with an established alternative diagnosis that explained all the clinical features as well as children already on TB treatment for >2 weeks at presentation. In the active case–detection arm, we recruited KHDSS-resident children <5 years of age sharing a household with persons with new cases of smear-positive pulmonary TB.

Clinical Procedures

All children underwent a similar structured history and examination, chest radiography, and tuberculin skin testing according to WHO guidelines (*14*) (online Technical Appendix). Children who were able to expectorate provided up to 3 spontaneous sputum samples. Sputum induction was performed on the remainder (*14*). Further investigations including extrapulmonary or repeat sputum sampling were performed at the discretion of the clinical team caring for the patient. Provider-initiated testing and counseling for HIV was performed according to national guidelines.

We classified children as having confirmed TB, highly probable TB, possible TB, or not TB (TB excluded) according to clinical, radiologic, and microbiological findings, based closely on stringent published definitions (online Technical Appendix Table 2) (*15,16*). For comparison, we also applied other published clinical definitions to our dataset (online Technical Appendix). Treatment protocols followed national guidelines. Children were followed up for 6 months or until a diagnosis of TB could be confidently excluded.

Laboratory Methods

Acid-fast bacilli microscopy and mycobacterial culture using the BACTEC MGIT system (BD Diagnostics, Sparks, MD, USA) were performed according to standard protocols (*17*). Positive cultures were further characterized using the BD MGIT TBc Identification Test (BD Diagnostics) and Hain Genotype line probe assays (Hain Lifescience GmbH, Nehren, Germany), including isoniazid and rifampin drug-susceptibility testing. We performed the Xpert MTB/RIF assay version G4 (Cepheid, Sunnyvale, CA, USA) at the end of the study on specimens from all children treated for confirmed, highly probable, or possible TB as well as from children for whom a TB diagnosis had been excluded. Laboratory procedures were externally

monitored using the United Kingdom National External Quality Assessment Service's quality-assurance scheme (http://ukneqas.org.uk).

Statistical Analysis

Incidence Estimates

We used clinical data from KCH and event data from KHDSS to compile for every KHDSS-resident child a series of chronological time-span records representing the periods between consecutive birth, migration, enumeration, hospital presentation, or death events during the study period. We split these periods of observation by age category and estimated crude TB incidence rates as the total number of new TB cases identified (by both active and passive case detection) divided by the total person-years of observation in each age stratum. We compared estimates generated using the study case definitions with incidence estimates derived by applying other published clinical definitions of childhood TB to our dataset (online Technical Appendix).

Estimating the CDR

Crude incidence estimates assume all incident cases among KHDSS residents are captured by the study; however, hospital-based surveillance of childhood illnesses is known to be insensitive in this setting (*18–20*). We defined the CDR as the proportion of KHDSS-resident TB cases captured by the study. Because the actual number of children with TB is unknown, we used 3 different methods to estimate the CDR independently (detailed description in online Technical Appendix).

TB Notification Data

We linked clinical data with National Tuberculosis Programme notification data and KHDSS census data. We estimated the CDR as 1) the proportion of KHDSS-resident smear-positive childhood TB cases reported to the National Tuberculosis Programme that were captured by passive case detection at KCH, and 2) the proportion of children's household contacts of new smear-positive pulmonary TB cases captured by active contact tracing.

Hospital-Based Mortality Surveillance

We linked KHDSS vital status data with KCH admission data. We then calculated the proportion of all childhood deaths in the KHDSS area captured at KCH during the study period.

Verbal Autopsy

By using disease-specific mortality data from a contemporaneous verbal autopsy study of all deaths within the KHDSS (*21*), we estimated the proportion of childhood TB deaths captured by our study. Because the number of child TB cases diagnosed by verbal autopsy is small and healthcare-seeking behavior is usually determined by clinical features rather than diagnosis per se (*20*,*22*), we also estimated the CDR as the proportion of children who died having clinical features of suspected TB that were captured by the study.

To derive the most conservative estimates of the actual annual incidence of childhood TB, we divided crude incidence rates by the highest CDR estimate. We modeled the likely number of incident confirmed or highly probable TB (CHPTB) cases among children nationally by multiplying the total number of adult cases reported in Kenya in 2010 (*23*) by the ratio of child-to-adult cases in the KHDSS, assuming a similar ratio and adult CDR nationally. We then used denominator population data from the national census (*24*) to estimate the national incidence of childhood TB.

Risk Factors for Childhood TB

We explored risk factors for childhood TB in a nested case–control analysis of children with CHPTB (cases) and children for whom TB was excluded (controls). To mitigate ascertainment bias in analysis of TB contact history, we excluded the small minority of children identified through active contact tracing. For each association, we derived crude odds ratios (ORs) and 95% CIs. We then included in a multivariable logistic regression model those variables with at least a weak association with TB in the univariable analysis (likelihood ratio test; $p \leq 0.1$) and presented adjusted ORs and 95% CIs.

By using the number of KHDSS-resident adult cases reported to the National Tuberculosis Programme during the study period and the mean number of close contacts <5 years of age per case (*25*), we estimated the prevalence of household exposure to a person with confirmed TB among KHDSS-resident children <5 years of age. Using the contact status of CHPTB cases detected in the study, the child years at risk derived from the KHDSS census, and the exposure prevalence, we estimated the incidence of TB among contacts and noncontacts. The population attributable fraction for contact with a person with confirmed TB was calculated from the ensuing incidence rate ratio (IRR) and the exposure prevalence (p) by calculating $p(IRR - 1)/1 + p(IRR - 1)$ (online Technical Appendix).

Results

We identified 2,183 children with suspected TB during the study period and summarized patient enrollment and diagnostic assignments (Figure 2). We excluded 141 (6%) children who died, were discharged, or were lost to follow-up before their diagnostic workups, including specimen collection for mycobacterial culture, could be completed (Figure 2). We summarized baseline clinical characteristics of the remaining 2,042 children included in the analyses (Table 1).

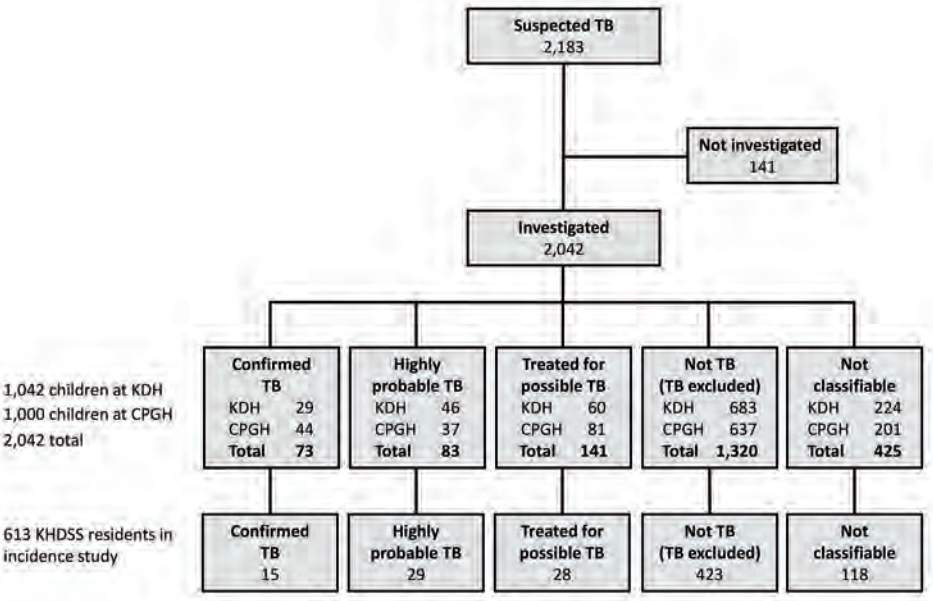

Figure 2. TB patient enrollment and disease classifications, Kilifi Health and Demographic Surveillance Survey, Kenya, August 2009–July 2011. A total of 141 children were not investigated (27 died, 40 were discharged, 3 were transferred, 1 self-discharged before workup completed, 30 defaulted outpatient follow-up, 40 had no reason documented). At KDH, 108/1,042 (10%) children investigated were identified through active contact tracing (2 confirmed TB, 4 highly probable TB, 87 not TB, 15 not classifiable). CPGH, Coast Provincial General Hospital; KDH, Kilifi District Hospital; KHDSS, Kilifi Health and Demographic Surveillance Survey; TB, tuberculosis.

Crude Incidence Estimates

We determined crude, hospital-based, age-specific incidence rates based on the study definitions (Table 2). The incidence of all childhood TB was 30.2 (95% CI 23.6–38.0) cases/100,000 children/year. The incidence of CHPTB was 18.4 (95% CI 13.4–24.7) cases/100,000 children/year; this estimate was very similar to that derived by retrospectively applying to our data consensus definitions of definite or probable TB that were published after completion of our study (*26*) (20.5 [95% CI 15.2–27.1]/100,000/year). Both figures are at the lower end of the range of estimates derived using published clinical definitions, which vary >30-fold (2.9–91.7/100,000/year) (Table 3).

CDR and Adjusted Incidence Estimates

CDR estimates derived using TB notifications, KHDSS census data, and verbal autopsy ranged from 0.2 to 0.35 (Table 4), substantially lower than the estimated CDR of 0.82 for adults in Kenya (*41*). Hospital-based mortality surveillance provided the largest and most precise estimate of the CDR (0.35 [95% CI 0.31–0.40]), so we used this to derive the most conservative estimates of the actual community incidence of childhood TB (Table 5). After adjustment for CDR, the incidence of CHPTB and all TB among children in the KHDSS was 53 (95% CI 38–71) and 86 (95% CI 67–109) cases/100,000/year, respectively.

Implications for the National Incidence of Childhood TB

During August 2009–July 2011, a total of 678 new cases of adult TB were reported to the National Tuberculosis Programme, and an estimated 126 new CHPTB cases were reported in children (Table 5) among KHDSS residents. Nationally 89,883 adult and 5,721 child TB cases were reported in 2010 (*41*) among a population that includes ≈17.6 million children <15 years of age (*24*). Applying the ratio of adult-to-child TB cases in the KHDSS to the national caseload yields an estimated 16,704 new CHPTB cases among children <15 years of age nationally in 2010, suggesting a national childhood TB CDR of 29% and incidence of 95 cases/100,000 children/year (online Technical Appendix Table 3).

Risk Factors for Childhood TB

We summarized associations of CHPTB and important putative risk factors (Table 6). A history of known close TB contact at presentation was strongly associated with CHPTB, with an effect gradient according to the contacts' smear status, proximity, relationship, and number (online Technical Appendix Table 4). No child case-patients with a close TB contact had received isoniazid chemoprophylaxis. We observed a weaker association with HIV and in young children with severe malnutrition but no association between the presence of a bacillus Calmette-Guérin (BCG) vaccination scar and TB, although power to detect an effect was low because of the small proportion of children without a BCG vaccination scar.

Preventable TB Burden among Child Household TB Contacts

Among KHDSS-resident children <5 years of age, an estimated 1,259 were close contacts of adults with new TB cases reported during the study period. The incidence of CHPTB was 596 cases/100,000/year among children with a close TB contact and 17 cases/100,000/year among those without a close TB contact, yielding a 49% population attributable fraction for having a recent and known TB contact (online Technical Appendix Table 5).

Table 1. Baseline characteristics of children with and without TB examined at Kilifi County Hospital and Coast Provincial General Hospital, Kenya, August 2009–July 2011*

Characteristic	Confirmed TB, n = 73	Highly probable TB, n = 83	Treated for possible TB, n = 141	Not TB/TB excluded, n = 1,320	Not classifiable, n = 425
Case ascertainment					
Passive case detection	71 (97)	79 (95)	141 (100)	1,237 (94)	410 (96)
Active case detection (contact tracing)	2 (3)	4 (5)	0 (0)	83 (6)	15 (4)
Patient demographics					
Median age (interquartile range), mo	52 (16–114)	32 (13–70)	17 (10–64)	17 (10–41)	17 (9–44)
0–4 y	38 (55)	59 (71)	99 (70)	1,119 (85)	345 (81)
5–9 y	17 (25)	15 (18)	27 (19)	140 (11)	56 (13)
10–14 y	18 (25)	9 (11)	15 (11)	61 (4)	24 (6)
Sex					
M	39 (53)	43 (52)	70 (50)	696 (53)	224 (53)
F	32 (47)	40 (48)	71 (50)	624 (47)	201 (47)
Risk factors for TB					
HIV infected	17 (23)	21 (25)	42 (30)	160 (12)	112 (26)
Severely malnourished	30 (41)	37 (45)	58 (41)	457 (35)	162 (38)
BCG vaccination scar	65 (89)	86 (71)	128 (91)	1,172 (89)	338 (80)
Close TB contact	36 (49)	33 (40)	27 (19)	246 (19)	78 (18)
Clinical features of suspected TB					
Cough >2 wks	48 (66)	48 (58)	95 (67)	572 (43)	225 (53)
Fever >2 wks	45 (62)	30 (36)	92 (65)	502 (38)	196 (46)
Weight loss or failure to thrive >4 wks	42 (58)	39 (47)	77 (55)	575 (44)	208 (49)
Pneumonia not responding to 1st-line ABX	27 (37)	25 (30)	42 (30)	308 (23)	159 (37)
TB clinical syndrome					
Smear-positive pulmonary TB	20 (27)	4 (5)	0	NA	NA
Smear-negative pulmonary TB	40 (55)	69 (83)	108 (77)	NA	NA
All pulmonary TB†	60 (82)	73 (88)	108 (77)	NA	NA
Extrapulmonary TB†	30 (41)	17 (20)	46 (33)	NA	NA
Miliary TB	6 (8)	3 (4)	5 (4)	NA	NA
TB meningitis	8 (11)	2 (2)	12 (9)	NA	NA
Pleural TB	6 (9)	2 (2)	7 (5)	NA	NA
TB lymphadenitis	6 (8)	6 (7)	6 (4)	NA	NA
Osteoarticular TB	2 (3)	3 (4)	1 (1)	NA	NA
Abdominal TB	9 (12)	2 (2)	10 (7)	NA	NA
Persistent fever without a focus	0	1 (1)	13 (9)	NA	NA
Drug resistance					
Isoniazid monoresistance	0	NA	NA	NA	NA
Multidrug-resistant TB	1 (1.4)	NA	NA	NA	NA

*Values are no. (%) unless otherwise indicated. ABX, antibiotics; BCG, bacillus Calmette-Guérin; NA, not applicable; TB, tuberculosis.
†Some children had >1 focus of infection, including some with pulmonary TB and extrapulmonary TB. Among children with confirmed TB, microbiologic confirmation was required from ≥1 site; diagnosis of other sites of disease was based on the definitions of highly probable TB (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/3/17-0785-Techapp1.pdf).

Discussion

This study provides rare prospective empiric data on the TB incidence and CDR among children <15 years of age in Kenya, a country with a high TB burden, and is one of few prospective incidence studies globally (*3*). This community-based study was nested in a demographic surveillance survey, underpinned by enhanced active and passive surveillance, mycobacterial culture facilities, and linked hospital, demographic, notification, and verbal autopsy data. We used a hierarchical diagnostic classification in keeping with recommendations for childhood TB surveillance and research (*26*,*35*). A comprehensive algorithm of clinical, radiologic, and laboratory investigations combined with careful follow-up of children enrolled in the KIDS TB Study ensured diagnostic classifications were optimized within the limitations of currently available diagnostic tools.

Although the diagnosis of confirmed TB has the highest specificity, the poor sensitivity of mycobacterial culture for childhood TB diagnosis means that incidence estimates based only on confirmed cases will underestimate the actual disease burden. Conversely, including possible TB cases in the numerator might overestimate incidence. Most children in the highly probable TB group probably did have TB, given the stringent diagnostic criteria, and although the sensitivity of this classification is not perfect, it probably captured many of the actual cases of active TB for which culture confirmation was not obtained. We therefore used a combination of confirmed or highly probable TB (CHPTB) as the measure most likely to optimize sensitivity and specificity for estimation of childhood TB incidence.

Compared with estimates based on published clinical definitions, our measure of CHPTB incidence is among the most conservative, similar to the estimate obtained by

Table 2. Crude hospital-based childhood TB incidence, by age group and diagnostic classification, Kilifi Health and Demographic Surveillance Survey, Kenya, August 2009–July 2011*

TB classification	Age group, y	No. person-years of observation	No. KHDSS-resident TB cases	Incidence, cases/100,000 children/y (95% CI)
Confirmed TB	0–4	89,503	7	7.8 (3.1–16.1)
	5–9	79,170	6	7.6 (2.8–16.5)
	10–14	70,073	2	2.9 (0.3–10.3)
	Total	238,746	15	6.3 (3.5–10.4)
Confirmed or highly probable TB	0–4	89,503	30	33.5 (22.6–47.9)
	5–9	79,170	11	13.9 (6.9–24.9)
	10–14	70,073	3	4.3 (0.9–12.5)
	Total	238,746	44	18.4 (13.4–24.7)
All TB	0–4	89,503	46	51.4 (37.6–68.6)
	5–9	79,170	21	26.5 (16.4–40.6)
	10–14	70,073	5	7.1 (2.3–16.7)
	Total	238,746	72	30.2 (23.6–38.0)

*KDHSS, Kilifi Health and Demographic Surveillance Survey; TB, tuberculosis.

retrospectively applying more recent consensus definitions for research (*26*). Even after inclusion of all TB cases, our measure remained among the lowest, suggesting that many published clinical definitions would overdiagnose TB in this and similar settings were they to be applied routinely in clinical practice. The huge range in incidence estimates derived using different case definitions emphasizes the difficulty in interpreting existing disease burden data and the need for high-quality prospective incidence studies to improve disease burden estimates.

Robust community incidence estimates depend on high-quality diagnosis to minimize misclassification as well as a high CDR. Broad screening criteria for all children admitted to hospital with any features of suspected TB, plus active case detection through contact tracing, ensured that case ascertainment at KCH was optimized. Nevertheless, the social, financial, and geographic barriers to obtaining hospital care in this setting mean that many ill KHDSS-resident children are not seen at KCH (*18–20*). Furthermore, challenges in childhood TB diagnosis, combined with limited diagnostic resources, make surveillance data from other health facilities unreliable. We therefore estimated the CDR of hospital-based surveillance at KCH by using 3 independent techniques. Each measure is necessarily a surrogate, and each has limitations, but the similarity of these estimates supports their validity.

Because we used the highest CDR estimate to generate conservative estimates of childhood TB incidence, the

Table 3. Incidence of childhood TB derived by applying other published clinical definitions, algorithms, and guidelines, in order of increasing incidence, Kilifi Health and Demographic Surveillance Survey, Kenya, August 2009–July 2011*

Author, year (reference)	Outcomes defined	No. cases	Incidence, cases/100,000 children/y (95% CI)†
WHO, 2006 (*27*)	(a) Strongly suggestive of TB‡	7	2.9 (1.2–6.0)
Stegen (*28*)	(a) Probable TB	18	7.5 (4.5–11.9)
Nair (*29*)	(a) "TB appears unquestionable"	28	11.7 (7.8–17.0)
WHO, 2006 (*27*)	(b) Requires investigation for TB‡	33	13.8 (9.5–19.4)
Graham (*26*)	Probable TB	42	17.6 (12.7–23.8)
Hawkridge (*30*)	Probable TB	54	22.6 (17.0–29.5)
Nair (*29*)	(b) TB probable or "unquestionable"	55	23.0 (17.4–30.0)
Stoltz (*31*)	Probable TB	73	30.6 (24.0–38.5)
Jeena (*32*)	Probable TB	107	44.8 (36.7–54.2)
Edwards (*33*)	Criteria for TB treatment	110	46.1 (37.9–55.5)
Ghidey (*34*)	(a) Criteria for TB treatment§	113	47.3 (39.0–56.9)
WHO, 1983 (*35*)	Probable TB	116	48.6 (40.2–58.3)
Ramachandran (*36*)	Criteria for TB treatment	118	49.4 (40.9–59.2)
Ghidey (*34*)	(b) Criteria for TB treatment§	130	54.5 (45.4–64.7)
Stegen (*28*)	(b) Probable or possible TB	136	57.0 (47.8–67.4)
Graham (*26*)	Probable or possible TB	145	60.7 (51.3–71.5)
Osborne (*37*)	Probable TB	159	66.6 (56.7–77.8)
Fourie (*38*)	High probability of TB¶	162	67.9 (57.8–79.2)
Cundall (*39*)	Probable TB	207	86.7 (75.3–99.4)
Kiwanuka (*40*)	Probable TB	219	91.7 (80.0–104.7)

*TB, tuberculosis; WHO, World Health Organization.
†Denominator for incidence calculations is the total person-years observation among children age <15 y (N = 238,746).
‡Results shown separately for (a) children whose clinical features "strongly suggest a diagnosis of TB" according to the guidelines, and (b) using broader criteria that included under "physical signs highly suggestive of TB" all the other "suggestive clinical signs" listed as requiring investigation for TB.
§Results for Ghidey and Habte tool (*34*) shown using both (a) ≥3 and (b) ≥2 signs and symptoms to define a "suggestive symptom complex of TB" (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/3/17-0785-Techapp1.pdf).
¶For the purposes of our analyses, we used "score 2" proposed by Fourie et al (*38*), which was derived in high TB burden settings in South Africa, Madagascar, and Nicaragua.

Table 4. Case detection rate estimates derived by using TB notifications, Kilifi Health and Demographic Surveillance Survey census data, and verbal autopsy methods, Kenya, August 2009–July 2011*

Method	Calculation of CDR estimate	CDR estimate (95% CI)
TB notifications		
Passive case detection	$\frac{\text{No. KHDSS-resident smear-positive child TB cases captured by KIDS TB Study}}{\text{Total no. KHDSS-resident smear-positive child TB cases}} = \frac{3}{10}$	0.30 (0.07–0.65)
Active contact tracing	$\frac{\text{No. index cases captured at KCH}}{\text{Total no. index cases}} \times \frac{\text{No. eligible child contacts investigated}}{\text{Total no. eligible child contacts identified at KCH}} = \frac{195}{362} \times \frac{108}{297}$	0.20 (0.13–0.26)
KHDSS census		
Mortality surveillance	$\frac{\text{No. KHDSS-resident children who died at KCH}}{\text{Total no. childhood deaths among KHDSS residents}} = \frac{182}{514}$	0.35 (0.31–0.40)
Verbal autopsy		
TB deaths	$\frac{\text{No. TB deaths in VA study that occurred in children captured by KIDS TB Study}}{\text{No. TB deaths in VA study}} = \frac{2}{10}$	0.20 (0.03– 0.56)
TB suspected deaths	$\frac{\text{No. TB suspected deaths in VA study that were captured by KIDS TB Study}}{\text{No. TB suspected deaths in VA study}} = \frac{x}{y}$	0.22 (0.15–0.32)

*CDR, case detection rate; KCH, Kilifi County Hospital; KHDSS, Kilifi Health and Demographic Surveillance Survey; TB, tuberculosis, VA, verbal autopsy.

projected national incidence was 3 times higher than that reported. Nevertheless, the projected ratio of adult-to-child TB cases is still consistent with other studies in Africa (*43*,*44*) and with recent global estimates (*1*,*5*,*6*,*9*), although lower than some regional and global figures (*3*). Other estimates of the global TB burden have indicated a lower proportion of childhood cases (*7*,*8*). However, in the absence of data from children, those estimates assume a similar CDR for adults (*8*) or impute missing data based on reported proportions of smear-negative and extrapulmonary TB by age group (*7*), assumptions that have been challenged (*11*,*45*). Our study provides important empirical data on the probable CDR among children. The results suggest that the CDR among children is substantially lower than among adults and support estimates derived using other modeling approaches (*5*,*6*), including recently revised WHO estimates of global childhood TB incidence that assume a CDR of 36% (*9*).

The strong association of childhood TB with a history of close TB contact has 2 important implications for clinical practice and public health policy. First, eliciting a history of TB contact should be a standard part of the assessment of every ill child in TB-endemic settings. Among inpatients in our study, 1 in 5 with a known close TB contact had CHPTB. Early identification and investigation of this high-risk group might improve clinical outcomes through earlier diagnosis and treatment.

Second, and most important, our finding that 49% TB cases among children <5 years of age were attributable to a known household TB contact suggests that half the CHPTB cases in young children might have been prevented by chemoprophylaxis. Estimating the population attributable fraction of contact with a person with confirmed TB provides a novel approach for assessing the potential impact of TB chemoprophylaxis at the population level that might be applied to other settings. Our results from Kenya support recent global estimates of TB burden among child TB contacts (*25*). By demonstrating a large potential impact on childhood TB incidence, our findings provide further strong endorsement for existing policy recommendations for TB chemoprophylaxis (*25*,*46*).

Extrapolation of results from a single district must be interpreted with caution. Childhood TB incidence and the contribution of childhood TB cases to the total TB burden are likely to be affected by factors that vary geographically,

Table 5. Estimated annual caseload and incidence of childhood TB after adjustment for the case detection rate, Kilifi Health and Demographic Surveillance Survey, August 2009–July 2011*

TB classification	Age group, y	No. cases	Adjusted incidence, cases/100,000 children/y (95% CI)
Confirmed TB	0–4	20	22 (9–46)
	5–9	17	22 (8–47)
	10–14	6	9 (1–29)
	Total	43	18 (10–30)
Confirmed or highly probable TB	0–4	86	96 (65–137)
	5–9	31	39 (20–71)
	10–14	9	13 (3–36)
	Total	126	53 (38–71)
All TB	0–4	131	146 (107–196)
	5–9	60	76 (47–116)
	10–14	14	20 (7–48)
	Total	205	86 (67–109)

*To generate the most conservative estimates of community childhood TB incidence, we used the highest case detection rate estimate of 0.35 derived from hospital-based mortality surveillance. TB, tuberculosis.

Table 6. Crude and adjusted odds ratios for risk factors associated with confirmed or highly probable TB among children examined at Kilifi County Hospital and Coast Provincial General Hospital, Kenya, August 2009–July 2011*

	Cases		Controls					
Age group	Factor present	Factor absent	Factor present	Factor absent	Crude OR for TB (95% CI)	p value	aOR for TB (95% CI)	p value
Children <5 y								
HIV infection†	17	73	112	872	1.8 (1.0–3.2)	0.036	1.3 (0.7–2.4)	0.321
Severe malnutrition‡	56	35	413	620	2.4 (1.5–3.7)	<0.001	2.6 (1.6–4.1)	<0.001
BCG vaccination scar	82	9	921	112	1.1 (0.5–2.3)	0.779	–	
Close TB contact	33	58	125	908	4.1 (2.6–6.6)	<0.001	5.1 (3.1–8.3)	<0.001
Children 5–14 y								
HIV infection†	21	38	47	143	1.7 (0.9–3.2)	0.103	1.5 (0.8–2.9)	0.229
Severe malnutrition‡	9	50	43	157	0.7 (0.3–1.4)	0.294	–	
BCG vaccination scar	48	11	173	27	0.7 (0.3–1.5)	0.327	–	
Close TB contact	30	29	34	166	5.1 (2.6–9.9)	<0.001	5.2 (2.7–9.8)	<0.001
All children <15 y								
HIV infection†	38	111	159	1,015	2.2 (1.5–3.3)	<0.001	1.9 (1.2–2.9)	0.003
Severe malnutrition‡	65	85	456	777	1.3 (0.9–1.8)	0.130	–	
BCG vaccination scar	130	20	1,094	139	0.8 (0.5–1.4)	0.455	–	
Close TB contact	63	87	159	1,074	5.0 (3.4–7.3)	<0.001	5.0 (3.4–7.2)	<0.001

*aOR, adjusted odds ratio; BCG, bacillus Calmette-Guérin; OR, odds ratio; TB, tuberculosis.
†HIV status was missing for 1/150 (0.7%) cases and 59/1233 (4.8%) controls.
‡Severe malnutrition defined according to World Health Organization guidelines as weight-for-age z-score of ≤3 or the presence of nutritional edema (*42*).

including community TB prevalence; social and demographic factors, such as urbanization, that affect the annual risk for TB infection; prevalence of host factors, such as BCG vaccination, HIV infection, and malnutrition; and local population structures. Therefore, we did not attempt simply to age-standardize the Kilifi incidence rates to the national population of children in Kenya.

We reasoned instead that the proportion of the total TB caseload accounted for by children is probably less prone to geographic variation, and estimated the national burden of childhood TB by assuming that the CDR among adults and the ratio of adult-to-child cases is the same in the KHDSS and nationally. Importantly, the age structures of the KHDSS and Kenya are very similar (*13*,*24*), suggesting that age is unlikely to confound this approach. Compared with Kilifi, the higher estimate of TB incidence nationally is consistent with greater urbanization (*13*,*24*) and a higher annual risk for TB infection (*47*), HIV prevalence (*24*), and overall TB incidence (*1*). Because ecologic data suggest that the pediatric proportion of cases actually increases with increasing overall TB incidence (*6*,*12*), this approach might underestimate the actual national childhood TB burden. Our restriction of TB cases to those that met the stringent criteria of CHPTB and our adjustment of hospital-based incidence rates using the highest CDR estimate also suggest that our estimates are conservative.

In conclusion, by using a combination of clinical, laboratory, and epidemiologic resources not usually available for routine surveillance, we have estimated the incidence of childhood TB in Kenya. Although this study is very resource-intensive, the wide range of incidence estimates based on existing clinical definitions highlights the difficulty in interpreting routine notification data and reinforces the need for similar studies in a range of different epidemiologic settings. In a setting where routine facilities for childhood TB diagnosis are typical of most countries with a high TB burden, our results also provide important empirical data on the TB CDR among children. The results support recently improved WHO estimates of global childhood TB incidence based on modeling approaches, which assume a very similar CDR (*1*,*9*). Our findings also reinforce the urgent need to improve case detection among children to reduce childhood TB mortality (*48*). Crucially, they suggest that half the TB cases in young children might be prevented by implementing existing WHO guidelines for contact tracing and chemoprophylaxis.

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Author contributions: A.J.B., J.A.G.S., and M.L. designed the study with input from C.R.J.N., T.N.W., C.N., E.B., J.S., and K.P. A.J.B., J.L., C.M., and J.W. recruited and followed up children with suspected TB. Chest radiographs were read and interpreted by A.J.B., J.S., and K.P. A.J.B. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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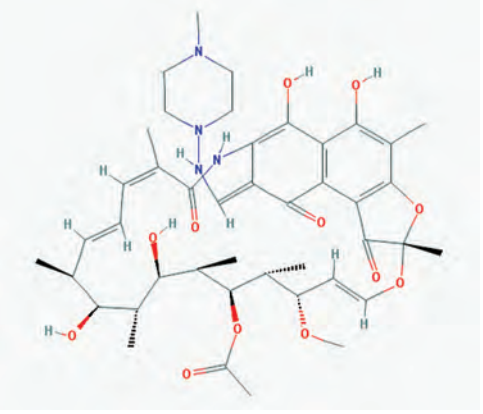
etymologia

Ronnie Henry

Rifampin *[rif-am'pin]*

In 1957, Piero Sensi and colleagues isolated a new bacterium, *Streptomyces mediterranei* (now *Amycolatopsis rifamycinica*), from a soil sample from a pine forest in France. Material extracted from fermentation broths of *A. rifamycinica* contained microbiologically active substances that, as a group, were nicknamed Rififi. *Rififi* (French slang for "trouble") was a 1955 French gangster film that was popular at the time and became the root of the name "rifamycin" for this group of antimicrobial agents. (Similarly, matamycin was originally nicknamed Mata Hari.) Rifampin (also known as rifampicin) is the N-amino-N′-methylpiperazine (AMP) derivative of rifamycin.



Chemical structure of Rifampin. Data deposited in or computed by PubChem, source: PubChem; https://pubchem.ncbi.nlm.nih.gov/compound/5381226#section=2D-Structure

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Acquired Resistance to Antituberculosis Drugs in England, Wales, and Northern Ireland, 2000–2015

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Among tuberculosis (TB) patients, acquired resistance to anti-TB drugs represents a failure in the treatment pathway. To improve diagnosis and care for patients with drug-resistant TB, we examined the epidemiology and risk factors associated with acquired drug resistance during 2000–2015 among TB patients in England, Wales, and Northern Ireland. We found acquired resistance in 0.2% (158/67,710) of patients with culture-confirmed TB. Using multivariate logistic regression, we identified the following factors associated with acquired drug resistance: having pulmonary disease; initial resistance to isoniazid, rifampin, or both; a previous TB episode; and being born in China or South Africa. Treatment outcomes were worse for patients with than without acquired resistance. Although acquired resistance is rare in the study area, certain patient groups are at higher risk. Identifying these patients and ensuring that adequate resources are available for treatment may prevent acquisition of resistance, thereby limiting transmission of drug-resistant strains of mycobacteria.

In 2015, of an estimated 10.4 million incident tuberculosis (TB) cases worldwide, 4.6% were multidrug resistant (MDR) (*1*). MDR TB is a major challenge for TB control, and strategies to reduce it underpin the 3 pillars of the World Health Organization End TB Strategy (*1*,*2*). The main challenges are preventing acquisition of drug resistance and ensuring early detection and appropriate treatment to prevent further transmission of drug-resistant TB. As a result, in the Collaborative TB Strategy for England 2015–2020, reducing drug-resistant TB is a key area of action for ensuring that adequate resources needed to prevent and treat MDR TB exist (*3*).

In the United Kingdom, the proportion of MDR TB cases has remained stable over the past decade, accounting for 1.5% of all culture-confirmed TB cases in 2015, similar to the proportions in other low-incidence countries, including the United States (1.2%) and France (1.7%) (*1*,*4*). Other drug-resistance profiles affect treatment and include isoniazid resistance without MDR TB (attributable to 5.6% of TB cases in the United Kingdom) and rifampin resistance without MDR TB (accounts for only 0.2% of TB cases) (*4*). In addition to the clinical challenges of long and complex treatment regimens often involving adverse reactions for patients with drug-resistant TB, social risk factors and concurrent conditions may complicate treatment and worsen outcomes (*5*).

Drug-resistant TB can arise in 2 ways: through transmission of a drug-resistant strain (primary drug resistance) or through acquisition of drug resistance (acquired drug resistance, in which a person is infected by a strain that is initially sensitive to a particular drug but resistance to that drug evolves later) (*6*,*7*). Acquired drug resistance can result from inadequate treatment, which may be caused by interruptions to receipt of the full drug regimen (*8*,*9*). Treatment interruption because of drug unavailability, poor adherence, or side effects contributes to insufficient dosing or treatment duration (*7*).

Acquired drug resistance has consequences for public health through the spread of drug-resistant TB, poorer health outcomes for patients, and cost to the healthcare system (*10*–*12*). Understanding the risk factors associated with acquired resistance could improve prevention measures. Our objective for this study was to describe the epidemiology of TB among patients in England, Wales, and Northern Ireland, in whom drug resistance was acquired while they were receiving treatment. We also examined the frequency and timing of acquired resistance, sociodemographic and clinical factors associated with acquired resistance, and treatment outcomes for these patients.

Methods

Study Population and Definitions

We conducted a retrospective cohort study of all patients with culture-confirmed TB (pulmonary and

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extrapulmonary-only disease) notified to the Enhanced TB Surveillance System in England, Wales, and Northern Ireland during 2000–2015 for whom initial drug-susceptibility testing (DST) results for at least isoniazid and rifampin were available. Acquired resistance was defined as a sensitive DST result for a drug on 1 isolate and a subsequent resistant DST result for the same drug on another. We excluded TB patients known to have not started treatment.

Laboratory Methods and Data

The United Kingdom follows the global guidance that samples should be obtained for culture and DST at least every 1, 2, and 5 months, whereas samples from patients whose TB was initially MDR TB should be obtained every month (*13–15*). Samples are collected more often from patients who do not clinically respond to treatment as expected.

Samples from all presumptive TB patients are sent from hospital laboratories in England, Wales, and Northern Ireland to a *Mycobacterium* reference laboratory for speciation, phenotypic DST, and 24-loci MIRU-VNTR (mycobacterial interspersed repetitive unit–variable-number tandem repeat) strain typing (*16*). Results from culture-confirmed *M. tuberculosis* complex samples were routinely extracted from laboratory information management systems and imported into the Enhanced TB Surveillance System. The strain lineage was derived from the MIRU-VNTR strain types for cases notified during 2010–2015 (*17*). The reference laboratories routinely performed DST for first-line drugs (isoniazid, rifampin, ethambutol, and pyrazinamide) on all *M. tuberculosis* complex samples. DST for second-line injectables (amikacin, capreomycin, and kanamycin) and fluoroquinolones (ofloxacin, moxifloxacin, and ciprofloxacin) was performed on rifampin-resistant isolates or at the request of the treating clinician. DST results for ethambutol and pyrazinamide can vary (*18*), which was mitigated at the reference laboratory by repeat testing of isolates that initially showed resistance.

Data Collection

Clinical and demographic data for TB patients were collected in the Enhanced TB Surveillance System and probabilistically matched to *M. tuberculosis* complex isolates (*5*,*19*). We analyzed individually the 8 countries of birth with the highest number of TB patients with acquired drug resistance. Data on patient social risk factors (current or history of drug misuse, alcohol misuse, homelessness, and imprisonment) were available for cases notified during 2010–2015. Treatment outcome was reported for cases notified during 2001–2013.

Descriptive Analyses

We described cases with acquired resistance by year of notification. The proportion of TB patients in whom resistance to each drug was acquired was calculated from the number of TB patients for whom DST results for that drug were available. TB patients with acquired resistance were categorized according to their initial drug resistance profile (Table 1) and acquired drug resistance profile (Table 2) throughout the analysis. Each new identification of resistance in different cultures from the same patient was defined as an episode of acquired resistance.

The time to diagnosis of acquired resistance (referred to as acquiring drug resistance) was calculated from the treatment start date to the specimen collection date for the first DST result that showed evidence of acquired resistance. The time to diagnosis of acquired resistance was calculated for TB patients who acquired resistance after starting an appropriate treatment; therefore, we excluded from this section of analysis those patients who acquired resistance before starting an appropriate treatment or who did not have a known start date for appropriate treatment.

Statistical Analyses

We compared clinical and demographic data among patients with any acquired resistance with those without acquired resistance by using univariable logistic regression to calculate odds ratios (ORs). We created a multivariable model by using a forward stepwise approach, including factors with $p<0.5$ from the univariable analysis and using data from the entire cohort (2000–2015) (model 1). We created a second multivariable model limiting the data to 2010–2015 because of the availability of social risk factors and strain type lineage data (model 2).

To account for the variability in resistance results for ethambutol and pyrazinamide, we performed a sensitivity analysis by excluding from logistic regression model 1 those TB patients who acquired resistance to only ethambutol and pyrazinamide. Because of low numbers and loss of power, we did not perform a sensitivity analysis on model 2.

Table 1. Initial drug resistance profiles used to categorize resistance to anti-TB drugs*

Initial drug resistance profile	Description of drug-resistance profile†
Drug sensitive	Initially sensitive to both isoniazid and rifampin
Isoniazid resistance without MDR TB	Initially resistant to isoniazid and sensitive to rifampin
Rifampin resistance without MDR TB	Initially resistant to rifampin and sensitive to isoniazid
MDR TB	Initially resistant to both isoniazid and rifampin

*MDR, multidrug-resistant; TB, tuberculosis.
†These profiles refer specifically to resistance to isoniazid and/or rifampin, but resistance to other drugs may also be present.

Table 2. Description of acquired drug resistance profiles used to categorize resistance to anti-TB drugs*

Acquired drug resistance profile	Description of drug-resistance profile
TB patients who did not have MDR TB before or after acquiring resistance	Initially sensitive to isoniazid, rifampin, or both and may have acquired resistance to either isoniazid or rifampin but not both or may have acquired resistance to other anti-TB drug(s).
TB patients who acquired resistance that resulted in MDR TB	Initially sensitive to isoniazid, rifampin, or both and acquired resistance to isoniazid, rifampin, or both to have MDR TB.
Patients with initial MDR TB and acquired resistance to additional drugs	Initially resistant to at least isoniazid and rifampin and on subsequent culture acquired resistance to other anti-TB drug(s).

*MDR, multidrug-resistant; TB, tuberculosis.

We used χ^2 tests to compare treatment outcomes among those with and without acquired drug resistance. All analyses were conducted by using Stata version 13.1 (StataCorp LLC, College Station, TX, USA).

Results

During 2000–2015, a total of 69,300 culture-confirmed TB cases were notified in England, Wales, and Northern Ireland; among these, 99.1% (68,686/69,300) had DST results for at least isoniazid and rifampin (Figure 1). We excluded 976 TB patients known to have not started treatment. Overall, 0.2% (158/67,710) of culture-confirmed TB patients with DST results acquired drug resistance. The median number of TB patients with acquired resistance per year was 11 (range 0–21) (Figure 2). The number of TB patients with acquired resistance increased over time (p = 0.002).

Among patients tested for each drug, the highest numbers acquired resistance to rifampin (50/67,710), isoniazid (48/67,710), ethambutol (32/67,645), and pyrazinamide (28/67,061). The highest percentage of patients had acquired resistance to ethionamide (1.1%, 15/1,345) and prothionamide (0.8%, 15/1,345) (Figure 3).

Initial Drug-Resistance Profile

Isolates from most TB patients with acquired resistance were initially sensitive to both isoniazid and rifampin (46.8%, 74/158) (Table 3). Resistance to additional drugs was acquired by 0.1% (74/62,795) of TB patients with isolates initially sensitive to isoniazid and rifampin, 0.9% (34/3,864) of TB patients with isoniazid resistance and without rifampin resistance, 1.5% (3/203) of TB patients with rifampin resistance and without isoniazid resistance, and 5.5% (47/848) of MDR TB patients.

For 44 TB patients, acquired resistance resulted in MDR TB; among these patients, initial isolates from 20 were sensitive to both isoniazid and rifampin, 22 were resistant to isoniazid without MDR TB, and 2 were resistant to rifampin without MDR TB. A total of 67 TB patients acquired resistance without it being MDR TB (initial or acquired); the highest proportion was resistant to isoniazid (32.8%, 22/67), followed by streptomycin (22.4%, 15/67) and pyrazinamide (14.9%, 10/67).

Episodes of Acquired Resistance

Resistance was acquired to 1–6 drugs; most (69.0%, 109/158) resistance was to only 1 drug, 19.6% (31/158) to 2 drugs, and 6.3% (10/158) to ≥3 drugs. Of those who

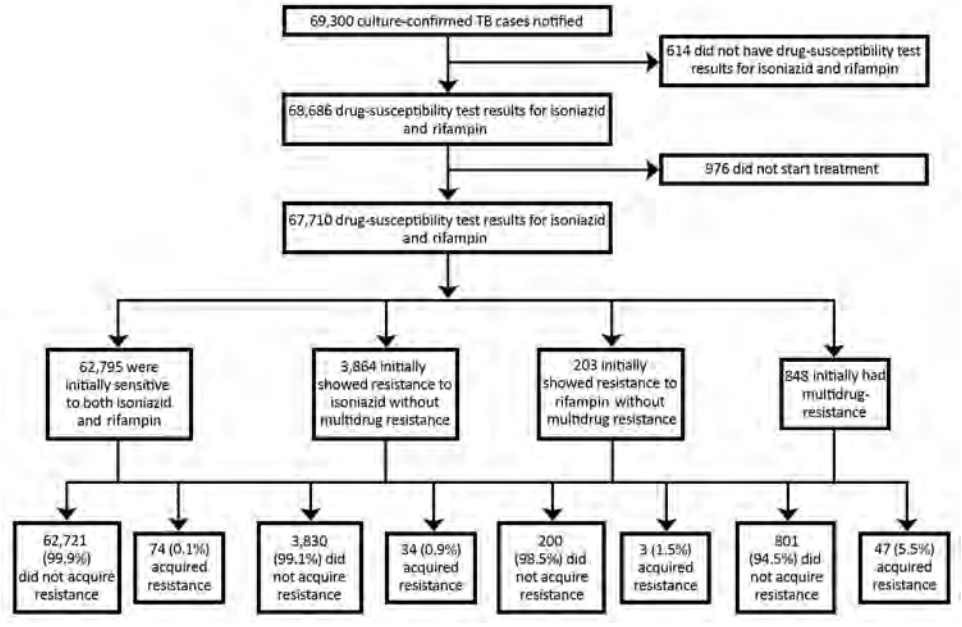

Figure 1. Process used to select tuberculosis (TB) patients with acquired drug resistance among all TB patients with culture-confirmed TB, England, Wales, and Northern Ireland, 2000–2015.

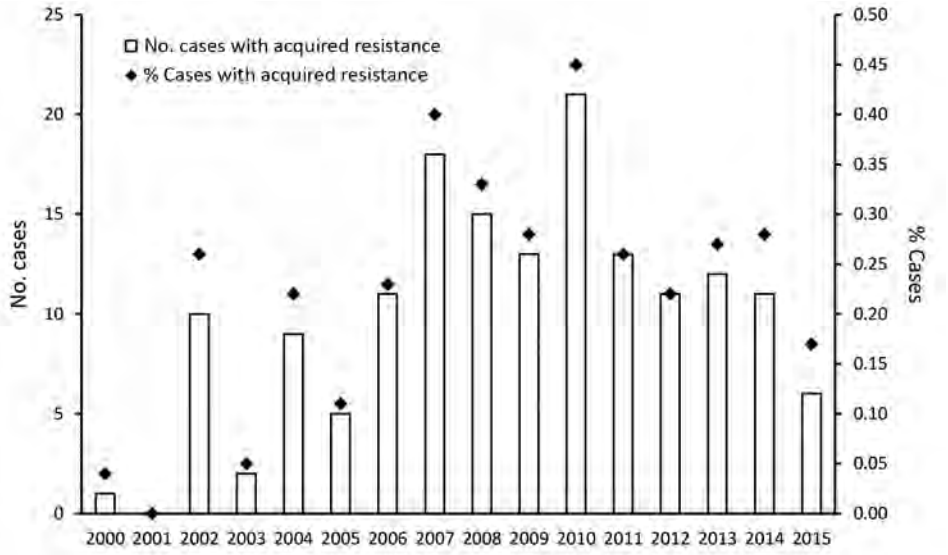

Figure 2. Number and proportion of tuberculosis patients with acquired drug resistance, by year, England, Wales, and Northern Ireland, 2000–2015.

acquired resistance to >2 drugs, 65.3% (32/49) acquired resistance in 1 episode, 28.6% (14/49) over 2 episodes, and 6.1% (3/49) over 3 episodes. The median time between the first and second episode for those who had at ≥2 episodes was 3.5 months (interquartile range [IQR] 2.4–11.9 months), and the median time between the second and third episode for those who had 3 repeat episodes was 5.3 months (IQR 4.7 months–2 years).

Time between Starting Treatment and Acquiring Resistance

Ten MDR TB patients acquired resistance between starting an initial regimen and switching to the appropriate regimen for drug resistance. These patients acquired resistance to ethambutol (4), pyrazinamide (1), ethionamide (4), and prothionamide (1).

A total of 136 TB patients acquired resistance after starting an appropriate treatment regimen; median time between starting treatment and acquiring resistance to ≥1 drug or to the first drug was 3.4 months (IQR 1.5–8.3 months). Resistance was acquired by 12% (17/136) of patients 1 year after starting treatment (Table 4).

Most (64.1%, 25/39) TB patients in whom acquired resistance developed into MDR TB had received treatment for >6 months before resistance was acquired (Table 4). Conversely, 76.2% (32/42) of MDR TB patients in whom resistance to additional drugs was acquired had received treatment <6 months before acquiring additional resistance.

Risk Factors for Acquiring Resistance

Among TB patients with acquired resistance, most were 15–44 years of age (70.3%, 111/158), were male (59.5%, 94/158), were foreign born (75.2%, 115/153), and had pulmonary disease (82.9%, 131/158). A previous TB diagnosis was reported for 23% (32/141). Among those who had acquired resistance >1 year after starting

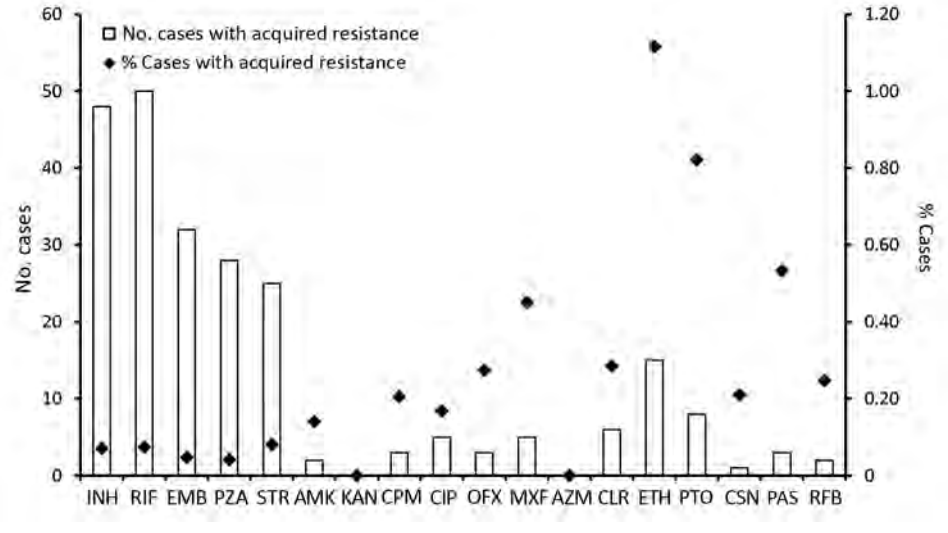

Figure 3. Number and proportion of tuberculosis patients with acquired drug resistance, by drug, England, Wales, and Northern Ireland, 2000–2015. AMK, amikacin; AZM, azithromycin; CIP, ciprofloxacin; CLR, clarithromycin; CPM, capreomycin; CSN, cycloserine; EMB, ethambutol; ETH, ethionamide; KAN, kanamycin; INH, isoniazid; MXF, moxifloxacin; OXF, ofloxacin; PAS, para-aminosalicylic acid (bacteriostatic); PTO, prothionamide; PZA, pyrazinamide; RIF, rifampin; RFB, rifabutin; STR, streptomycin.

Table 3. TB cases according to drug-resistance profile, England, Wales, and Northern Ireland, 2000–2015*

Initial drug-resistance profile, no. patients with acquired drug resistance	Acquired resistance and MDR TB developed later, no. (%)	Initial MDR TB with additional resistance acquired later, no. (%)	No MDR TB before or after acquiring resistance, no. (%)
Drug sensitive, n = 74	20 (27.0)	NA	54 (73.0)
Isoniazid resistant without MDR, n = 34	22 (64.7)	NA	12 (35.3)
Rifampin resistant without MDR, n = 3	2 (66.7)	NA	1 (33.3)
MDR TB, n = 47	NA	47 (100)	NA
Total, n = 158	44 (27.8)	47 (29.7)	67 (42.4)

*MDR, multidrug-resistant; NA, not applicable; TB, tuberculosis.

treatment, the demographic profile differed: most were female (58.8%, 10/17) and born in the United Kingdom (66.7%, 5/15), and although they were similar in age, most (58.8%, 10/17) were 15–44 years of age. Among TB patients notified during 2010–2015, a total of 28% (18/65) had ≥1 social risk factor.

Multivariable model 1 showed that acquired resistance was more likely among TB patients with than without the following characteristics: pulmonary disease (adjusted OR [aOR] 2.1, 95% CI 1.3–3.3); initial resistance to isoniazid (aOR 6.2, 95% CI 3.9–10.0), rifampin (aOR 10.8, 95% CI 3.3–35.3), or both (aOR 41.8, 95% CI 27.0–64.7); and a previous TB episode (aOR 2.3, 95% CI 1.5–3.5) (Table 5). TB patients born in China were 3.4 times more likely (95% CI 1.3–8.8) and those born in South Africa were 2.8 times more likely (95% CI 1.1–7.5) to acquire resistance than those born in the United Kingdom; patients born in India were less likely to acquire resistance than those born in the United Kingdom (aOR 0.4, 95% CI 0.2–0.8). Sensitivity analysis of multivariable model 1, which excluded TB patients who acquired resistance to ethambutol only, pyrazinamide only, or both, showed trends of demographic characteristics that were more or less likely to be associated with acquired resistance similar to those in the original model (Table 6).

Multivariable model 2, which included social risk factors and strain type lineage, showed that acquisition of drug resistance was more likely among TB patients with than without the following characteristics: age 45–64 years (aOR 1.9, 95% CI 1.0–3.6); pulmonary disease (aOR 2.7, 95% CI 1.2–6.0); and initial resistance to isoniazid (aOR 6.5, 95% CI 2.9–14.4), rifampin (aOR 14.7, 95% CI 1.8–1213.0), or both (aOR 77.3, 95% CI 39.1–152.8) (Table 7). Although a higher proportion of TB patients with (27.7%, 18/65) than without (11.6%, 2,632/22,795) acquired resistance had a social risk factor, after the other factors in model 2 were adjusted for, having a social risk factor was not significantly associated with acquiring resistance. No country of birth was significantly associated with acquired resistance, but TB patients born in India were less likely to acquire resistance than those born in the United Kingdom (aOR 0.3, 95% CI 0.1–0.8). TB patients infected with a Beijing lineage *M. tuberculosis* strain (aOR 3.4, 95% CI 1.6–7.4) or *M. bovis* (aOR 10.3, 95% CI 2.1–49.8) were more likely to acquire drug resistance. Among this subpopulation of TB cases notified during 2010–2015, lineage and country of birth were collinear (p<0.001) and thus not included in the model.

Treatment Outcomes

Treatment was completed by a lower proportion of TB patients who did (61.4% [86/140]) than did not (79.9% [45,690/57,209]) acquire drug resistance (p<0.001), and the median duration of treatment was longer among those who did (19.2 months [IQR 12–30 months) than did not (6.2 months [IQR 6–8.8 months]) acquire drug resistance (Table 8). A higher proportion of TB patients with (7.1% [10/140]) than without (4.6% ([2,651/57,209]) acquired resistance died (p<0.001), but time to death was longer for those with acquired resistance (6.8 months [IQR 5.8 months–2.1 years]) than for those without acquired resistance (1.3 months [IQR 0.4–3.2 months]). Among TB patients with acquired resistance, outcomes were worse for those in whom MDR developed than for those with other categories of drug resistance (p = 0.03) (Table 8).

Table 4. Number and proportion of TB cases, by time between treatment initiation and drug resistance acquisition and by acquired drug resistance categories, England, Wales, and Northern Ireland, 2000–2015*

Time between treatment start and acquired resistance	Acquired drug resistance category			
	No MDR TB before or after acquiring resistance, no. (%)	Acquired resistance and MDR TB developed later, no. (%)	Initial MDR TB with additional resistance acquired later, no. (%)	Total with acquired drug resistance, no. (%)
<1 mo	20 (36.4)	2 (5.1)	5 (11.9)	27 (19.9)
2 mo	10 (18.2)	0	13 (31.0)	23 (16.9)
3 mo	8 (14.5)	3 (7.7)	4 (9.5)	15 (11.0)
4–6 mo	6 (10.9)	9 (23.1)	10 (23.8)	25 (18.4)
7–9 mo	4 (7.3)	8 (20.5)	5 (11.9)	17 (12.5)
10 mo–1 y	4 (7.3)	7 (18.0)	1 (2.4)	12 (8.8)
>1 y	3 (5.5)	10 (25.6)	4 (9.5)	17 (12.5)
Total	55 (100)	39 (100)	42 (100)	136 (100)

*MDR, multidrug-resistant; TB, tuberculosis.

Table 5. Univariable and multivariable logistic regression model 1 for acquired resistance to anti-TB drugs in England, Wales, and Northern Ireland, 2000–2015*

Characteristic	No acquired drug resistance, no. (%)	Acquired drug resistance, no. (%)	Univariable analysis		Multivariable analysis	
			OR (95% CI)	p value	OR (95% CI)	p value
Age, y						
0–14	1,391 (2.1)	2 (1.3)	1.0	NA	NA	NA
15–44	44,227 (65.5)	111 (70.3)	1.7 (0.4–7.1)	0.4	1.6 (0.4–6.7)	0.5
45–64	12,859 (19.0)	40 (25.3)	2.2 (0.5–9.0)	0.3	2.5 (0.6–10.7)	0.2
≥65	9,071 (13.4)	5 (3.2)	0.4 (0.1–2.0)	0.2	0.6 (0.1–3.2)	0.5
Sex						
F	28,453 (42.2)	64 (40.5)	1.0	NA	NA	NA
M	38,985 (57.8)	94 (59.5)	1.1 (0.8–1.5)	0.7	1.2 (0.8–1.6)	0.4
Site of disease						
Extrapulmonary only	24,217 (35.9)	27 (17.1)	1.0	NA	NA	NA
Pulmonary with or without extrapulmonary	43,313 (64.1)	131 (82.9)	2.7 (1.8–4.1)	<0.001	2.1 (1.3–3.3)	0.003
Initial drug resistance						
Drug sensitive	62,721 (92.9)	74 (46.8)	1.0	NA	NA	NA
Isoniazid resistance without MDR	3,830 (5.7)	34 (21.5)	7.5 (5.0–11.3)	<0.001	6.2 (3.9–10.0)	<0.001
Rifampin resistance without MDR	200 (0.3)	3 (1.9)	12.7 (4.0–40.7)	<0.001	10.8 (3.3–35.3)	<0.001
MDR	801 (1.2)	47 (29.7)	49.7 (34.3–72.2)	<0.001	41.8 (27.0–64.7)	<0.001
Country of birth						
United Kingdom	16,184 (26.3)	38 (25.7)	1.0	NA	NA	NA
China	465 (0.8)	6 (4.1)	5.5 (2.3–13.1)	<0.001	3.4 (1.3–8.8)	0.01
India	11,227 1(8.3)	11 (7.4)	0.4 (0.2–0.8)	0.01	0.4 (0.2–0.8)	0.01
Lithuania	238 (0.4)	9 (6.1)	16.1 (7.7–33.7)	<0.001	1.8 (0.8–4.4)	0.2
Pakistan	7,696 (12.5)	15 (10.1)	0.8 (0.5–1.5)	0.6	0.9 (0.4–1.7)	0.6
Somalia	4,184 (6.8)	9 (6.1)	0.9 (0.4–1.9)	0.9	0.9 (0.4–1.9)	0.7
South Africa	759 (1.2)	5 (3.4)	2.8 (1.1–7.1)	0.03	2.8 (1.1–7.5)	0.04
Nigeria	1,324 (2.2)	4 (2.7)	1.3 (0.5–3.6)	0.6	0.8 (0.2–2.6)	0.7
Other	19,417 (31.6)	51 (34.5)	1.1 (0.7–1.7)	0.6	0.9 (0.6–1.5)	0.8
Previous TB episode						
No	54,346 (93.3)	109 (77.3)	1.0	NA	NA	NA
Yes	3,910 (6.7)	32 (22.7)	4.0 (2.7–6.1)	<0.001	2.3 (1.5–3.5)	<0.001

*MDR, multidrug-resistant; NA, not applicable; OR, odds ratio; TB, tuberculosis.

Discussion

In England, Wales, and Northern Ireland, acquiring drug resistance while receiving TB treatment is rare (0.2% of cases) but more likely among those with pulmonary disease; with initial resistance to isoniazid, rifampin, or both; who experienced a previous TB episode; or who were born in China or South Africa. Our description of the characteristics of TB patients provides useful insight into the care required for these patients.

For most (57.6%) of the TB patients in our study, either their acquired resistance resulted in MDR TB or they initially had MDR TB and acquired additional resistance, thereby limiting treatment options. Our study demonstrates the value of identifying and preventing acquired drug resistance in patients without MDR TB, one third of whom acquire resistance to isoniazid, one of the main drugs used in standard treatment. A study in China showed that acquired drug resistance among any patients with culture-confirmed TB was much higher (3.7%) than that for our study population (*20*). Studies from India have shown high rates of MDR TB among previously treated patients with drug-sensitive isolates, some of which would be accounted for by acquired resistance to the first-line drugs (*21*).

Most other studies from other low-incidence countries report on TB patients with MDR TB in whom further resistance to second-line drugs is acquired; for example, in the United States, drug resistance was acquired by 5.0% of MDR TB patients, similar to the rate of 5.5% of MDR TB patients identified in our study (*22–24*). Numerous studies highlight the need for individualized treatment regimens for patients with MDR TB (*13*,*25*,*26*). In the United Kingdom, the national TB control strategy advocates increasing resources to strengthen a multidisciplinary clinical advisory service that will ensure that MDR TB is treated effectively (*3*). Our study adds to the growing evidence for the need to more closely supervise and monitor MDR TB patients by regular use of DST for second-line drugs and the need to ensure that adequate resources are available to meet patient needs.

In our study, acquired resistance was more likely in TB patients with pulmonary disease and, if ineffectively treated, could lead to the transmission of drug-resistant strains. Another study found that the presence of pulmonary cavitation increased the risk of acquiring resistance (*23*). In our study and others, acquired resistance was more likely in

Table 6. Sensitivity analysis of univariable and multivariable logistic regression model 1 for acquired resistance to anti-TB drugs in England, Wales, and Northern Ireland, 2000–2015*

Characteristic	No acquired drug resistance, no. (%)	Acquired drug resistance, no. (%)	Univariable analysis		Multivariable analysis	
			OR (95% CI)	p value	aOR (95% CI)	p value
Age, y						
0–14	1,392 (2.1)	1 (0.9)	1.0 (NA)	NA	1.0 (NA)	NA
15–44	44,263 (65.5)	75 (69.4)	2.4 (0.3–17.0)	0.4	2.4 (0.3–17.4)	0.4
45–64	12,871 (19.0)	28 (25.9)	3.0 (0.4–22.3)	0.3	3.0 (0.4–23.2)	0.3
≥65	9,072 (13.4)	4 (3.7)	0.6 (0.1–5.5)	0.7	0.9 (0.1–7.8)	0.9
Sex						
F	28,476 (42.2)	41 (38.0)	1.0 (NA)	NA	1.0 (NA)	NA
M	39,012 (57.8)	67 (62.0)	1.2 (0.8–1.8)	0.4	1.4 (0.9–2.1)	0.2
Site of disease						
Extrapulmonary only	24,233 (35.9)	11 (10.2)	1.0 (NA)	NA	1.0 (NA)	NA
Pulmonary with or without extrapulmonary	43,347 (64.1)	97 (89.8)	4.9 (2.6–9.2)	<0.001	3.0 (1.6–5.7)	0.001
Initial drug resistance						
Drug sensitive	62,740 (92.8)	55 (50.9)	1.0 (NA)	NA	1.0 (NA)	NA
Isoniazid resistance without MDR	3,839 (5.7)	25 (23.2)	7.4 (4.6–11.9)	<0.001	6.5 (3.7–11.2)	<0.001
Rifampin resistance without MDR	200 (0.3)	3 (2.8)	17.1 (5.3–55.1)	<0.001	15.2 (4.6–50.2)	<0.001
MDR	823 (1.2)	25 (23.2)	34.7 (21.5–55.9)	<0.001	32.7 (18.9–56.8)	<0.001
Country of birth						
United Kingdom	16,189 (26.3)	33 (33.0)	NA	NA	1.0 (NA)	NA
China	467 (0.8)	4 (4.0)	4.2 (1.5–11.9)	0.01	2.5 (0.8–7.7)	0.1
India	11,232 (18.2)	6 (6.0)	0.3 (0.1–0.6)	0.003	0.2 (0.1–0.6)	0.004
Lithuania	243 (0.4)	4 (4.0)	8.1 (2.8–23.0)	<0.001	0.8 (0.2–2.8)	0.7
Pakistan	7,702 (12.5)	9 (9.0)	0.6 (0.3–1.2)	0.1	0.8 (0.4–1.7)	0.5
Somalia	4,190 (6.8)	3 (3.0)	0.4 (0.1–1.1)	0.1	0.4 (0.1–1.3)	0.1
South Africa	759 (1.2)	5 (5.0)	3.2 (1.3–8.3)	0.02	3.3 (1.2–8.8)	0.02
Nigeria	1,326 (2.2)	2 (2.0)	0.7 (0.2–3.1)	0.7	0.3 (0.04–2.1)	0.2
Other	19,43 (31.6)	34 (34.0	0.9 (0.5–1.4)	0.5	0.7 (0.4–1.2)	0.2
Previous TB episode						
No	54,379 (93.3)	76 (79.2)	1.0 (NA)	NA	1.0 (NA)	NA
Yes	3,922 (6.7)	20 (20.8)	3.6 (2.2–6.0)	<0.001	2.2 (1.3–3.8)	0.01

*aOR, adjusted OR; MDR, multidrug-resistant; NA, not applicable; OR, odds ratio; TB, tuberculosis.

TB patients who had had a previous TB episode, which suggests that these cases were neither successfully treated nor cured during the previous TB episode, most likely because of previous compliance issues (*23*,*27*–*29*). These findings demonstrate the need to efficiently diagnose resistance and ensure that appropriate drug regimens are provided and adhered to.

Our study also found that acquired resistance was more likely in TB patients born in China and South Africa; however, this association was lost when strain lineage was considered. This relationship between country of birth and lineage may be explained by the geographic stratification of *M. tuberculosis* complex lineages. Therefore, the country of birth could instigate greater monitoring of TB treatment for patients in whom acquired resistance is more likely (e.g., those infected with Beijing strains, who acquire MDR TB more readily [*30*,*31*]). Acquired resistance was more likely in patients with TB caused by *M. bovis*; and although the number of patients in our study was small, only 2 patients with acquired resistance had TB caused by *M. bovis*, and both of these patients acquired resistance to isoniazid. This association with *M. bovis* has also been shown by a study in the United States, where *M. bovis* was independently associated with acquiring resistance to isoniazid (*32*).

Our study indicated that resistance was acquired by a higher proportion of TB patients with than without a social risk factor. However, studies in other countries did not consistently find an association between acquired resistance and social risk factors (*7*,*23*,*27*). Our findings indicate that outcomes were poorer (e.g., death or loss to follow-up) among TB patients with than without acquired resistance. Similar results have been reported elsewhere (*9*,*20*,*33*–*35*). Acquisition of resistance occurred later in treatment among patients who acquired resistance that resulted in MDR TB; one quarter acquired resistance >12 months after starting treatment, and their outcomes were worse than those for patients with other types of acquired drug-resistance profiles. Treatment can continue beyond 12 months for several reasons (e.g., initial drug resistance, acquired resistance, adherence issues, or clinical signs that the patient has not been cured), which by international reporting standards would be reported as treatment failed, but in the United Kingdom these patients are reported as still receiving treatment, and outcomes beyond 12 months are reported. Treatment failure has been shown to be

Table 7. Univariable and multivariable logistic regression model 2 for acquired resistance to anti-TB drugs in England, Wales, and Northern Ireland, 2010–2015*

Characteristic	No acquired drug resistance, no. (%)	Acquired drug resistance, no. (%)	Univariable analysis		Multivariable analysis	
			OR (95% CI)	p value	aOR (95% CI)	p value
Age, y						
0–14	467 (1.8)	0	NA	NA	NA	NA
15–44	17,119 (64.6)	48 (64.9)	1.0	NA	1.0	NA
45–64	5,429 (20.5)	23 (31.1)	1.5 (0.9–2.5)	0.1	1.9 (1.0–3 6)	0.04
≥65	3,503 (13.2)	3 (4.1)	0.3 (0.1–0.9)	0.05	0.6 (0.2–2.2)	0.5
Sex						
F	10,664 (40.3)	24 (32.4)	1.0	NA	1.0	NA
M	15,820 (59.7)	50 (67.6)	1.4 (0.9–2.3)	0.2	1.3 (0.7–2.3)	0.4
Site of disease						
Extrapulmonary only	10,069 (38.0)	10 (13.5)	1.0 NA	NA	1.0	NA
Pulmonary with or without extrapulmonary	16,443 (62.0)	64 (86.5)	3.9 (2.0–7.6)	<0.001	2.7 (1.2–6.0)	0.01
Initial drug resistance						
Drug sensitive	24,659 (93.0)	25 (33.8)	1.0	NA	1.0	NA
Isoniazid resistance without MDR	1,446 (5.4)	13 (17.6)	8.9 (4.5–17.4)	<0.001	6.5 (2.9–14.4)	<0.001
Rifampin resistance without MDR	50 (0.2)	1 (1.4)	19.7 (2.6–148.4)	0.004	14.7 (1.8–123.0)	0.01
MDR	363 (1.4)	35 (47.3)	95.1 (56.3–160.5)	<0.001	77.3 (39.1–152.8)	<0.001
Country of birth						
United Kingdom	6,239 (24.6)	21 (29.2)	1.0	NA	1.0	NA
China	183 (0.7)	3 (4.2)	4.9 (1.4–16.5)	0.01	2.1 (0.5–9.5)	0.3
India	5,470 (21.6)	5 (6.9)	0.3 (0.1–0.7)	0.01	0.3 (0.1–0.8)	0.02
Lithuania	181 (0.7)	8 (11.1)	13.3 (5.7–30.0)	<0.001	0.6 (0.2–1.8)	0.3
Pakistan	3,270 (12.9)	5 (6.9)	0.5 (0.2–1.2)	0.1	0.7 (0.2–2.5)	0.6
Somalia	1,191 (4.7)	3 (4.2)	0.8 (0.2–2.5)	0.7	0.6 (0.1–2.7)	0.5
South Africa	174 (0.7)	1 (1.4)	1.7 (0.2–12.8)	0.6	1.3 (0.1–11.7)	0.8
Nigeria	579 (2.3)	2 (2.8)	1.0 (0.2–4.4)	0.9	0.9 (0.1–6.9)	0.9
Other	8,080 (31.8)	24 (33.3)	0.9 (0.5–1.6)	0.7	0.7 (0.3–1.4)	0.3
Previous TB episode						
No	23,799 (94.5)	57 (80.3)	1.0	NA	1.0	NA
Yes	1,391 (5.5)	14 (19.7)	4.2 (2.3–7.6)	<0.001	1.7 (0.8–3.4)	0.2
Social risk factors						
0	20,163 (88.4)	47 (72.3)	1.0	NA	1.0	NA
≥1	2,632 (11.6)	18 (27.7)	2.9 (1.7–5.1)	<0.001	1.0 (0.5–2.0)	1.0
Organism lineage						
Euro-American	8,631 (39.3)	18 (26.5)	1.0	NA	1.0	NA
Central Asian	6,013 (27.4)	9 (13.2)	0.7 (0.3–1.6)	0.4	1.0 (0.4–2.8)	0.9
East-African-Indian	3,098 (14.1)	6 (8.8)	0.9 (0.4–2.3)	0.9	1.3 (0.5–3.9)	0.6
Beijing	1,230 (5.6)	23 (33.8)	9.0 (4.8–16.7)	<0.001	3.4 (1.6–7.4)	0.002
Mycobacterium africanum	192 (0.9)	0	NA	NA	NA	NA
M.bovis	124 (0.6)	2 (2.9)	7.7 (1.8-33.7)	0.01	10.3 (2.1–49.8)	0.004
Multiple	599 (2.7)	2 (2.9)	1.6 (0.4–7.0)	0.5	1.4 (0.3–6.7)	0.7
None	2,076 (9.4)	8 (11.8)	1.8 (0.8–4.3)	0.1	1.9 (0.8–4.9)	0.2

*aOR, adjusted OR; MDR, multidrug-resistant; NA, not applicable; OR, odds ratio; TB, tuberculosis.

associated with late emergence of acquired resistance (*20*), which is consistent with our findings. This evidence illustrates how acquired resistance impedes successful treatment outcomes.

Our study benefits from having a large national cohort followed over an extended period. In the United Kingdom, culture-positive results are routinely matched to case data, enabling the study of drug susceptibility combined with demographic and clinical factors. However, our study does have several limitations. First, some local laboratories may process follow-up cultures without sending the samples on to the *Mycobacterium* reference laboratories, leading to underestimation of acquired resistance. It is also possible that patients with initial resistance (to isoniazid, to rifampin, or MDR TB) may receive more frequent sampling, enabling acquisition of resistance to be more readily detected. Sampling may also differ by site of disease because obtaining multiple samples from patients with extrapulmonary disease may be more difficult. However, these situations are unlikely to occur commonly because failure to respond to treatment would prompt further sample collection. In addition, if there is a delay in collecting samples because of missed/rescheduled appointments, the reported time to diagnosis of acquired resistance may be longer than the actual time to diagnosis.

Although TB guidance in the United Kingdom recommends collecting specimens when TB is suspected, and

Table 8. Treatment outcomes for patients with and without acquired drug resistance to anti-TB drugs, England, Wales, and Northern Ireland, 2001–2013*

Outcomes	Acquired drug resistance category				
	No MDR TB before or after acquiring resistance, no. (%)	Acquired resistance that resulted in MDR TB, no. (%)	Initial MDR TB with additional acquired resistance, no. (%)	Total with acquired drug resistance, no. (%)	No acquired drug resistance, no. (%)
Treatment completed	39 (65)	20 (48.8)	27 (69.2)	86 (61.4)	45,690 (79.9
Died	1 (1.7)	8 (19.5)	1 (2.6)	10 (7.1)	2,651 (4.6)
Lost to follow-up	5 (8.3)	5 (12.2)	3 (7.7)	13 (9.3)	3,046 (5.3)
Still receiving treatment	10 (16.7)	7 (17.1)	5 (12.8)	22 1(5.7)	1,253 (2.2)
Treatment stopped	2 (3.3)	1 (2.4)	3 (7.7)	6 (4.3)	410 (0.7)
Not evaluated	3 (5)	0	0	3 (2.1)	4,159 (7.3)

*MDR, multidrug resistant; TB, tuberculosis.

therefore the initial specimen should be taken before starting treatment, 37% of patients in our study had a treatment start date before the initial specimen collection date. Some of these patients may have acquired resistance before the initial sample was taken, leading to underestimation of acquired resistance. DST results for ethambutol and pyrazinamide often vary between sensitive and resistant because of laboratory and biological artifacts. The effect of this variability is probably minimal because DST was repeated for any isolate that showed resistance to these agents. In addition, our sensitivity analysis, which excluded TB patients who acquired resistance to these agents, showed results consistent with the original model. Last, other factors, such as treatment adherence, may have affected the acquisition of resistance, and those factors associated with acquired resistance identified in this study may not be independent of adherence; however, adherence data are not routinely collected nationally.

Strategies for reducing drug-resistant TB are being implemented in England and globally (*2*,*3*). Whole-genome sequencing has been implemented as a diagnostic tool throughout the United Kingdom, which may bring further insight into the drivers of acquired resistance through identification of mutations (*36*). Whole-gene sequencing will also improve monitoring of acquired resistance to all drugs, first- and second-line, identified for each sample, and will reduce variation in results. The World Health Organization has endorsed a shorter (9-month) treatment regimen for MDR TB, which could potentially improve compliance and reduce acquired resistance (*37*).

These improvements in TB diagnostics and treatment, along with the emphasis placed on reducing MDR TB in national and global strategies, have increased awareness regarding the complex needs of MDR TB patients. Because the likelihood of acquiring resistance is increased among patients with initial drug resistance, these patients have a greater need for more specialized facilities and social services to ensure that appropriate treatment is started quickly and monitored thoroughly. Ongoing monitoring of the level of acquired drug resistance is one indicator of TB control and should be incorporated into routine TB surveillance, especially in countries with higher levels of MDR and acquired resistance (*27*). These results indicate that other countries should undertake similar studies to understand risk factors for acquired drug resistance in their own countries and to ensure that drug resistance is diagnosed and transmission is limited.

About the Author

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Characteristics Associated with Negative Interferon-γ Release Assay Results in Culture-Confirmed Tuberculosis Patients, Texas, USA, 2013–2015

Duc T. Nguyen, Larry D. Teeter, Julie Graves, Edward A. Graviss

Interferon-γ release assays (IGRAs) are the preferred diagnostic test for tuberculosis (TB) infection in at-risk populations in developed countries. However, IGRAs have high false-negative rates in patients with TB disease. Population-based studies assessing the factors associated with negative IGRA results in TB patients have not been performed. Using statewide TB surveillance data of culture-confirmed TB patients in Texas, USA, during 2013–2015, we describe the patient characteristics and treatment outcomes associated with false-negative IGRA results. Among 2,854 TB patients, 1,527 (53.5%) had an IGRA result; 97.4% (1,487/1,527) of those had a positive (87.7%) or negative (12.3%) result. Older age, HIV co-infection, non-Hispanic white race/ethnicity, and being tested with T-SPOT.*TB* were associated with negative IGRA results. TB patients with negative IGRA results had a higher mortality, potentially due to delayed treatment. Healthcare providers should consider these risk factors when making decisions for patients with suspected TB and negative IGRA results and potentially provide treatment.

Interferon-γ release assays (IGRAs) are blood tests that measure immune reactivity to *Mycobacterium tuberculosis*–specific antigens and aid in the diagnosis of latent tuberculosis (TB) infection (LTBI). During the period of this study, 2 IGRAs, which have been approved by the US Food and Drug Administration, were commercially available for use in the United States: QuantiFERON-TB Gold In-Tube (QFT; QIAGEN, Germantown, MD, USA); and T-SPOT.*TB* (Oxford Immunotec, Inc., Marlborough, MA, USA) (*1*). In Texas, a state with one of the highest TB burdens in the United States, IGRAs have been used in targeted testing among persons at high risk for LTBI or at high risk of developing TB disease once infected with TB (*2*). Although IGRAs are not recommended to be used as rule-out tests for TB disease because of their inability to differentiate LTBI from TB disease and inconsistent sensitivity and specificity in different populations with TB disease (*3*–*7*), IGRAs are still preferred tools used by healthcare providers to identify LTBI in persons being evaluated for TB disease before biological confirmation. In the absence of other positive rapid diagnostic test results for TB disease, a negative IGRA result might inappropriately lower the clinical suspicion for TB and result in delayed treatment initiation. Previous studies have suggested that older age, underweight, HIV co-infection, extrapulmonary TB, and increased number of human leukocyte antigen DRB1*0701 alleles were associated with negative IGRA results (*8*–*11*). However, these studies had small sample sizes or were performed at a single center. In addition, little information is available on deaths associated with negative IGRA results in a population with confirmed TB. The main objectives of our population-based analysis were to identify the demographics, clinical characteristics, and patient outcomes associated with negative IGRA results in patients with culture-confirmed TB.

Methods

We acquired deidentified TB surveillance data of patients with TB disease reported in the Texas National Electronic Disease Surveillance System (NEDSS) database during January 1, 2013–December 31, 2015. NEDSS is a TB surveillance data system used by all state jurisdictions within the United States to facilitate the electronic transfer of public health surveillance data from the healthcare system to public health departments (*12*). The downloaded data included sociodemographic, clinical, laboratory, and radiographic characteristics. We downloaded genotype information from the Centers for Disease Control and Prevention TB

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Genotyping Information Management System (*13*). We included for analysis only the TB patients who were confirmed TB positive by *M. tuberculosis* culture and had either a positive or negative IGRA result available. We excluded TB patients with negative culture results and those for whom specimens were not taken for TB culture (clinical TB patients), as well as those with unavailable or unknown IGRA types or indeterminate (for QFT) or failed (for T-SPOT.*TB*) IGRA results. We defined the diagnosis date as the specimen collection date of the first *M. tuberculosis*–positive culture and the time to TB treatment as the number of days from the diagnosis date to the date when the TB treatment started.

Statistical Analysis

We reported demographic and clinical characteristics as medians and interquartile ranges (IQRs) for continuous variables and as numbers and percentages for categorical variables. We compared the differences between groups (e.g., IGRA-positive group vs. IGRA-negative group) using the Kruskal-Wallis test for continuous variables and the χ^2 test for categorical variables. We used logistic regression with the robust standard error option to obtain unbiased standard errors and calculated odds ratios (ORs) and 95% CIs to identify potential associations between negative IGRA results and demographic, clinical, or laboratory characteristics. We further investigated variables having a p value of <0.2 in the univariate logistic regression by multiple logistic regression modeling to identify characteristics significantly associated with negative IGRA results. We analyzed the survival during the 1-year period after TB diagnosis and stratified by IGRA type using the Kaplan-Meier method. We compared the survival differences between IGRA types using the log-rank test. We used Cox proportional hazards modeling to determine the risk factors associated with death during the 1-year period after TB diagnosis. We conducted variable selection for the multiple logistic regression and Cox proportional hazards models using the Bayesian model averaging method (*14*,*15*). In brief, we ran Stata's Bayesian model averaging program to evaluate possible model sets for all variables having a p value of <0.2 in the univariate analysis, and Stata suggested good models that included the variables with a high posterior probability of being a risk factor. We used the likelihood ratio test to further reduce the model subsets and selected the best model on the basis of its small Bayesian information criterion. We also tested the selected models for the proportional hazard assumption and evaluated model discrimination using the Harrell C statistic. We performed all analyses with Stata version 14.2 (StataCorp LLC, College Station, TX, USA) and considered p values <0.05 statistically significant.

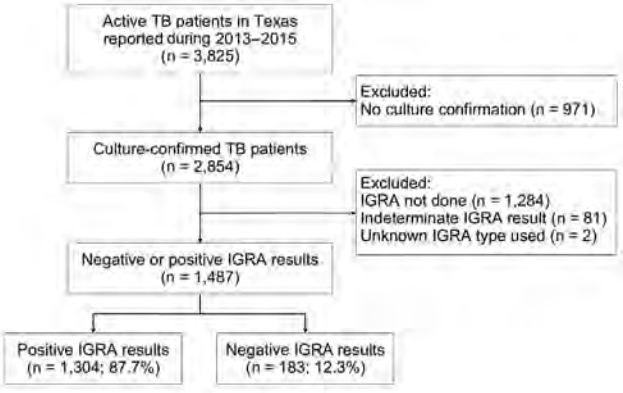

Figure 1. Flowchart showing selection of culture-confirmed TB patients with IGRA results, Texas, USA, 2013–2015. IGRA. IGRA, interferon-γ release assay; TB, tuberculosis.

Results

Among the 3,825 patients with TB disease reported in Texas during 2013–2015, a total of 2,854 (74.6%) were confirmed positive by *M. tuberculosis* culture (Figure 1). Of the 2,854 patients with culture-confirmed TB, 1,527

Table 1. Characteristics of 1,487 culture-confirmed TB patients with negative and positive IGRA results, Texas, USA, 2013–2015*

Characteristic	Value
Age, y, median (IQR)	47.0 (30.0–61.0)
Age ≥60 y	402 (27.0)
Sex	
M	942 (63.3)
F	545 (36.7)
Race/Ethnicity	
Non-Hispanic white	152 (10.2)
Black	275 (18.5)
Hispanic	762 (51.2)
Asian	288 (19.4)
Other	10 (0.7)
Foreign-born	897 (60.3)
Resident of long-term care facility	20 (1.3)
Homeless	100 (6.7)
Excess alcohol user	257 (17.3)
Injection drug user	38 (2.6)
Chest radiograph result	
Normal	160 (10.8)
Consistent with TB	1,240 (83.4)
Unknown/Not done	87 (5.9)
TB site	
Pulmonary	1,136 (76.4)
Extrapulmonary	196 (13.2)
Both	155 (10.4)
HIV status	
Negative	1,279 (86.0)
Positive	90 (6.1)
Unknown	118 (7.9)
MDR	13 (0.9)
Days from diagnosis to treatment, median (IQR)	3.0 (0–13.0)
Died within 1 year of TB diagnosis	105 (7.1)

*Values are no. (%) unless otherwise indicated. Percentages were calculated on the basis of the number of patients with data available. IGRA, interferon-γ release assay; IQR, interquartile range; MDR, multidrug resistance; TB, tuberculosis.

(53.5%) had an IGRA result, with 97.4% (1,487/1,527) having either a positive (87.7%, 1,304/1,487) or negative (12.3%, 183/1,487) result. Of the 2,854 culture-confirmed TB patients, 1,367 (47.9%) were excluded from analysis, either because the IGRA was not done or reported or the IGRA was done but the result was indeterminate or the test type was not specified in the surveillance record (Figure 1). Compared with the patients included in analysis, the patients excluded from analysis were less likely to be Asian and more likely to be men, foreign-born persons, persons who used alcohol in excess or were injection drug users, persons with TB-related radiographic abnormalities, and persons who died (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/3/17-1633-Techapp1.pdf).

The patients included in the study sample (N = 1,487) had a median age of 47 (IQR 30–61) years, and most were foreign-born (60.3%); 63.3% were men (Table 1). More than half (51.2%) of the population was Hispanic. Most of the patients had a chest radiograph consistent with TB disease (83.4%). A total of 105 (7.1%) TB patients died within 1 year of TB diagnosis (Table 1).

Compared with patients having positive IGRA results (median age 46 years), those having negative IGRA results were older (median age 55 years; p<0.001), typically ≥60 years of age (OR 2.13, 95% CI 1.55–2.93). Patients

Table 2. Crude association between negative IGRA results and culture-confirmed TB patient characteristics, Texas, USA, 2013–2015*

Variable	Positive IGRA, n = 1,304	Negative IGRA, n = 183	Crude OR (95% CI)	p value
Age, y, median (IQR)	46 (29–60)	55 (40–68)	1.02 (1.01–1.03)	<0.001
Age ≥60 y	326 (25.0)	76 (41.5)	2.13 (1.55–2.93)	<0.001
Sex				
F	479 (36.7)	66 (36.1)	Reference	
M	825 (63.3)	117 (63.9)	1.03 (0.75–1.42)	0.861
Non-Hispanic white	116 (8.9)	36 (19.7)	2.50 (1.66–3.77)	<0.001
US born	505 (38.7)	85 (46.4)	1.37 (1.01–1.87)	0.046
Homeless	85 (6.5)	15 (8.2)	1.28 (0.72–2.27)	0.40
Resident of correction institution	50 (3.8)	4 (2.2)	0.56 (0.20–1.57)	0.27
Resident of long-term care facility	18 (1.4)	2 (1.1)	0.79 (0.18–3.43)	0.75
Injection drug user	35 (2.7)	3 (1.6)	0.60 (0.18–1.99)	0.41
Excessive alcohol user	218 (16.7)	39 (21.3)	1.35 (0.92–1.98)	0.13
Contact with TB patient within past 2 y	73 (5.6)	3 (1.6)	0.28 (0.09–0.90)	0.03
Received TNF-α antagonist therapy	2 (0.2)	1 (0.5)	3.58 (0.32–39.64)	0.30
Solid organ transplant recipient	1 (0.1)	1 (0.5)	7.16 (0.45–114.96)	0.17
Diabetes	265 (20.3)	39 (21.3)	1.06 (0.73–1.55)	0.76
Chronic kidney disease	16 (1.2)	3 (1.6)	1.34 (0.39–4.65)	0.64
Immunosuppression	30 (2.3)	7 (3.8)	1.69 (0.73–3.90)	0.22
Previous TB	31 (2.4)	1 (0.5)	0.23 (0.03–1.66)	0.14
Chest radiograph result				
Normal	137 (10.5)	23 (12.6)	Reference	
Consistent with TB	1,092 (83.7)	148 (80.9)	0.81 (0.50–1.30)	0.38
Unknown/Not done	75 (5.8)	12 (6.6)	0.95 (0.45–2.02)	0.90
TB site				
Pulmonary	997 (76.5)	139 (76.0)	Reference	
Extrapulmonary	169 (13.0)	27 (14.8)	1.15 (0.74–1.79)	0.55
Both	138 (10.6)	17 (9.3)	0.88 (0.52–1.51)	0.65
HIV status				
Negative	1,134 (87.0)	145 (79.2)	Reference	
Positive	70 (5.4)	20 (10.9)	2.23 (1.32–3.78)	0.003
Unknown/Not done	100 (7.7)	18 (9.8)	1.41 (0.83–2.39)	0.21
Epidemiologically linked	118 (9.0)	8 (4.4)	0.46 (0.22–0.96)	0.04
Tested by T-SPOT.*TB*	436 (33.4)	76 (41.5)	1.41 (1.03–1.94)	0.03
IGRA sample collected after TB treatment initiated	415 (31.8)	74 (40.4)	1.45 (1.06–20)	0.02
MDR	12 (0.9)	1 (0.5)	0.59 (0.08–4.58)	0.62
Genotyped	1,260 (96.6)	175 (95.6)	0.76 (0.35–1.65)	0.49
East Asian family lineage	231 (17.7)	27 (14.8)	0.80 (0.52–1.24)	0.32
Clustered	555 (44.1)	85 (48.6)	1.20 (0.87–1.65)	0.26
GENType G00010	34 (2.6)	6 (3.3)	1.27 (0.52–3.06)	0.60
Days from diagnosis to treatment initiation, median (IQR)	2.0 (0–12.0)	5.0 (0.5–26.0)	1.00 (1.00–1.01)	0.04
Follow-up, mo, median (IQR)†	9.4 (7.3–10.7)	9.2 (7.0–10.7)	0.96 (0.92–1.01)	0.11
Year of diagnosis				
2013	373 (28.6)	56 (30.6)	Reference	
2014	428 (32.8)	53 (29.0)	0.82 (0.55–1.23)	0.35
2015	503 (38.6)	74 (40.4)	0.98 (0.68–1.42)	0.92

*Values are no. (%) unless otherwise indicated. GENType, tuberculosis genotype; IGRA, interferon-γ release assay; IQR, interquartile range; MDR, multidrug resistance; OR, odds ratio; TB, tuberculosis; TNF-α, tumor necrosis factor α.
†From diagnosis date until the end of treatment or patient death, whichever came first.

Table 3. Multivariate analysis of association between negative IGRA results and culture-confirmed TB patient characteristics, by IGRA type, Texas, USA, 2013–2015*

	All IGRA results, N = 1,338†		QFT only, n = 875†		T-SPOT.*TB* only, n = 463†	
Variable	aOR (95% CI)	p value	aOR (95% CI)	p value	aOR (95% CI)	p value
Age in years	1.02 (1.01–1.03)	<0.001	1.02 (1.01–1.04)	<0.001	1.02 (1.00–1.03)	0.01
Non-Hispanic white race/ethnicity	2.61 (1.70–4.02)	<0.001	2.76 (1.60–4.76)	<0.001	2.29 (1.11–4.73)	0.03
HIV-positive status	2.72 (1.56–4.77)	<0.001	3.59 (1.86–6.94)	<0.001	1.34 (0.44–4.07)	0.60
Tested by T-SPOT.*TB*	1.58 (1.12–2.24)	0.01	–	–	–	–
Time from diagnosis to treatment	1.00 (1.00–1.01)	0.046	1.00 (1.00–1.01)	0.22	1.01 (1–1.01)	0.03

*aOR, adjusted odds ratio; IGRA, interferon-γ release assay; QFT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; –, analysis could not be performed.
†Multiple logistic models were run by using data from patients having complete data sets for all the included variables; aORs were from the multiple logistic regression models.

with negative results were also more likely than those with positive results to be non-Hispanic white (OR 2.50, 95% CI 1.66–3.77), US-born (OR 1.37, 95% CI 1.01–1.87), and HIV-infected (OR 2.23, 95% CI 1.32–3.78) (Table 2). Culture-positive, IGRA-negative patients were more likely than culture-positive, IGRA-positive patients to have been tested with the T-SPOT.*TB* assay (OR 1.41, 95% CI 1.03–1.94). Culture-positive, IGRA-negative patients had a significantly longer median time from diagnosis to treatment initiation (5 [IQR 0.5–26.0] days) than culture-positive, IGRA-positive patients (2 [IQR 0–12] days). The proportion of specific genotype clusters between patients having negative (48.6%) and positive (44.1%) IGRA results was not significantly different (p = 0.26). The *M. tuberculosis* genotype cluster G00010 was the most common found in study patients, with no significant difference found between the negative (3.3%) and positive (2.6%) IGRA groups (p = 0.60) (Table 2). Fewer IGRA-negative patients reported contact with an infectious TB patient during the previous 2 years (OR 0.28, 95% CI 0.09–0.90) and, therefore, were less likely to be epidemiologically linked to a TB patient (OR 0.46, 95% CI 0.22–0.96). Multivariate analysis results suggested that older age, non-Hispanic white race/ethnicity, HIV co-infection, being tested with the T-SPOT.*TB* assay, and longer time to TB treatment were significantly associated with having a negative IGRA result (Table 3). In stratified analyses by IGRA type, a negative QFT result was significantly associated with older age, non-Hispanic white race/ethnicity, and HIV co-infection, and a negative T-SPOT.*TB* result was significantly associated with older age, non-Hispanic white race/ethnicity, and longer time to TB treatment (Table 3).

Patients with negative IGRA results had a higher overall mortality than those with positive IGRA results both at the time of diagnosis (2.7% [5/183] vs. 0.7% [9/1,304]; crude OR 4.04, 95% CI 1.34–12.2) and during TB treatment (11.2% [20/178] vs. 4.9% [64/1,295]; crude OR 2.43, 95% CI 1.43–4.13). The 1-year survival rate from diagnosis was 83% for patients with negative IGRA results and 94% for patients with positive IGRA results (p<0.001) (Figure 2), with an adjusted hazard ratio of 1.99 (95% CI 1.18–3.33; p = 0.01) (Table 4). When stratified by IGRA type, only negative T-SPOT.*TB* results remained significantly associated with death. Older age, HIV co-infection, and time to TB treatment were identified by Cox proportional hazards modeling as independent risk factors for death; however, only age remained significant after stratifying by IGRA

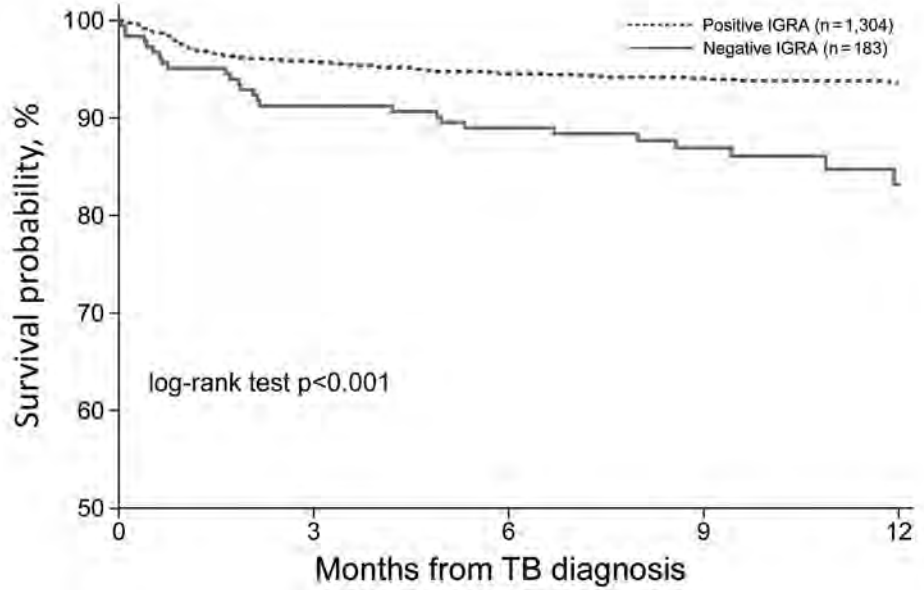

Figure 2. One-year survival from date of TB diagnosis for culture-confirmed TB, stratified by IGRA result, Texas, USA, 2013–2015. IGRA. IGRA, interferon-γ release assay; TB, tuberculosis.

Table 4. Cox proportional hazards model for association between 1-year survival from date of tuberculosis diagnosis and characteristics of culture-confirmed TB patients with negative IGRA results, by IGRA type, Texas, USA, 2013–2015*

	All IGRA results, N = 1,343†		QFT only, n = 880†		T-SPOT.*TB* only, n = 463†	
Variable	aHR (95% CI)	p value	aHR (95% CI)	p value	aHR (95% CI)	p value
Negative IGRA result	1.99 (1.18–3.33)	0.01	1.64 (0.88–3.08)	0.120	3.36 (1.27–8.90)	0.02
Age in years	1.06 (1.04–1.07)	<0.001	1.06 (1.04–1.08)	<0.001	1.05 (1.02–1.08)	0.001
Alcohol	1.55 (0.92–2.60)	0.10	1.75 (0.97–3.15)	0.06	1.19 (0.39–3.63)	0.76
HIV positive	5.00 (2.68–9.33)	<0.001	4.79 (2.43–9.47)	<0.001	4.38 (0.90–21.44)	0.07
Time from diagnosis to treatment	0.99 (0.99–1.00)	0.002	0.99 (0.99–1.00)	0.01	0.99 (0.98–1.00)	0.15

*aHR, adjusted hazard ratio; IGRA, interferon-γ release assay; QFT, QuantiFERON-TB Gold In-Tube.
†Cox proportional hazards models were run with data from patients having complete data sets for all the included variables; aHRs were from the multivariate Cox proportional hazards models.

type (Table 4). The 1-year mortality did not differ significantly by year of TB diagnosis (9.1% for 2013, 6.0% for 2014, and 6.4% for 2015; p = 0.15) (data not shown). Multivariate Cox modeling assessing the survival after the initiation of TB treatment also suggested that negative IGRA results were significantly associated with a higher 1-year mortality (Table 5).

Discussion

Our results suggest that TB patients with false-negative IGRA results had significantly poorer outcomes, with nearly 2 times the odds for death within 1 year of TB diagnosis, compared with patients with positive IGRA results. False-negative IGRA results were more likely to occur in older, non-Hispanic white, and HIV-infected patients or patients tested with the T-SPOT.*TB* assay.

Our study not only confirms the World Health Organization and Centers for Disease Control and Prevention recommendation of not using IGRAs as rule-out tests for TB disease (*7*,*16*) but also indicates that patients with confirmed TB diagnoses but negative IGRA results have poor outcomes. Our study results highlight the need for having a systematic and extensive management strategy for suspected TB patients who have negative IGRA results to minimize misdiagnosis and improve patient outcomes. In symptomatic patients being evaluated for TB disease, further diagnostic evaluation and close follow-up for TB disease should be considered, especially in persons ≥60 years of age, non-Hispanic white, HIV-infected, or tested by the T-SPOT.*TB* assay.

Although false-negative IGRA results were associated with a nearly 2-fold increase in risk for death within 1 year of TB diagnosis, only a false-negative T-SPOT.*TB* result remained significantly associated with death after stratification by IGRA type. This finding suggests that patients who had false-negative T-SPOT.*TB* results in our study might be sicker than those who had false-negative QFT results. This explanation is consistent with the significant delay in treatment initiation after TB diagnosis, especially for patients in the T-SPOT.*TB* group. The significant association between time to treatment and death among all patients and patients by IGRA type suggests that delayed treatment might contribute to the higher mortality of patients with false-negative IGRA results. This finding demonstrates the programmatic implications for treating patients who have negative IGRA findings. Consistent with previously published data, we also found a significantly higher risk for death among patients of older age or with HIV co-infection (*17*–*19*).

Consistent with results of previous studies (*8*–*10*,*20*), in our study, older age was also significantly associated with a false-negative result for both the QFT and T-SPOT.*TB* assays. An explanation for this association could be the gradual decrease of IFN-γ production that occurs in response to *M. tuberculosis*–specific antigens ESAT-6 and CFP-10 with age (*9*,*10*). Other authors have observed a higher rate of false-negative IGRA results in young children (*21*–*23*). However, all 17 children ≤5 years of age in our study had positive IGRA results.

HIV co-infection was associated with a false-negative QFT result but not a false-negative T-SPOT.*TB* result. This finding is consistent with the current literature, which has suggested that the T-SPOT.*TB* assay has higher sensitivity than QFT among HIV co-infected persons, especially

Table 5. Cox proportional hazards model for association between 1-year survival from tuberculosis treatment start date and characteristics of culture-confirmed TB patients with negative IGRA results, by IGRA type, Texas, USA, 2013–2015*

	All IGRA results, N = 1,343†		QFT only, n = 880†		T-SPOT.*TB* only, n = 463†	
Variable	aHR (95% CI)	p value	aHR (95% CI)	p value	aHR (95% CI)	p value
Negative IGRA result	2.12 (1.24–3.63)	0.01	1.84 (0.98–3.47)	0.06	3.41 (1.18–9.88)	0.02
Age in years	1.06 (1.04–1.07)	<0.001	1.06 (1.04–1.07)	<0.001	1.05 (1.02–1.09)	0.002
Alcohol use	1.42 (0.82–2.48)	0.21	1.57 (0.85–2.93)	0.15	1.06 (0.30–3.80)	0.92
HIV positive	4.68 (2.40–9.12)	<0.001	4.60 (2.26–9.34)	<0.001	2.75 (0.32–23.40)	0.36
Time from diagnosis to treatment	0.99 (0.99–1.00)	0.002	0.99 (0.99–1.00)	0.01	0.99 (0.98–1.00)	0.10

*aHR, adjusted hazard ratio; IGRA, interferon-γ release assay; QFT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis.
†Cox proportional hazards models were run with data from patients having complete data sets for all the included variables; aHRs were from the multivariate Cox proportional hazards models.

those with low CD4+ T-cell counts (*24*,*25*). As observed in a previous study (*26*), in our study, non-Hispanic white race/ethnicity was also associated with a false-negative QFT result. In our analysis, extrapulmonary TB was not significantly associated with a false-negative IGRA result or patient outcomes.

Our study has several limitations. We could not completely rule out some provider selection bias, considering nearly 48% (1,367/2,854) of the culture-confirmed TB patients were excluded from the analysis, most (94%, 1,284/1,367) because an IGRA was not performed. Because we used deidentified administrative data obtained from NEDSS, quantitative test results for QFT and T-SPOT.*TB* were not available for analytic evaluation. In addition, by selecting only nonindeterminate results (for QFT) and nonfailed results (for T-SPOT.*TB*), the IGRAs' true sensitivities were overestimated (89.0% [868/975] for QFT and 85.2% [436/512] for T-SPOT.*TB*; p = 0.031). If indeterminate and failed results are included with the negative results when measuring the sensitivity, then the true sensitivity of the QFT and TSPOT.*TB* assays in this population-based screening becomes 84.4% for QFT and 80.9% for T-SPOT.*TB*, and the difference in the sensitivity between QFT and T-SPOT.*TB* becomes nonsignificant (p = 0.095). Immunologic and genetic details, which can help explain the cause of false-negative IGRA results, were also unavailable. Last, because some of the data were originally obtained from interviewing TB patients, the possibility of recall bias cannot be ruled out.

In conclusion, we identified negative IGRA results as a significant predictor for death within 1 year of TB diagnosis among culture-confirmed *M. tuberculosis*–infected patients. Older age, non-Hispanic white race/ethnicity, HIV co-infection, T-SPOT.*TB* assay results, and longer time to TB treatment were significantly associated with false-negative IGRA results. Healthcare providers should consider these risk factors when making decisions on whether to initiate further diagnostic evaluations for TB patients with negative IGRA results.

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About the Author

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Genetic Spatiotemporal Anatomy of *Plasmodium vivax* Malaria Episodes in Greece, 2009–2013

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An influx of immigrants is contributing to the reemergence of *Plasmodium vivax* malaria in Greece; 1 persistent focus of transmission is in Laconia, Pelopónnese. We genotyped archived blood samples from a substantial proportion of malaria cases recorded in Greece in 2009–2013 using 8 microsatellite markers and a *PvMSP-3*α gene fragment and plotted their spatiotemporal distribution. High parasite genetic diversity with low multiplicity of infection was observed. A subset of genetically identical/related parasites was restricted to 3 areas in migrants and Greek residents, with some persisting over 2 consecutive transmission periods. We identified 2 hitherto unsuspected additional foci of local transmission: Kardhítsa and Attica. Furthermore, this analysis indicates that several cases in migrants initially classified as imported malaria were actually locally acquired. This study shows the potential for *P. vivax* to reestablish transmission and counsels public health authorities about the need for vigilance to achieve or maintain sustainable malaria elimination.

The global strategies for malaria control and elimination have led to substantial decreases in malaria incidence worldwide (*1*). In countries outside of Africa, *Plasmodium vivax* often predominates, making this species the most widespread malaria parasite. A combination of *P. vivax* biologic characteristics hamper its elimination and facilitate its reintroduction. The hypnozoite (i.e., the parasite during the dormant liver stage) can activate after months and cause new episodes, extending the period of carriage and transmission. The wide range of anopheline vectors that are susceptible to *P. vivax* and the capability of sporozoites to mature in lower temperatures extends this parasite's geographic range. Also, compared with *P. falciparum*, *P. vivax* infections have low peak parasitemias and clinical immunity (protection against clinical manifestations) is rapidly acquired, resulting in infections that are often asymptomatic, which can prevent prompt diagnosis and treatment and prolong the duration of infection (*2*).

Episodes of rapid resurgence of *P. vivax* malaria have occurred as a consequence of substantial disruption of public health services and large-scale population displacement triggered by war, civil unrest, or socioeconomic crises (*3*,*4*). In addition, with the increase in international travel and migration, sporadic cases of autochthonous infections due to transmission from carriers are noted yearly in various nonendemic countries (*5*). In South Korea (declared malaria-free in 1979), reintroduction of *P. vivax* malaria followed by sustained transmission was observed. Starting with a few cases recorded in the early 1990s in military personnel posted at the demilitarized zone that bisects the Korean Peninsula, the numbers increased to a peak of ≈4,100 by year 2000, with soldiers, veterans, and civilians nearby the demilitarized zone equally affected (*6*). Infected mosquitoes from North Korea were responsible for the introduction of *P. vivax* and probably still contribute to transmission (*7*).

During 2009–2012, episodes of reintroduced autochthonous *P. vivax* malaria occurred in Greece, the first such episodes in Europe since the 1970s. Greece was hyperendemic for malaria before an intense malaria eradication program (in 1946–1960), which led to the country becoming malaria-free in 1974, with a small number of imported malaria cases recorded annually thereafter (*8*). A threat to Greece's malaria-free status arose with a major upsurge in the number of undocumented migrants from

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P. vivax–endemic countries, principally the Indian subcontinent (estimated 200,000 migrants during 2006–2012) (*9*). During 2009–2013, nearly one fourth (27/113) of migrants with imported cases of *P. vivax* malaria reported a history of malaria in their country of origin (C. Hadjichristodoulou, unpub. data). The Hellenic Center for Disease Control and Prevention (HCDCP; Athens, Greece) declared these 113 cases as imported malaria (defined as occurring in persons arriving from endemic areas). HCDCP also defined *P. vivax* cases occurring in migrants <3 years after their arrival to Greece as relapses (recrudescence from persistent subpatent blood infections [i.e., *P. vivax* infections in which the pathogen is present but not detectable by standard laboratory methods] was considered unlikely, although it could not be formally excluded). The potential for local transmission increased substantially in receptive rural areas where migrants settled to serve as seasonal agricultural workers, thereby bringing carriers in contact with the local anopheline vectors during the transmission season. Of note, vector control activities after 1974 (after Greece was declared malaria-free) were not focused on *Anopheles* mosquitoes but rather on those that were considered a nuisance to the local population.

In 2009, an initial cluster of *P. vivax* malaria in 6 Greece residents (defined here as persons living in Greece with no travel history to *P. vivax* malaria–endemic areas) in a small agricultural locality in southern Pelopónnese (regional unit Laconia, municipality Evrótas) alerted the HCDCP. Only 1 such locally acquired (autochthonous) case was reported in Evrótas in 2010 (*10*,*11*), but in 2011, a further 36 locally acquired cases in Greece residents and 21 cases classified as imported in migrant workers were recorded in the same area; these 57 cases represented ≈60% of all cases recorded in Greece that year. Despite prompt deployment of classic malaria prevention measures (mosquito control, distribution of insecticide-treated bed nets, fever screening of immigrants, and investigation of each malaria case) during the 2011 and 2012 transmission seasons, 27 more cases (17 imported in migrants and 10 in Greece residents) occurred in Evrótas in 2012. The next year, the same measures were reinforced by the deployment of mass drug administration targeting the migrant workers in the area (*12*), a strategy that seemed to have contributed to successfully thwarting the reestablishment of the parasite, considering that no autochthonous cases were recorded from this regional unit in 2013 and 2014. During the same period (2009–2013), 74 imported *P. falciparum* cases were recorded in Greece, but none were in Laconia.

In another area, a village in regional unit Kardhítsa, Thessalía, 11 *P. vivax* malaria cases (2 locally acquired in Greek residents and 9 in migrants) were recorded in 2012. In addition, 1 locally acquired case was recorded in 2013.

The availability of archived blood samples from a substantial proportion of all the *P. vivax* malaria cases recorded in Greece during 2009–2013, including those from Laconia, provided the opportunity to investigate in detail the spatiotemporal distribution of *P. vivax* introduction during this period through a molecular description of select parasite polymorphic genetic markers (*13*). Here we report on the spatiotemporal genotypic data from these cases and discuss the insights the data provide on the potential for *P. vivax* to establish sustained transmission in vulnerable receptive areas.

Materials and Methods

P. vivax Blood Samples

During 2009–2013, a total of 311 malaria cases were reported to the HCDCP, of which 209 were caused by *P. vivax* as confirmed by microscopy or PCR analysis. All cases were symptomatic and recorded either through the passive surveillance system (hospitals, health centers) or through active surveillance activities, which included focus investigations of autochthonous cases and fever screening among immigrants in Laconia (*12*). Of the 209 recorded *P. vivax* cases, 122 blood samples collected from 118 patients were available for genotyping (Figure 1). Of the 53 Greece residents, 4 were Romanian, 4 were Moroccan, and 1 was Polish. The study protocol was approved with a waiver of informed consent by the Scientific Committee of the Postgraduate Program of Applied Public Health and Environmental Hygiene, Faculty of Medicine, University of Thessaly, Larissa, Greece.

Genomic DNA Isolation

We isolated genomic DNA from blood samples using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), the Magcore automated system (RBC Bioscience, New Taipei City, Taiwan), or iPrep PureLink gDNA Blood Kit (Invitrogen, Carlsbad, CA, USA). The last method was used exclusively for the samples collected late in 2012 and in 2013.

Genotyping Analysis

We subjected *P. vivax* populations to genotyping targeting 9 main polymorphic markers: 8 microsatellite loci and a region of the gene encoding *PvMSP-3α*. We pooled data available from a previous study for a subset of the samples (genotyped for a subset of markers) with the data we obtained using additional samples and microsatellite markers (*13*). We performed genotyping for *PvMSP-3α* and microsatellite loci m1501 and m3502 as previously described (*13*) and for microsatellite loci MS1, MS5, MS7, MS8, MS12, and MS20 as previously described (*14*). For a subset of samples (n = 54) that yielded similar or closely

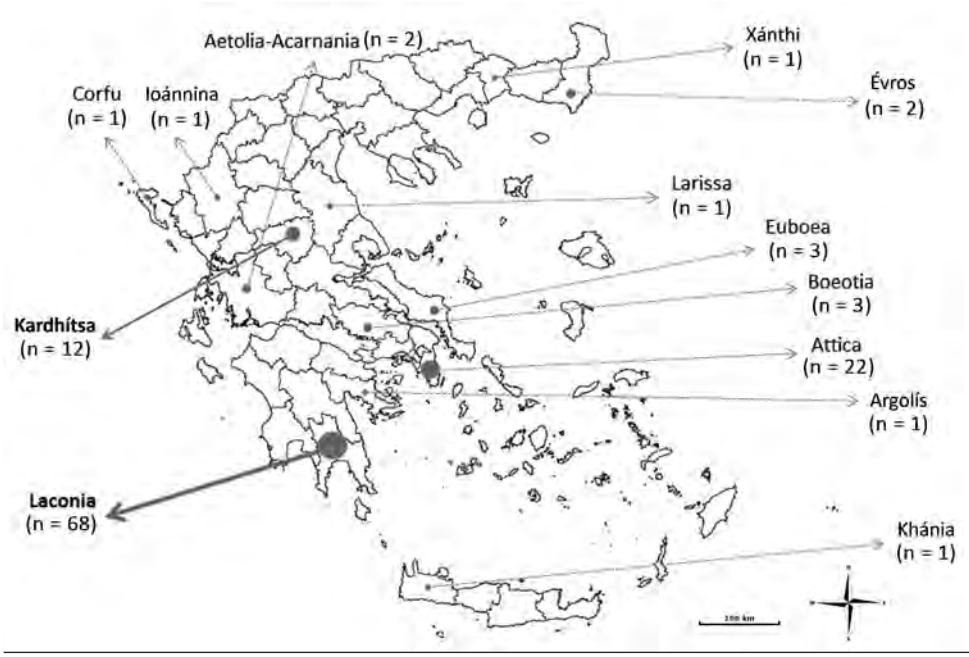

Figure 1. Geographic origin of *Plasmodium vivax* cases analyzed, Greece, 2009–2013. The 2 foci of transmission are Laconia and Kardhítsa (in bold). Size of dots is proportional to number of cases. Samples from Attica were distributed widely throughout this large regional unit, which includes Athens.

related haplotypes, we performed genotyping for 4 additional polymorphic markers (3 microsatellite loci MS10, MS16, and Pv3.27, and the polymorphic F3 domain of *PvMSP1*), which are hereafter referred to as additional markers, as previously described (*15*). For all microsatellite markers, we analyzed PCR products by capillary electrophoresis using 500 LIZ or 1200 LIZ (Applied Biosystems, Foster City, CA, USA) as size standards. We used GeneMarker 2.6.0 (https://www.softgenetics.com/GeneMarker_4.php) for allele calling and considered peaks <500 relative fluorescence units noise. We checked samples manually to confirm true alleles.

We excluded samples from further analysis if <7 of the initial 9 genetic markers did not yield valid results. We classified a sample as having multiple genotype infections if ≥1 allelic variant with a peak height of at least a third of the main allelic variant peak was observed or, for *PvMSP-3α*, if >1 DNA band was visible in the PCR products. We considered 2 haplotypes the same only when all 9 markers shared the same allelic variant. In cases of mixed genotype infections, the dominant allelic variant for each marker was alone considered to determine the haplotype. We used the term family to denote parasites that shared ≥7 out of the 9 main markers. We constructed the phylogenetic tree using Populations 1.2.31 (http://bioinformatics.org/populations/) using the shared allele distance and UPGMA algorithm and visualized with Dendroscope 3 (*16*).

Results

Of the 122 samples, we genotyped 104 successfully; 14 had 1 or 2 of the 9 markers fail to amplify, and 4 were excluded because <7 markers amplified. These 118 samples were obtained from 114 separate patients (4 patients had *P. vivax* infection and were tested in 2011 and 2012). This set comprised a substantial proportion of all the *P. vivax* cases recorded during 2009–2013 (Table). The samples were divided into 3 subsets by their geographic location (Figure 1): the first 2, Laconia (with 68 samples) and Kardhítsa (with 12 samples), were foci of transmission where multiple cases were recorded over time in a relatively restricted area, and the third encompassed the samples from the rest of Greece (with 38 samples).

Table. Reported *Plasmodium vivax* malaria cases, Greece, 2009–2013

	Laconia				Kardhítsa and rest of Greece			
	Greece resident		Migrant*		Greece resident		Migrant	
Year of onset	Cases	Samples	Cases	Samples	Cases	Samples	Cases	Samples
2009	6	4	2	0	1	0	11	0
2010	1	0	1	1	3	0	23	0
2011	34	19	23	11	6	5	14	3
2012†	15‡	15	18	17	10	9	39	29
2013	0	0	1	1	4§	3	8	1

*Population includes 6 persons with a travel history to endemic countries.
†Includes 5 relapse cases and samples: 3 Greece residents and 1 migrant in Laconia and 1 Greece resident in the rest of Greece.
‡Includes 2 locally acquired cases attributed to the 2011 transmission period.
§Includes 1 locally acquired case attributed to the 2012 transmission period.

The extent of allelic diversity observed differed for the main genetic markers (range 5–23 distinct variants/marker; online Technical Appendix 1 Tables 1–3, https://wwwnc.cdc.gov/EID/article/24/3/17-0605-Techapp1.xlsx), and the corresponding allelic frequency distributions differed by collection site (Figure 2, panels A–I). Mixed clone infections were detected in 18 samples, in which only 2 allelic variants were observed; among these 36 variants, 27 were the same 2 variants (12 of 1 variant and 15 of the other). Thus, most *P. vivax* cases diagnosed in Greece appeared to harbor monoclonal infections.

A total of 67 distinct haplotypes were observed when all 9 genetic markers were considered (online Technical Appendix 1 Table 1), of which 53 were unequivocal and 14 corresponded to samples in which 1 or 2 of the markers failed to amplify. Of the 68 samples from the Laconia outbreak, we observed 28 unique *P. vivax* haplotypes only once, and we detected only 1 of these haplotypes (family Greece 4 [Gr4]) in samples collected from another region of Greece. Fourteen of the haplotypes from Laconia (observed in 51 of 68 samples) fell into 4 families: 1) family Laconia 1 (La1), which was observed in 4 samples collected in 2009; 2) La4, which had 8 subtypes and was observed in 32 samples (24 of which were subtype La4–2); 3) La5, which had 2 subtypes, each observed only once; and 4) La6, which had 3 subtypes observed in 13 samples

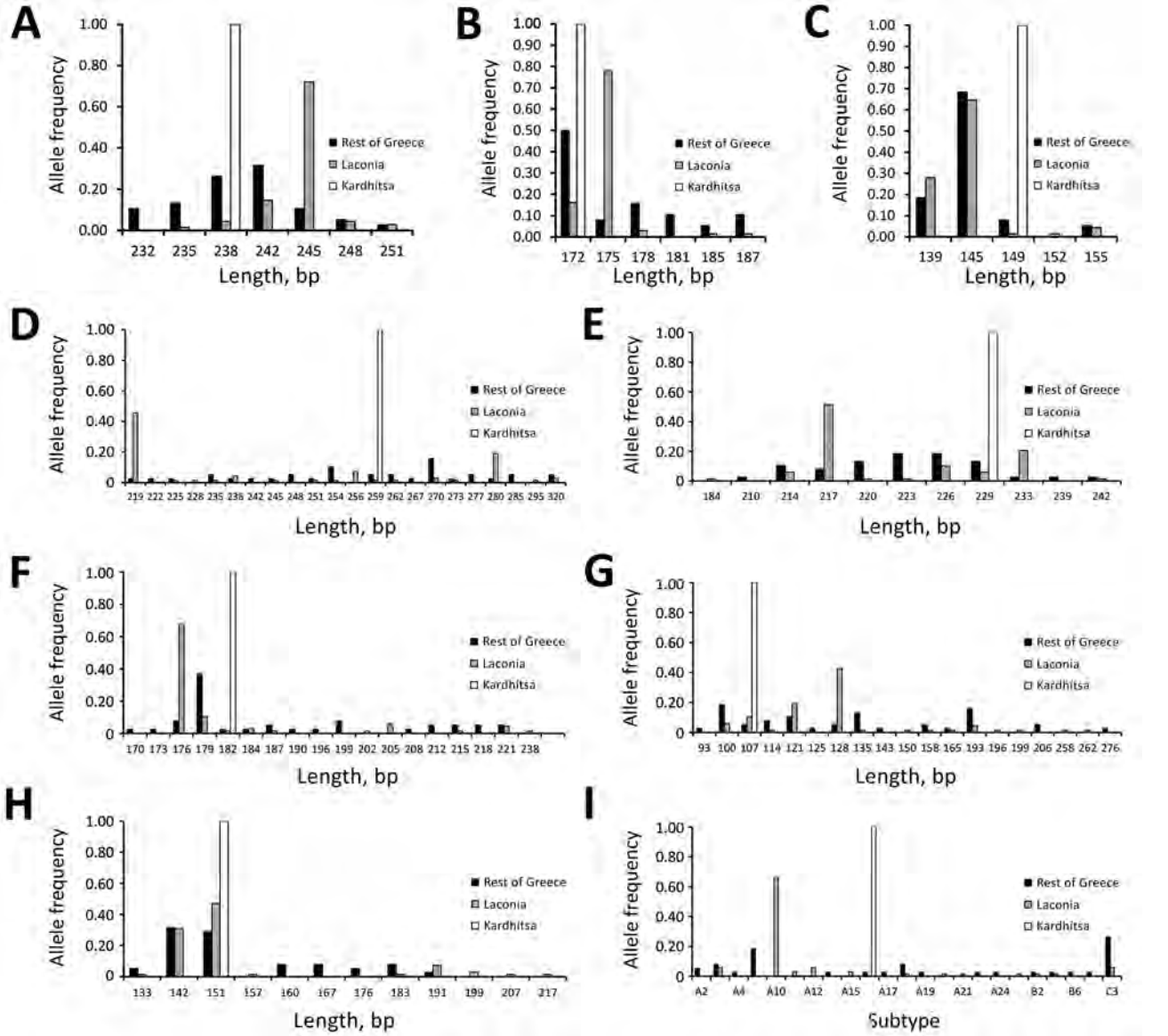

Figure 2. Frequency distribution of *Plasmodium vivax* allelic variants and lengths or subtype of genetic markers, by geographic location, Greece, 2009–2013. The frequencies for microsatellite markers MS1 (A), MS5 (B), MS7 (C), MS8 (D), MS12 (E), MS20 (F), m1501 (G), and m3502 (H) and gene fragment msp3 (I) are calculated separately for the samples from each of the 3 geographic sets: Laconia (n = 68), Kardhítsa (n = 12), and the rest of Greece (n = 38).

(11 of which were subtype La6–1). One fourth (17/68) of the samples from Laconia had unique haplotypes that did not belong to these 4 haplotype families. Of the 35 haplotypes recorded outside Laconia, 1 haplotype of the family Kardhítsa (Ka) was observed solely in the 12 samples (from 3 Greece residents and 9 migrants) in regional unit Kardhítsa. Two related haplotypes (of family Gr1) were observed in the same person who had a relapse in February 2012 seven months after the first episode, and another 2 haplotypes (Gr31 and Gr32, also related) were found in 2 Greece residents from regional unit Évros who had symptoms 1 month apart from each other. Finally, 2 haplotypes of the Gr4 family were observed in 6 persons in the east of regional unit Attica (5 Greece residents and 1 migrant). The remaining 28 haplotypes were each observed only once in samples obtained throughout the rest of Greece (Figure 3; online Technical Appendix 2 Figures 1, 2, https://wwwnc.cdc.gov/EID/article/24/3/17-0605-Techapp2.pdf).

To obtain a dynamic picture of the *P. vivax* isolates that were circulating in Greece throughout the episodes of reintroduction, we displayed the haplotypes in their order of temporal appearance (Figure 3). The dispersal pattern of *P. vivax* parasites, in particular those of the major haplotype families (Gr4 in the eastern part of Attica; Ka in Kardhítsa; and La1, La4, La5, and La6 in Laconia), could be discerned. The genetic analysis of the parasites from Laconia, the main focus of reintroduced *P. vivax*, showed that the parasite haplotype found in Greek residents in 2011 and 2012, when most cases occurred, represented a restricted subset of the haplotypes found in the migrants. All but 3 of the samples acquired from 31 Greek residents (34 samples) belonged to 3 haplotype families: La4, La5, and La6. Fifteen of the 27 samples acquired from migrants during the same years also belonged to families La4–La6, with the remaining 12 samples from migrants each harboring a unique but unrelated parasite haplotype.

In Attica, 6 of the cases recorded during August 2011 and summer 2012 among Greek residents from 4 different locations were the same haplotype (Gr4); thus, Attica can be considered an additional focus of local transmission. We observed the same haplotype in an Afghani migrant who had *P. vivax* in early July 2012 in Laconia. Finally, in regional unit Kardhítsa, Thessalía, all 12 *P. vivax* samples (11 from 2012 and 1 from 2013) collected from a single village (6 of which were from a focus investigation) shared the same haplotype. Eleven of these samples were from patients of different nationalities (7 Afghani, 2 Bangladeshi, and 2 Romanian) who lived in close proximity and sought treatment within 2 months of each other.

Given that the differences between the haplotypes within each of these 6 families were minor, the corresponding samples were subjected to genotyping with 4 additional genetic markers: 3 microsatellite loci MS10, MS16, and Pv3.27, and the polymorphic F3 domain of *PvMSP1*. This analysis revealed that the Gr4 family comprises 4 subtypes that differ with respect to 1 or 2 of the additional markers. We were able to distinguish 4 subtypes of haplotype family Ka and 3 subtypes of haplotype family La4 with 1 of these 4 additional loci. The members of the La6 family were homogeneous at all these loci (online Technical Appendix 2 Figures 1, 2).

Discussion

Using archived blood samples, we determined the genetic anatomy of many of the *P. vivax* isolates introduced into Greece during 2009–2013 and investigated the dynamics of the transition of a location from a source of imported cases to an area with established, sustained autochthonous transmission. These transitions are increasingly likely to occur in areas where malaria control efforts have eliminated or will eliminate the parasite. This fact is of particular concern for *P. vivax*, whose ability to form hypnozoites substantially enhances its potential to transmit over extended periods. Standard approaches of screening and treatment are inapplicable for *P. vivax* because screening for hypnozoite carriage is not possible. Moreover, the toxicity of primaquine phosphate, the only hypnozoitocidal drug available, precludes its use without prior onerous testing for glucose-6-phosphate dehydrogenase deficiency. Thus, the often-asymptomatic or clinically mild relapses that might occur in migrants over time provide additional opportunities for local anopheline mosquitoes to transmit the parasite locally.

A total of 1,320 cases of malaria were reported in Greece during 1975–2008, a mean of 39 (range 16–79) cases/year; 475 of these cases were *P. vivax* diagnosed in visitors from the Indian subcontinent. Over this period only 13 autochthonous cases (2 in 1975, 3 in 1976, 5 in 1991, 1 in 1999, and 2 in 2000) occurred in diverse regions of Greece (*8*). In contrast, during 2009–2013, *P. vivax* malaria was diagnosed in 139 migrants or travelers from endemic countries and 76 Greece residents (Table 1). Most likely, the substantial increase after 2006 in numbers of Indian subcontinent migrants, with many employed over the spring and summer months as agricultural workers, led to the reintroduction of *P. vivax* in some rural areas. The focus of introduced *P. vivax* malaria was considered to be in regional unit Laconia because 53 of the 76 cases in Greece residents were from 6 villages in Laconia (municipality Evrótas) within a 2.5-km radius, with the remaining 23 being from diverse and distant regions of Greece. The identification of the same haplotypes as in an earlier study, in which a combination of only 3 markers were used to analyze samples from 2 consecutive years (2011 and 2012), suggested that sustained transmission in the area was possible and further measures were needed to control the disease. Thus, mass drug administration with the immigrant

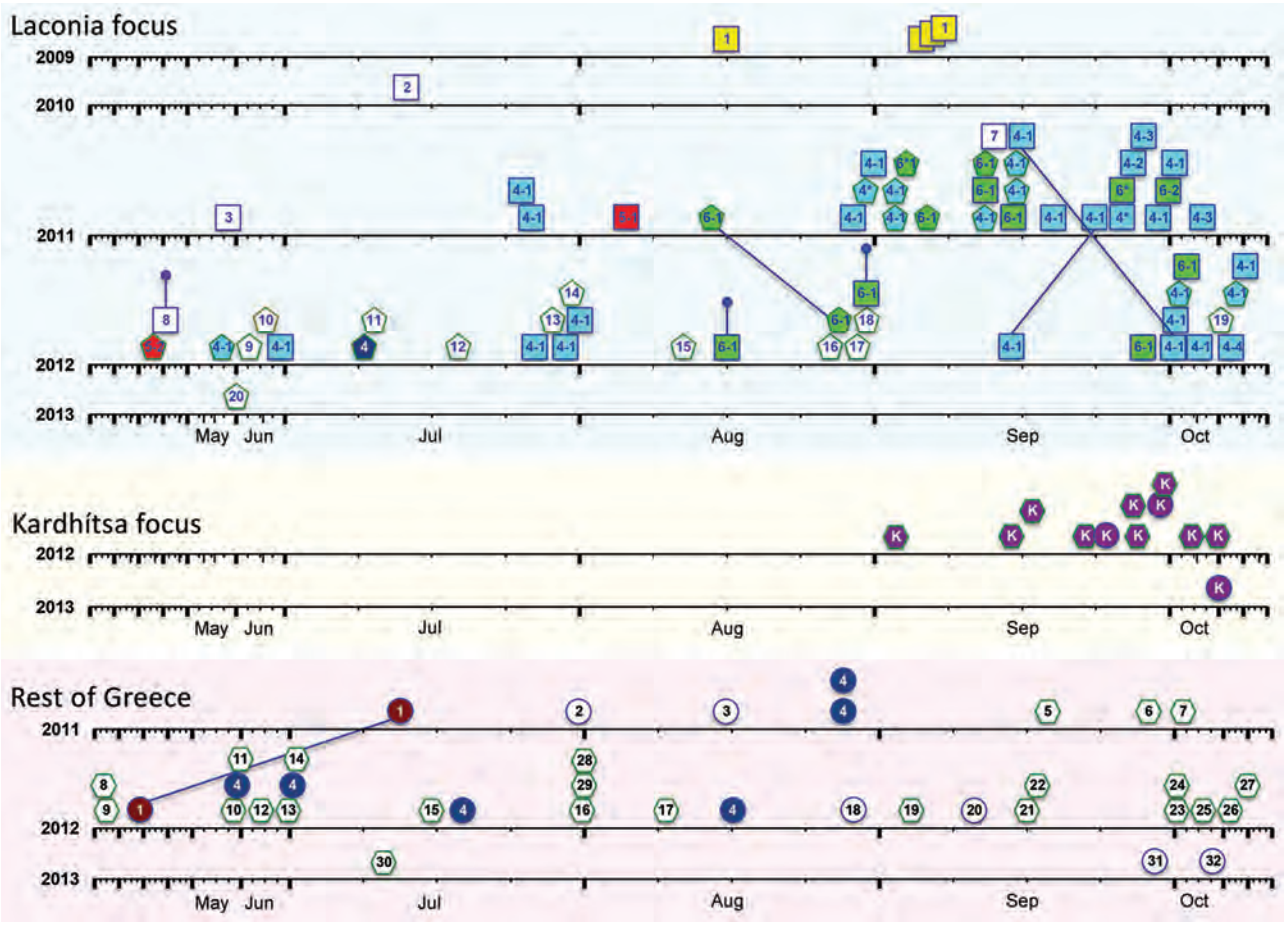

Figure 3. Temporal representation of the occurrence of *Plasmodium vivax* haplotypes, Greece, 2009–2013. Data for each region (Laconia, Kardhítsa, and rest of Greece) are depicted. Samples are indicated by shapes: in Laconia, squares indicate Greece residents and pentagons migrants; in Kardhítsa and rest of Greece, circles indicate Greece residents and hexagons migrants. Colors indicate haplotype families: La-1, yellow; La-4, blue; La-5, red; La-6, green; Gr-1, maroon; Gr-4, dark blue; and Ka, purple. Haplotype numbers are given inside shapes. The *x*-axis time scale for each year was stretched to accommodate the higher number of cases reported during the transmission season, with January, February, March, April, November, and December compressed. Lines joining 2 samples indicate isolates collected from the same person. Truncated vertical lines indicate the 3 samples collected in 2012 from persons considered exposed in the previous year: 1 relapse of a 2011 case and 2 patients with symptom onset in 2012 whose exposure was attributed to the 2011 transmission period. Dates of symptom onset of 2 patients tested in 2012 (green hexagons, haplotypes 28 and 29) were unknown and were arbitrarily assigned date August 1. Further details of samples are provided in online Technical Appendix 1 Tables 1–3 (https://wwwnc.cdc.gov/EID/article/24/3/17-0605-Techapp1.xlsx).

population in Laconia was decided on and performed as a supplementary measure (*12*).

Our detailed spatiotemporal genetic analysis, in which we identified and compared the *P. vivax* haplotypes in patient samples, helped shed light on the epidemiology of reintroduced *P. vivax* episodes. Some of the infections in migrants in Laconia in 2011–2012 were most probably acquired locally and should consequently be reclassified as autochthonous. Careful analysis of the genotyping data uncovered 2 additional foci of local transmission and confirmed the need to reevaluate the classification of cases as autochthonous and imported. Despite the high number of cases, the focus in Attica was unsuspected because cases were scattered throughout the region. Genotyping revealed that parasites from 6 unrelated Greece residents (8 samples, 2 in 2011 and 6 in 2012) living within a 15-km radius of each other shared the same haplotype (Gr4). This haplotype was also found in 2012 in 1 migrant living in Laconia, but further investigation did not reveal any epidemiologic connection between this case and those from Attica.

The cluster of 11 cases recorded in 2011 in Kardhítsa was not thought of as a focus of local transmission because 9 of these cases were in migrants from the Indian subcontinent. However, genotyping revealed that the parasites from all 11 migrants were of the same haplotype, as were those from a Greece resident with no history of malaria or travel

in 2013. This infection might have been acquired during the previous months from an asymptomatic carrier, or the infection might represent a delayed primary attack after inoculation by an infected mosquito the previous year. Thus, Kardhítsa represents a third focus of local transmission, where many of the migrant cases classified as imported malaria by using classic epidemiologic criteria should be reclassified as locally acquired.

From an epidemiologic point of view, *P. vivax* parasites have a high potential for initiating and maintaining local transmission within a short period of time from carriers reaching receptive areas hitherto free of malaria. In such areas, the nonimmune status of the local population will facilitate rapid detection of cases, considering that infected, previously malaria-naive residents would generally have clinical symptoms that are sometimes severe. However, in regions where *P. vivax* malaria has been eliminated or brought to very low incidence, the imported parasite strains might persist as subpatent infections because of residual levels of acquired immunity in the local population, which would enhance the likelihood of wider dissemination. Nonetheless, data from the reintroduction episodes we present suggest that the potential for establishing transmission might differ by parasite strain, considering that relatively few *P. vivax* strains that were introduced into Greece during the 5 years of our analysis led to clusters of sustained local transmission. The difference in transmission might be attributable to biologic or environmental factors or a combination of both. The local mosquito populations might be inefficient at transmitting some of the imported strains (*17*), and variation in the vector midgut bacterial biota might also restrict transmission (*18*). However, the 3 foci we identified involved genetically diverse parasites (online Technical Appendix Figure 2), which could be explained by the parasites having differential transmission efficacies for the local anopheles populations. Finally, the degree of human–anopheline contact also plays a crucial role, such that the parasites in carriers residing in a rural area with a high mosquito density are more likely to be transmitted than parasites in carriers living in an urban environment with a low mosquito density.

The reintroduction of *P. vivax* malaria into Greece during 2009–2013 should serve as a warning to those engaged in efforts to eliminate malaria (*19*) in countries where *P. vivax* predominates, especially for those in countries close to elimination and in malaria-free areas neighboring *P. vivax*–endemic regions. The prompt and flexible countermeasures brought to bear by the Greek public health infrastructure were effective in containing and then extinguishing the foci of transmission (*12*). In our genotypic analyses, we highlight 2 epidemiologic features: first, the high potential for imported *P. vivax* to establish sustainable transmission within a short time; and second, the difficulty in predicting the locations where such transmission might occur. *P. vivax* is a recognized obstacle for malaria elimination (*20*). Maintaining vigilance will be crucial to ensure that this parasite will not threaten those countries that achieve it.

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Invasive Infections Caused by *Nannizziopsis* spp. Molds in Immunocompromised Patients

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We report 2 new cases of invasive infections caused by *Nannizziopsis* spp. molds in France. Both patients had cerebral abscesses and were immunocompromised. Both patients had recently spent time in Africa.

Nannizziopsis spp. molds have been reported in extremely rare cerebral and disseminated infections (*1*,*2*), (Table). We describe 2 cases of *Nannizziopsis* infection diagnosed in France during the past 2 years. Both case-patients were immunocompromised and had recently returned from Africa.

The Cases

Case-patient 1 was a 63-year-old woman from France who had been treated for T-cell prolymphocytic leukemia diagnosed in December 2014. She initially received alemtuzumab, switching to bendamustine in March 2015 after tumor progression. That treatment failed, and idelalisib was started on July 11, 2015. The patient was hospitalized 2 days later for acute renal failure with mild fever. She became confused and drowsy, and cerebral computed tomography (CT) scan showed systematized subcortical hypodense areas. Lumbar puncture showed neoplastic cells in the cerebrospinal fluid (CSF) and glucose concentration within reference range. Bacteriological and fungal cultures were sterile. A large volume of ascites fluid remained, despite iterative punctures with negative bacteriological cultures. We initiated intrathecal chemotherapy with methotrexate/cytarabine/methylprednisolone. However, the patient's condition worsened, with heart failure and loss of consciousness. On July 18, we took new specimens of CSF, bronchial wash, ascites fluid, and blood cultures and sent them for bacteriological investigation. We started empiric treatment with imipenem/aminoglycosides, but the patient died on July 19 of septic shock. No autopsy was performed. Extended-spectrum β-lactamase–producing *Escherichia coli* sensitive to imipenem grew quickly in 1 pair of blood cultures. A second pair was positive 4 days later, with the presence of large septate fungal hyphae and arthroconidia. White and thin cottony mold colonies grew on Sabouraud media incubated at 35°C (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/3/17-0772-Techapp1.pdf). We performed best model determination and phylogenetic analyses in MEGA6 (http://www.megasoftware.net). We identified *N. obscura* by sequencing the 18S-internal transcribed spacer (ITS) 1–5.8S-ITS2 region (online Technical Appendix Figure 2). The strain had low MICs for antifungals as defined by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/): amphotericin B 0.06 μg/mL, itraconazole 0.25 μg/mL, voriconazole 0.03 μg/mL, posaconazole 0.06 μg/mL, caspofungin 0.5 μg/mL, and micafungin 0.015 μg/mL. We performed mycologic investigations of CSF and ascites fluid a posteriori on frozen aliquots and conducted PCR assays targeting the ITS region on CSF sampled on July 15 and July 19 and on ascites fluid sampled on July 19. We observed positive amplifications in all samples; subsequent sequencing confirmed the presence of DNA from *N. obscura*. We investigated the origin of the patient's contamination. She had made several trips to Senegal, the latest in January 2015, during which an ulcerative inflammatory lesion developed on her left little finger. However, Grocott stain and PCR on paraffin-embedded tissue of skin biopsy were negative, and we attributed the lesion to the hematological malignancy.

Case-patient 2 was a 52-year-old woman from France living in Mali, who was hospitalized in Bamako in November 2016 for cough, fever, alteration of general state, and headache. She tested seropositive for HIV (CD4 3/μL; HIV-1 viral load 45.300 copies/mL). Chest radiograph showed bilateral pneumonia, and cerebral CT scan showed a single process on the left temporal lobe. Antiretroviral therapy was initiated with a combination of efavirenz/lamivudine/tenofovir associated with isoniazid, metronidazole, amoxicillin/clavulanate, and trimethoprime/sulfamethoxazole. Because of worsening of her neurologic status, she was repatriated to France. At hospital admission on January 12, 2017, she had a lesion on the left middle fingernail suggestive of onychomycosis, hemiparesis, and paralysis of the right side of the face

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Table. Characteristics of *Nannizziopsis* spp. infection in humans*

Year (reference)	Age, y/sex	Country	Underlying condition or context	Species	Localization	Positive samples	Treatment	Outcome
2017 (this study)	52/F	France	HIV, living in Mali	*Nannizziopsis* sp.	Brain abscess	Cerebral biopsy, CSF	AmpB for 1 mo, then VCZ	Recovery but neurologic sequela after 2 mo
2015 (this study)	63/F	France	Leukemia, recent travel to Senegal	*N. obscura*	Brain abscess	Blood culture, CSF, ascites fluid	Not treated (death before diagnosis)	Death before diagnosis
2005 (*2*)	38/M	Germany	HIV, travel to Nigeria	*N. obscura*	Brain abscess	Needle aspiration of brain lesion	VCZ	Recovery without sequelae after 4 mo
2005 (*5*)	40/M	United States	HIV	*N. infrequens*	Lung	Bronchial washing	Not treated, considered as a contaminant	Recovery after treatment of CMV infection
2000 (*3,4*)	32/M	United States	Travel to Nigeria	*N. hominis*	Lymph nodes, heart, lungs, spleen, kidneys	3 lymph nodes	ITRA for 2 y	NA
1994 (*3*)	NA/M	United States	HIV	*N. hominis*	Right thigh mass	Deep muscle mass on the right thigh, right groin, buttock, and lung	ITRA	Death after 8 mo
1982 (*1*)	24/M	United States	Travel to Africa	*N. obscura*	Abscess in right ankle, osteomyelitis	2 biopsies of abscess in tibia	AmpB for 4 mo	Recovery after 4 mo

*AmpB, amphotericin B; CMV, cytomegalovirus; CSF, cerebrospinal fluid; ITRA, itraconazole; NA, not available; VCZ, voriconazole.

associated with Broca's aphasia. A thoracic-abdominal-pelvic scan revealed a nodular lesion in the right lung (Figure, panel A) and multiple partly calcified pleural lesions. Bacteriological assays, including investigation for mycobacteria on bronchoalveolar lavage (BAL) fluid, showed negative results. A *Penicillium* grew rapidly on mycological medium, and *Pneumocystis jiroveci* PCR results were slightly positive. Cranial tomodensitometry showed multiple gadolinium-enhancing nodules surrounded by edema (Figure, panel B). We initiated fluconazole and pyrimethamine/sulfadiazine and switched antiretroviral therapy to raltegravir/abacavir/lamivudine after the onset of acute renal insufficiency. Hemiplegia developed 15 days later. A new CT scan showed stable cerebral lesions but an increase in surrounding edema. We performed a lumbar puncture and started intravenous corticotherapy. CSF contained 127 leukocytes (61% lymphocytes) and showed hypoglycorrhachia. Investigations for toxoplasmosis, cryptococcosis, histoplasmosis, tuberculosis, and CMV showed negative results, but ITS-targeting PCR results were positive on CSF. The sequence was closely related to *N. obscura*, but mycological cultures were sterile (online Technical Appendix Figure 2). The results of a β-D-glucan assay of serum was positive (983 pg/mL; Fungitell, Associates of Cape Cod, Inc., http://www.acciusa.com/clinical/fungitell/index.html) and galactomannan antigen was negative. On February 6, we performed a cerebral biopsy. Histopathologic examination showed granuloma containing hyphae (Figure, panels C, D), and on the fourth day of incubation, white mold grew on Sabouraud media at 25°C and 35°C (online Technical Appendix Figure 1). The phylogenetic analyses of the 18S-ITS1–5.8S-ITS2 region confirmed that the fungus belonged to *Nannizziopsis* spp. Although the 18S region was closely related to *N. obscura*, the ITS1 region had a large insertion, suggesting the strain does not belong to the described *Nannizziopsis* species (online Technical Appendix Figure 2). Strain MICs as defined by the European Committee on Antimicrobial Susceptibility Testing were amphotericin B 0.25 μg/mL, itraconazole 0.03 μg/mL, voriconazole 0.125 μg/mL, posaconazole 0.25 μg/mL, caspofungin 0.25 μg/mL, and micafungin ≤0.008 μg/mL. We initiated liposomal amphotericin B (5 mg/kg). On July 16, the patient became drowsy with a bilateral pyramidal syndrome and moderate reactive mydriasis. Cranial CT scan showed an increase in the abscesses and edema with brain displacement. We performed a craniotomy. One month after diagnosis, the patient's general status had improved, with regression of the lung lesions and cerebral abscesses; her CD_4 cell count was 50/μL and HIV-1 load <40 copies/mL. We switched her antifungal treatment to voriconazole.

Conclusions

The molds of the *N. vriesii* complex (*Chrysosporium*-like anamorph, CANV) are members of the *Nannizziopsis* genus (*Onygenales*, *Eurotiomycetidae*, *Eurotiomycetes*, *Ascomycota*).

CANV includes the keratinophilic species, which causes skin and fatal disseminated infections in reptiles (*3*,*4*). There is no documented evidence of zoophilic species involvement in human infections, but 3 other CANV species have been recovered from human samples (Table). Of the 5 previous cases of *Nannizziopsis* infections, 3 involved HIV patients. One of our patients was seropositive for HIV and the other had T-cell prolymphocytic leukemia, which suggests that lymphopenia could be a key risk factor. All patients with reported *N. obscura* infection had recently traveled in Africa. Results for case-patient 1 showed that the fungus grows in blood cultures and thus has high potential for dissemination. Case-patient 2 had pulmonary lesions, but BAL cultures were rapidly invaded by a *Penicillium* fungus. Although we could not detect *Nannizziopsis* in BAL, the lesion evolved favorably after antifungal therapy. Because *Nannizziopsis* spp. are keratinophilic molds, we looked for cutaneous lesions. Both case-patients had recently developed cutaneous or nail lesions during their time in Africa, but we did not detect *Nannizziopsis* from these lesions. These molds have not been isolated in our laboratory in other kinds of samples (clinical or environmental).

CSF cultures from both our case-patients and ascites cultures from case-patient 1 were negative, but panfungal PCR successfully detected molds (*6*). Data on biomarkers are scarce. *N. infrequens* cross-reacts with the *Histoplasma* AccuProbe test and *N. hominis* with the *Blastomyces* AccuProbe test (Hologic, San Diego, CA, USA) (*5*). In case-patient 2, β-D-glucan was positive in CSF and serum but galactomannan antigen was not. *N. obscura* seems to be sensitive to most antifungal agents (*2*).

These observations show how difficult this infection is to detect, which could explain why so few cases of human infections have been reported. However, the diagnosis of these 2 cases since 2015 suggests that the prevalence of *Nannizziopsis* infections may be underestimated.

Acknowledgments

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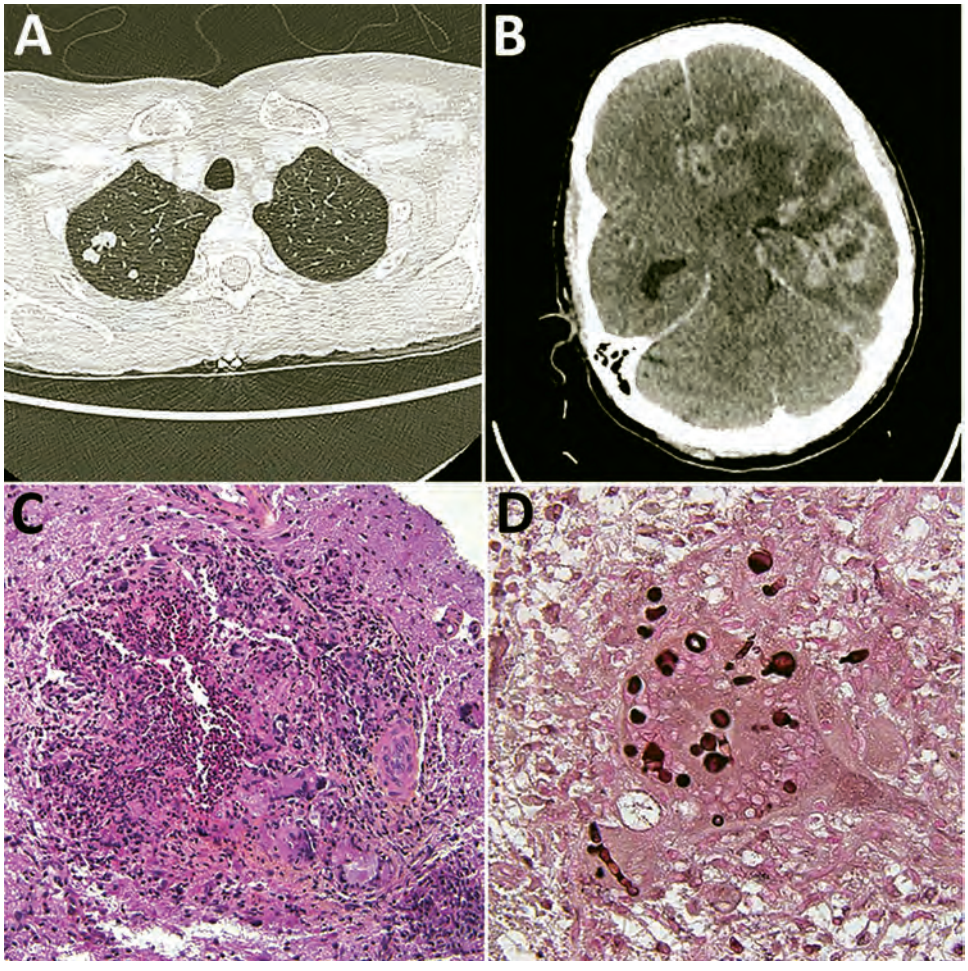

Figure. Diagnostic testing of a 52-year-old woman from France living in Mali who had *Nannizziopsis* spp. fungal infection. A) Thoracic-abdominal-pelvic scan shows pseudo-nodular lesions in the apex of the right lung, of which one is excavated. B) Cerebral computed tomography scan shows contrast enhancement on several hemispheric nodules on the left and in frontal, parietal, and temporal regions, responsible for large surrounding edema and compression of the left lateral ventricle. The median line is deviated to the right with a subfalcorial herniation. C) Hematoxylin-eosin-saffron stain of brain biopsy containing mononuclear inflammatory infiltrates; giant cell granulomas; histiocytes, sometimes with an epithelioid appearance; and neutrophils (original magnification ×200). D) Grocott stain showing thick bulbous mycelial filaments in the cytoplasm of certain giant cells/histiocytes (original magnification ×600). Round shapes correspond to cross-sections of bulbous territories.

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Cache Valley Virus in *Aedes japonicus japonicus* Mosquitoes, Appalachian Region, United States

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We detected Cache Valley virus in *Aedes japonicus*, a widely distributed invasive mosquito species, in an Appalachian forest in the United States. The forest contained abundant white-tailed deer, a major host of the mosquito and virus. Vector competence trials indicated that *Ae. j. japonicus* mosquitoes can transmit this virus in this region.

Cache Valley virus (CVV; family *Bunyaviridae*, genus *Orthobunyavirus*) is widespread throughout North and Central America and infects many species of domestic ungulates (sheep and cattle), but white-tailed deer are a likely reservoir (*1*). Although this virus has been isolated from >30 mosquito species in several genera, the principal vectors remain unknown (*2*,*3*). However, on the basis of field isolations and laboratory transmission studies, *Anopheles quadrimaculatus* and *An. punctipennis* mosquitoes probably play major roles in its transmission cycle (*1*,*4*).

CVV infection is common in sheep and causes spontaneous abortion, stillbirth, and congenital defects (*5*). The virus is neuroinvasive in humans, and there have been 3 confirmed cases and 1 death in the United States (*2*,*6*). Medical laboratories rarely test for CVV, which underestimates its true incidence and effect on human health, but serologic studies have reported high human infection rates (up to 18%) in virus-endemic areas (*7*). We report detection of CVV in the invasive mosquito *Aedes japonicus japonicus* in Blacksburg, Virginia, USA, and demonstrate that this species is a competent vector of the virus.

The Study

We collected adult mosquitoes during June 1–August 21, 2015, by using gravid traps in a forested area (area 196,115 m^2) (Figure 1). We collected 1,197 *Ae. triseriatus* and 690 *Ae. j. japonicus* adult female mosquitoes; identified them to species on the basis of morphology; and pooled them by species, trap number, and date (Table 1). Pools (626 of *Ae. triseriatus* and 442 of *Ae. j. japonicus*) consisting of 1–50 mosquitoes were stored at –80°C. We screened samples on Vero cells for cytopathic effect and confirmed the presence of virus in positive samples by using a plaque assay (*8*).

We amplified virus isolates on Vero cells to a titer of 10^5 PFU/mL and extracted virus RNA from infected cell supernatants by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). We used reverse transcription PCR and *Bunyaviridae*-specific universal primers BCS82C and BCS332V to produce a 251-bp amplicon of the small RNA segment, which was then sequenced (*9*). Sequencing was performed by Eton Bioscience, Inc. (San Diego, CA, USA). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) query indicated that the isolates were CVV.

We amplified large RNA segments by using reverse transcription PCR and CVV-specific primers CVV_L (5′-AGTCAGCCAAAACAGCCACT-3′) and CVV_R (5′-TACAAATCTAGGGGGCATGG-3′) and amplified medium RNA segments by using primers M14C and M4510R (*9*,*10*). Resulting amplicons were sequenced and identified as CVV by performing a BLAST query. Medium RNA segments encoding the Gc protein were amplified by using the primer pair CVV_M_L (5′-CTGTCACGGTGCTAGTAGGAAAGATGTG-3′) and CVV_M_R (5′-AGTAGTGTGCTACCGGTATCAAAAACAGC-3′) and then sequenced.

We detected CVV in 2 *Ae. j. japonicus* female mosquitoes collected from different traps on August 7 (Table 1). For the week of August 4–11, we calculated the CVV minimum infection rate to be 11.5/1,000 mosquitoes (173 mosquitoes tested individually). This late-season occurrence of CVV is consistent with results of a study in Connecticut, USA (*4*). Although *Ae. triseriatus* mosquitoes were more abundant than *Ae. j. japonicus* mosquitoes and have a similar biology as the invasive mosquito, we did not detect CVV in *Ae. triseriatus* mosquitoes.

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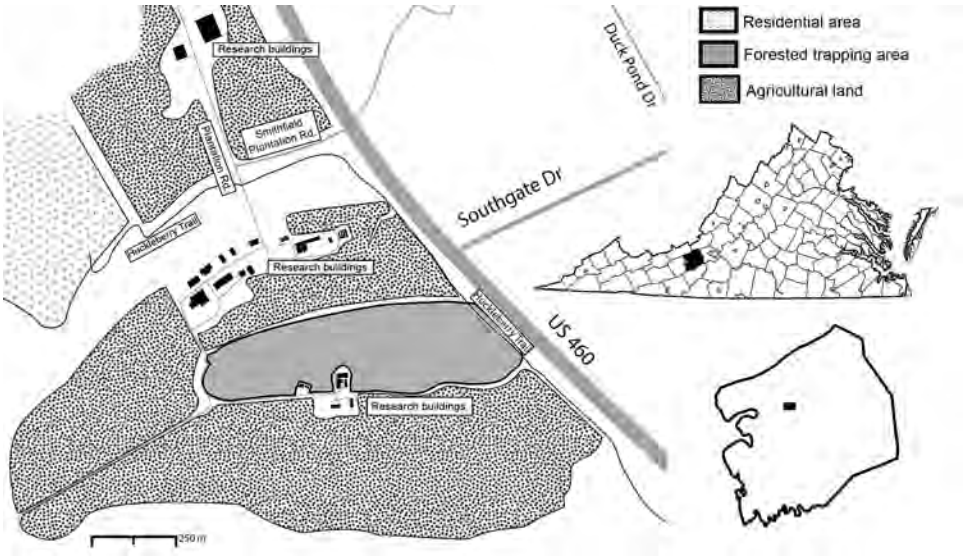

Figure 1. Study site used for detection of Cache Valley virus in *Aedes japonicus japonicus* mosquitoes, Blacksburg, Virginia, USA, 2015. Insets show location of Blacksburg in Montgomery County (black box) and the county in Virginia (black shading).

We used the medium RNA segment to infer phylogeny with CVV isolates reported by Armstrong et al. (*10*). We translated RNA sequences to amino acid sequences and aligned them visually, which was trivial because of invariant length and lack of insertion–deletion events. The alignment was composed of 1,803 bp from 100 isolates. We partitioned sequences by codon position and evaluated alternative models of nucleotide site substitution. We inferred phylogenetic trees by using a Markov chain Monte Carlo method in MrBayes 3.2.5 with a simultaneous estimation of topology, branch lengths, and other parameters (*11*). Stabilization of 4 concurrent chains occurred at 1 million generations, and the first 250,000 trees were discarded as a burn-in. We averaged branch lengths and other parameters and constructed a consensus tree (Figure 2) from the posterior distribution that contained support values for each clade in posterior probabilities.

CVV isolates were grouped into 2 clades or lineages (Figure 2) (*10*). Lineage 1 viruses were from the United States and Canada during 1952–2011 and lineage 2 were more recent strains from the northeastern United States. Virus isolates from Virginia were genetically similar to each other (3-bp differences) and grouped in the newly emergent lineage 2 of CVV. This monophyletic lineage shares a most recent common ancestor with a virus from Mexico isolated in 1961 (GenBank accession no. AF231118). This sister-group relationship suggests a derivation of this group from Mexico (*10*). However, additional historical samples from the region in Veracruz, Mexico, and elsewhere during the history of its introduction into the northeastern United States would further provide understanding of the phylogeography of the virus.

To determine vector competence of local mosquitoes for CVV, we established a laboratory strain from uninfected *Ae. j. japonicus* mosquitoes. Week-old female mosquitoes from the F_2 generation were offered an infectious blood meal in a membrane feeder. This blood meal contained 1 mL of the CVV-4B isolate and 9 mL of sheep blood (Colorado Serum Company, Denver, CO, USA). We transferred postfeeding, engorged mosquitoes to 0.7-L cages and held them for 14 days at 25°C, a relative humidity of 75%, and a 16:8 (L:D) photoperiod and provided 10% sucrose. We measured rates of nondisseminated and disseminated infection (virus present in legs and wings) and oral transmission (virus present in saliva). We conducted this experiment 3 times.

Infectious blood meal titers ranged from 1.6×10^5 to 4.6×10^6 PFU/mL (Table 2). *Ae. j. japonicus* female mosquitoes were susceptible to oral infection with CVV and capable of transmitting the virus. After a 14-day incubation, CVV was present in 41% of abdomens, 38% of legs and wings, and 28% of saliva samples (Table 2). We found no

Table 1. Screening of *Aedes triseriatus* and *Aedes japonicus* adult mosquitoes from gravid traps for arthropod-borne virus by using plaque assays, Appalachian Region, United States, 2015

	Aedes triseriatus		*Ae. japonicus*		
Collection date	No. mosquitoes	No. pools*	No. mosquitoes	No. pools*	No. positive samples
Jun 1–30	569	14	196	26	0
Jul 1–31	383	367	257	240	0
Aug 1–19	245	245	237	176	2†
Total	1,197	626	690	442	2†

*After July 21, most mosquitoes were tested individually rather than in pools.
†Two Cache Valley virus–infected adult *Ae. j. japonicus* mosquitoes were collected and tested individually on August 4.

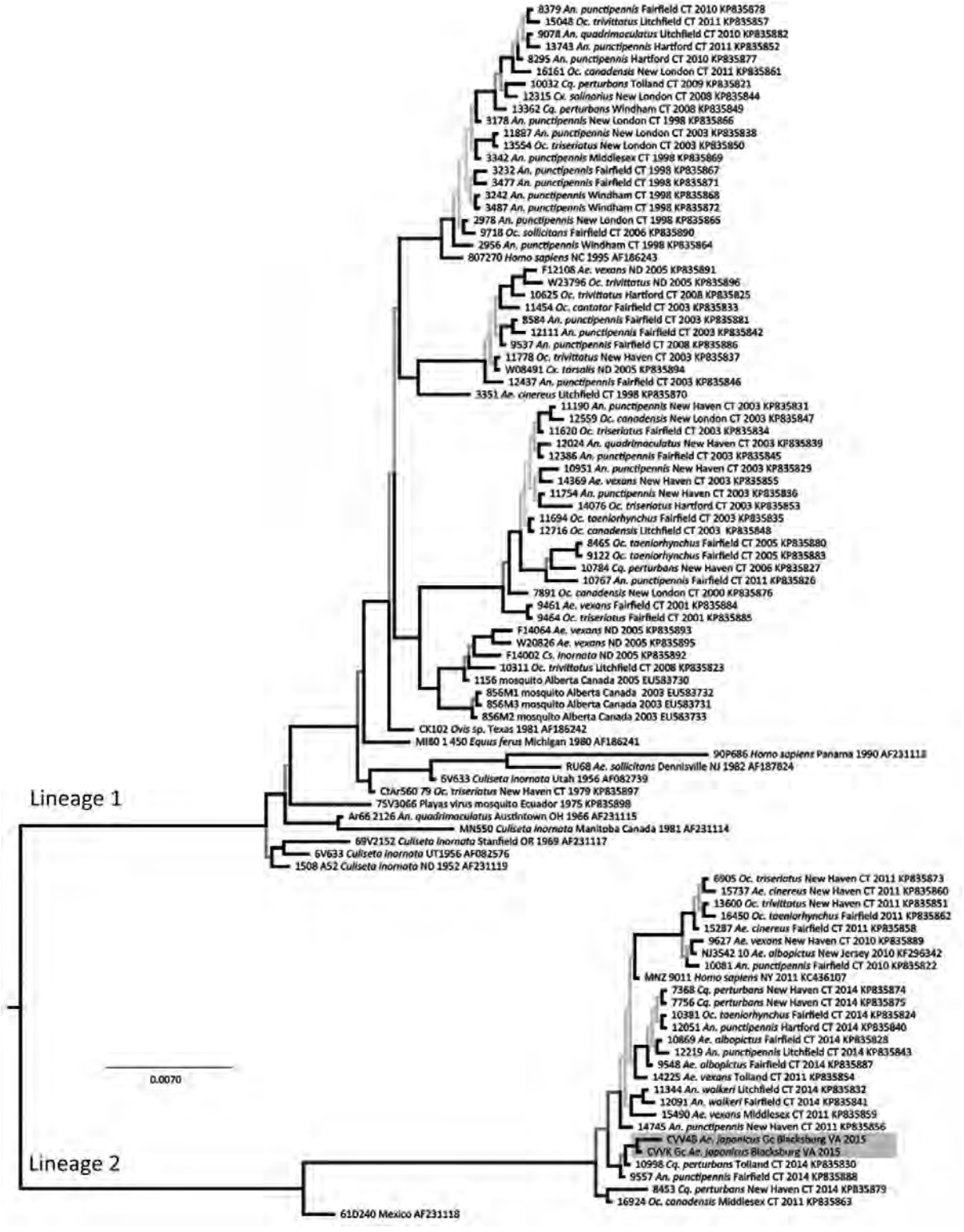

Figure 2. Phylogeny of Cache Valley virus (CVV) isolates in mosquitoes collected in Blacksburg, Virginia, USA (GenBank accession nos. KX583998 and KX583999), and reference isolates. The tree was inferred from the medium RNA segment of the virus polyprotein gene and estimated by using mixed model partitioned Bayesian analysis. State, year, host, and GenBank accession number are listed for each isolate. Historical lineages (1 and 2) of CVV are indicated. Shading in lineage 2 indicates strains isolated in this study. The closely related Fort Sherman virus from Panama (accession no. AF234767) is not included. Scale bar indicates expected nucleotide substitutions per site. *Ae., Aedes*; *An*., *Anopheles*; *Cq*., *Coquillettidia*; *Cx*., *Culex*; *Oc*., *Ochlerotatus*.

Table 2. Rates of midgut infection, dissemination, and oral transmission of Cache Valley virus by *Aedes japonicus japonicus* mosquitoes after a 2-week extrinsic incubation, Appalachian Region, United States, 2015*

Replicate	Infectious blood meal titer, log_{10} PFU/mL	No. tested	Nondisseminated infections, %	Disseminated infections, %	Transmission, %
1	1.2×10^6	18	44	39	33
2	1.6×10^5	26	42	42	27
3	4.6×10^6	30	37	33	27
Total	Not applicable	74	41	38	28

*Virus recovered from the midgut only was classified as a nondisseminated infection. Mosquitoes with virus-positive legs and wings were considered to have a disseminated infection. If virus was detected in salivary expectorate, it was classified as transmitting.

significant differences among the 3 replicates for infection or transmission rates ($p>0.05$ by χ^2 test).

Conclusions

Ae. j. japonicus mosquitoes are an invasive species that has spread throughout most of the eastern United States and are a competent vector of several endemic viruses (*12*). Although CVV was previously isolated from *Ae. j. japonicus* mosquitoes in the northeastern United States (*4*,*13*), we report isolation of CVV from this species in Appalachia and show that it is a competent vector of the virus. In the laboratory, vector competence of *Ae. j. japonicus* mosquitoes was equivalent to that for other species believed to be part of the CVV transmission cycle. For example, transmission rates for *An. quadrimaculatus* mosquitoes ranged from 20% to 33% after imbibing infectious blood meals with virus titers similar to those used in our study (*1*).

Ae. j. japonicus mosquitoes readily feed on humans and large animals, such as white-tailed deer (*12*). Consequently, this species probably contributes to local transmission of CVV. The study site is in close proximity to humans and pastured sheep and is frequented by deer (Figure 1). Therefore, all components for establishment of a focus of CVV are present. If *Ae. j. japonicus* mosquitoes are capable of transovarial transmission, as is the case with La Crosse virus, another bunyavirus (*14*), these mosquitoes could then contribute to concentrating the virus within this limited geographic area. Emergence of La Crosse virus in the Appalachian region of the United States has been associated with invasions by *Ae. j. japonicus* and *Ae. albopictus* mosquitoes (*15*). Thus, additional studies are needed to determine the role of *Ae. j. japonicus* mosquitoes in the transmission, maintenance, and presence of CVV.

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Seroprevalence of Dengue and Chikungunya Virus Antibodies, French Polynesia, 2014–2015

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We investigated dengue and chikungunya virus antibody seroprevalence in French Polynesia during 2014–2015. Dengue virus seroprevalence was ≈60% among schoolchildren and >83% among the general population; chikungunya virus seroprevalence was <3% before and 76% after Zika virus emergence (2013). Dengue virus herd immunity may affect Zika virus infection and pathogenesis.

In French Polynesia, the only recognized actively circulating arboviruses were the 4 dengue viruses (DENV; family *Flaviviridae,* genus *Flavivirus*) (*1*,*2*) until Zika virus (family *Flaviviridae,* genus *Flavivirus*) emerged there in 2013 (*3*), followed by chikungunya virus (family *Togaviridae*, genus *Alphavirus*) in 2014 (*4*). Serosurveys conducted among blood donors in French Polynesia during 2011–2013, before these outbreaks, confirmed the absence of Zika and chikungunya virus circulation and assessed DENV antibody seroprevalence at ≈80% at that time (*5*,*6*). Another study conducted after the emergence of Zika virus showed Zika virus antibody seroprevalence rates ± 95% CIs to be 49% ± 7% among the general population and 66% ± 5% among schoolchildren in 2014 and 22% ± 6% among the general population in 2015 (*7*). We report seroprevalence of antibodies against the 4 DENVs and chikungunya virus in French Polynesia in 2014–2015 and discuss the possible role of anti-DENV herd immunity on Zika virus infection and pathogenesis.

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The Study

To assess antibody seroprevalence against DENV types 1–4 and chikungunya virus in the population of French Polynesia, we conducted 3 cluster samplings (*7*). During February–March 2014, we sampled 196 participants from the general population of the 8 most inhabited islands of the 5 French Polynesia archipelagos: Tahiti and Moorea (Society), Rangiroa and Makemo (Tuamotu), Nuku Hiva and Hiva Oa (Marquesas), Rurutu (Australs), and Rikitea (Gambier). To better estimate seroprevalence of antibodies against these viruses among children, during May–June 2014, we recruited 476 schoolchildren from primary and high schools on the most populous island (Tahiti). To increase accuracy of seroprevalence data for DENVs and assess postoutbreak chikungunya virus seroprevalence, during September–November 2015, we sampled 700 members of the general population from the most inhabited archipelago (Society). All participants were asked whether they had had symptoms suggestive of past dengue disease. Because chikungunya virus emerged in French Polynesia in late 2014, symptoms suggestive of past disease were recorded only from participants sampled in 2015. The study was conducted in accordance with the French Polynesia Ethics Committee (agreement 60/CEPF_06/27/2013).

We performed detection of DENV and chikungunya virus IgG on blood samples collected in 2014 by using a recombinant-antigen–based indirect ELISA (*5*,*6*) and tested samples collected in 2015 by microsphere

immunoassay (MIA) (*8*) using the same recombinant antigens as for the ELISA. Among the samples collected in 2015, we selected 20 to be a representative panel of the different antibody profiles found by MIA and tested them for neutralizing antibodies against each dengue, chikungunya, and Zika virus (*8*). We analyzed data by using GraphPad Prism version 6.03 software (https://www.graphpad.com/) and the Fisher exact test. We set significance at $p<0.05$.

Overall seropositivity rates for antibodies against ≥1 DENV were 96% ± 3% among the general population and 60% ± 5% among schoolchildren in 2014 and 83% ± 3% among the general population in 2015 (Table 1). Seroprevalence of DENV antibodies did not differ significantly between archipelagos, except for DENV-3, which differed between Society (76% ± 18%) and Austral-Gambier (53% ± 15%) Islands ($p = 0.034$). In all 3 groups of participants, we found the highest seropositivity rates for DENV-1 antibodies and the lowest for DENV-2. Seropositivity rates in 2014 and 2015 for all DENV antibodies were significantly lower for schoolchildren (median age 11 years) than for the general population (median age 41 years in 2014 and 43 years in 2015) (all $p<0.0001$). Seroprevalence of chikungunya virus antibodies was 3% ± 3% among the general population and 1% ± 1% among schoolchildren in 2014 and 76% ± 5% among the general population in 2015 after the outbreak.

According to information provided by a questionnaire, the percentages of asymptomatic DENV infections were 65% ± 7% among the general population and 81% ± 5% among schoolchildren in 2014 and 51% ± 4% among the general population in 2015. The percentage of asymptomatic chikungunya virus infections in participants recruited in 2015 was 13% ± 2%. Among the 20 samples positive by MIA for IgG against DENV-1–4 and chikungunya virus in this study and against Zika virus in the previous serosurvey (*7*), the proportions showing neutralizing activity were 8/9 (89%) for DENV-1, 5/5 (100%) for DENV-2, 6/6 (100%) for DENV-3, 6/6 (100%) for DENV-4, and 7/8 (88%) each for chikungunya and Zika viruses (Table 2).

Conclusions

The finding of DENV IgG in >80% of the general population sampled in 2014 and 2015 corroborates past high transmission rates for these viruses in French Polynesia (*1*,*2*,*6*,*9*). The lower seropositivity rates for antibodies against DENVs among children is consistent with seroprevalence rates for antibodies against viruses endemic to the region, which are expected to increase with duration of exposure (*6*). Finding the highest seroprevalence rate against DENV-1 in the general population and schoolchildren is consistent with the long-term circulation of this virus in French Polynesia during 2001–2009 and since 2013 (*1*,*9*). DENV-2 has not circulated in French Polynesia since 2000 (*9*), which is consistent with the lowest seroprevalence rate against this virus. The reduced population immunity against DENV-2 coupled with the circulation of this virus in neighboring Pacific Islands and detection of imported infections in travelers entering French Polynesia from Vanuatu in February 2017 (*9*) indicates that French Polynesia is at risk for a new epidemic. Although seroprevalence rates for antibodies against DENVs are consistent with epidemiologic data, bias associated with the presence of cross-reactive antibodies produced during a previous flavivirus infection could have resulted in detection of antibodies against viruses to which the persons were never exposed. The proportion of asymptomatic DENV infections assessed in this study seems to be consistent with reported rates (*10*). However, because the questionnaire asked about clinical manifestations suggestive of past DENV infection over a lifetime or during time spent in French Polynesia, the estimates may be strongly limited by recall bias.

The low seroprevalence rates for chikungunya virus antibodies for the general population (3% ± 3%) and

Table 1. Seropositivity for antibodies against dengue and chikungunya viruses among participants randomly recruited, French Polynesia, 2014–2015*

	Seropositivity, no. (% ± 95% CI)						
	General population, Feb–Mar 2014					Schoolchildren, May–Jun 2014, Society, n = 476	General population, Sep–Nov 2015, Society, n = 700
Virus	Society, n = 49	Tuamotu, n = 49	Marquesas, n = 49	Austral – Gambier, n = 49	Total, n = 196		
Dengue							
≥1 type	46 (94 ± 8)	49 (100 ± 0)	46 (94 ± 8)	47 (96 ± 6)	188 (96 ± 3)	285 (60 ± 5)	582 (83 ± 3)
Type 1	42 (86 ± 16)	47 (96 ± 5)	41 (84 ± 13)	42 (86 ± 11)	172 (88 ± 6)	239 (50 ± 5)	562 (80 ± 4)
Type 2	23 (47 ± 17)	22 (45 ± 13)	26 (53 ± 12)	28 (57 ± 12)	99 (51 ± 7)	0	127 (18 ± 4)
Type 3	37 (76 ± 18)	33 (67 ± 21)	35 (71 ± 14)	26 (53 ± 15)	131 (67 ± 9)	72 (15 ± 3)	384 (55 ± 4)
Type 4	31 (63 ± 8)	32 (65 ± 21)	29 (59 ± 15)	27 (55 ± 15)	119 (61 ± 8)	69 (14 ± 3)	293 (42 ± 7)
Asymptomatic	36/46 (78 ± 12)	29/49 (59 ± 14)	24/46 (52 ± 14)	34/47 (72 ± 13)	123/188 (65 ± 7)	230/285 (81 ± 5)	299/582 (51 ± 4)
Chikungunya	3 (6 ± 8)	1 (2 ± 4)	1 (2 ± 4)	1 (2 ± 4)	6 (3 ± 3)	2 (1 ± 1)	529 (76 ± 5)
Asymptomatic	ND	ND	ND	ND	ND	ND	70/529 (13 ± 2)

*95% CIs were calculated by taking into account the cluster sampling design and using the Fisher exact test. Median ages of participants among the general population, 38–47 y; of schoolchildren, 11 y. ND, not determined.

Table 2. Results of microsphere immunoassay and neutralization assay for dengue, chikungunya, and Zika viruses for 20 participants sampled from the Society archipelago, French Polynesia, September–November, 2015*

	Years in	Virus											
	French	Dengue 1		Dengue 2		Dengue 3		Dengue 4		Chikungunya		Zika‡	
Participant ID	Polynesia	MIA	NTA†	MIA	NTA	MIA	NTA	MIA	NTA	MIA	NTA	MIA‡	NTA
Pueu-6	1	–	<10	–	<10	–	<10	–	<10	+	80	–	<10
Papeete-62	2	–	<10	–	<10	–	<10	–	<10	–	<10	–	10
Papeete-63	2	–	<10	–	<10	–	<10	–	<10	–	<10	–	40
Papeete-35	6	–	<10	–	<10	–	<10	–	<10	–	<10	–	20
Punaauia-96	6	–	<10	–	<10	–	<10	–	<10	–	<10	–	10
Pirae-6	10	–	<10	–	<10	–	<10	–	80	–	<10	+	160
Mahina-26	13	–	<10	–	<10	–	<10	–	<10	+	320	+	160
Punaauia-61	15	–	640	–	<10	–	<10	–	<10	–	<10	–	<10
Faaa-54	17	+	10	–	<10	–	<10	–	<10	+	160	+	>1,280
Toahotu-1	18	–	<10	–	<10	–	<10	+	80	–	<10	–	<10
Punaauia-93	21	–	<10	–	640	–	<10	–	<10	–	<10	–	20
Punaauia-36	24	–	40	–	640	–	40	–	80	+	160	–	20
Papenoo-10	28	+	640	+	>1,280	+	>1,280	+	640	–	<10	–	<10
Moorea-59	36	+	320	–	<10	+	>1,280	+	>1,280	–	<10	+	640
Paea-48	38	+	160	–	<10	–	80	–	80	–	<10	+	160
Papeari-17	44	+	320	+	>1,280	+	80	+	160	+	20	+	320
Papenoo-1	49	+	160	+	>1,280	+	20	+	80	+	640	+	<10
Faaa-22	54	+	80	+	640	–	320	–	160	+	40	–	<10
Papeete-10	55	+	320	+	>1,280	+	80	+	640	+	<10	+	160
Afaahiti-7	67	+	80	–	640	+	320	–	160	–	<10	–	<10

*ID, identification; MIA, microsphere immunoassay; NTA, neutralization assay; +, positive; –, negative.
†Neutralization activity was considered positive for serum samples with a 50% neutralization antibody titer ≥20.
‡Results from Aubry et al. (7).

schoolchildren (1% ± 1%) in 2014 corroborate previous findings for blood donors sampled during 2011–2013 (3%) (*5*), suggesting that this virus did not actively circulate in French Polynesia before 2014. The seroprevalence rate for the general population in 2015 (76% ± 5%) was higher than the initial estimate of 25% of chikungunya virus infections, which was based on the number of patients who sought medical care during the outbreak (*5*). The rate of asymptomatic chikungunya virus infections identified in this study (13% ± 2%) was comparable to rates reported by other countries (*10*).

Chikungunya virus antibody seroprevalence was much higher (76% ± 5%) than Zika virus antibody seroprevalence for the general population in 2015 (22% ± 6%) (*7*), despite the fact that both were virgin soil outbreaks occurring at the same place during 2 consecutive years (*5*,*6*). This discrepancy could be the result of distinct vector competence for Zika and chikungunya viruses in French Polynesia, as demonstrated in local mosquito species (*11*,*12*). Another possible explanation is that past exposure of the population to dengue viruses, as corroborated by the high level of anti-DENV neutralizing responses detected in samples collected in 2015, may have provided cross-protection against Zika virus infection (*13*). However, the occurrence of many cases of Guillain-Barré syndrome and Zika virus infection–associated congenital abnormalities (*8*,*14*) might also suggest that Zika virus immunopathogenesis is enhanced in the setting of high seroprevalence of DENV antibodies (*15*).

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Molecular and Epidemiologic Analysis of Reemergent *Salmonella enterica* Serovar Napoli, Italy, 2011–2015

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Human infections with *Salmonella enterica* serovar Napoli are uncommon in Europe. However, these infections represented 5.9% of salmonellosis cases in Italy during 2014–2015. The source of infection is unknown. We analyzed surveillance data and compared strain genetic similarities and found that contaminated vegetables and surface water are probable sources of human infection.

Salmonella enterica serovar Napoli has attracted interest since 1982, when chocolate contaminated with this serovar and imported from Italy caused 2 foodborne outbreaks (*1*,*2*). During 2000–2006, a 140% increase in *Salmonella* Napoli cases was reported in Europe (*3*), most (87%) in France, Italy and Switzerland. Since 2004, the Rapid Alert System for Food and Feed (https://webgate.ec.europa.eu/rasff-window/portal/) has received 8 reports of *Salmonella* Napoli infection, which involved 6 countries and vegetables from Italy as the source of infection.

In Italy, sporadic cases of infection with *Salmonella* Napoli were reported before and after the first outbreak was reported in 1984 (*4*). The number of cases increased by 28% during 2000–2011 (*5*). Recently, *Salmonella* Napoli has caused waterborne (*6*) and foodborne (*7*) outbreaks. Although studies in other countries (*3*) and Italy (*8*–*10*) investigated the mode of infection transmission, the zoonotic reservoir is still unknown.

To update epidemiologic trends for *Salmonella* Napoli in Italy, we identified the population at greatest risk for infection and defined putative vehicles of infection, which could assist possible control measures. We also analyzed 2011–2015 surveillance data for human, animal, environmental, food, and feed *Salmonella* Napoli isolates; evaluated associated factors; and compared genetic profiles.

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The Study

We extracted data on February 16, 2017, from the passive, voluntary, laboratory-based, surveillance system Enter-Net (http://www.iss.it/ente) for *Salmonella* spp. isolates from 2011–2015; patient identity was anonymous. Enter-Net is coordinated by the Istituto Superiore di Sanità (Rome, Italy) and contains information on strains from human and environmental samples. We excluded samples from nonhuman and nonenvironmental sources (n = 43), unspecified sample types (n = 21), and undefined *Salmonella* serovars (n = 1,066) from analysis. We obtained data (aggregated for 2011–2012 and 2013–2014) for *Salmonella* spp. isolated from food, animals, and feed by using the passive and voluntary veterinary surveillance system Enter-Vet (http://www.izsvenezie.it/temi/malattie-patogeni/salmonella/enter-vet/).

We evaluated potential epidemiologic risks associated with human and environmental *Salmonella* Napoli isolates and the 5 most frequent nontyphoidal *Salmonella* (NTS) serovars other than Napoli (monophasic variant of Typhimurium, Typhimurium, Derby, Enteritidis, and Infantis) by using simple 2-way frequency tables. Univariate analysis ($p<0.05$ by χ^2 test) was based on comparing categories for each variable and excluding missing values. We developed a multiple logistic regression model (Stata version 12; StataCorp LLC, College Station, TX, USA) to evaluate potential confounding among variables. We also used data for patients hospitalized with *Salmonella* Napoli infections or bacteremia as outcomes adjusted for other factors. The top 5 other NTS represented 73% of human *Salmonella* serovars isolated during 2011–2015. Results were consistent when we compared *Salmonella* Napoli with all reported NTS.

We serotyped *Salmonella* spp. isolates by using the White–Kauffmann–Le Minor scheme (*11*) and evaluated genetic relatedness among *Salmonella* Napoli isolates by using pulsed-field gel electrophoresis according to the PulseNet protocol (https://www.cdc.gov/pulsenet/pathogens/protocols.html). We performed dendrogram and cluster analyses by using BioNumerics 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). We scored similarity between chromosomal fingerprints by using the Dice coefficient and used the unweighted pair-group method with arithmetic means, 1.00% tolerance limit, 1.00% optimization, and coefficient of similarity ≥80% to define clonal relationships between strains.

The monophasic variant of Typhimurium (37.3%), Typhimurium (21.6%), Enteritidis (9.3%), Napoli (4.7%), Derby (3.1%), and Infantis (1.9%) were the most frequent of 331 *Salmonella* serovars identified for 21,486 human records. The proportion of *Salmonella* Napoli cases increased from 4.3% in 2011 to 5.8% in 2015 (p<0.001). *Salmonella* Napoli infection was more common in men, infants, children, and the elderly in northern and southern Italy during July–September than other reported serovars (Table 1). For *Salmonella* Napoli–infected patients with recent travel histories, those who traveled within Italy were more likely infected with this serovar (adjusted odds ratio 7.82, 95% CI 1.00–61.25; p = 0.050) than patients who traveled abroad. Although patients infected with *Salmonella* Napoli were hospitalized at rates similar to those infected with other serovars (Table 1), they had a greater risk for development of bacteremia (adjusted odds ratio 4.25, 95% CI 3.07–5.88; p<0.001).

Among nonhuman isolates, 15,579 *Salmonella* spp. were reported (7,563 animal, 5,496 food, 2,078 environmental, and 442 feed). These isolates included 187 *Salmonella* Napoli (89 animal, 76 environmental, 21 food, and 1 feed).

Salmonella Napoli ranked fifth (3.7%) among environmental *Salmonella* spp. and showed a significant increase from 1.6% in 2011 to 2.9% in 2015 (p<0.005). A total of 85% of Napoli isolates were from surface water. Multivariate analysis (Table 2) confirmed that Napoli strains were more commonly isolated during April–September (p<0.001) and less commonly in northern and southern Italy than in central Italy.

Among *Salmonella* spp. animal isolates, the frequency of *Salmonella* Napoli increased from 0.7% in 2011 to 1.3% in 2015. A total of 50% of Napoli strains were obtained in the wild, most (70%) from wild boars in northern Italy. The low proportion (0.4%) from food remained stable during 2011–2015. Of 21 Napoli samples, 7 were from shellfish. One alfalfa sample contaminated with Napoli was reported in southern Italy in 2014.

We performed pulsed-field gel electrophoresis for 182 *Salmonella* Napoli strains isolated from different sources. We detected 121 unique profiles; 32 identical profiles contained 2–13 isolates. Despite high genetic variability among strains, we identified 4 main clusters that had genetic homologies ≥80% and matched the 3 areas in Italy: cluster A in northern Italy, cluster B in central Italy, and clusters C and D in southern Italy (p<0.0001) (Figure). Most human and environmental isolates from northern Italy belonged to cluster A, and most in central Italy belonged to cluster B (p<0.0001). In

Table 1. Characteristics of patients infected with *Salmonella enterica* serovar Napoli and patients infected with the 5 most common other nontyphoidal serovars, Italy, 2011–2015*

Characteristic	Serovar Napoli, no. (%)	Other serovars, no. (%)	Total	p value	aOR (95% CI)
Sex					
M	525 (5.2)	7,470 (73.8)	10,119	0.001	1.17 (1.02–1.35)
F	383 (4.3)	6,521 (73.5)	8,876		1
Unknown	111 (4.5)	1,757 (70.5)	2,491		0.94 (0.75–1.18)
Age, y					
<1	69 (8.8)	491 (62.6)	784	<0.001	3.05 (2.22–4.18)
1–14	584 (5.6)	8,378 (80.2)	10,442		1.57 (1.28–1.92)
15–64	127 (3.1)	2,667 (65.6)	4,063		1
≥65	195 (5.0)	2,447 (63.3)	3,863		1.76 (1.39–2.22)
Unknown	44 (1.9)	1,765 (75.6)	2,334		0.65 (0.45–0.92)
Geographic area					
North	893 (5.1)	12,708 (73.0)	17,397	<0.001	1.77 (1.43–2.20)
Central	102 (3.0)	2,540 (74.1)	3,428		1
South	24 (3.6)	500 (86.4)	661		1.63 (1.02–2.61)
Time of sampling					
Jan–Mar	12 (0.3)	3,623 (78.3)	4,626	<0.001	1
Apr–Jun	203 (4.4)	3,356 (73.2)	4,584		17.98 (10.02–32.26)
Jul–Sep	594 (8.8)	4,622 (68.5)	6,752		38.00 (21.41–67.44)
Oct–Dec	210 (3.8)	4,147 (75.1)	5,524		15.15 (8.45–27.17)
Period of sampling					
2011–2013	643 (4.3)	11,152 (73.9)	15,081	<0.001	1
2014–2015	376 (5.9)	4,596 (71.8)	6,405		1.4 (1.22–1.61)
Hospitalization					
Yes	368 (4.9)	5,453 (72.4)	7,533	0.583	ND
No	358 (4.9)	5,533 (75.1)	7,367		ND
Unknown	293 (4.4)	4,762 (72.3)	6,586		ND
Bacteremia					
Yes	962 (4.6)	15,527 (74.1)	20,962	<0.001	ND
No	57 (10.9)	221 (42.2)	524		ND
Unknown	6 (4.9)	75 (61.0)	123		ND
Total	1,019 (4.7)	15,748 (73.3)	21,486	ND	ND

*The 5 most common other serovars were the monophasic variant of Typhimurium, Typhimurium, Derby, Enteritidis, and Infantis. Hospitalization and bacteremia were not included in evaluation of risk factors because they were considered a consequence of the infection, rather than risk factors. aOR, adjusted odds ratio; ND, not determined.

Table 2. Characteristics of environmental samples contaminated with *Salmonella enterica* serovar Napoli and samples with the 5 most common other nontyphoidal serovars, Italy 2011–2015*

Characteristic	Serovar Napoli, no. (%)	Other serovars, no. (%)	Total	p value	aOR (95% CI)
Geographic area					
Northern	37 (2.5)	531 (35.6)	1,493	<0.001	0.13 (0.06–0.26)
Central	16 (11.2)	38 (26.6)	143		1
Southern	23 (5.2)	151 (34.2)	442	0.001	0.25 (0.11–0.57)
Time of sampling					
Jan–Mar	4 (1.0)	200 (49.8)	402		1
Apr–Jun	28 (4.9)	169 (29.8)	567	<0.001	9.45 (3.15–28.35)
Jul–Sep	30 (4.7)	153 (24.2)	632	<0.001	10.8 (3.62–32.25)
Oct–Dec	14 (2.9)	198 (41.5)	477	<0.05	3.83 (1.22–12.06)
Period of sampling					
2011–2013	43 (3.6)	411 (34.5)	1,193		1
2014–2015	33 (3.7)	309 (34.9)	885	0.066	0.59 (0.33–1.04)
Total	76 (100.0)	720 (100.0)	2,078	ND	ND

*The 5 most common other serovars were the monophasic variant of Typhimurium, Typhimurium, Derby, Enteritidis, and Infantis. aOR, adjusted odds ratio; ND, not determined.

southern Italy, all human strains isolated belonged to clusters A or D, and 90% of environmental strains belonged to cluster C (p<0.0001). The 4 isolates from vegetables in southern Italy belonged to clusters A, C, and D. Seven profiles with genetic homologies <80% were obtained from different sources in northern and southern Italy.

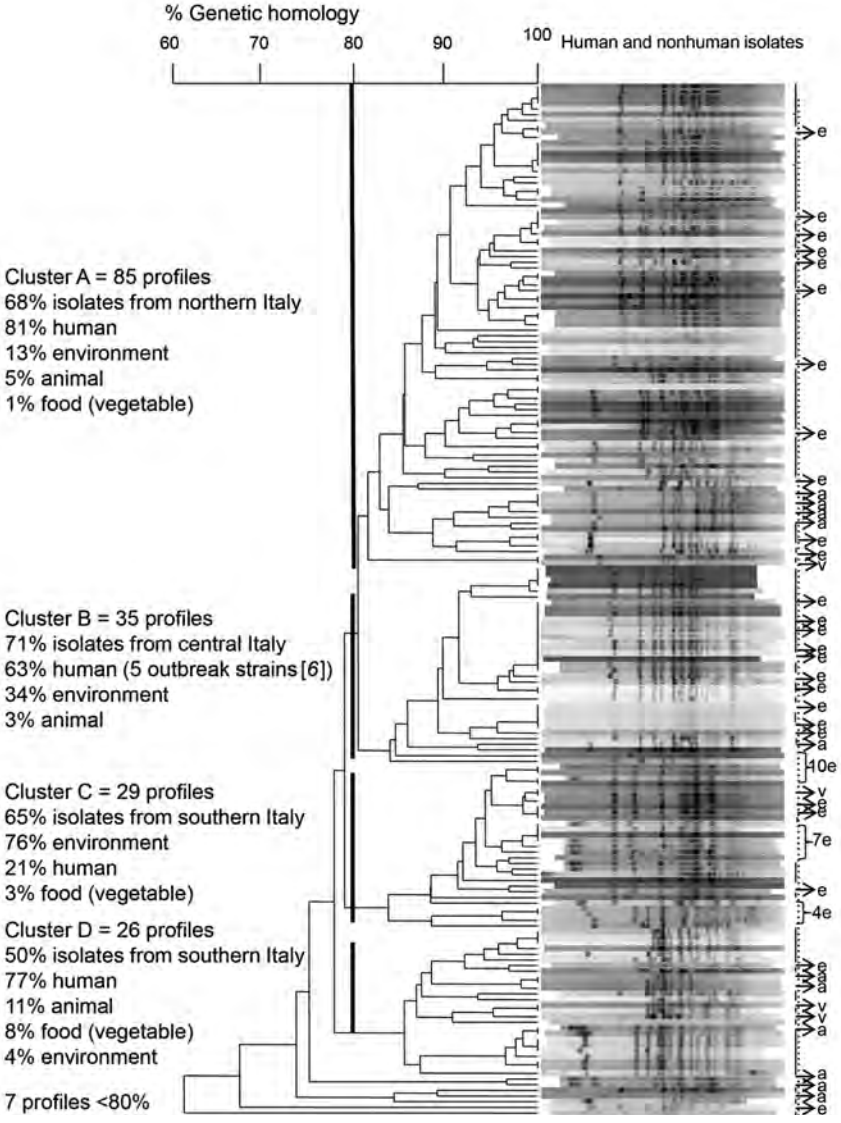

Figure. Dendrogram of 182 pulsed-field gel electrophoresis–based profiles of *Salmonella enterica* serovar Napoli strains isolated from human, environmental, animal, and food samples in Italy, 2011–2015. Four main clusters matched with the 3 main geographic areas in Italy (cluster A in northern Italy, cluster B in central Italy, and clusters C and D in southern Italy). Genetic analysis was based on 80% homology. Human strains (n = 124) are indicated by a solid vertical line. e indicates environmental strains (n = 46), a indicates animal strains (n = 8), and v indicates food (vegetable) strains (n = 4).

Conclusions

Outbreaks of *Salmonella* Napoli infection reemerged in Italy during 2011–2015. In Italy, *Salmonella* Napoli patients accounted for 5.9% of salmonellosis cases reported during 2014–2015. France had highest percentage of infections (1.2%) during this period (http://atlas.ecdc.europa.eu/public/index.aspx). Consistent with a study conducted in Italy (*9*), we found that surface water, including irrigative water, rather than animal food, was the main cause of infection with *Salmonella* Napoli. On the basis of an outbreak in Sweden (http://www.epinorth.org/eway/default.aspx?pid_230&trg_Area_5328&MainArea_5260_5328:0:&4148_5326:58&Area_5328_5273:46553::0:5326:574:::0:0), reports of the Rapid Alert System for Food and Feed on vegetables from Italy contaminated with *Salmonella* Napoli, and that the fact that ≈30% of irrigative surface water in Italy is used for vegetables (https://www.istat.it/it/files/2014/11/Utilizzo_risorsa_idrica.pdf), we believe that ready-to-eat vegetables irrigated with contaminated surface water are the main food vehicle for *Salmonella* Napoli.

Marked seasonality of cases matching environmental reports might be caused by increased irrigation and consumption of ready-to-eat vegetables during summer and fall, and more frequent human contact with contaminated surface water during summer and fall. We also reported frequent isolation of *Salmonella* Napoli from wild boars, which suggests a role for dissemination through the environment (*12*). Human and environmental strains of *Salmonella* Napoli belonging mainly to cluster A in northern Italy and cluster B in central Italy suggest direct infection. Different clusters for human and environmental strains from southern Italy might indicate wider spread in the environment.

Heterogeneous sampling of nonhuman isolates through Italy was a limitation of this study. Travel history within Italy, rather than abroad, of patients infected with *Salmonella* Napoli increases public health concern in Italy. Uncontrolled spread of *Salmonella* Napoli through the environment and associated foodborne and waterborne outbreaks, the increasing proportion of young patients, and high risks for development of bacteremia make infections with *Salmonella* Napoli a serious public health concern in Italy.

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Increase in Hospital Admissions for Severe Influenza A/B among Travelers on Cruise Ships to Alaska, 2015

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An increase in hospital admissions for influenza occurred during the summer of 2015 at an acute care facility in Vancouver, British Columbia, Canada. Investigation identified 25 patients with recent history of cruise ship travel to Alaska. All characterized influenza A viruses were A(H3N2). We describe patient treatment regimens and outcomes.

Disorders of the respiratory tract are the most common cause of medically attended illness (29%) among cruise ship passengers and crew (*1*). Influenza outbreaks associated with travel on cruise ships, including those sailing to Alaska in the summer months (*2*–*5*), have been described previously. The number of travelers on cruises to Alaska is large, with ≈900,000 documented in 2011 (*6*). We report an increase in hospital admissions for severe influenza among travelers on cruise ships to Alaska during the summer of 2015 at an acute care facility in Vancouver, British Columbia, Canada.

The Study

During the 2015 summer season (June–September), an increase in admissions for severe influenza was detected at our hospital, despite minimal influenza activity in the community (*7*,*8*). We determined that 25 cruise ship travelers to Alaska tested positive by PCR for influenza at hospital admission, 24 for influenza A and 1 for influenza B. We included all 25 of these patients in our study. We reviewed patient charts to determine date of illness onset, vaccination status, risk factors, treatment with antivirals or antibiotics, and patient outcomes (*9*). We sent influenza A–positive samples to the provincial public health reference laboratory for typing and performed gene sequencing on an additional subset to establish phylogenetic clade.

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The study group consisted of 11 women and 14 men (average age 72.5 years). Twenty-two travelers were from the United States, 2 from Canada, and 1 from the United Kingdom. Only 4 patients had receipt of the 2014–15 influenza vaccine documented in their medical chart. All patients had ≥1 risk factor for severe influenza, with an average of 2.5 recognized risk factors per patient. The most common were age ≥65 years (84%), cardiovascular disease (48%), and pulmonary disease (44%). Symptom onset occurred on average 4.1 days before admission. Eight patients were documented to have an earlier influenza diagnostic test performed on board the ship; 7 were positive, and 1 was negative. All 7 of these influenza-positive patients were started on oseltamivir before hospital admission. However, none of the patients who were negative or not tested for influenza on the cruise ship were documented to have been empirically treated with oseltamivir. Before admission, antibiotics were prescribed to 12 patients, with an average course lasting 2.2 days.

Admission blood culture results were negative for all 25 patients. Admission sputum cultures were collected from 17 patients: 1 showed heavy growth of methicillin-resistant *Staphylococcus aureus*, and the other 16 showed no growth or normal respiratory flora. During hospitalization, oseltamivir treatment was administered to 19 patients, with average duration (including discharge prescriptions) of 6.1 days. Twenty-two patients were empirically started on antibiotics at hospital admission. The average length of antibiotic treatment, including discharge prescriptions, was 7.4 days from the date of admission.

In-hospital complications included 1 case of *Pseudomonas aeruginosa* ventilator-associated pneumonia, 1 myocardial infarction, and 1 tracheostomy. No in-hospital deaths occurred. The average length of hospitalization was 7.1 days. Patients with influenza diagnosed on the cruise ship and started on oseltamivir (n = 7) had an average length of stay of 5.3 days versus 7.8 days for patients with influenza diagnosed in hospital only (n = 18), but this difference was not statistically significant (p = 0.42 by 2-tailed *t*-test).

All influenza A virus samples were sent to a reference laboratory for subtyping and were identified as H3N2. In addition, 16/24 isolates underwent gene sequencing and were identified as clade 3C.2a, the dominant genetic

and antigenic drift variant responsible for the 2014–15 influenza epidemic in the Northern Hemisphere. Clade 3C.2a viruses are distinguished from the 2014–15 A/Texas/50/2012 (clade 3C.1) vaccine strain by multiple (10–12) amino acid substitutions at antigenic sites of the surface hemagglutinin protein, notably F159Y and adjacent K160T mutations within immunodominant antigenic site B, the latter conferring a potential gain of glycosylation further relevant to antibody binding (*10*,*11*). The antigenic drift of clade 3C.2a viruses and their mismatch to the 2014–15 vaccine were widely recognized that season, with historically low vaccine effectiveness reported and recommendations issued for adjunct protective measures (*11*).

Conclusions

We present a review of 25 cases of laboratory-confirmed influenza illness requiring hospital admission during the summer of 2015 and associated with cruise ship travel to Alaska. The study was initiated in response to the perception by hospital staff of an atypical surge in admissions for severe influenza in summer 2015, with 27 of 33 influenza A–positive patients having illness associated with cruise ship travel, compared with 1 of 3 cases in summer 2014 and 8 of 19 cases in summer 2016.

The US Centers for Disease Control and Prevention (CDC) has published guidelines for the management of influenza-like illness (ILI) on cruise ships (*12*). For prevention, CDC recommends that crew and passengers receive the season's recommended influenza vaccination, postpone travel when ill, and comply with respiratory etiquette and that persons who are ill be appropriately isolated. In addition, CDC recommends antiviral treatment in cases of confirmed or suspected influenza in ILI patients with severe manifestations or risk factors for severe disease. Cruise ship passengers, particularly those at high risk for severe influenza complications, should be advised of these recommended measures before travel to mitigate their risk.

In our review, 7 patients had influenza A diagnosed on the cruise ship; 1 patient had tested negative for influenza A/B on the ship. Only patients who tested positive for influenza A on the cruise ship were documented to have been started on oseltamivir; however, a positive test is not required for initiation of treatment, as per CDC guidelines, especially in the context of a documented outbreak (*12*). Point-of-care influenza A/B antigen tests have poor sensitivity; therefore, in the appropriate clinical scenario, treatment should not be withheld on the basis of a negative test (*13*). In this small case series, patients who received treatment with oseltamivir before admission had a shorter duration of stay (Δ = 2 days), although this difference was not statistically significant. Definitive conclusions cannot be drawn from this observation, although it corresponds with a previous metaanalysis that noted a 21% decrease in the time to alleviation of symptoms in the oseltamivir treatment versus placebo group (*14*).

Our case series has several limitations. First, minimal follow-up information was available after discharge from hospital. Second, all of our patients had countries of origin in the Northern Hemisphere, but propagated viruses might have been introduced from elsewhere. Cruise ships to Alaska include travelers from the Southern Hemisphere, where influenza peaks during June–September. Third, we relied on the cruise ship medical records to provide information on treatment before admission. For many patients, this information was not provided, and if so, we assumed that no treatment or further diagnostics occurred. Finally, our review focused on a single facility; patients admitted to other facilities in the region would add to the total disease burden but were not captured in this study.

In summary, we report a series of severe influenza cases requiring hospitalization among cruise ship travelers to Alaska during the summer of 2015. Cruise ship passengers should be advised of such influenza risks and preventive measures before travel. Our findings reinforce the need for surveillance monitoring to inform timely initiation of antiviral treatment during cruise ship outbreaks. Clinicians caring for passengers with ILI should consider empiric influenza therapy, particularly because cruise ship travelers can include a large proportion of persons at risk of severe influenza complications.

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Drug Resistance of *Mycobacterium tuberculosis* Complex in a Rural Setting, Angola

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We found high prevalence rates of multidrug-resistant tuberculosis among retreatment patients (71.1%) and persons with new cases (8.0%) in Angola. These findings are of concern but should be interpreted with caution. A national drug-resistance survey is urgently needed to determine the actual prevalence of multidrug-resistant tuberculosis in Angola.

Angola is among the 30 countries with the highest incidence of tuberculosis (TB) and multidrug-resistant (MDR) TB worldwide (*1*). However, drug-resistance prevalence is unknown in the absence of a national survey or laboratory drug-resistance surveillance systems (*1*). The objectives of our study were to determine the proportion of TB drug resistance in isolates from pulmonary TB patients and describe molecular mechanisms accounting for drug resistance in these isolates.

The Study

We conducted a survey during April 2014–July 2015 at the Nossa Senhora da Paz Hospital (HNSP), a reference center for the diagnosis and treatment of TB in the town of Cubal, Benguela Province, Angola. Patients >16 years of age with a diagnosis of pulmonary TB (i.e., patients with clinical symptoms and a positive smear result) and those infected with HIV who had suggestive clinical signs of pulmonary TB but negative sputum samples for acid-fast bacilli were eligible for enrollment in the study. We collected data on age, sex, HIV status, and any previous TB treatment.

Before the start of treatment, we collected sputum specimens from all case-patients and provided them to the Mycobacteriology Unit (a World Health Organization Supranational TB Reference Laboratory) at Vall d'Hebron University Hospital in Barcelona, Spain, for culture and drug-susceptibility testing. Positive cultures were tested by using GenoType MTBDR*plus* 2.0 (Hain Lifescience GmbH, Nehren, Germany). Isolates identified as *Mycobacterium tuberculosis* complex (MTBC) underwent drug-susceptibility testing with BD-MGIT-960 SIRE and PZA kits (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Isolates that were resistant to ≥1 drug were subjected to drug-susceptibility testing for second-line TB drugs by using the BD-MGIT-960 SIRE system.

We performed statistical analysis by using Stata 12 (StataCorp LLC, College Station, TX, USA). We considered a p value <0.05 to be statistically significant. We calculated the percentage of patients with resistance patterns to first- and second-line TB drugs on the basis of total number of cases and the total number of MDR TB cases, respectively.

We included 422 cases; 44 were excluded because sputum specimen was not obtained (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/3/17-1562-Techapp1.pdf). Of these cases, we classified 311 as new and the remaining 111 as retreatment cases. We isolated MTBC in 225 of the new cases. We observed no difference in the distribution of age, sex, or HIV status between case-patients with suspected or confirmed TB disease among the new cases. We isolated MTBC in 83 of the retreatment cases. We found case-patients in whom MTBC was not isolated were more frequently HIV-positive (14.3% compared with 4.8% in whom TB was confirmed; p = 0.09). We observed no difference in sociodemographic characteristics between patients with new and retreatment culture-positive cases (online Technical Appendix Table 1).

Eighteen (8.0%) of the 225 MTBC isolates from new cases demonstrated multidrug resistance. Other combinations of drug resistance were identified in 40 (17.8%) of new cases. The incidence of primary resistance was as follows: isoniazid, 47 cases (20.9%); streptomycin, 25

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cases (11.1%); rifampin, 20 cases (8.9%); pyrazinamide, 13 cases, (5.8%); and ethambutol, 10 cases (4.4%) (Table 1). No isolates showed extensively drug-resistant TB (online Technical Appendix Table 2).

Among the 47 isoniazid-resistant isolates, *katG* mutations occurred in 26 (55.3%) and *inhA* mutations in 2 (4.3%); the remaining 19 isolates (40.4%) were classified as susceptible (Table 2). Among the 20 rifampin-resistant isolates, *rpoB* mutations occurred in 19 (95.0%), and 1 (5.0%) was classified as susceptible. Mutations detected included S531L (12 cases, 60.0%); D516V (4 cases, 20.0%); and H526Y (2 cases, 10.0%) (Table 2).

Fifty-nine (71.1%) of the 83 MTBC isolates from retreatment case-patients demonstrated multidrug resistance, and 33.9% of these case-patients had isolates that were resistant to all first-line drugs. Other combinations of drug resistance were identified in 10 case-patients (12.0%) (Table 1). No case-patients had extensively drug-resistant TB (online Technical Appendix Table 2).

Among the 66 isoniazid-resistant isolates, *katG* mutations occurred in 47 (71.2%) and *inhA* mutations in 4 (6.1%); the remaining 15 (22.7%) isolates were classified as susceptible (Table 2). Among the 61 rifampin-resistant isolates, *rpoB* mutations occurred in 58 (95.1%), and the remaining 3 (4.9%) were classified as susceptible. Mutations detected included S531L (37 cases, 60.7%), D516V (11 cases, 18.5%), and H526D (3 cases, 3.7%) (Table 2).

Conclusions

We found a high prevalence of MDR TB among retreatment (71.1%) and new (8.0%) cases. These rates are >4 times the estimated prevalence of MDR TB for Angola (21% for retreatment cases, 2.8% for new cases) (*1*). The rates we describe represent the highest rates of MDR TB reported in sub-Saharan Africa (*2*,*3*); not even South Africa has reported a higher prevalence of MDR TB (*4*).

Our findings are part of a larger project to reinforce the capacities of the diagnostic laboratory by incorporation of the Xpert MTB/RIF test (Cepheid, Maurens-Scopont, France) (*5*). At the beginning of the project, none of the 18 provinces in Angola had access to the test; moreover, Nossa Senhora da Paz Hospital is a reference center for the diagnosis and treatment of TB, and these 2 factors might have generated a pull effect in more severe cases. Patients in the study might have largely consisted of TB patients referred because of poor treatment response or availability of second-line treatment, thus overrepresenting patients with resistance patterns, particularly among retreatment patients. This suggestion is supported by the high proportion of retreatment patients in the eligible study population

Table 1. Resistance to first-line antituberculosis drugs among *Mycobacterium tuberculosis* complex isolates, Cubal, Angola, April 2014–July 2015*

	Isolates from new cases, n = 225		Isolates from retreatment cases, n = 83	
Phenotypic drug susceptibility	No.	% (95 CI)	No.	% (95 CI)
Susceptible to all 5 first-line drugs	167	74.2 (68.1–79.5)	14	16.9 (10.3–26.3)
Resistance to any drug	58	25.8 (20.5–31.9)	69	83.1 (73.7–89.7)
Any resistance to the following				
INH	47	20.9 (16.1–26.7)	66	79.5 (69.6–86.8)
RIF	20	8.9 (5.8–13.3)	61	73.5 (63.1–81.8)
STM	25	11.1 (7.6–15.9)	42	50.6 (40.1–61.1)
EMB	10	4.4 (2.4–8.0)	32	38.6 (28.8–49.3)
PZA	13	5.8 (3.4–9.6)	37	44.6 (34.4–55.3)
Overall monodrug resistance	31	13.8 (9.9–18.9)	7	8.4 (4.1–16.4)
INH only	21	9.3 (6.2–13.8)	4	4.8 (1.9–11.7)
RIF only	1	0.4 (0.1–2.5)	2	2.4 (0.7–8.4)
STM only	8	3.6 (1.8–6.9)	1	1.2 (0.2–6.5)
PZA only	1	0.4 (0.1–2.5)	0	0.0 (0.0–4.4)†
Overall multidrug resistance	18	8.0 (5.1–12.3)	59	71.1 (60.6–79.7)
INH + RIF	4	1.8 (0.7–4.5)	12	14.5 (8.5–23.6)
INH + RIF + STM	2	0.9 (0.2–3.2)	5	6.0 (2.6–13.3)
INH + RIF + EMB	0	0.0 (0.0–1.7)†	3	3.6 (1.2–10.1)
INH + RIF + PZA	2	0.9 (0.2–3.2)	2	2.4 (0.7–8.4)
INH + RIF + STM + EMB	3	1.3 (0.5–3.8)	4	4.8 (1.9–11.7)
INH + RIF + STM + PZA	1	0.4 (0.1–2.5)	9	10.8 (5.8–19.3)
INH + RIF + EMB + PZA	1	0.4 (0.1–2.5)	4	4.8 (1.9–11.7)
INH + RIF + STM + EMB + PZA	5	2.2 (1.0–5.1)	20	24.1 (16.2–34.3)
Overall polydrug resistance	9	4.0 (2.1–7.4)	3	3.6 (1.2–10.1)
INH + STM	5	2.2 (1.0–5.1)	0	0.0 (0.0–4.4)†
INH + EMB	1	0.4 (0.1–2.5)	0	0.0 (0.0–4.4)†
INH + PZA	2	0.9 (0.2–3.2)	0	0.0 (0.0–4.4)†
INH + STM + EMB	0	0.0 (0.0–1.7)†	1	1.2 (0.2–6.5)
INH + STM + PZA	0	0.0 (0.0–1.7)†	2	2.4 (0.7–8.4)
RIF + STM + PZA	1	0.4 (0.1–2.5)	0	0.0 (0.0–4.4)†

*EMB, ethambutol; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; STM, streptomycin.
†1-sided, 97.5% CI.

Table 2. Distribution of gene mutations associated with INH and RIF resistance, Cubal, Angola, April 2014–July 2015*

Phenotypic resistance	GenoType MTBDR*plus*			Isolates from new cases	Isolates from retreatment cases
	katG	*inhA*	*rpoB*		
INH, n = 113				47	66
	Δwt, S315T1	–		23	42
	Δwt	–		3	5
	–	Δwt1, C15T		2	2
	–	Δwt1		0	1
	–	C15T		0	1
	–	–		19	15
RIF, n = 81				20	61
			–	1	3
			Δwt2	0	2
			Δwt2,3,4, D516V	1	8
			Δwt3,4, D516V	3	3
			Δwt4,5	0	1
			Δwt7	0	1
			Δwt7, H526Y	2	0
			Δwt7, H526D	0	2
			Δwt7,8, H526D	0	1
			Δwt8	1	3
			Δwt8, S531L	12	37

*INH, isoniazid; RIF, rifampin; –, no mutation inside region.

(26.3% where the expected population proportion would be 10%–15%) and the extremely high prevalence of MDR TB in this group (in particular compared with new patients). Also, for new patients, such selection bias might have occurred, for example, because TB patients who were contacts of known or suspected MDR TB patients were preferentially referred to this facility.

Regarding associated mutations, previous studies have shown that ≈95% of resistance mutations to rifampin are associated with the *rpoB* gene mutations, which cluster mainly in the region of codon 507–533. In our study, the distribution of gene mutations among rifampin-resistant isolates was 60.4% Ser531Leu, 18.5% Asp516Val, 3.7% His526Asp, and 2.5% His526Tyr; in 9.9% of cases, the mutation was detected by the absence of the wild-type hybridization signal. This distribution is different from that previously reported, reflecting different distribution of gene mutations associated with rifampin resistance in different geographic locations (*6*) or different levels of maturation of the MDR TB epidemic. In areas with high MDR TB prevalence and a high proportion of MDR TB cases attributed to transmission, mutations that confer resistance without loss of reproductive fitness will be selected out (*7*,*8*).

Whereas 40%–95% of isoniazid-resistant isolates are defined as having high-level drug resistance because of *katG* gene mutations, 75%–90% of which are recognized as mutations in the 315 codon of the *katG* gene, in our study, 57.5% of isoniazid-resistant isolates were associated with mutations in the 315 codon of the *katG* gene. Approximately 8%–43% of isoniazid-resistant isolates are defined as having low-level drug resistance because of mutations in the promoter region of *inhA*. In our study, this proportion was 5.3%. Furthermore, 10%–25% of isoniazid-resistant isolates are thought to have mutations outside the *katG* and *inhA* loci (*9*–*11*).

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Statistical Method to Detect Tuberculosis Outbreaks among Endemic Clusters in a Low-Incidence Setting

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We previously reported use of genotype surveillance data to predict outbreaks among incident tuberculosis clusters. We propose a method to detect possible outbreaks among endemic tuberculosis clusters. We detected 15 possible outbreaks, of which 10 had epidemiologic data or whole-genome sequencing results. Eight outbreaks were corroborated.

We previously reported use of data from the National Tuberculosis Genotyping Service in the United States to predict outbreaks among incident clusters of tuberculosis (TB), defined as clusters in which the initial case was preceded by at least 24 months of no genotype-matched cases within a geographic area (*1*). This method cannot be applied to endemic clusters (i.e., reported since current TB genotype surveillance began in 2009 with at least 1 case every 24 months) because the initial case cannot be determined. These endemic clusters may be a combination of cases that are the consequence of reactivation of TB in persons who were previously infected and recent transmission of TB.

In this article, we postulate that a statistically driven method can determine the beginning of a TB outbreak in endemic clusters, referred to here as prevalent clusters. Our method searches for instances of excessive unexpected cluster growth above a background rate. We validated our approach by using a combination of epidemiologic data acquired during field investigations and whole-genome sequencing (WGS), which provides higher resolution of the bacterial genome than current genotyping methods (*2*,*3*). Our method systematically reviews data collected at the national level and local epidemiologic data when reported to the Centers for Disease Control and Prevention (CDC).

The Study

We used the US National Tuberculosis Surveillance System and the National Tuberculosis Genotyping Service datasets for 2009–2016 for this analysis (*4*). We defined prevalent clusters as having ≥1 TB case with a genotype-matched case also reported in that county during 2009–2010, and subsequent cases reported at least once every 24 months (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1613-Techapp1.pdf). Clusters were reviewed during 2011–2013 for cluster growth. Case counts were aggregated by 3-month time periods, or the first through fourth quarters of each calendar year. We fit negative binomial hurdle models to each consecutive group of eight quarter time intervals and calculated the 95th percentile of the resulting fit (online Technical Appendix). Unexpected growth in a prevalent cluster was defined as the earliest quarter where the number of TB cases exceeded the 95th percentile on the basis of fit to the previous 8 quarters (baseline period). For those prevalent clusters identified with unexpected growth, we defined a possible outbreak as a cluster that accrued ≥10 cases in excess of the quarterly average number of cases in the baseline period during the 3-year follow-up period after unexpected growth was first identified.

When available, we used epidemiologic data from onsite investigations by CDC scientists in conjunction with local TB programs and WGS results to refute or corroborate our classification of possible outbreaks. Studies of epidemiologically linked pairs have estimated *Mycobacterium tuberculosis* to accumulate ≈0.5 single-nucleotide polymorphism (SNP) differences per genome per year (or 1.5 SNPs per 3-year observation period) and found that divergence rarely exceeds 5 SNPs in 3 years between pairs (*5*,*6*). As a conservative estimate in this study, we defined isolates with 2 SNP differences within 3 years to indicate recent transmission (online Technical Appendix). We constructed a standard list to meet our definition of an outbreak, consisting of outbreaks investigated by CDC (*7*) and clusters with epidemiologic data and WGS results. Clusters with epidemiologic links or closely related WGS results (i.e., ≤2 SNP differences) among ≥50% of cases were corroborated as outbreaks.

Of 2,723 clusters determined during 2011–2016, a total of 706 clusters had ≥1 TB case in the initial baseline period (2009–2010). Among these 706 prevalent clusters, unexpected growth was identified in 174 (24.6%). Of these clusters, 15 accumulated ≥10 cases above the baseline average during the 3-year follow-up period after unexpected growth, meeting our definition of a possible outbreak. Of these clusters, 10 had WGS results and epidemiologic data, of which 8 met our definition of an outbreak, 1 was refuted

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on the basis of diverse WGS results, and 1 was marginal in meeting our definition. The remaining 5 clusters were indeterminate because neither WGS results nor epidemiologic data were available to CDC at the time of publication. When we excluded indeterminate clusters, we found that our methods had a positive predictive value of 80%.

Our standard list included 3 outbreaks that were not detected by our method. Two undetected outbreaks were initially reported during the baseline period (2009–2010) and grew quickly, setting a high starting baseline average. Although our method flagged these clusters for unexpected growth, they did not accumulate sufficient cases during the follow-up period to meet our threshold for excessive growth. Our method identified no unexpected growth in the remaining known outbreak.

We provide an epidemiologic curve (Figure 1) of the marginal cluster detected as a possible outbreak with WGS results and epidemiologic links reported. Our method identified unexpected growth in the second quarter of 2011, with 5 cases exceeding the 95th percentile of the hurdle model for the previous 8 quarters, calculating a baseline average of 1.25 cases per quarter. From this time point, we counted the number of cases that exceeded the baseline average: 3.75 in the second and third quarters of 2011, 1.75 in the second quarter of 2012, 0.75 in the fourth quarter of 2012, and 0.75 in the fourth quarter of 2013. The cluster accumulated 10.75 excess cases within 3 years of unexpected growth and met our criteria as a possible outbreak.

WGS results showed that, of the 20 isolates reported 3 years after unexpected growth, a closely related group of 9 isolates were within 2 SNPs of each other (Figure 2). Two additional isolates within the closely related group were outside the unexpected growth time window, and 2 other isolates, 1 reported during and 1 outside the time window, were within 3 SNPs of the closely related group.

Conclusions

This research continues our development of alerting clusters of public health concern (*8*–*10*). We describe a statistical method that accurately detected TB outbreaks among endemic clusters. Our method, based on routinely collected surveillance data, can be prospectively implemented to detect possible TB outbreaks. CDC plans to conduct universal WGS for all culture-confirmed TB case specimens, which would provide more precise molecular data for possible outbreaks. Our method will still be helpful in identifying when cluster growth exceeds an expected rate.

Genotype surveillance of TB cases is limited to culture-confirmed cases, which represent 78% of all cases (*11*). Therefore, we excluded non–culture-confirmed cases. In a similar manner, our validation was limited to epidemiologic data available to CDC. In addition, our approach searches for outbreaks within a single county, but TB transmission can cross county borders.

Our method for determining unexpected growth, based on the 95th percentile for a negative binomial hurdle model, serves only as an initial screening. Although our method can identify excessive unexpected growth, to confirm an outbreak requires epidemiologic investigation and increasingly relies on WGS results. Even with universal WGS, outbreak confirmation requires epidemiologic investigations to distinguish recent transmission from reactivation of remotely acquired TB (*12*).

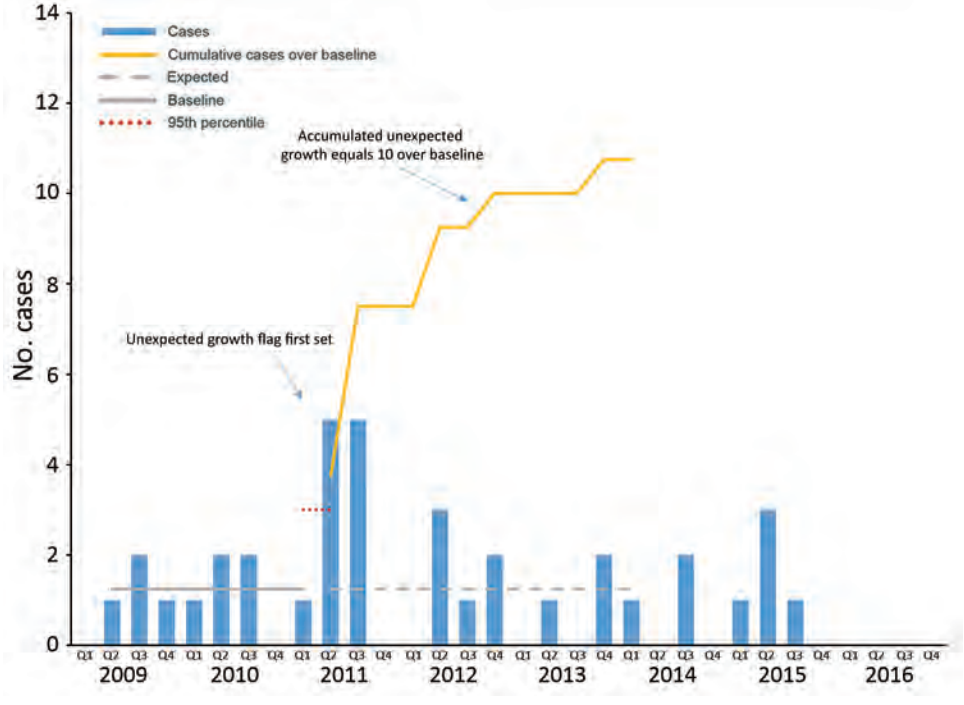

Figure 1. Epidemiologic curve showing a prevalent (endemic) outbreak of tuberculosis, by case counts per 3-month period, United States, 2009–2016. Q, quarter.

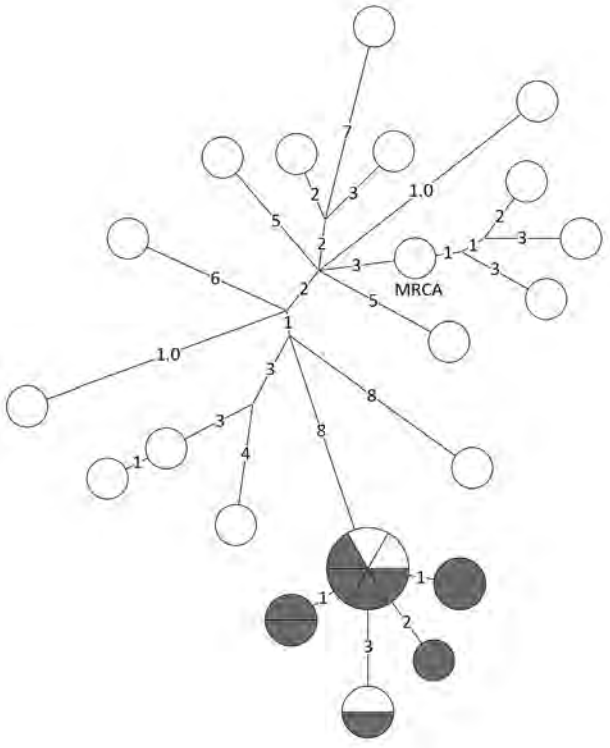

Figure 2. Whole-genome sequencing results for a prevalent (endemic) cluster detected as a possible tuberculosis outbreak, United States, 2009–2016. Values indicate number of SNPs. Shown is a closely related (≤2 SNPs) group of 11 isolates (lower section of phylogenetic tree). Isolates reported during a 3-year window of unexpected growth are indicated in gray. One isolate reported 1 quarter before and 1 isolate reported 1 quarter after the 3-year window of unexpected growth detection are indicated in white. An additional 2 isolates were 3 SNPs from this closely related group, 1 during (gray) and 1 outside (white) the unexpected growth window. MRCA, most recent common ancestor; SNP, single-nucleotide polymorphism.

Our methods provide an approach to detect possible outbreaks among prevalent clusters. We expect to incorporate these methods into CDC's existing surveillance system for large outbreaks of TB in the United States (*13*). We will explore additional approaches to evaluate initial cases of unexpected growth in all clusters, incident and prevalent, to develop an algorithm that can predict which clusters are most likely to become outbreaks.

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Global Health Estimate of Invasive *Mycobacterium chimaera* Infections Associated with Heater–Cooler Devices in Cardiac Surgery

Rami Sommerstein, Barbara Hasse, Jonas Marschall, Hugo Sax, Michele Genoni, Matthias Schlegel, Andreas F. Widmer, the Swiss Chimaera Taskforce[1]

Investigations of a worldwide epidemic of invasive *Mycobacterium chimaera* associated with heater–cooler devices in cardiac surgery have been hampered by low clinical awareness and challenging diagnoses. Using data from Switzerland, we estimated the burden of invasive *M. chimaera* to be 156–282 cases/year in 10 major cardiac valve replacement market countries.

Invasive *Mycobacterium chimaera* infection associated with heater–cooler devices (HCDs) in cardiac surgery was identified as a new disease entity in 2014 (*1*). The most likely pathogenesis involves aerosols transmitted from the HCD to the patient during surgery (*2*). As of September 2017, ≈120 cases have been recognized globally; mycobacterial device contamination at the manufacturing site of the LivaNova 3T (LivaNova, London, UK), the HCD market leader, seems to represent the most likely point source for the outbreak (*3*,*4*). The incubation period after exposure is long, with a median of 17 months (range 3–72 months). Signs and symptoms are generally nonspecific and include fatigue, fever, and weight loss. There is no established therapy, and the case-fatality rate is ≈50% (*3*,*5*). The disease appears to be rare and most commonly affects patients after valve replacement or other implant procedures in open heart surgery. Currently, the extent of the epidemic is unknown. We aimed to estimate the global epidemiology of disseminated *M. chimaera* disease associated with open heart surgery. The study followed the Guidelines for Accurate and Transparent Health Estimates Reporting (*6*).

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The Study

Case Definition

Switzerland was the leading country in recognizing and researching the global outbreak of *M. chimaera* disease associated with open heart surgery (*1*,*2*,*5*,*7*,*8*). This recognition included early establishment of a nationwide interdisciplinary expert group consisting of hospital epidemiologists, infectious disease physicians, cardiac surgeons, perfusionists, microbiologists, and consultants from the Swiss Agency for Therapeutic Products and the Federal Office of Public Health (collectively called the Swiss Chimaera Taskforce). This expert group has issued nationwide recommendations on case definition, lookback mechanisms, and infection prevention measures and releases updates on proven cases (*9*,*10*). We identified cases for our study through review of clinical and microbiological data, based on a previously published case series (*7*) and on reports to the Swiss Chimaera Taskforce. A proven case was defined as a case in a patient with previous open heart surgery, including implant surgery, and the subsequent detection of the *M. chimaera* outbreak strain from a sterile site.

Valve Replacements and HCDs in Use

Data on annual valve replacements performed during 2008–2016 were available from the Swiss National Registry for Cardiac Surgery. We report aggregated data for mitral and aortic valves. Currently active HCDs with the reporting date of January 15, 2015, were submitted via an electronic spreadsheet to the Swiss Chimaera Taskforce.

Statistical Analyses

We obtained data on demographic development from the Swiss Federal Office of Statistics (*11*). By applying a linear regression model, we determined estimates of the annual prevalence per 1,000 valve replacement procedures for index surgeries from 2008 (the presumed beginning of the outbreak) through 2014 (taking the latency of disease manifestation into account). Likewise, we calculated the yearly incidence of detected cases per 1,000,000 inhabitants from

[1]Additional members of the Swiss Chimaera Taskforce are listed at the end of this article.

2011 (the year of the first diagnosed case) through 2016. We determined best fit values and 95% CIs.

We identified 11 proven cases during January 2008–September 2017 (Figure, panel A). The number of annual valve replacement procedures increased from 1,632 in 2008 to 2,581 in 2016. During the same period, the population of Switzerland increased from 7,593,494 to 8,327,126 (Table). The annual estimated incidence of proven cases per 1 million inhabitants increased from 0.16 (95% CI 0–0.37) in 2011 to 0.25 (95% CI 0.05–0.45) in 2016 (p = 0.49). The prevalence per 1,000 valve replacement surgeries increased from 0.52 (95% CI 0–1.41) in 2008 to 0.94 (95% CI 0.05–1.83) in 2014 (p = 0.49; Figure, panel B). The LivaNova 3T was the predominant HCD, in use by 15 of 18 reporting cardiac surgery centers in Switzerland as of January 15, 2015, with 28 of 44 HCDs in use (64%). This rate is comparable with the worldwide LivaNova 3T market share of 70% (*3*).

The prevalence we found is similar to that in a previously published report of a relatively crude estimation (*12*) and about 4 to 7 times higher than a UK study based on national laboratory and hospital admissions data (*13*). The difference from the UK study can be explained by the active case detection strategy in Switzerland, compared with the laboratory investigation in the United Kingdom (*10*,*13*).

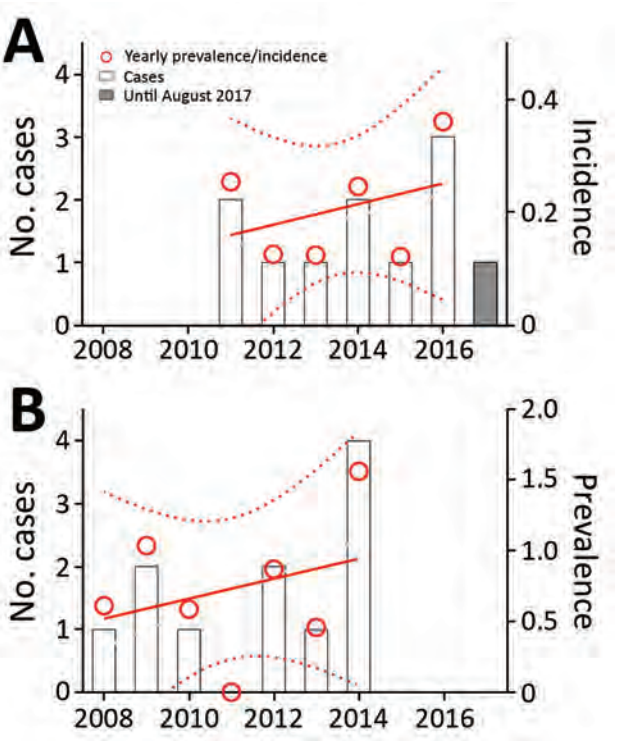

Figure. Incidence and prevalence of invasive *M. chimaera* cases in Switzerland since 2008. A) Yearly incidence per 1 million inhabitants according to the date of detection. B) Prevalence per 1,000 valve replacement surgeries according to the date of index surgery. Numbers of cases are projected on the left *y*-axis (bars); prevalence and incidence on the right *y*-axis (red circles). Gray bar indicates no. cases for January–August 2017. Linear regressions (red lines) were modeled for the respective window periods of interest; dotted lines indicate 95% CIs.

Conclusions

The risk for *M. chimaera* in patients undergoing heart surgery is similar to the risks for more widely known diseases such as parathyroid carcinoma (0.2/million/y), adrenal carcinoma (0.3/million/y), and congenital rubella syndrome (0.3/million/y) (*14*) that draw more attention but may have a smaller preventable proportion.

Considering an estimated 300,000 global annual valve replacement surgeries in the 10 major market countries and a US population of 323 million (*12*,*15*), we can extrapolate our findings to an annual incidence of 156–282 cases for the 10 major valve replacement markets and 51–80 cases in the United States alone. The currently known cases of invasive *M. chimaera* disease related to valve replacement reported from around the globe (n ≈120) are well below our estimate (*3*,*4*). Many countries in Europe did not report cases during the past few years, which is likely due to underreporting or underdiagnosing (*3*,*4*).

The study has limitations. Invasive *M. chimaera* is not a mandatory reportable disease in Switzerland. However, the small size of the country and the close collaboration of the Swiss Chimaera Taskforce with the professional societies, as well as the broad coverage of the outbreak in television and newspapers, decreases the likelihood of underreporting. In addition,

Table. Numbers of *Mycobacterium chimaera* cases, annual valve replacement procedures, and population in Switzerland, 2008–2017*

Year	Cases by date of index surgery	Cases by date of detection	No. aortic valves	No. mitral valves	Total no. valves	Total population
2008	1	0	1,291	341	1,632	7,593,494
2009	2	0	1,238	691	1,929	7,701,856
2010	1	0	1,306	398	1,704	7,785,806
2011	0	2	1,468	274	1,742	7,870,134
2012	2	1	2,032	273	2,305	7,954,662
2013	1	1	2,017	164	2,181	8,039,060
2014	4	2	2,120	441	2,561	8,139,631
2015	0	1	2,039	472	2,511	8,237,666
2016	0	3	2,112	469	2,581	8,327,126
2017	0	1	NA	NA	NA	NA

*NA, not available.

the Swiss National Center for Mycobacteria in Zurich receives most, if not all, strains for confirmation. As demonstrated previously, some patients may still be misinterpreted as having a rheumatologic disease (*7*), a potential reason for reporting bias. With valve replacement surgery as the denominator, we may overestimate the incidence/prevalence of invasive *M. chimaera*, as it is possible that some cases resulted from a procedure other than valve replacement surgery. All cases in Switzerland were associated with valve replacement surgery, however. Furthermore, many factors may influence incidence/prevalence on a global level, such as the predominant HCD brands in use, the degree of mycobacterial contamination of these HCDs, the built hospital environment, positioning in the operating room, and the number and type of cardiac surgery procedures performed. Infection after HCD exposure is likely to be unevenly distributed and may be quite high in 1 hospital but near zero in a nearby hospital, even though each hospital uses the implicated HCD. It is essential to investigate this variability and understand the relative contribution of some of these variables to the risk of infection. Nevertheless, it is likely that the aggregated risk derived from these factors is similar across national healthcare systems.

In summary, our data provide an estimate of the global burden of *M. chimaera* associated with open heart surgery, enabling policy makers to guide actions and to decrease the risk for transmission from HCDs. Our data suggest implementation of systematic lookback approaches in each country where LivaNova 3T HCDs have been used to optimize case finding. In addition, countries may consider mandatory reporting of invasive nontuberculous mycobacterial infections.

Additional members of the Swiss Chimaera Taskforce are Jolanda Consiglio, Samuel Erny, Céline Gardiol, Achim Häussler, Peter M. Keller, Daniel Koch, Virginie Masserey, Rafael Moreno, Bettina Schulthess, and Markus Wälti.

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Whole-Genome Analysis of *Mycobacterium tuberculosis* from Patients with Tuberculous Spondylitis, Russia

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Whole-genome analysis of *Mycobacterium tuberculosis* isolates collected in Russia (N = 71) from patients with tuberculous spondylitis supports a detailed characterization of pathogen strain distributions and drug resistance phenotype, plus distinguished occurrence and association of known resistance mutations. We identify known and novel genome determinants related to bacterial virulence, pathogenicity, and drug resistance.

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, which typically affects the lungs but can affect other sites. In 2016, an estimated 10.4 million new TB cases and 1.6 million TB-related deaths were documented worldwide (*1*). The Russian Federation reported >120,000 TB cases and ≈13,700 TB deaths in 2016 (*1*). TB strains with multidrug resistance (MDR TB), characterized by resistance to isoniazid or rifampin, are common in the Russian Federation. The estimated rate of MDR TB was 27% among TB case-patients newly diagnosed in 2016 and 65% among previously treated case-patients in 2016 (*1*). Most TB cases are associated with pulmonary localization of the disease; however, in some cases, extrapulmonary TB develops. In Russia, the rate of extrapulmonary TB cases among new TB cases was 3.3% in 2014; most extrapulmonary TB cases are osteoarticular and genitourinary (*2*). Approximately 70% of osteoarticular TB cases are tuberculous spondylitis (TBS), which causes severe specific lesions of ≥1 components of the spine (*2*).

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We report whole-genome sequencing (WGS) and variant analyses of *M. tuberculosis* isolates from patients treated in Russia for TBS during 2007–2014.

The Study

The isolates were randomly collected from 71 TBS patients who received treatment at clinics of the Research Institute of Phthisiopulmonology in 32 regions of the Russian Federation (Figure 1). In these cases, *M. tuberculosis* isolates were cultured from extrapulmonary clinical material and stored. We assessed the susceptibility of these stored isolates to streptomycin, isoniazid, rifampin, ethambutol, pyrazinamide, ethionamide, ofloxacin, kanamycin, amikacin, cycloserine, capreomycin, and paraaminosalicylic acid according to World Health Organization recommendations (*3*). We isolated genomic DNA from cultured bacteria by using phenol/chloroform extraction and subjected bacterial DNA to WGS by using the MiSeq platform (Illumina, San Diego, CA, USA) to a mean coverage of 47× (range 18×–170×), covering ≥99% of the *M. tuberculosis* H37Rv reference genome (GenBank accession no. NC_000962.3). We deposited WGS reads in the NCBI Sequence Read Archive (accession no. PRJNA352769).

We aligned sequenced reads to the reference genome and called variants (single-nucleotide polymorphisms [SNPs] and short insertions/deletions) by using bioinformatics software: bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml); SAMtools (http://samtools.sourceforge.net); VCFtools (http://vcftools.sourceforge.net); and FreeBayes (https://github.com/ekg/freebayes). We used mutations that had q-scores ≥20 for comprehensive analysis. We used concatenated SNPs for phylogenetic analysis by using the GTRCAT (general time-reversible model with rate heterogeneity accommodated by using discrete rate categories) maximum-likelihood algorithm from the RAxML software package (*4*) to calculate an approximation model and 100 bootstrap replications. To avoid misalignments, we annotated SNPs in repetitive genome regions and in genes encoding proteins that contain proline-glutamate or proline-proline-glutamate motifs and filtered them from analysis. We used PhyTB (*5*) and SpoTyping tools (*6*) for phylogenetic classification of *M. tuberculosis* genomes and verified SpoTyping output by using previously conducted conventional spoligotyping analysis for 20 isolates that were previously described (*7*).

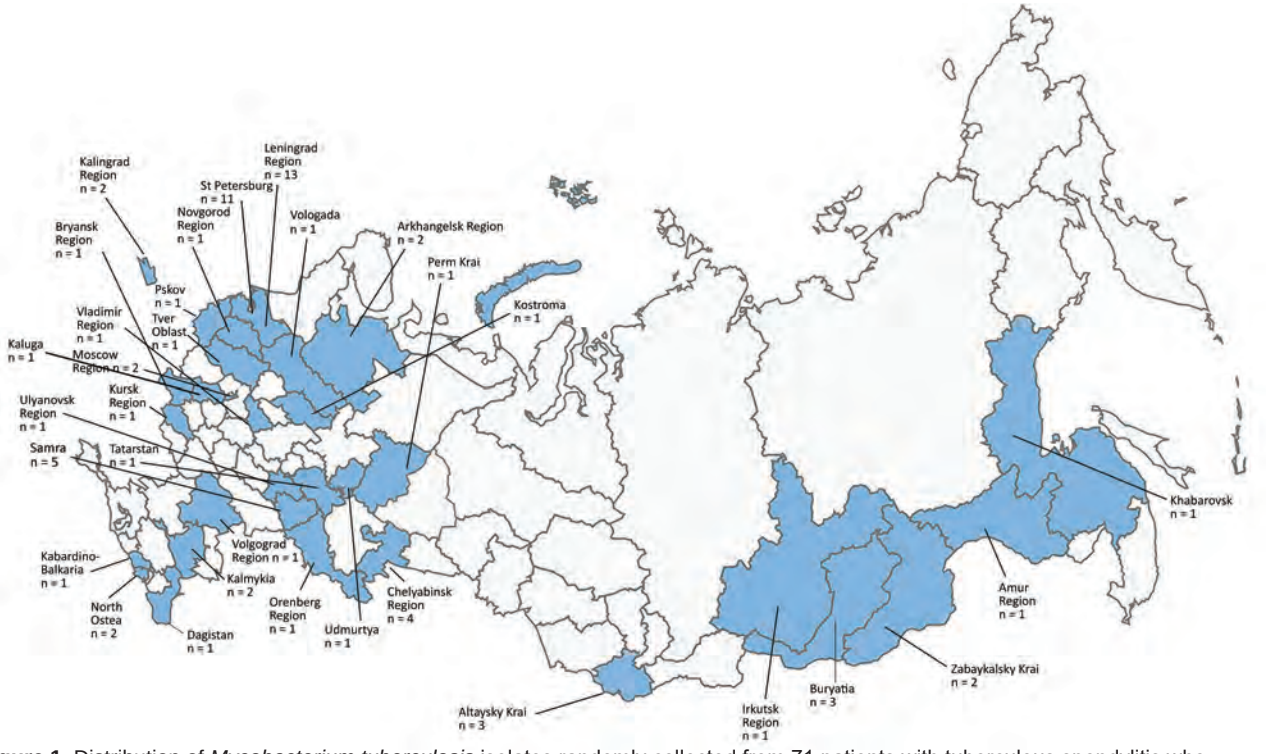

Figure 1. Distribution of *Mycobacterium tuberculosis* isolates randomly collected from 71 patients with tuberculous spondylitis who received treatment at clinics of the Research Institute of Phthisiopulmonology in 32 regions of the Russian Federation, 2007–2014.

We identified 2 principal phylogenetic lineages among *M. tuberculosis* isolates, lineage 2 and lineage 4; further, we detected evolutionary ancient and modern sublineages within major lineage 2 (Beijing; Figure 2) according to previously described classifications (*8*). The largest subgroup within the Beijing clade belonged to the B0/W148 clonal cluster (Figure 2) (*8*). Lineage 4 was represented by 4 genetic families: Ural, 4.2; Latin-American/Mediterranean (LAM), 4.3; and T, 4.1 and 4.8. The 58 Beijing genotype isolates contained 38 MDR (65.5%), 5 extensively drug-resistant (XDR; resistant to isoniazid and rifampin plus any fluoroquinolone and >1 of 3 injectable second-line drugs) (8.6%), 7 polyresistant but not MDR (12%), 1 monoresistant (1.9%), and 7 susceptible (12%) TB isolates. The MDR TB frequency in the Beijing group (65.5%) was higher than that for other genetic groups pooled ($p<0.0096$ by Fisher exact test). The *M. tuberculosis* Beijing B0/W148 cluster was represented by 1 susceptible (4.8%), 2 polyresistant (9.5%), 3 XDR (14.3%), and 15 MDR (71.4%) TB isolates. The B0/W148 genetic group demonstrates an association with MDR TB ($p = 0.03$), shown previously (*9*,*10*). The other genetic groups (T, LAM, and Ural) included too few isolates to test for association with MDR TB.

Specimens of 50 TBS patients were HIV negative; 21 were HIV positive. Although we found no significant association of *M. tuberculosis* genetic groups to HIV infection, 42% of patients infected by B0/W148 strains were HIV positive whereas among patients infected by non-B0/W148 Beijing strains, only 22% were HIV positive. Further, only 14% patients infected with non-Beijing *M. tuberculosis* strains were HIV positive (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/3/17-0151-Techapp1.pdf).

We examined *M. tuberculosis* isolates for the presence of published variants associated with resistance to TB drugs (Table). We found a high level of concordance of phenotypic and genetic data for reported isoniazid- and rifampin-resistant isolates. We detected mutations in *rpsL*, *gid*, and *rrs* genes in 96.4% of streptomycin-resistant isolates; 81.8% of ofloxacin-resistant isolates had mutations in *gyrA* gene (there were no mutations in *gyrB* gene). Most ethambutol-resistant isolates (72.7%) showed mutations in the *embA* promoter region or *embB* region between codons 296 and 497. However, 3 ethambutol-susceptible isolates had mutations M306I (n = 1) and G406A (n = 2) in the *embB* gene. We detected mutations in genes *pncA* (*11*) and *rpsA*, associated with pyrazinamide resistance in 55.6% of pyrazinamide-resistant isolates, and 55% of kanamycin-resistant strains had mutations in the *eis* promoter. We detected no mutations in *alr* and *ddl* genes among cycloserine-resistant isolates, nor in *thyA* gene among paraaminosalicylic acid–resistant isolates.

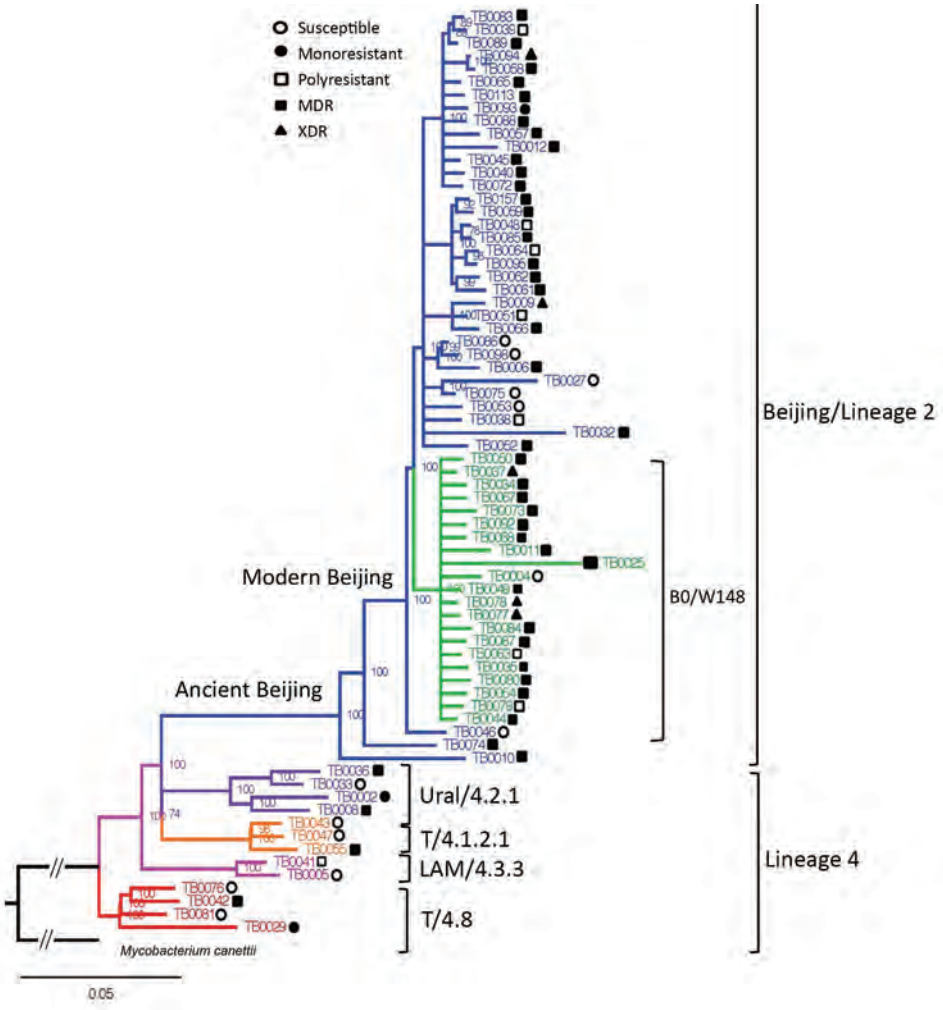

Figure 2. Phylogenetic analysis of *Mycobacterium tuberculosis* isolates obtained from patients with tuberculous spondylitis, Russian Federation, 2007–2014. Symbols indicate drug resistance: susceptible to all tested drugs; monoresistant to 1 drug; polyresistant, resistant to multiple drugs other than isoniazid or rifampin; MDR, resistant to isoniazid or rifampin; XDR, resistant to isoniazid or rifampin plus any fluoroquinolone and >1 of 3 injectable second-line drugs. Number of isolates and parsimony informative sites by genetic group: All groups, 71/2,656; Beijing, 58/919; Beijing B0/W148, 21/213; Beijing non-B0/W148, 37/722; T (4.8), 4/38; Ural, 4/204. The truncated root branch connects the studied *M. tuberculosis* lineages with an outgroup represented by *M. cannetii*. Scale bar indicates nucleotide substitutions per site.

Our analysis for small insertions and deletions detected 15 and 9, respectively, among the Beijing group (online Technical Appendix Table 2). A deletion in *kdpD* and an insertion in *Rv1258c* were previously described (*12*,*13*). The other 22 mutations are novel: 18 were specific to the Beijing group; 2 to the modern Beijing group; 1 to the ancient Beijing group; and 3 to the B0/W148 group. We identified most mutations in genes encoding membrane-associated proteins, although several mutations were in regulatory genes, genes involved in cell metabolism, probable transposase genes, and genes with unknown function.

One insertion and 2 deletions were significantly associated with B0/W148 genetic group in *kdpD, mmr* and Rv1995 ($p = 2.5 \times 10^{-17}$) genes. Merker et al. (*12*), who proposed a pathogenic influence for B0/W148 strains, described a frameshift deletion in *kdpD* among Beijing B0/W148 strains. Deletion in the *kdpD* gene can lead to the formation of nonfunctional proteins KdpD and KdpE. Parish et al. showed that *M. tuberculosis* lacking KdpD and KdpE function express increased virulence in a mouse model of infection (*14*), which supports that the *kdpD* deletion detected in our study may influence Beijing B0/W148 strain's rapid expansion and virulence. A mutation in the promoter region of the *mmr* gene (*Rv3065*), encoding multidrug-transport integral membrane protein, might contribute to drug resistance in Beijing B0/W148 strains. Sriraman et al. recently showed that *mmr* is upregulated in rifampin-resistant and MDR TB strains, even in the presence of target gene mutations (*15*). Insertion in *Rv1258c* is common to all Beijing strains except ancient TB0010. In their study, Villellas et al. found the cytosine nucleotide insertion between positions 580 and 581 in the *Rv1258c* gene in all Beijing isolates among streptomycin-resistant *M. tuberculosis* strains (*13*).

In conclusion, we examined the phylogenetic and drug-resistance properties of *M. tuberculosis* isolates collected from 71 TBS patients in 32 locales across Russia. Our analyses confirmed the phylogenetic separation of pathogenic *M. tuberculosis* strains and support the prevalence of Beijing strains showing high levels of multidrug

Table. Mutations associated with drug resistance detected in *Mycobacterium tuberculosis* genomes

Tuberculosis drug	Gene variant	No. strains with confirmed drug resistance	Drug-resistant strains, %
Isoniazid	*katG* S315T	52	100
	fabG-inhA-15	1	
	katG S315T	2	
	fabG-inhA-15	2	
Rifampin	*rpoB* S450L	40	100
	rpoB D435V	2	
	rpoB H445D	1	
	rpoB H445R	1	
	rpoB D574E	1	
Streptomycin	*rpsL* K43R	33	96.4
	rpsL K88R	5	
	gid G48G	1	
	gid G34G	1	
	*rrs*516	12	
Ofloxacin	*gyrA* D94N	1	81.8
	gyrA D94Y	1	
	gyrA D94G	5	
	gyrA D94A	2	
Ethambutol	*embA*-16	4	72.7
	embA-8	1	
	embB M3061	2	
	embB S3471	1	
	embB N399T	1	
	embB G406D	2	
	embB G406A	2	
	embB A453A	1	
	embB Q497R	2	
Pyrazinamide	*rpsA* D123A	1	55.6
	rpsA A412V	1	
	pncA L159R	1	
	pncA C138R	1	
	pncA T135P	1	
	pncA V130E	1	
	pncA Q122Stop	1	
	pncA Y103Stop	2	
	pncA G97S	1	
Kanamycin	*eis*-37	4	55.0
	eis-14	3	
	eis-10	4	

resistance among TBS isolates. Further, we found known SNP variants that had high concordance with suggested drug resistance. Finally, novel insertions/deletions were apparent, which we suggest are candidates for conferring drug resistance pending independent replication studies. Our analysis of WGS data identified known and novel genetic determinants that could or do influence bacterial virulence, pathogenicity, and drug resistance.

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E.C., V.Z., and S.O.B. designed the experiment. N.S., V.Z., and P.Y. provided *M. tuberculosis* genomic DNA, information on drug resistance, and clinical data. E.C. performed whole-genome sequencing. E.C., M.R., K.K., and A.Y. performed bioinformatics and statistical analysis of whole-genome sequencing data. A.V. and I.M. conducted *M. tuberculosis* spoligotyping. E.C., I.M., and S.O.B. wrote the manuscript.

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Artificial Differences in *Clostridium difficile* Infection Rates Associated with Disparity in Testing

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In 2015, *Clostridium difficile* testing rates among 30 US community, multispecialty, and cancer hospitals were 14.0, 16.3, and 33.9/1,000 patient-days, respectively. Pooled hospital onset rates were 0.56, 0.84, and 1.57/1,000 patient-days, respectively. Higher testing rates may artificially inflate reported rates of *C. difficile* infection. *C. difficile* surveillance should consider testing frequency.

Persons testing positive for *Clostridium difficile* by molecular methods might not always have *C. difficile* disease. Up to 10% of hospitalized patients carry toxigenic *C. difficile*; carriage rates of 35%–50% have been described in certain high-risk settings (*1*–*4*). In contrast, the risk for *C. difficile* infection (CDI) among hospitalized patients is 1%–2% (*5*).

Nucleic acid amplification testing (NAAT) has revolutionized diagnostic microbiology. Rapid, highly sensitive results can be returned to clinicians within hours, helping them make timely management decisions. The exquisite sensitivity of the test, however, has created an unexpected problem for *C. difficile* diagnosis: the test cannot distinguish patients with active disease from those who are asymptomatic carriers. Several clinical studies have shown that persons who test positive by NAAT without concomitant detection of toxin on conventional assays have milder disease than toxin-positive persons (*6*–*8*). The conclusion from these studies is straightforward: NAATs are highly sensitive but have an unacceptably low positive predictive value. Unnecessary clinical testing will generate many false cases; several downstream sequelae are potentially detrimental for patients and add to superfluous healthcare-associated costs.

The Study

We hypothesized that, in the era of NAAT-based testing for CDI, unevenness in testing rates introduces variability in measurement of CDI among different healthcare settings. We designed our survey to capture information about hospital characteristics and various aspects of *C. difficile* testing from a convenience sample of 38 medical centers, including 17 members of the Comprehensive Cancer Center Infection Control Network, 13 community hospitals, and 9 university-affiliated multispecialty centers. The survey

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Table. Hospital characteristics of study centers and methods used for the diagnosis of *Clostridium difficile* infection, 2015*

Characteristic	Hospital type, N = 30		
	Community hospital, n = 11	Large multispecialty academic center, n = 9	Tertiary-care cancer center, n = 10
Average no. beds (range)	294 (156–472)	869 (563–1,525)	221 (20–660)
No. annual admissions (range)	12,297 (2,897–22,000)	38,711 (14,589–86,658)	9308 (459–28,400)
No. annual patient-days (range)	56,322 (12,249–88,241)	241,034 (155,140–469,664)	65,263 (5,832–202,483)
Average length of stay, d	4.76	7.2	7.95
Transplantation, no.			
Hematopoietic stem cell	0	7	9
Solid organ	0	8	0
Diagnostic test, no.			
NAAT, 1-step	7	6	5
NAAT, 2-step	2	3	3
No. rejections of formed fecal samples	10	9	8
Pooled HO-CDI rate/1,000 patient-days	0.56	0.87	1.57

*HO-CDI, hospital-onset *C. difficile* infection; NAAT, nucleic acid amplification testing.

consisted of 13 questions for the year 2015: total number of beds, admissions, oncology beds, and transplants; *C. difficile* testing practices (inpatient testing volume, diagnostic method, and rejection policies); and rate of hospital-onset CDI (HO-CDI). We compared rates using ordinary 1-way analysis of variance or Kruskal–Wallis and determined the Pearson coefficient to measure the strength and direction of linear relationships between testing and *C. difficile* infection rates. We considered $p<0.05$ as significant. Each hospital provided information after responding to the Institutional Review Board and Health Insurance Portability and Accountability Act considerations at its institution.

Of the 31 (82%) hospitals responding to the survey, 30 provided complete data (Table). Overall, NAAT use was 87% (82% of community hospitals, 100% of multispecialty centers, and 80% of cancer centers). Among centers with 2-step testing, the commonly used initial test was glutamate dehydrogenase with or without toxin enzyme immunoassay, followed by NAAT for indeterminate samples. The overall fecal positivity rate among centers that used a 1-step NAAT was 16% for community hospitals, 15% for multispecialty centers, and 12% for cancer centers. Among NAAT users, the positivity rate for 1-step versus 2-step tests did not differ (13.9% vs. 14.3%, respectively). Formed fecal samples were rejected at 28 of 30 hospitals. Fifteen centers implemented a formal policy to avoid testing replicate samples; 2 rejected samples from patients receiving laxatives.

We determined the testing rate for each study hospital (Figure 1). The mean number of tests per 100 admissions for 2015 was 6.4 for community hospitals, 12.2 for multispecialty centers, and 29.1 for cancer centers ($p = 0.0003$). The mean number of tests per 1,000 patient-days was 14.0,

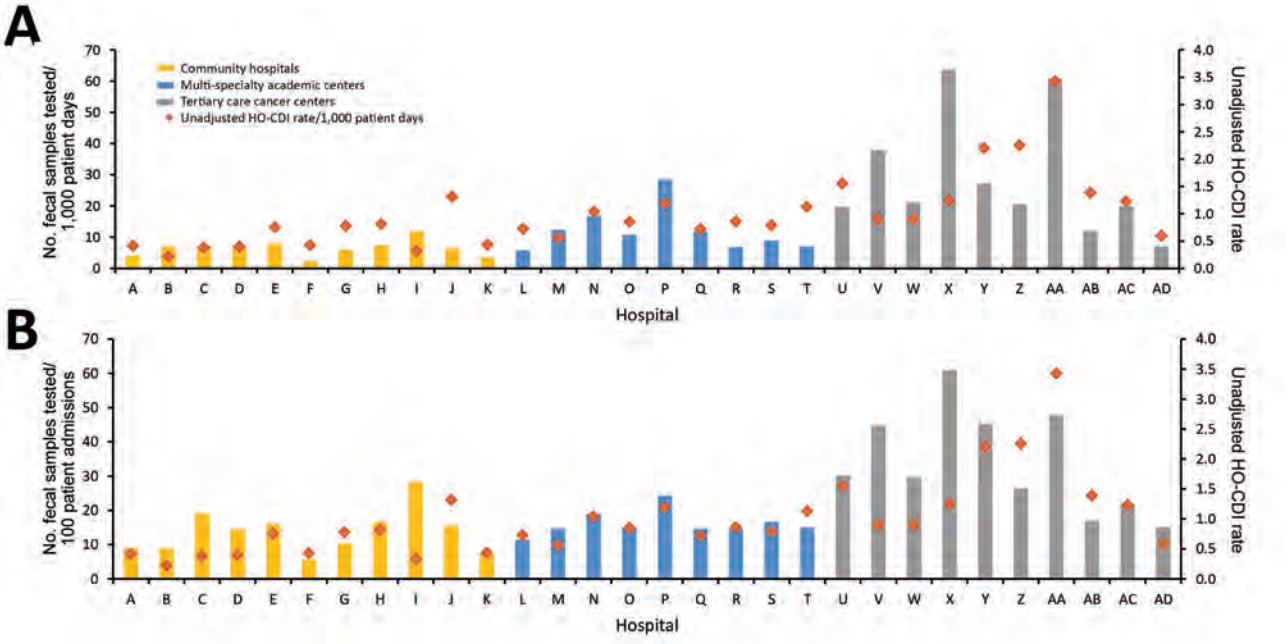

Figure 1. Hospital-specific rates of testing for *Clostridium difficile* standardized by patient-days of admission (A) and number of admissions (B), with HO-CDI rates (cases/1,000 patient-days), 30 US hospitals, 2015. HO-CDI, hospital-onset *C. difficile* infection.

16.3, and 33.9, respectively (p = 0.0016). A separate analysis on the subset of centers using PCR-based testing yielded similar results: 6.1, 12.6, and 28.0 tests/100 admissions (p = 0.0058) and 14.1, 16.3, and 33.4 tests/1,000 patient-days, respectively (p = 0.015).

The mean rate of hospital-onset *C. difficile* infection for the community hospitals, multispecialty centers, and cancer centers was 0.57, 0.88, and 1.57 cases per 1,000 patient-days (p = 0.0007). The correlation between testing rates, number of hospital beds, and average length of stay (Figure 2, panels A–D) illustrated a positive linear relationship between testing rates and length of stay. HO-CDI rates were highest for cancer centers that use NAAT (Figure 2, panel E).

Conclusions

We demonstrated that hospitals that test for *C. difficile* more frequently, such as cancer hospitals, have significantly higher CDI rates. This finding has 2 logical explanations. First, patients with cancer and patients in highly specialized hospitals have higher rates of HO-CDI because of the complexity of their underlying conditions and treatment. Second, and of particular importance in the era of reported healthcare-associated infections, overtesting can overdiagnose carriers of *C. difficile* as *C. difficile* cases, when in fact these patients have nondisease conditions of lesser clinical and epidemiologic significance.

Our study findings suggest that much of the excess *C. difficile* diagnosis in tertiary and cancer centers might be attributable to overtesting. We base our conclusion on the significant association found between testing rates and level of specialized care, particularly among cancer centers, as well as a positive correlation between testing and rates of HO-CDI. Among cancer centers, the likely explanation for excessive testing despite good diagnostic stewardship is the higher prevalence of diarrhea caused by effects of chemotherapy, newer immunotherapeutic modalities, and transplant-related gastrointestinal complications.

During the early 2000s, a hypervirulent NAP1 (North American pulsed-field gel electrophoresis type 1)

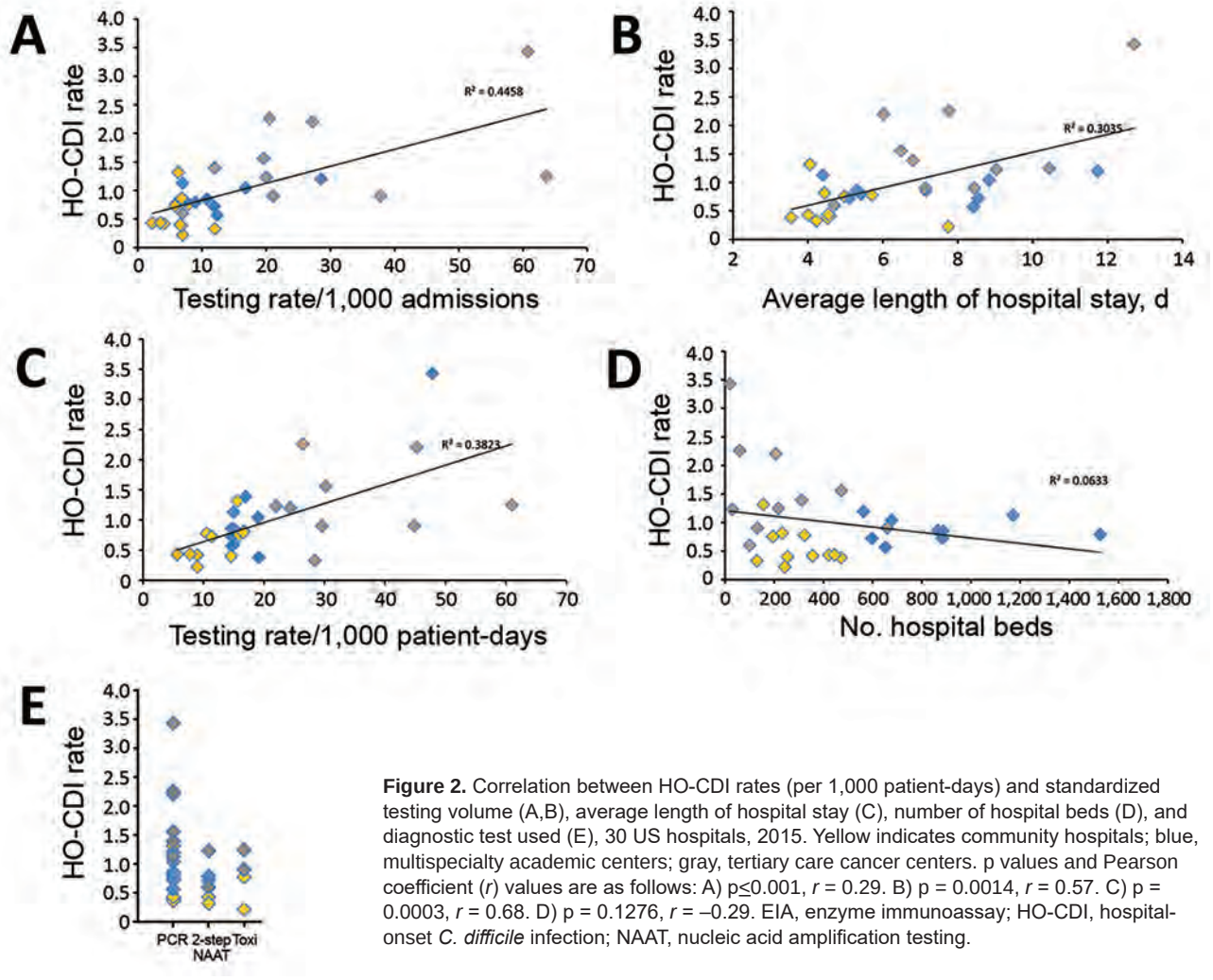

Figure 2. Correlation between HO-CDI rates (per 1,000 patient-days) and standardized testing volume (A,B), average length of hospital stay (C), number of hospital beds (D), and diagnostic test used (E), 30 US hospitals, 2015. Yellow indicates community hospitals; blue, multispecialty academic centers; gray, tertiary care cancer centers. p values and Pearson coefficient (*r*) values are as follows: A) p≤0.001, *r* = 0.29. B) p = 0.0014, *r* = 0.57. C) p = 0.0003, *r* = 0.68. D) p = 0.1276, *r* = –0.29. EIA, enzyme immunoassay; HO-CDI, hospital-onset *C. difficile* infection; NAAT, nucleic acid amplification testing.

C. difficile strain emerged in North America and Europe (*9*,*10*). With the premise that early and reliable detection of CDI will enhance control, the first Food and Drug Administration–approved NAATs for *C. difficile* became available in 2008 (*5*,*11*). NAAT use increased sharply during 2011–2013. As of 2016, ten different molecular tests for *C. difficile* have been Food and Drug Administration approved. More than half of all acute care settings that report CDI rates to the National Healthcare Safety Network use NAAT-based testing (*12*). Reports of oversensitivity of NAAT followed the increase in use, leading to a 43%–100% increase in reported incidence rates of CDI (*7*,*11*,*13*).

From a reporting perspective, risk adjustments have been made during interfacility comparisons of rates of HO-CDI to account for differences in diagnostic testing methods (*11*). Despite the apparent association between testing rates and likelihood of false-positive results with NAATs, testing frequency for *C. difficile* has been overlooked as a reporting metric by the National Healthcare Safety Network.

Based on the most recent estimates from 2014, rates of HO-CDI have declined by 8% in the United States since 2011 (*14*). Whether some component of this is due to less testing than in previous years remains unknown. Hospitals that use NAAT that have performed better over time may have truly reduced infections or simply adjusted to the new test. Current surveillance methods do not consider the effect of testing volume on reported *C. difficile* rates, a common cause of artificial changes.

Our report has several limitations. First, we used a convenience sample of 30 hospitals in which cancer centers were overrepresented. Second, we did not include clinical characteristics of patients tested for *C. difficile* because our survey focused on comparing testing rates in several representative hospitals across the healthcare continuum.

In conclusion, CDI infection surveillance programs must recognize that testing methods and testing frequency need to be considered independently when comparing infection rates. In addition, frequency of diarrhea in hospitalized patients is an important determinant that might vary by patient–case mix and affect testing and rates of HO-CDI. Testing frequency is not only important for local quality improvement but also should be made an essential component of *C. difficile* reporting to standardize disease measurement, monitor effectiveness of prevention strategies, and establish meaningful trends.

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Severe Pneumonia Caused by Toxigenic *Corynebacterium ulcerans* Infection, Japan

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Corynebacterium ulcerans infection was recently recognized as a zoonosis. We present 2 cases of severe pneumonia complicated by diffuse pseudomembrane formation on the bronchus caused by *C. ulcerans*–producing diphtheria toxin. Our purpose is to alert medical professionals to the virulence of *Corynebacterium* species other than *C. diphtheriae*.

The well-known *Corynebacterium* species is *C. diphtheriae*, which causes diphtheria. *C. ulcerans* has recently been identified as a causative agent of zoonotic infection and isolated from a wide range of domestic and wild animals (*1*). The bacterium has increasingly been reported in western Europe (*2*,*3*), and the number of reported cases of respiratory infection with *C. ulcerans* has been increasing (*4*–*6*). Certain strains produce diphtheria toxin and can cause a serious condition similar to diphtheria infection (*7*). This infection has a predilection for the upper respiratory tract; pneumonia caused by *C. ulcerans* is rare but can be fatal (*8*). We describe 2 cases of severe pneumonia caused by *C. ulcerans* with confirmed production of diphtheria toxin.

The Study

Case-patient 1, a 67-year-old woman with hepatocellular carcinoma, hypertrophic obstructive cardiomyopathy, and type 2 diabetes mellitus, came to Nagasaki University Hospital with severe dyspnea. She kept a cat and a dog in her house and also had close contact with stray cats in her neighborhood. Four days previously, she had noticed pharyngeal pain, cough, and nasal discharge. The dyspnea developed 2 days before the admission. The patient's body temperature was 38.0°C, blood pressure was 110/82 mm Hg, and respiratory rate was 32 breaths/min; oxygen saturation was 56% on room air. We gave her 10 L of oxygen via face mask with reservoir bag (Table 1). We observed no patches of exudates over the pharynx. Chest radiography showed right lung infiltrates (Figure 1, panel A). Computed tomography (CT) revealed consolidation, ground-glass opacity in the right upper and lower lobe, and thickened right main bronchus wall (Figure 1, panel B). Due to the rapid progression of respiratory failure and shock on the day of admission, we admitted the patient to intensive care and intubated her. The right lung collapsed within a few hours (Figure 1, panel C). Bronchoscopic observation revealed obstruction of the right main bronchus with massive white and yellowish exudate (pseudomembrane). We removed part of the pseudomembrane using forceps, reopening the right main bronchus and improving oxygenation. We observed club-shaped gram-positive rods on Gram staining of the exudate (Figure 1, panel D) and isolated *C. ulcerans*. We initiated intravenous vancomycin, tazobactam/piperacillin, and azithromycin (AZM) empirically, followed by sulbactam/ampicillin (SBT/ABPC). The bacterium was sensitive to ampicillin, gentamicin, erythromycin, and levofloxacin. The patient responded well to the treatment without administration of diphtheria antitoxin and was extubated on day 5 after admission. We discharged her ambulatory on day 30 after admission.

Case-patient 2, a 66-year-old woman with borderline diabetes, was admitted to Kawakita General Hospital in Tokyo for febrile dyspnea. She kept 3 cats in her house and had close contact with them. Throat pain and cough had developed 10 days before the admission. The patient's body temperature was 37.6°C, blood pressure was 135/78 mm Hg, and respiratory rate was 16 breaths/min; oxygen saturation was 87% on room air (Table 1). There was no lymphadenopathy or white spots on the pharynx. Chest radiography showed left lung infiltrates (Figure 1, panel E). We observed tracheal and bronchial wall thickening causing occlusive atelectasis of the left lower lobe (Figure 1, panels F, G). We administered SBT/ABPC and AZM. Because of rapidly exacerbating respiratory distress on the second hospital day, we intubated the patient. We administered SBT/ABPC continuously but replaced AZM with ciprofloxacin (CPFX). Bronchoscopy revealed white-yellowish exudate covering the whole left main bronchus and narrowing the lumen (Figure 1, panel H). We found massive gram-positive rods on Gram staining of the exudate. *C. ulcerans* sensitive to ampicillin, gentamicin, erythromycin,

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Table 1. Laboratory data at admission for 2 case-patients with severe pneumonia, Japan

Test	Case-patient 1	Case-patient 2
Leukocytes, cells/mm^3	13,800	23,200
Neutrophils, %	85	90
Lymphocytes, %	4	4.2
Erythrocytes, $\times 10^4$/μL	448	428
Hemoglobin, g/dL	12.6	13.1
Hematocrit, %	41.2	38.4
Platelets, $\times 10^4$/μL	24	26.2
Total bilirubin, mg/dL	0.1	0.6
Aspartate aminotransferase, IU/L	26	18
Alanine aminotransferase, IU/L	20	14
Lactate dehydrogenase, IU/L	238	249
Blood urea nitrogen, mg/dL	43	11
Creatinine, mg/dL	1.31	0.7
C-reactive protein, mg/dL	13.58	31.8
Administered oxygen	Mask, 10 L oxygen	Nasal, 2 L oxygen
Arterial blood gas levels		
$PaCO_2$, mm Hg*	56.1	36.7
PaO_2, mm Hg†	69.1	72.2
pH	7.263	7.45
Bicarbonate, mmol/L	24.5	25
Base excess, mmol/L	−2.7	1.3
Lactate, mmol/L	0.9	Not tested

*Arterial carbon dioxide partial pressure.
†Arterial oxygen partial pressure.

and levofloxacin was isolated. The patient responded to the treatment without administration of diphtheria antitoxin and was extubated on the 12th hospital day. The wall thickness and atelectasis improved. Consolidation and ground glass opacity, compatible with organizing pneumonia, persisted and improved gradually. We discharged the patient with no serious sequelae on the 35th day after admission.

We performed environmental surveillance for patients' companion animals and their belongings. We conducted swab sample collections for culture at the sites where the cats were housed and performed sampling from animals with their owners' informed consent and in their presence. We performed toxigenicity analysis and determined anti-diphtheria toxin neutralization titers by Vero cell cytotoxicity assay, as described by Katsukawa et al. (*9*,*10*). We performed ribotyping of isolated strains and conducted *rpoB* gene sequencing with the primers C2700F and C3130R as described by Khamis et al. (*11*).

Case-patient 1 had fed her pet cat and dog and 1 stray cat. At the patient's house, we detected *C. ulcerans* in the feeding tray, on the surface of the furniture on which the cat lay, on the cat's blanket, and on the telephone receiver. Samples from

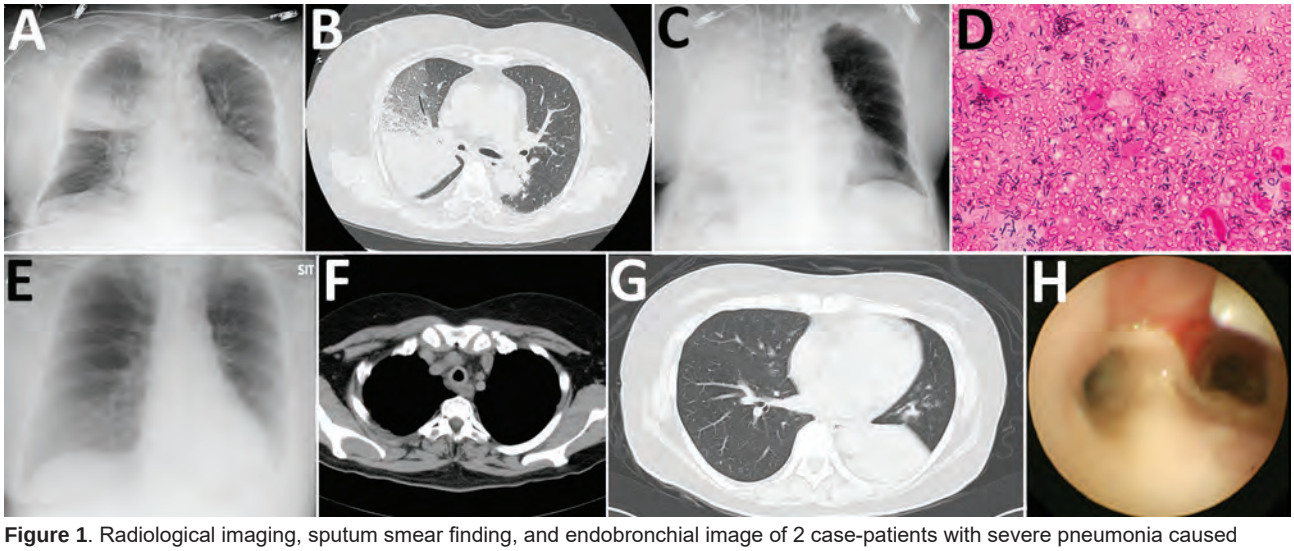

Figure 1. Radiological imaging, sputum smear finding, and endobronchial image of 2 case-patients with severe pneumonia caused by *Corynebacterium ulcerans* infection, Japan. Case-patient 1: A) Atelectasis in middle lung field at admission; B) consolidation and atelectasis in right upper lobe; C) rapid development of diffuse atelectasis; D) gram-positive rods in all fields of endotracheal aspiration sputum sample (original magnification x1,000). Case-patient 2: E) Infiltrates in left lower lung field; F) thickened tracheal membrane; G) atelectasis of left lower lobe; H) pseudomembrane formation on trachea and bronchus.

Table 2. Characteristics of *Corynebacterium ulcerans* isolates identified from swabs of 2 case-patients with severe pneumonia and their environments, Japan*

Sample	Culture	*rpoB* gene	*tox* gene	Vero cell cytotoxicity
Case-patient 1				
Patient sputum	Positive	*C. ulcerans*	+	+
Husband throat swab	Negative			
Cat throat swab	Negative			
Swab from cat's feeding tray	Positive	*C. ulcerans*	+	Not tested
Swab from cat's blanket	Positive	*C. ulcerans*	+	Not tested
Swab from telephone receiver	Positive	*C. ulcerans*	+	Not tested
Case-patient 2				
Patient sputum	Positive	*C. ulcerans*	+	+
Cat 1 throat swab	Positive	*C. ulcerans*	+	+
Cat 2 throat swab	Positive	*C. ulcerans*	+	+
Cat 3 throat swab	Negative			

*Detectable by PCR (*tox* gene) or Vero cell cytotoxicity assay. +, positive.

stray cats were not available. For case-patient 2, we treated her 2 cats that tested positive for *C. ulcerans* with erythromycin. Isolates from case-patient 1, case-patient 2, and case-patient 2's cats were all toxigenic (Table 2). We analyzed antitoxin titers from case-patients 1 and 2. Case-patient 1 showed a nonprotective level of antitoxin (<0.01 IU/mL) on day 12 after onset; however, her husband showed a protective level (>0.1 IU/mL). Case-patient 2 showed a protective level 1.5 months after onset (334 IU/mL). Case-patient 1 received an additional vaccination with diphtheria toxoid, but case-patient 2 did not.

Ribotyping of *C. ulcerans* isolates from the cases matched that of the cats and cat environment in their households. We categorized ribotyping for isolates from case-patient 1 as R3 group and for case-patient 2 as R1 group (Figure 2).

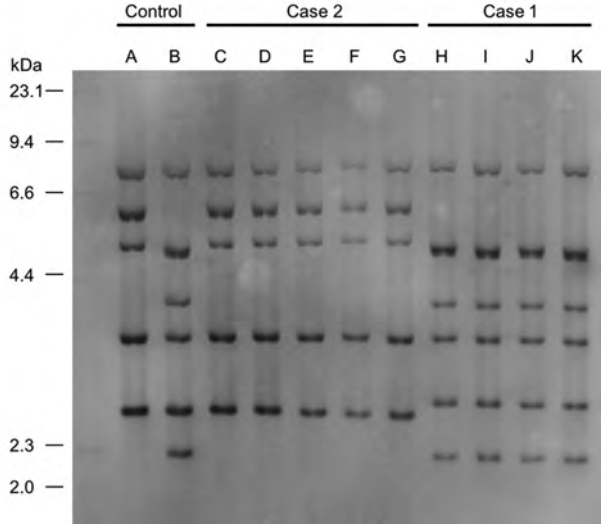

Figure 2. Ribotyping tests for isolates from 2 case-patients with severe pneumonia caused by *Corynebacterium ulcerans* infection, Japan. Case-patient 1 isolate was categorized as R3 group and case-patient 2 isolate as R1 group. A, 0102 (R1 group); B, 0211 (R4 group); C, isolate from case-patient 2's exudate on bronchus; D, isolate from cat 1's throat in case 2; E, isolate from cat 2's nasal cavity in case 2; F, isolate from cat 2's throat in case 2; G, isolate from cat 2's conjunctiva in case 2; H, isolate from case-patient 1's exudate on bronchus; I, isolate from cat's feeding tray in case-patient 1's house; J, isolate from cat's blanket in case-patient 1's house; K, isolate from telephone receiver in case-patient 1's house.

Conclusions

The mode of transmission of *C. ulcerans* to humans via infected companion animals was well documented in our cases. We demonstrated diphtheria toxin from both case-patients and isolation of *C. ulcerans* from case-patients and their companion animals. An immunization program for diphtheria in Japan started at the time of these case-patients' births; therefore, their immunization histories for diphtheria were uncertain. The antitoxin level of case-patient 1 was considered nonprotective according to World Health Organization guidelines, whereas her husband's antitoxin level was considered fully protective (*12*). This finding may explain why her husband did not have the same severe condition develop. Although case-patient 1 received the diphtheria vaccine after discharge, little is known about the efficacy of the diphtheria toxoid vaccine in preventing *C. ulcerans* infection, and recommendation of its prophylaxis with diphtheria antitoxin remains controversial (*13–15*).

Both cases showed similar patterns of acute respiratory failure. Thickened bronchial wall and pseudomembrane formation caused airway obstruction and severe atelectasis, leading to rapid progress of respiratory insufficiency. Release of the obstruction improved oxygenation dramatically. We considered acute atelectasis development and not damage to lung parenchyma, which is usually seen in severe pneumonia, to be the main cause of respiratory failure.

Facilities in which diphtheria toxin analysis is available remain limited. In some areas where reporting systems for *C. ulcerans* have not been introduced, accurate diagnosis is often missed. When Gram stain findings and endotracheal appearance point to infection, clinicians must pay careful attention to respiratory management for airway obstruction.

In summary, severe pneumonia caused by *C. ulcerans* is uncommon but life-threatening. It is important to recognize that *C. ulcerans* can be pathogenic in humans and that outcomes could be fatal.

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Tuberculosis—the Face of Struggles, the Struggles We Face, and the Dreams That Lie Within

Patrick K. Moonan

Tuberculosis disease, or phthisis (φθίσις, the Greek word for consumption), was named by the father of allopathic medicine, Hippocrates (c. 460–370 BCE), because the disease appeared to consume the affected person through substantial weight loss and wasting (*1*). Hippocrates warned his students against treating persons in late stages of tuberculosis, because nearly all of their patients would die, which would likely tarnish their reputations as healers (*2*).

Today, >10 million persons become ill with tuberculosis, and 2 million die from the disease each year (*3*). India accounts for the largest number of persons with tuberculosis and tuberculosis-related deaths in the world. In 2016, 1.9 million tuberculosis cases were reported to the Revised National Tuberculosis Programme of India (*3*). An additional 1.2–5.3 million patients were estimated to have received treatment for the disease in the private sector of India but remained unrecognized by the global surveillance system (*4*). Many millions of future case-patients will emerge from the huge reservoir of an estimated 354 million persons currently infected with tuberculosis in India (*5*).

India has a large burden of poverty and malnutrition among both adults and children. Malnutrition has wide-ranging effects on health, including increased susceptibility to infectious diseases such as tuberculosis (*6*). In 2016, malnutrition was the leading risk factor for 14.6% of the total disability-adjusted life-years for all-cause illness and death in India (*7*). More than half of all cases of active tuberculosis among women (55%, 95% CI 27%–76%) and men (54%, 95% CI 26%–75%) in India are attributable to susceptibility caused by malnutrition (*8*).

Having been inspired by the faces behind tuberculosis, the artist Stefan Prakash Eicher (http://www.stefaneicher.com/), born in Maharashtra, India, captures the essence of the term "consumption" through his portrait of an emaciated and wasting man rescued from the streets of New Delhi. In *What Dreams Lie Within* (Figure), dark tones represent muscle atrophy recessed against a bony torso and sunken cheeks to highlight the debilitating cost of advanced stages of tuberculosis, partly due to catabolic losses by a cachetin-induced mechanism (a macrophage-secreted cytokine) (*9*) and malnutrition (*10*). Billowing clouds and a blue sky of hope within his eyes subtly support the struggle of survival found within the intensity of his furrowed brow.

Each year on March 24, we commemorate World TB Day (https://wwwnc.cdc.gov/EID/page/world-tb-day) in honor of the day Robert Koch announced at the University of Berlin Institute of Hygiene that he

Figure. *What Dreams Lie Within* by Stefan Prakash Eicher. Oil on canvas, 2009.

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discovered the cause of consumption, the tuberculosis bacillus. World TB Day is a time to remember the millions of faces of persons who suffer from tuberculosis, to reflect on struggles we face as public health practitioners to end the epidemic, and to find hope in the eyes of the patients we treat and in the dreams that lie within our surviving patients searching for a better tomorrow.

About the Author

Dr. Moonan is a senior epidemiologist in the Division of Global HIV and Tuberculosis, Center for Global Health, Centers for Disease Control and Prevention, and has provided technical support to the Revised National Tuberculosis Programme of India for nearly a decade. His public health interests include tuberculosis transmission, operational research, and strengthening national tuberculosis programs to better manage drug-resistant forms of tuberculosis.

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Delftia tsuruhatensis, an Emergent Opportunistic Healthcare-Associated Pathogen

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Delftia tsuruhatensis, which was first isolated in environmental samples, was rarely associated with human infections. We report on pneumonia caused by *D. tsuruhatensis* in an infant who underwent cardiac surgery. Retrospective analyses detected 9 other isolates from 8 patients. *D. tsuruhatensis* is an emergent pathogen, at least for immunocompromised patients.

Delftia tsuruhatensis, a member of the *Comamonadaceae* family, was first isolated from sludge in Japan in 2003 (*1*). Mainly studied for environmental purposes (*2*,*3*), *D. tsuruhatensis* has rarely been identified in humans (*4*,*5*). We present a case report of a respiratory infection caused by *D. tsuruhatensis* in a premature infant.

A female infant, born premature at 36 weeks' gestation, had a cardiac congenital pathology for which resection of the ductus arteriosus and pacemaker placement were performed at 4 months of age. During the immediate follow-up period, she developed acute renal failure, which was treated by peritoneal dialysis. Her undernutrition status required enteral and parenteral nutrition. Laboratory tests showed slight leukocytosis, with elevated neutrophils at 9.2 G/L (reference range 1.4–8.5 G/L), monocytosis at 2.1 G/L (reference 0.2–2.0 G/L), and an elevated C-reactive protein at 15.1 mg/L (reference 0–5 mg/L). Two days after surgery, the infant developed pneumonia associated with ventilator-associated hypoxia, which prompted bronchial aspiration sampling that was sent to the clinical microbiology laboratory for analysis, which was performed as previously described (*6*). Colonies grew after 24 hours' incubation on both Polyvitex and Columbia media (bioMérieux, Craponne, France) in pure culture at 10^7 CFU/mL. We correctly identified the isolate using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Microflex, Bruker, Leipzig, Germany), as previously described (*7*). We maintain a custom MALDI-TOF mass spectrometry database that is updated regularly and enabled identification of the colony as *D. tsuruhatensis*, with an identification score of 2.082. We confirmed identification of the strain using 16S rDNA amplification coupled to sequencing, as previously reported (*8*). We obtained an amplicon of 1,296 bp and identified it as *D. tsuruhatensis* with a similarity of 99.70% with GenBank sequence no. KC572558. Because the strain was negative for D-mannitol assimilation as highlighted using API NE (bioMérieux), we excluded possible misidentification with *D. lacustris.*

We tested for antimicrobial drug susceptibility according to EUCAST 2017 recommendations (*9*) using the Etest gradient method. We categorized the strain as resistant to amoxicillin (MIC >256 mg/L) and amoxicillin/clavulanate (MIC >256 mg/L) but susceptible to ceftriaxone (MIC 0.5 mg/L), ertapenem (MIC 0.5 mg/L), imipenem (MIC 0.5 mg/L), and ofloxacin (MIC 0.047 mg/L). We administered ceftazidime to the patient for 10 days. Further collected samples were negative on culture.

One month later, the infant became febrile (temperature 39°C); a chest radiograph revealed pneumonia, and testing showed a still-elevated C-reactive protein (15 mg/L). A new bronchial aspiration was obtained and inoculated, as described previously. Twenty-four hours after incubation, we observed 2 isolates, each growing 10^5 CFU/mL, and analyzed them by MALDI-TOF mass spectrometry. One isolate was identified as *D. acidovorans* (score 2.207). Faced with the discrepancy with the previous results, we performed 16S rRNA PCR coupled with sequencing, as previously reported, enabling the identification of *D. tsuruhatensis* with a sequence identity of 99.70% to GenBank sequence no. KC572558. We identified the other isolate as *Neisseria macacae*, with a score of 2.033.

We initiated therapy with imipenem, vancomycin, and amikacin before we received the microbiology results, after which we readjusted the regimen, this time administering only tobramycin aerosol. The patient's health gradually deteriorated; she developed bradycardia and refractory hypoxia. She died at 6 months of age, 12 days after the last isolation of *D. tsuruhatensis.*

We report isolation of *D. tsuruhatensis* in respiratory samples from a 6-month-old infant, born at 36 weeks' gestation. Recurrent isolations of the microorganism from the same patient, including 1 time in pure culture, exclude potential contamination. In addition, clinical signs, such as pneumonia with ventilator-associated hypoxia, support infection rather than colonization. However, the patient had recurrent pneumonia, despite a successful first therapy with ceftazidime.

We also looked at the number of strains of *D. tsuruhatensis* isolated in our university hospitals in Marseille, France, during 2008–2015 and in the literature (Table). The microorganism has been isolated 13 times from 11 patients, including the case we describe here, mainly from blood

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Table. Characteristics of 11 patients with *Delftia tsuruhatensis* infection, 2 from the literature and 9 from university hospitals in Marseille, France*

Year, patient age, y/sex	Underlying conditions	Intravascular device	Specimen (description)	Clinical features; drug regimen	ID method; ID (score)	Amplification of 16 rDNA (similarity)	Ref
2010, 53/F	Metastatic breast cancer	Yes	Blood culture	Port-related bacteremia with fever; ceftriaxone	Phenotypic methods; *Comamonas testosteroni*	*D. tsuruhatensis* (99%)	(*4*)
2012, 53/F	Severe pulmonary hypertension	Yes	Blood culture	Catheter-related bacteremia with chills; oral ciprofloxacin	Phenotypic methods; *D. acidovorans*	*D. tsuruhatensis* (100%)	(*5*)
2008, 77/M	Liver cancer, colic adenocarcinoma	Yes	Bronchial aspirate (10^5 CFU/mL, pure)	Considered by physicians as colonization	Phenotypic methods; *D. acidovorans*	*D. tsuruhatensis* (100%)	Marseille hospitals (this study)
2009, 70/F	Unknown	Unknown	Bronchial aspirate (10^5 CFU/mL, pure)	Not available	MALDI-TOF MS; *D. acidovorans* (1.968)	*D. tsuruhatensis* (99.9%)	Marseille hospitals (this study)
2010, 59/F	Alcoholism, chronic end-stage renal failure	Yes	Blood culture	Catheter-related bloodstream infection; piperacillin, tazobactam, gentamycin	Not available	*D. tsuruhatensis* (99.9%)	Marseille hospitals (this study)
2010, 6/M	Cystic fibrosis	No	Sputum (10^3 CFU/mL, not pure)	Not available	MALDI-TOF MS; *Arthrobacter castelli*	*D. tsuruhatensis* (99.1%)	Marseille hospitals (this study)
2013, 42/M	Homeless, chronic renal failure, alcoholic hepatitis	Yes	Urine (10^6 CFU/mL, pure)	Not available	MALDI-TOF MS; *D. tsuruhatensis* (2.19)	*D. tsuruhatensis* (99.8%)	Marseille hospitals (this study)
2014, 13/F	Liver transplant	Yes	Blood cultures (N = 2)	Post-transplant fever; piperacillin, tazobactam	Not available	*D. tsuruhatensis* (99.9%)	Marseille hospitals (this study)
2015, 47/M	Kidney transplant	Yes	Blood culture	Fever	MALDI-TOF MS; *D. tsuruhatensis* (2.38)	Not performed	Marseille hospitals (this study)
2015, 82/M	Hemodialysis, vascular dementia	Yes	Blood culture 1	Catheter-related bloodstream infection; ceftazidime	MALDI-TOF MS; *D. acidovorans* (2.02)	*D. tsuruhatensis* (100%)	Marseille hospitals (this study)
			Blood culture 2		MALDI-TOF MS; *D. tsuruhatensis* (2.21)	*D. tsuruhatensis* (100%)	
2015, <1/F	Preterm birth	Yes	Respiratory sample 1 (10^7 CFU/mL)	Ventilator-associated pneumonia; ceftazidime, second-line treatment with imipenem and amikacin	MALDI-TOF MS; *D. tsuruhatensis* (2.08)	*D. tsuruhatensis* (99.9%)	Marseille hospitals (this study)
			Respiratory sample 2 (10^5 CFU/mL)		MALDI-TOF MS; *D. acidovorans* (2.21)	*D. tsuruhatensis* (100%)	

*Ref, reference; ID, identification; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

cultures (5/11 cases) and respiratory specimens (5/11), but also from 1 urine sample. Overall, the underlying conditions were observed for 10 cases, including 2 transplant recipients. No information was available for 1 patient. Considering the presence of a vascular catheter, hospital stay longer than 48 hours, or both, all reported infections were healthcare associated. In addition, of the 6 patients in whom the bacterium had been isolated from blood cultures, all 6 had an intravascular device. These data are consistent with the 2 cases of bacteremia involving *D. tsuruhatensis* already reported in the literature for which intravascular device–related and underlying conditions were found (*4*,*5*).

Bacterial identification systematically failed when using phenotypic methods. Since its implementation in routine laboratory tests, MALDI-TOF mass spectrometry has correctly identified *D. tsuruhatensis* in 4 of 8 tested isolates. For the 4 other isolates, *D. tsuruhatensis* was misidentified as *D. acidovorans* in 3 cases. Accurate identification was definitively performed using 16S rDNA sequencing.

In conclusion, *D. tsuruhatensis* is an opportunistic emergent healthcare-associated pathogen that can be easily misidentified. Clinicians should consider this bacterium particularly in immunocompromised patients and those with intravascular devices.

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Mycobacterium avium subsp. *hominissuis* Infection in a Domestic Rabbit, Germany

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Mycobacterium avium subsp. *hominissuis* is an opportunistic pathogen present in soil and dust. We report *M. avium* subsp. *hominissuis* infection found in a domestic rabbit in Hannover, Germany, in May 2017.

Mycobacterium avium subsp. *hominissuis* is an opportunistic pathogen with zoonotic potential (*1*,*2*) that is present in soil and dust. Animals are seen as a reservoir and potential threat for human infection, but the route and source of human infection remains unknown in most cases (*3*–*6*). We report on a 4-year-old intact male rabbit from a private breeder in Germany that died suddenly in May 2017. Several months before death, the rabbit showed intermittent diarrhea, and the veterinarian suspected coccidiosis. Necropsy findings included cachexia with small amounts of a clear fluid in body cavities due to hypoproteinemia; dehydration; multifocal intramural nodules ≤4 mm in diameter in the jejunum and ileum; highly liquefied intestinal contents without molding; and enlarged mesenteric lymph nodes. We detected single coccidia parasites in a native intestinal smear. Histologically, the nodular lesions in the ileum

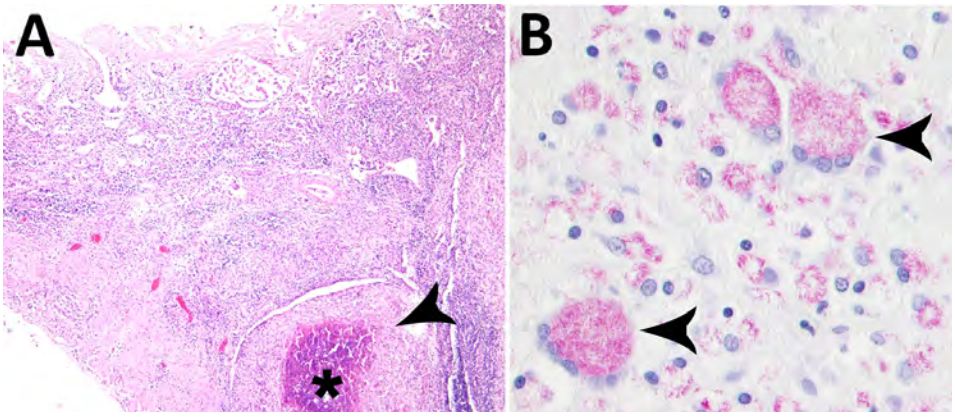

Figure. Results of histologic testing of samples from a domestic rabbit with *Mycobacterium avium* subsp. *hominissuis* infection, Germany. A) Hematoxylin and eosin stain reveals multifocal severe granulomatous enteritis in the ileum with focally extensive necrosis (asterisk) and numerous surrounding macrophages (arrowhead). Scale bar indicates 300 µm. B) Ziehl-Neelsen stain shows numerous acid-fast bacilli in the cytoplasm of macrophages and multinucleated giant cells (arrowheads). Scale bar indicates 20 µm.

showed severe granulomatous enteritis with large areas of necrosis and numerous multinucleated giant cells (Figure, panel A). Ziehl-Neelsen stain demonstrated large numbers of acid-fast bacilli in macrophages and multinucleated giant cells in the intestine (Figure, panel B). The mesenteric lymph node also exhibited a granulomatous inflammation with multinucleated giant cells. Additionally, the rabbit had mild suppurative splenitis, mild lymphohistiocytic to granulomatous hepatitis, mild focal lymphocytic interstitial orchitis, and a hyperplasia of the myeloic cell line in the femoral and sternal bone marrow. We detected no acid-fast bacilli in the mesenteric lymph nodes, the spleen, or the liver.

We decontaminated sections of the small intestine using NALC-NaOH and cultivated on Löwenstein-Jensen (Artelt-Enclit GmbH, Germany), Stonebrink (Artelt-Enclit), and Herrold's Egg Yolk (Becton Dickinson GmbH, Germany) agar slants (the last supplemented with Mycobactin J), as well as in Kirchner medium (Artelt-Enclit). We extracted DNA from grown colonies after heat inactivation by ultrasonic cell lysis and analyzed the DNA by PCRs targeting insertion sequence (IS) *1245*, IS*900*, and IS*901*. The presence of IS*1245*-specific and absence of IS*900*- and IS*901*-specific PCR products identified the bacilli as *Mycobacterium avium* subsp. *hominissuis*. Additionally, DNA sequencing of a *rpoB* gene PCR fragment yielded 100% sequence identity to *rpoB* from *M. avium* ssp. *hominissuis* strain IWGMT49 (GenBank accession no. EF521911) (*7*).

M. avium subsp. *hominissuis* is not currently a reported pathogen for rabbits. It has been reported only once in a slaughtered rabbit, but that animal showed no clinical or pathological abnormalities (*8*). In our investigation, as in reports in other host species, the source and route of infection was unclear. The presence of enteric inflammatory lesions with presence of acid-fast bacilli, however, suggests an oral route of infection.

It has been reported that a mycobacterial infection is dependent on the immunity and nutritional status of the host (*2*,*9*). In this case, the infestation with coccidia, common intestinal parasites in rabbits that can cause emaciation, may have contributed to the massive mycobacterial infection. Nevertheless, clinically manifest mycobacterial infection is a rare finding in domestic rabbits. We encourage awareness of a potential zoonosis, such as infection with *M. avium* subsp. *hominissuis*, in rabbits with intermittent diarrhea and chronic weight loss.

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Acetobacter indonesiensis Pneumonia after Lung Transplantation

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We report a case of *Acetobacter indonesiensis* pneumonia in a 51-year-old woman after bilateral lung transplantation. We found 2 other *A. indonesiensis* pneumonia cases reported in the literature. All 3 cases involved complex patients exposed to broad-spectrum antimicrobial drugs, suggesting that this pathogen may be opportunistic and highly drug-resistant.

A 51-year-old woman who had a medical history of hypersensitivity pneumonitis, extrinsic allergic alveolitis, and short telomere syndrome was admitted to a local hospital in Massachusetts, USA, for hypoxemic respiratory failure. The patient was transferred to the tertiary care hospital in which we practice in Boston, where she ultimately underwent a bilateral lung transplant from a high-risk donor without induction immunosuppression. The donor lungs grew group C *Streptococcus*, *Peptostreptococcus micros*, and *Candida albicans*. The native lungs were culture-negative.

The patient's postoperative hospital course was complicated by fever, leukocytosis, anemia, thrombocytopenia, and acute kidney injury. The clinical treatment team treated the patient with trimethoprim/sulfamethoxazole (TMP/SMX) and vancomycin; the latter was discontinued and piperacillin/tazobactam (14 days total) was administered after identification of *P. micros* in the donor's lungs. On postoperative day 21, 4 days after completion of her antimicrobial drug therapy, the patient continued to have respiratory symptoms, and we cultured samples from a tracheostomy suction. A Gram stain of the tracheostomy suction fluid revealed gram-variable coccobacilli. The next day, we also found 2 bronchioalveolar lavage specimens to be positive for gram-variable coccobacilli and considered them to be of the same phenotype. The patient's symptoms, along with the presence of the organism in 3 separate and sequential samples, argued against contamination.

Standard microbiological culture techniques revealed a slow-growing organism that was catalase-positive, oxidase-negative, L-pyrrolidonyl-β-napthylamide hydrolysis–negative, and vancomycin-resistant. We did not identify the organism by using exhaustive phenotypic techniques. We sequenced 16S rRNA (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/3/17-0409-Techapp1.pdf) and identified the organism as *Acetobacter indonesiensis*; we deposited this sequence in GenBank (accession no. KP330469). Because of the rare occurrence of this pathogen in humans (*1*,*2*), we achieved additional biochemical testing by using short- and medium-chain fatty acid analysis, which provided additional evidence supporting sequence-based identification.

At the time of the infection, the clinical microbiology laboratory at our hospital was not equipped with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry bacterial identification platform. However, this organism is not in any Food and Drug Administration–approved databases and therefore would not have been identified by using this instrumentation. Antimicrobial drug susceptibility testing using disk diffusion revealed an organism that failed to demonstrate in vitro activity to ampicillin, levofloxacin, ciprofloxacin, cephalothin, cefotetan, ceftadazime, cefepime, chloramphenicol, ertapenem, meropenem, piperacillin, aztreonam, thiosulfil/sulfamethizole, TMP/SMX, or colistin. The isolate did, however, demonstrate in vitro activity against aminoglycosides, tetracyclines, imipenem, and ceftriaxone. This drug susceptibility profile was similar to the profile found against the *A. indonesiensis* organism identified in a previously reported case (*2*).

Although this patient's isolate was resistant to the antimicrobial drugs she had received, her symptoms ultimately resolved. On postoperative day 33, her respiratory function had improved, and she was prescribed TMP/SMX (prophylaxis) and fluconazole at discharge.

At the time the bacteria was speciated by 16S rRNA gene sequencing, the patient's infection had already resolved. The clinical record does not document any additional antimicrobial treatments she may have received from other clinical teams, including the infectious disease, transplant, and nephrology departments. This organism appeared in 3 consecutive respiratory specimens collected when the patient's symptoms worsened and raised concerns among the attending clinical teams of potential infection with an innately drug-resistant species. However, we cannot definitively rule out the potential for colonization because a combination of factors likely led to clinical improvement in the patient. The organism was not detected in any subsequent bronchoscopies.

The genus *Acetobacter* encompasses a group of acetic acid–producing organisms that can survive at low pH, largely occupy environmental niches, are used industrially to produce acetic acid products, and are not generally thought to be human pathogens (*1*). Analysis of the medical literature revealed 2 other documented clinical cases of *A. indonesiensis* infection among humans (*2*,*3*). The first case involved a patient with cystic fibrosis who had undergone a recent lung transplant (*2*). Similar to our case-patient, the patient had undergone bilateral lung transplants and *A. indonesiensis* pneumonia subsequently developed in both after a long course of broad-spectrum antimicrobial drugs. The second case involved a child with metachromatic leukodystrophy who was found to have *A. indonesiensis* bacteremia after extensive nursing care and invasive devices, including a port catheter thought to be the source of the infection (*3*). As with the patient we report, the patient in that report had been treated with a 2-week course of pipericillin/tazobactam, although her initial diagnosis was bacteremia rather than pneumonia.

The case of *A. indonesiensis* human infection we report and both previous cases we found in the literature involved chronically ill patients with complex medical conditions who were exposed to a long course of broad-spectrum antimicrobial drugs. Although the source of the infecting organism in all 3 cases could not be definitively determined, the similarities between the cases raise the possibility that *A. indonesiensis* may represent a novel and emerging opportunistic and highly drug-resistant pathogen. Furthermore, the use of specific genotypic techniques such as 16S rRNA sequencing may aid in the identification of environmental organisms that are not identified by using traditional microbiological techniques.

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New Lineage of Lassa Virus, Togo, 2016

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We describe a strain of Lassa virus representing a putative new lineage that was isolated from a cluster of human infections with an epidemiologic link to Togo. This finding extends the known range of Lassa virus to Togo.

Lassa virus is endemic to the West Africa countries of Guinea, Sierra Leone, Liberia, Mali, Côte d'Ivoire, and Nigeria (*1*–*3*). The virus causes Lassa fever, a hemorrhagic disease with a case-fatality rate ≈30% in the current hospital setting in West Africa. So far, 4 lineages of Lassa virus are firmly established: lineages I, II, and III circulate in Nigeria, and lineage IV circulates in Guinea, Sierra Leone, Liberia, Mali, and Côte d'Ivoire (*1*–*3*). Recently, strains from Mali and Côte d'Ivoire were proposed to represent a separate lineage V (*4*). The newly discovered Lassa virus strain Kako from *Hylomyscus pamfi* rodents trapped in Nigeria is designated lineage VI for the purpose of this article (*5*).

Lassa virus has not been previously detected in humans or rodents in Togo; therefore, the virus was not considered endemic to this country. We describe a strain of Lassa virus representing a new lineage that was isolated from a cluster of human infections with an epidemiologic link to Togo (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1905-Techapp1.pdf) (*6*,*7*). The clinical courses of the 3 case-patients and medical and public health interventions are described elsewhere (*8*–*10*).

The Lassa virus infections in the index case-patient, secondary case-patient 1, and secondary case-patient 2 were confirmed by laboratory investigations at Bernhard Nocht Institute (Hamburg, Germany); Centers for Disease Control and Prevention (Atlanta, GA, USA); and Philipps University (Marburg, Germany), respectively. The viruses from all 3 patients were isolated in Vero E6 cell culture in the respective Biosafety Level 4 laboratories. Full-length virus sequences were generated directly from clinical specimens, from the isolates, or both using next-generation sequencing technology in combination with Sanger sequencing (sequences deposited into GenBank under accession nos. KU961971, KU961972, LT601601, LT601602, MF990886–MF990889) (online Technical Appendix). The sequence from the index case-patient was submitted to GenBank on March 23, 2016, and immediately made publicly available to support the laboratory and public health response in Togo and the other affected countries.

The virtually identical viruses from the 3 patients confirmed the transmission chains suggested by the epidemiologic data. Only the virus from secondary case-patient 2 showed differences in coding regions—a deletion of 3 nt and a nucleotide exchange in the polymerase (L) gene—from the viruses in the other 2 case-patients. These differences were confirmed by sequencing the virus in the clinical specimens. Differences among the 3 strains in the highly structured intergenic regions might represent artifacts created by the difficulty in sequencing these regions.

The phylogeny was inferred using BEAST2 (https://www.beast2.org/) with nucleotide sequences of full-length nucleoprotein (NP), glycoprotein precursor (GPC), and L and Z genes of the Togo strain in conjunction with representative sequences of Lassa virus and other Old World arenaviruses. The most stable reconstruction was obtained for the L gene with the Togo strain being placed in sister relationship with lineage II (all branches with posterior support values ≥0.97) (Figure). In the NP- and GPC-based phylogenies, the Togo strain clusters with lineages I and VI (Pinneo and Kako strains); however, the branching order is not well supported (posterior values 0.51–0.86) (Figure). The phylogeny based on the small Z gene further supports a relationship of the Togo strain with lineages I, II, and VI (online Technical Appendix Figure 1). The ambiguous position of the Togo strain relative to lineages I, II, and VI is consistent with a recombination analysis showing that most of the L gene sequence is related to lineage II, and NP and GPC comprise sequence stretches mainly related to lineages I and VI (online Technical Appendix Figure 2). This mosaic structure might be the result of recombination, reassortment, or both or might have evolved by chance.

The long branch (i.e., large phylogenetic distance) separating the Togo strain from known lineages suggests that it represents a new lineage. Because Lassa virus lineages were originally established on the basis of uncorrected sequence distances (*1*), we used the same method here. The frequency distribution of pairwise amino acid distances in GPC, NP, and L between the Togo strain and all other Lassa virus strains perfectly overlaps with the distribution of distances between Lassa virus lineages I, II, III, IV, and VI indicating that the Togo strain is a separate lineage (online Technical Appendix Table 1). However, we noted that the distance between the proposed lineage V and lineage IV rather corresponds to intralineage distances, and therefore, we considered lineage

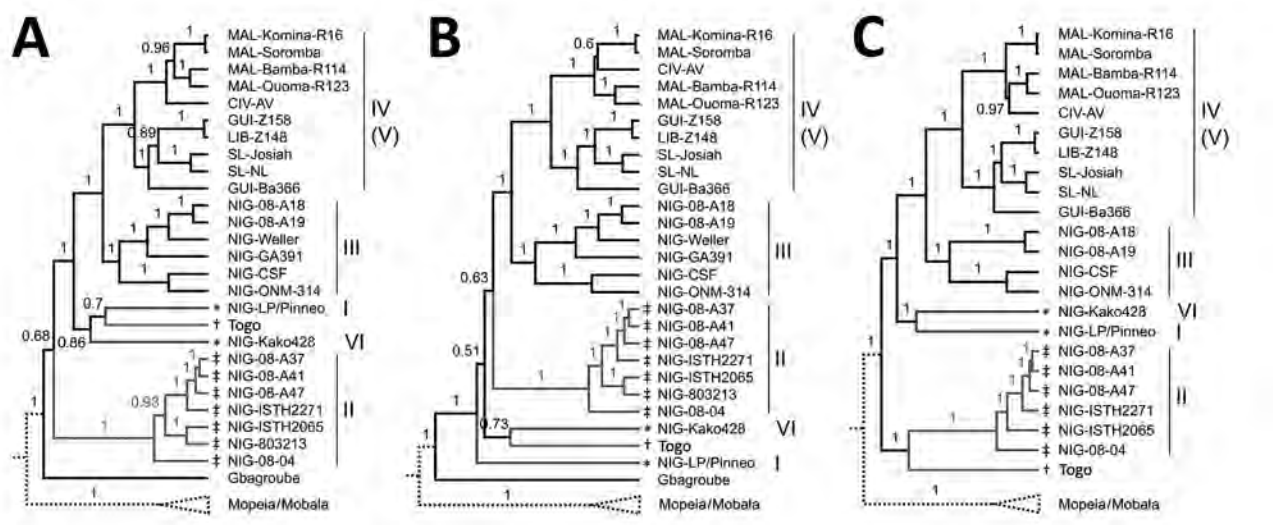

Figure. Phylogeny of the Lassa virus strain from Togo, 2016. Phylogenetic trees were inferred by using BEAST2 (https://www.beast2.org/) for full-length glycoprotein precursor (A), nucleoprotein (B), and polymerase (C) genes. The analysis included representative Lassa virus strains and other Old World arenaviruses (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/3/17-1905-Techapp1.pdf). Posterior support values are shown at the branches. Lassa virus lineages are indicated by roman numbers on the right. The branch for Mopeia and Mobala virus is shown schematically and the branches for the remaining Old World arenaviruses have been removed for clarity of presentation. The branches for the Togo strain and most closely related Lassa virus lineages are labeled (†, Togo; ‡, lineage II; *, lineages I and VI). The origins of the Lassa virus strains are abbreviated as follows: CIV, Côte d'Ivoire; GUI, Guinea; LIB, Liberia; MAL, Mali; NIG, Nigeria; SL, Sierra Leone. A color version of this figure is available online (https://wwwnc.cdc.gov/EID/article/24/3/17-1905-F1.htm).

V a subclade of lineage IV in our analysis (online Technical Appendix Table 1). We propose that formal recognition of Lassa virus lineages should be decided by the International Committee on Taxonomy of Viruses.

In conclusion, sequencing Lassa virus from a cluster of imported infections, with the index case-patient originating from Togo, reveals a new lineage of Lassa virus in West Africa. It seems to be related to lineage II or lineages I/VI, which are all circulating in Nigeria.

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Evidence for Previously Unidentified Sexual Transmission of Protozoan Parasites

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Knowing the mode of transmission of a disease can affect its control and prevention. Here, we identify 5 protozoan parasites with demonstrated presence in seminal fluid, only 1 of which has been identified as a sexually transmitted disease among humans.

A recent publication by Salam and Horby (*1*) identified at least 27 viruses present in human semen, some potentially transmissible through sexual contact. Trichomonas vaginalis is a protozoan parasite recognized as sexually transmissible among humans (*2*). Similar to that which occurs with viruses, parasites could reach seminal fluid by passing from the bloodstream to the male genital tract or by directly infecting reproductive organs. In this context, more parasitic infections might also be transmitted sexually. Considering that parasitic diseases represent one of the most common infections worldwide, mainly in developing countries, sexual transmission of parasitic diseases could represent a major global problem in terms of public health.

To investigate whether parasites could enlarge the broad list of potential sexually transmitted infections (STIs), we conducted an online search on November 3, 2017, by using PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), EMBASE (https://www.elsevier.com/solutions/embase-biomedical-research), and the Cochrane Library (http://www.cochranelibrary.com/) with no language restrictions. We used the terms "parasites OR parasitic disease" and "semen OR seminal plasma." We also made a manual search of the references of selected reports. Two reviewers independently screened the 512 returned results of titles, abstracts, and full text in selected articles.

Our search resulted in 5 parasite species with demonstrated presence in seminal fluid of humans: *Entamoeba hystolytica* (*3*), *Schistosoma haematobium* (*4*), *Trichomonas vaginalis* (*2*), *Trypanosoma cruzi* (*5*), and *Toxoplasma gondii*; the latter has been documented as sexually transmitted among animals, but not humans (*6*) (Table). *E. hystolytica* is a worldwide anaerobic protozoan; its prevalence increases disproportionately in areas of poor sanitation in low-income countries. *E. hystolytica* has been identified in the testicles, epididymis, and seminal fluid (*3*,*7*), can reportedly cause infertility as a result of reproductive organ damage (*8*), and is transmitted by sexual contact (both oral-anal and oral-genital sexual practices) (*7*).

Urogenital schistosomiasis caused by *S. haematobium* infection affects male and female children and adults mainly in Africa, the Middle East, and Corsica, France. After the larval *S. haematobium* cercarias penetrate intact skin from contaminated fresh water, they migrate and mature into adult worms, predominantly in the venous plexus of the bladder. These worms can then travel to the seminal vesicles and prostate, causing local pathology (*9*). *S. haematobium* eggs have been found in up to 43% of 44 semen samples and in 33.3% of cervix biopsies obtained from 36 women from endemic area populations (*4*,*10*); nevertheless, sexual transmission has not been reported.

T. vaginalis protozoa are the most common nonviral STI in the world, and incidence is increasing (*11*). The genital tract of humans is the natural habitat for this parasite, which can cause urogenital tract infection. *T. vaginalis* has been identified in seminal fluid and has been related to decreased sperm quality (*2*,*8*).

Chagas disease is caused by *T. cruzi* protozoa and affects nearly 6 million persons in Latin America countries.

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Table. Results of literature search for reports of parasites found in semen samples

Parasite (reference)	Method for parasite identification	Type of study	Evidence of sexual transmission	Evidence of causing infertility
Entamoeba hystolytica (*2*)	Microscopy	Case report	In humans	In humans
Schistosoma haematobium (*3*)	Microscopy	Cohort study	No evidence	In humans
Trichomonas vaginalis (*4*)	PCR	Cohort study	In humans	In humans
Trypanosoma cruzi (*5*)	PCR	Cohort study	Experimental mouse model	In animals
Toxoplasma gondii (*6*)	Microscopy	Cohort study	Experimental sheep model	In humans

Parasitic involvement of the male genital tract, alteration in semen characteristics (*7*), and the presence of the parasite's DNA in semen have recently been identified in chronically infected patients (*5*). Furthermore, an experimental mouse model has demonstrated infection through intravaginal infusion of semen from infected humans, posing the possibility of sexual transmission among humans (*5*).

Toxoplasmosis is a protozoan disease caused by *T. gondii* infection, with a worldwide prevalence from 20% to 80%. *T. gondii* has been found in the semen of infected men (*6*), and infection has been related to a decrease in semen quality (*8*). An experimental sheep model demonstrated infection after vaginal infusion of vegetative cysts of *T. gondii* (*7*).

Despite evidence of the presence of parasites in semen, the specific mechanism by which the parasite reaches the semen has not been clearly elucidated. The male genital tract can be invaded through a connection from the urethra to the testis or epididymis (*T. vaginalis, S. haematobium*), invasion from an adjacent structure (*E. hystolytica*), or as a result of a disseminated infection (*T. cruzi, T. gondii*) (*7*).

Other parasites, such as *Plasmodium* spp. and *Trypanosoma brucei*, can affect human spermatogenesis and impair fertility (*7*,*8*). *Leishmania* spp. have been detected in the testes in autopsies of patients who died of visceral leishmaniasis, and infection with these species has induced infertility (*8*). Although the presence of the *Leishmania*, *T. brucei*, and *Plasmodium* spp. in the male genital tract have been demonstrated, they have not been detected in human semen (*7*).

According to our review, it might be crucial to consider the sexual route of infection among parasitic diseases with seminal presence in addition to viral diseases previously identified (*1*). Infertility might be a consequence of parasitic infection regardless of its presence in seminal fluid (*8*).

These parasitic infections primarily affect vulnerable populations in developing countries. Many of these diseases are classified as neglected tropical diseases because of the scarcity of resources for their study; thus, potential sexual transmission of them is underresearched. The lack of available scientific information about the role of parasites in genital fluids leaves room for confusion about the relative importance of sexual activity as a route of transmission. Further studies are needed to implement better public health strategies.

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Introduction of the *Anopheles bancroftii* Mosquito, a Malaria Vector, into New Caledonia

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In June 2017, an *Anopheles* mosquito species was detected in New Caledonia. Morphologic identification and genomic sequencing revealed that the specimens tested belong to *An. bancroftii* genotype A1. This introduction underscores the risk for local malaria transmission and the vulnerability of New Caledonia to vector introduction.

New Caledonia, a French island in the southern Pacific Ocean, had been free of *Anopheles* mosquito species (*1*). The absence of all potential vectors of human *Plasmodium* spp. made New Caledonia free of malaria transmission. However, this situation is fragile because many neighboring countries (e.g., Papua New Guinea, Solomon Islands, and Vanuatu) are endemic for malaria and have highly competent malaria vectors (*2*) that could be introduced into New Caledonia. Entomologic surveillance conducted since 1979 has never found any *Anophelinae* mosquitoes in New Caledonia (*3*). In June 2017, however, a survey indicated the introduction of *An. bancroftii* mosquitoes into New Caledonia. This discovery prompted us to strengthen entomologic surveillance in the detection area to evaluate the situation.

During June 7–September 8, 2017, we trapped and identified 3,181 mosquitoes, including 27 *An. bancroftii* mosquitoes. All *An. bancroftii* mosquitoes were trapped in an area of 6 km^2, out of 50 km^2 covered by the survey. The first *An. bancroftii* specimen was trapped by using a Biogents Sentinel Trap (Biogents AG, Regensburg, Germany) at a plant nursery. Another specimen was detected 3 km away at the international airport, where traps designed at the US Centers for Disease Control and Prevention (CDC), equipped with UV light and CO_2, had been set. All other specimens were collected at the plant nursery by using CDC traps (14 females and 3 males) or the human landing catches technique (8 females). The collector (S.K.) noticed that, when he was in the shade, the *An. bancroftii* female mosquitoes were aggressive even during daytime (e.g., 2:00–4:00 PM). Despite all investigation efforts, no *Anopheles* mosquito larvae were found.

We used 2 morphologic identification keys to determine species (*4*,*5*). Both keys identified the individual specimens captured as belonging to the species *An. bancroftii*. The 2 species of the *bancroftii* group (*An. bancroftii* and *An. pseudobarbirostris*) can only be distinguished by pale patches on the wing fringe of the adult females. Because this morphologic criterion is controversial (*6*), we used 2 collected specimens to improve identification through sequencing. The primers we used distinguish between different genotypes in the *An. bancroftii* group by means of an internal transcribed spacer 2 gene sequence analysis (*7*), which indicated that both specimens collected in the nursery plant (GenBank accession nos. MF716525 and MF716526) match at 100% with *An. bancroftii* genotype A1 (GenBank accession no. AF203381; Figure). A 2001 study showed that *An. bancroftii* genotype A1 mosquitoes were found only in the Northern Territory of Australia, whereas other genotypes were found in Queensland, Australia, or in Papua New Guinea (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/3/17-1689-Techapp1.pdf) (*7*). This genetic analysis provides a small clue regarding the origin of the introduction of this species but does not indicate its route. This genotype might have been introduced in other countries during 2001–2017 before reaching New Caledonia.

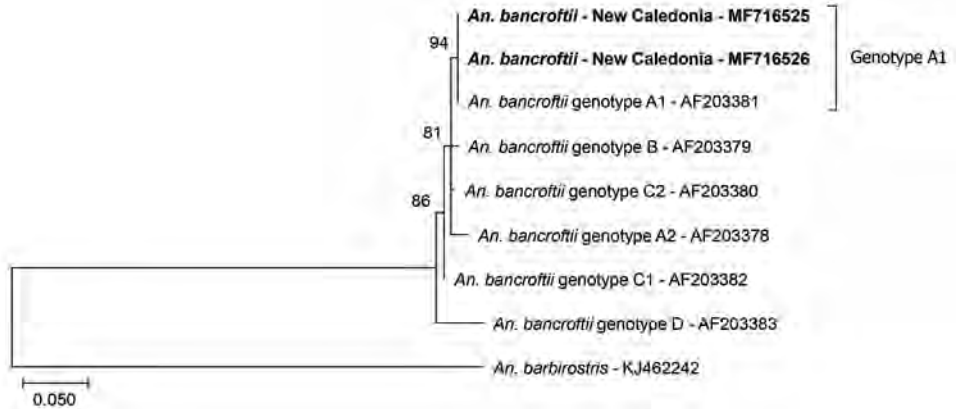

Figure. Phylogenetic analysis of *Anopheles* mosquito species introduced in New Caledonia. Phylogenetic trees were generated by the maximum-likelihood method based on the Kimura 2-parameter model using MEGA7 (http://www.megasoftware.net). Percentage bootstrap values shown at the nodes were calculated with 1,000 replicates. Bold indicates strains isolated in this study. Scale bar indicates nucleotide substitutions per site.

In Papua New Guinea, *An. bancroftii* mosquitoes are considered a secondary vector of malaria because of their weak anthropophilic feeding behavior and their small numbers in the regions studied. Furthermore, when investigated, the proportion of sporozoite-positive specimens was low (<1% in ELISA targeting circumsporozoite protein) (*8*,*9*). In the Northern Territory of Australia, where only genotype A1 is present (*7*), *An. bancroftii* mosquitoes are described as a major pest species because females readily and aggressively bite humans (*6*,*7*). This observation concurs with our preliminary observations made in New Caledonia, which might result in higher numbers of human–vector contacts than reported in Papua New Guinea. Considering that an average of 4 imported malaria cases were reported annually during 2000–2015 in New Caledonia (*10*), the introduction of a potential malaria vector in a new environment raises the specter of long-term local malaria transmission risk. In case of establishment, the risk for local transmission will be assessed by investigating the vector competence of the introduced genotype.

Although the bioecology of *An. bancroftii* mosquitoes remains poorly known in New Caledonia, the larval habitats have been previously described as floodplains and freshwater swamps (*6*). These ecosystems are abundant in the proximity of the plant nursery where adult mosquito specimens were initially detected. Although no larvae have been found, 27 adults were trapped during a period of 4 months, suggesting efficient colonization. Establishment of the species is a main concern, and sustainable entomologic surveillance might assist in the design and evaluation of an eradication plan.

In summary, we describe the introduction of *An. bancroftii* genotype A1 mosquitoes into New Caledonia, a territory previously known as free from *Anopheles* mosquito species. Although this species is not the most competent malaria vector, this sentinel event points to New Caledonia's vulnerability to the introduction of more competent vectors. Furthermore, because of its localization, its economic status, and the sea and air connections it shares with other Pacific islands, New Caledonia functions as a hub in the region. If *An. bancroftii* mosquitoes settle in New Caledonia, they could further spread to other *Anopheles*-free territories in the South Pacific.

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Fishborne Zoonotic Trematodes Transmitted by *Melanoides tuberculata* Snails, Peru

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We investigated the transmission of the fishborne trematodes *Centrocestus formosanus* and *Haplorchis pumilio* by *Melanoides tuberculata* snails in Peru. We report on results of experimental, morphological, and molecular approaches and discuss the potential risk for future human cases, given the existence of food habits in the country involving the ingestion of raw fish.

The World Health Organization has estimated that the number of humans infected with fishborne trematodes exceeds 18 million, and >500 million persons are at risk of infection (*1*). Among the causative agents of these trematodiases are representatives of the family Heterophyidae, which are small intestinal parasites from birds and mammals, including humans (*1*–*3*). Infection by heterophyids can be considered an emerging disease because of a set of factors, including high prevalence, reported mainly in Asia; outbreaks caused by *Ascocotyle longa* trematodes in Brazil; the introduction of *Centrocestus formosanus* trematodes and *Haplorchis pumilio* flukes from Asia into the Americas; and the involvement of larvae of *Procerovum varium* flukes as causative agents of human ocular disease (*3*–*5*).

Of the 30 species of heterophyids recognized worldwide (*4*), 7, including *C. formosanus* and *H. pumilio*, are transmitted by the red-rimmed melania or Malaysian trumpet snail, *Melanoides tuberculata* (*6*). Even though human infection by these 2 heterophyids has not been reported in the Americas, the possibility of future cases must be considered, especially in countries like Peru, whose inhabitants consume ceviche, a culinary dish prepared with raw fish. Thus, the evaluation of the involvement of *M. tuberculata* snails in the transmission of heterophyids in this country is needed given the potential public health concern related to these parasites.

In this study, we collected snails in 2 areas of the central coast of Peru, the Ventanilla Wetlands Regional Conservation Area (VWRCA) (11°52′31″S; 77°8′37″W) and the Pantanos de Villa Wildlife Refuge (PVWR) (12°12′33″S; 76°59′28″W), during December 2015 and January, June, and July 2016. Identification of the collected *M. tuberculata* snail specimens was based on conchiliological features according to previously published reports (*7*,*8*). We placed the specimens individually onto polystyrene plates containing dechlorinated water, subjected them to photostimulation, and examined them under a stereomicroscope. We examined cercariae, fluke larvae, in a light microscope after vital staining (0.05% Nile blue sulfate) and preliminarily identified 2 cercarial types, pleurolophocercous (Figure, panel A) and parapleurolophocercous (Figure, panel E). We used samples of the cercariae for experimental infection of vertebrate hosts to obtain other developmental stages for identification. Experiments were conducted in accordance with the local animal experimentation ethics committee (Comissão de Ética no Uso de Animais, Universidade Federal de Minas Gerais, protocol 20/2016).

We exposed 2 groups of *Poecilia reticulata* guppies (n = 30) individually to 50–100 cercariae of each larval type. We euthanized fish surviving at 30 days postinfection and collected metacercariae found in the gills (Figure, panels B, C) of fish infected with pleurolophocercous cercariae and in the bases of the fins (Figure, panels F and G) of fish exposed to parapleurolophocercous cercariae. We administered metacercariae orally to dexamethasone-immunosuppressed mice. Adult parasites recovered in the small intestines of mice at 6–7 days postinfection were fixed, stained, and mounted on permanent slides. We studied the morphology of the experimentally obtained stages using a light microscope for identification according to taxonomic works (*2*,*9*).

We used ethanol-fixed aliquots of cercarial types obtained in *M. tuberculata* snails for molecular characterization. We extracted DNA using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and amplified a fragment of the 28S rDNA by PCR using the primers Dig12 (forward) and 1500R (reverse) with PCR conditions as previously described (*10*). We purified the PCR products with 20% polyethylene glycol 8000 (Promega) and sequenced them in an ABI3730 automated sequencer using Pop-7 Polymer and the ABI BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). We edited the sequences we obtained using ChromasPro version 2.0.1 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia), compared them with data available in GenBank, and used them for phylogenetic analyses based on the maximum likelihood method using MEGA7 (http://www.megasoftware.net/) and Bayesian inference method using MrBayes 3.1.2 (http://mrbayes.sourceforge.net/). We deposited the obtained sequences in GenBank (accession nos. MG738251 and MG738252).

From the experimental infection of mice, we obtained adult parasites identified as *C. formosanus* (Figure, panel D) and *H. pumilio* (Figure, panel H). Molecular data revealed that the samples of *C. formosanus* and *H. pumilio* cercariae found in *M. tuberculata* snails from Peru are conspecific with isolates of these species from Vietnam and Thailand (99.8%–100% similarity), a finding supported by

phylogenetic analyses (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/24/3/17-2056-Techapp1.pdf). In total, we collected 6,731 *M. tuberculata* snails, of which 112 (1.66%) were found to be infected with heterophyid cercariae. We found *C. formosanus* cercariae in 71 (1.8%) of 3,874 snails collected in VWRCA and in 29 (1.0%) of 2,857 snails collected in PVWR. We found *H. pumilio* cercariae in 12 of 2,857 (0.4%) snails from PVWR.

The presence of *C. formosanus* and *H. pumilio* trematodes in *M. tuberculata* snails from Peru reveals the need to focus attention on the possible effect of these fishborne agents on human health. The increasing number of reports of these trematodes in the Americas indicates that the geographical areas they have invaded are expanding. This finding should serve as a warning, particularly given the popularity of dishes based on raw fish.

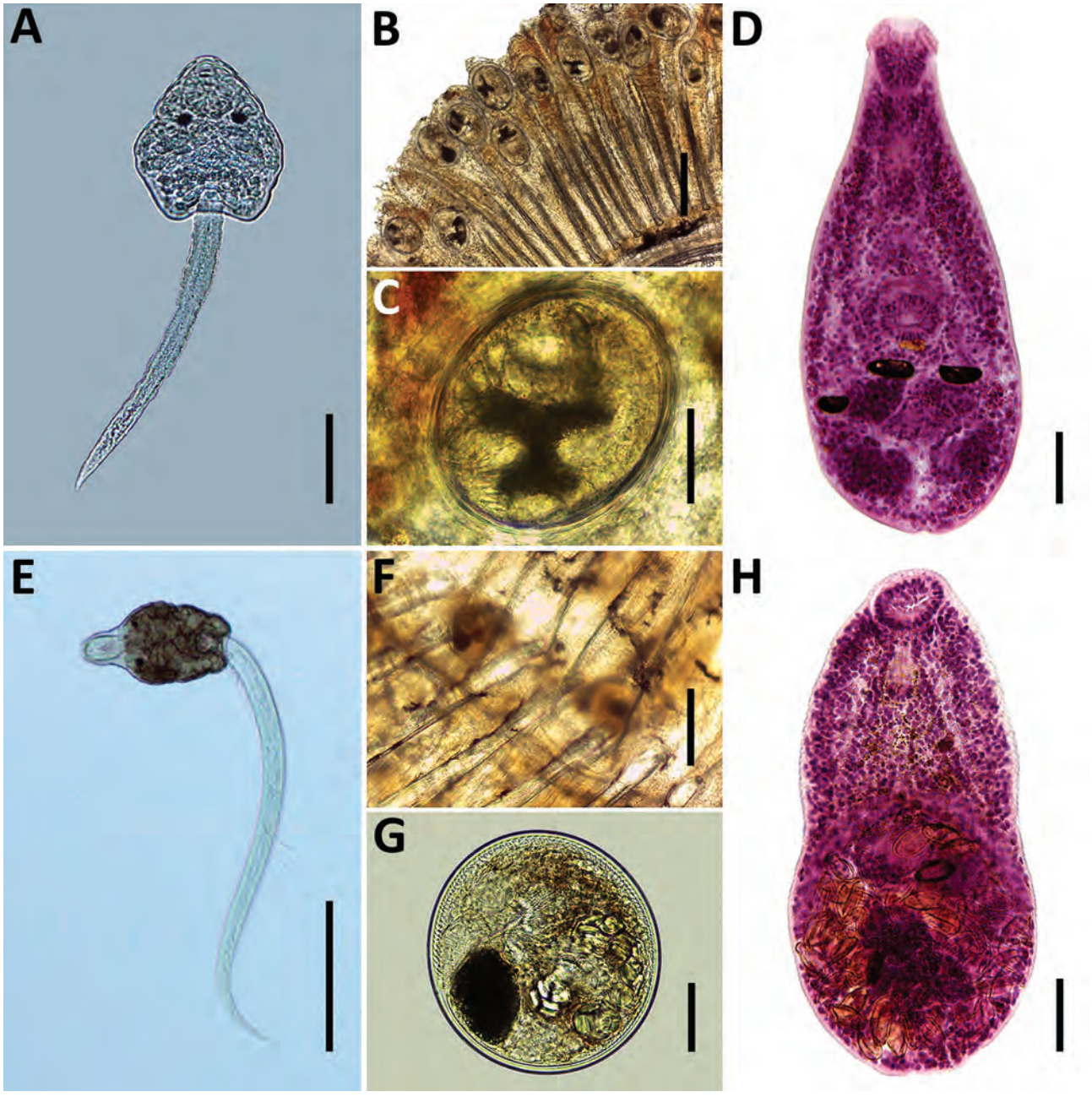

Figure. Species of heterophyids transmitted by *Melanoides tuberculata* snails in Peru. A–D) *Centrocestus formosanus*: cercaria (pleurolophocercous type) (A), encysted metacercariae in gills of *Poecilia reticulata* (B, C), and adult parasite obtained in experimentally infected mouse (D). E–H) *Haplorchis pumilio*: cercaria (parapleurolophocercous type) (E), metacercariae found at the base of the caudal fin of *P. reticulata* (F–G), and adult recovered in experimentally infected mouse (H). Scale bars indicate 50 μm in panels A, C, D, G, and H, 200 μm in panels B, E, and F.

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LETTER

Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Canada, 2017

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DOI: http://dx.doi.org/10.3201/eid2403.171892

To the Editor: I read with great interest the report by Lefebvre et al. about a *Neisseria gonorrhoeae* isolate identified in Canada demonstrating a ceftriaxone MIC of 1 mg/L (*1*). The authors note: "As of October 15, 2017, only 5 ceftriaxone-resistant *Neisseria gonorrhoeae* isolates had been reported worldwide (MIC range 0.5–2 mg/L)." The authors cite published reports from Spain, Japan, Australia, and France.

I would like to clarify that additional *N. gonorrhoeae* isolates have been identified with ceftriaxone MICs ≥0.5 mg/L. Since 1987, as part of the Gonococcal Isolate Surveillance Project, the Centers for Disease Control and Prevention has been testing *N. gonorrhoeae* isolates for ceftriaxone susceptibility. During 1987–2016, the Centers for Disease Control and Prevention identified and reported 5 isolates with ceftriaxone MICs of 0.5 mg/L in the United States. These isolates were found in San Diego, California (1987); Cincinnati, Ohio (1992 and 1993); Philadelphia, Pennsylvania (1997); and most recently, Oklahoma City, Oklahoma (2012) (*2*). Therefore, although the number of *N. gonorrhoeae* isolates with ceftriaxone MICs ≥0.5 mg/L identified globally to date has been small, these Gonococcal Isolate Surveillance Project findings should be acknowledged. Continued and enhanced global surveillance of gonococcal isolates for antimicrobial susceptibility testing is imperative.

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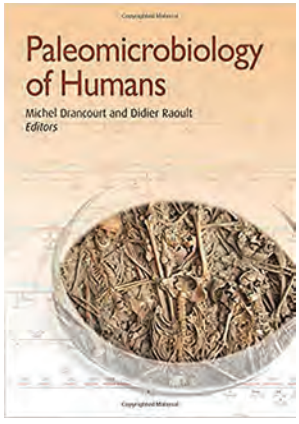
Paleomicrobiology of Humans

Michel Drancourt and Didier Raoult; ASM Press, Washington, DC, USA; ISBN: 978-1555819163; Pages: 212; Price: $90.00



We study history in part to avoid repeating it. This fascinating book reveals our past and informs our future. Each chapter is like an episode in a televised season of the kind of crime procedural drama that has become wildly popular with the general public, although here each story is both dramatic and factual, and should appeal to both generalists and specialists.

Early in this book, we learn how to assess archeological remains, what source materials to target (environmental samples, tissues, bone, teeth, coprolites), and historical and modern ways to investigate them. Common themes emerge: the weakness of negative results (perhaps the "right" site or sample has yet to be found or analyzed); the importance of negative controls and other standard practices; the types of damage experienced by biomolecules over time (*1*); the increasing role of molecular methods; and the continued need for improving them.

Later chapters focus mostly on specific infectious diseases, with some exceptions. High points of the book include evolution of the human immune system and its relationship to archaic humans, ancient antibiotic resistance, and an integrative tale of paleopathology (this one might fit better earlier in the book). The tuberculosis narrative is nicely coupled to the challenges of ancient samples and modern molecular methods. The integrative summary of leprosy raises important questions: Why can't we culture its causative mycobacterial agents? How will refinement of molecular methods contribute to present-day treatment? One also gets the sense that despite the sample preparation challenges, such methods also have much to contribute to paleoparasitology and present-day eradication efforts. Recent success with antibody-based and nucleic acid (including metagenomic) approaches are mentioned in several chapters, including in the discussion of malaria; these methods complement skeletal pathophysiology, which can be nonspecific.

Then, we move on to discover the roles of trade, slavery, and war in transmission of smallpox. Lingering questions remain: despite great successes, are we at risk for future outbreaks from a pathogen's relatives? What surveillance is needed? Late in the book, we encounter cholera and the famous John Snow. What will be the impact of climate change, given the linkage between natural disasters and recent outbreaks and epidemics? The final story, about lice, again highlights the importance of molecular methods and their use to infer human history and migrations.

Minor points: In one chapter, figure text is in French, and Raoult's story of lice in plague pandemics is welcomingly personal. The story of Bartonelloses could benefit from more detail on pathogenesis, transmission, and modern risk. And room remains for episodes about influenza and our co-evolutionary history with *Helicobacter pylori*. I also hungered for a final, even if hazy, canvas of the future.

Perhaps next season will go deeper into degradation of biomolecules, nascent molecular methods such as single-molecule or single-cell sequencing and immuno-PCR, and the future of paleomicrobiology. How can we avert the next major infectious disease pandemic? Perhaps with improved global surveillance, as stated by Gardy and Loman (*2*), better informed by paleomicrobiological knowledge. What better place to start than with this book?

Dr. Carr is a research scientist at the Massachusetts Institute of Technology and a research fellow at Massachusetts General Hospital in Boston. His research interests include application of new technologies to biological discovery on Earth and beyond.

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Correction: Vol. 24, No. 1

Figure 4 and part of the legend for Figure 1 were incorrect in Detection and Circulation of a Novel Rabbit Hemorrhagic Disease Virus in Australia (J.E. Maharet al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/24/1/17-0412_article).

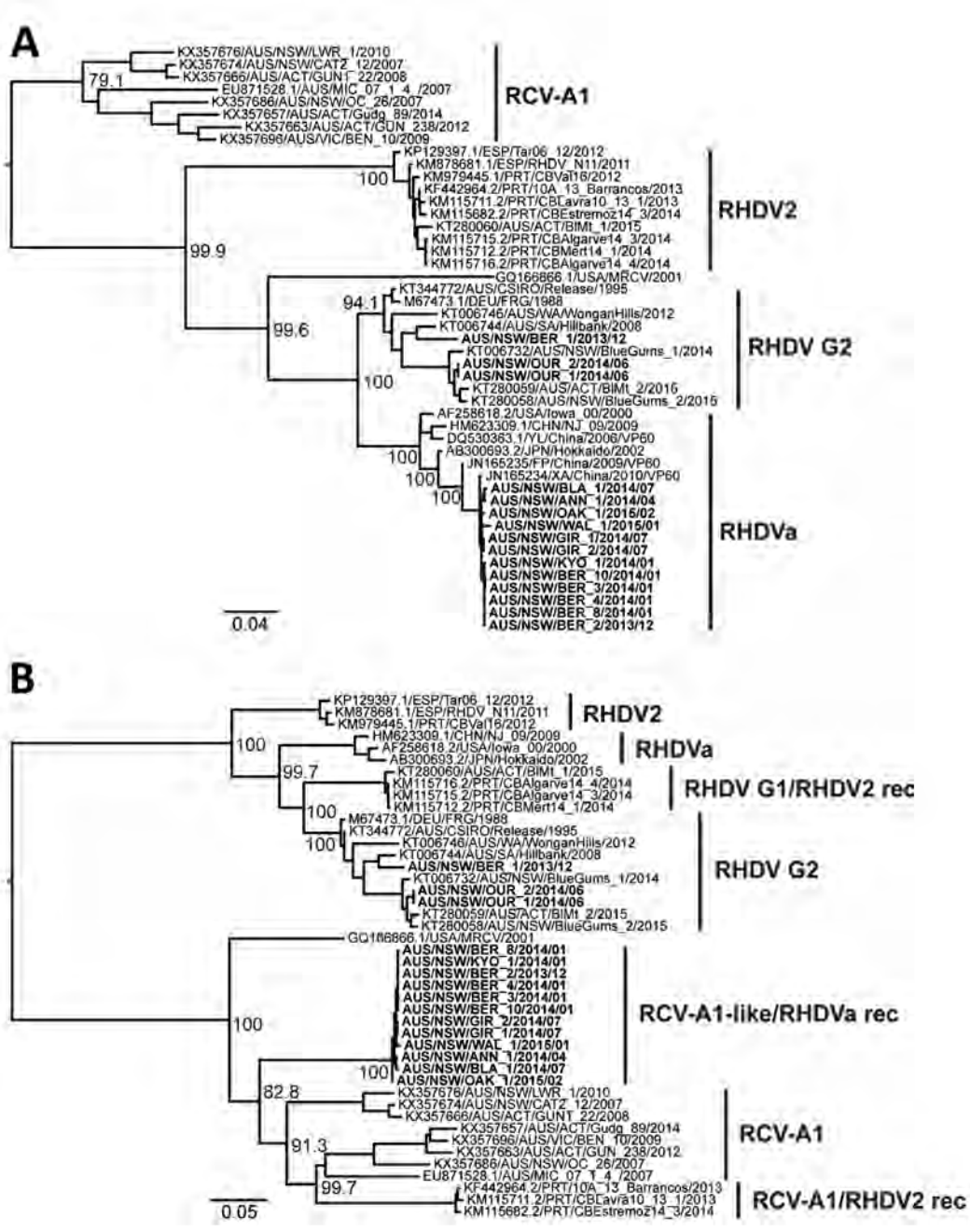

Figure 4. Phylogenetic analysis of viral protein 60 (VP60) capsid (n = 47) and nonstructural (n = 44) and VP60 capsid (n = 47) genes of RHDV strains from Australia and reference sequences. Maximum likelihood phylogenies of the A) VP60 capsid genes and B) nonstructural genes were prepared from an alignment of the newly sequenced RHDV samples (bold) along with published sequences (accession numbers of published sequences indicated in the taxa name). The JN165235/FP/China/2009 and JN165234/XA/China/2010 sequences were restricted to the capsid gene tree because nonstructural gene sequences are not available for these viruses. Variant names for each cluster are indicated. Recombinant (rec) variants are labeled as nonstructural/capsid gene type. Phylogenies were rooted using an early European brown hare syndrome virus isolate (not shown). Bootstrap support values are shown for the major nodes. Scale bars indicate nucleotide substitutions per site. RCV, rabbit calicivirus; RHDV, rabbit hemorrhagic disease virus.

Antonio de Francisci (1887–1964). US Silver Peace Dollar, 1935. Silver (90%), copper (10%), 1.5 in/38.1 mm. Photography by James Gathany.

Peace, Liberty, Mycobacteria, and Tuberculosis Mortality

Terence Chorba

In 1935, Gerhard Domagk and Josef Klarer, working with dyes at the Bayer Institute of Pathology and Bacteriology, published the results of several clinical investigations of sulfamidochrysoidine. This antibacterial drug was the first of the sulfonamide-containing or related products that transformed approaches to treatment of infection and heralded the antibiotic era. Before that, the only antimicrobials available were the arsenicals (arsphenamine and neosalvarsan), which were used to treat syphilis. Sulfamidochrysoidine, produced under the trademark name of Prontosil, was demonstrated to be effective for treating streptococcal and staphylococcal infections. In 1939, Domagk was recognized with the Nobel Prize in Physiology or Medicine; although the Third Reich forced him to decline the award, he subsequently received it in 1947.

The year 1935 also was the final one in which a US dollar made predominantly of silver (silver dollar) was minted for general circulation. The coin was first issued in 1921. Its design earned it the name "Peace Dollar"; its reverse side portrays an American bald eagle with an olive branch in its talons, intended to celebrate the long-lasting peace that was to have followed the Great War, World War I. The bird is at rest, its wings folded, perched on a craggy rock above the word "Peace," facing the dawn's rays rising beyond distant hills.

The front side of the coin depicts the profile of the head and neck of the goddess Liberty, a personification who had been represented on coins in ancient Greece as Eleutheria and in ancient Rome as Libertas. A departure from previous and more staid US representations of Liberty, the Peace Dollar goddess has hair pulled into a bun with stray locks loose and flowing, and wears a radiant tiara, likened to the sun god, Helios, as seen in depictions of rulers on coinage back to the third century BCE.

From the coin's outset, the identity of the Liberty model was disputed. Some experts contended that the model was Maria Teresa Carafelli, wife of the coin's designer, Antonio de Francisci, who had immigrated to America in 1905 and studied under several sculptors known for their designs of US coins of other denominations: James Fraser (the Buffalo Nickel, 1913–1935), Hermon MacNeil (the Standing Liberty Quarter, 1916–1930), Adolph Weinman

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(the Mercury Dime, 1916–1945), and Augustus Saint-Gaudens (the $20 Double Eagle, 1907–1933). However, de Francisci maintained that the visage was a "composite" face that "typified something of America." In her later years, Carafelli held that she was not the model for Liberty but "just an accessory."

The other rumored model for Liberty was a British-born actress, Maryland Morne, who was reputed to have won a contest for the job. Coincidentally, Morne died of tuberculosis in the same year that the minting of the Peace Dollar ceased; in 1935, newspaper notices of her death and funeral unequivocally declared her the "Peace Dollar Girl." Unfortunately, the newly discovered antimicrobial activity of sulfamidochrysoidine included little if any activity against *Mycobacterium tuberculosis.* It was not until 1944 that Albert Schatz, Elizabeth Bugie, and Selman Waksman identified streptomycin produced by *Streptomyces griseus* as the first antimicrobial with bactericidal activity against mycobacteria.

The understanding that neither poverty nor genetics was the primary cause of tuberculosis came after the discovery of the transmissible organism, *M. tuberculosis,* announced by Robert Koch, on March 24, 1882; for this discovery, he was duly recognized with the Noble Prize for Physiology or Medicine in 1905. In 1900, the reported US mortality rate from tuberculosis was in excess of 200 deaths per 100,000 population. Through the years that the Peace Dollar was minted, recognition of an infectious etiology contributed greatly to decreases in tuberculosis morbidity and mortality in the United States, even in the absence of antimicrobial drugs, as attention focused on containment and exclusion of persons symptomatic with disease from close contact with others. From the beginning of Peace Dollar coinage in 1921 to its end in 1935, the US mortality rates from tuberculosis decreased from 99.4 deaths per 100,000 population to 55.0 deaths per 100,000 population. Today, the US mortality rate for persons for whom tuberculosis was indicated as a cause of death is <0.1 deaths per 100,000 population, owing largely to understanding the need for respiratory precautions to disrupt transmission but owing also to appropriate use of antimicrobial drugs to treat active disease and latent infection, and to contact investigation and screening of persons in high-risk populations.

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Article Title

Epidemiology of Recurrent Hand, Foot and Mouth Disease, China, 2008–2015

CME Questions

1. Your patient is a 2-year-old Asian boy with hand-foot-and-mouth disease (HFMD). According to the surveillance study by Huang and colleagues, which of the following statements about the epidemiologic features of recurrent HFMD, using national surveillance data during 2008–2015 in mainland China, is correct?

A. Median time from primary episode to first recurrence was 24 months
B. Probability of HFMD recurrence was 1.9% at 12 months, 3.3% at 24 months, and 3.9% at 36 months, remaining stable at 4.0% at 38.8 months after the primary episode
C. The severity of HFMD was associated with probability of recurrence and with time intervals between HFMD episodes
D. Seasonal pattern was considerably different for patients with recurrent laboratory-confirmed HFMD than for those with only 1 episode of HFMD

2. According to the surveillance study by Huang and colleagues, which of the following statements about the virologic features of recurrent HFMD, using national surveillance data during 2008–2015 in mainland China, is correct?

A. Each episode of recurrent HFMD was more likely to be caused by the identical enterovirus serotype to that causing the primary episode
B. Patients with reinfection by different enterovirus serotypes differed in demographic factors from patients who were reinfected by the identical enterovirus serotype as in their primary episode
C. Of 1767 patients with laboratory-confirmed recurrence of HFMD, there were 99 with EV-A71 reinfections, 45 with CV-A16 reinfections, and 364 with other enterovirus reinfections
D. CV-A16 infections were more severe than EV-A71 infections and other EV infections

3. According to the surveillance study by Huang and colleagues, which of the following statements about the clinical implications of the epidemiologic and virologic features of recurrent HFMD, using national surveillance data during 2008–2015 in mainland China, is correct?

A. Only 1 monovalent EV-A71 vaccine has been administered in China
B. Children who receive EV-A71 vaccine are protected against HFMD
C. The burden of HFMD recurrence was likely overestimated in this study
D. To protect against an HFMD epidemic, multivalent vaccines are needed, such as EV-A71 combined with CV-A16, CV-A6, and other prevalent circulating viruses causing H

Visit www.cdc.gov/vaccines/parents. Learn about the vaccines your baby needs from a reliable source. The CDC's website explains the 14 diseases vaccines prevent, CDC's recommended schedule, possible side effects, how to comfort your baby during vaccine visits and more. Talk to your child's doctor, and visit our website to get the facts about vaccines.

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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.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. 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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