EMERGING INFECTIOUS DISEASES®

November 2012

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EMERGING INFECTIOUS DISEASES®

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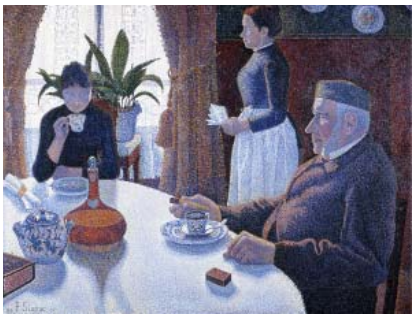

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Paul Signac (1863–1935)
La salle à manger, Breakfast (1886–1887)
Oil on canvas
(89.5 cm × 116.5 cm)

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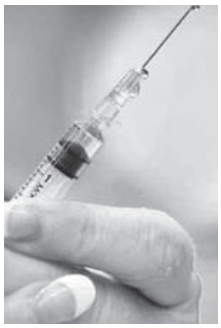

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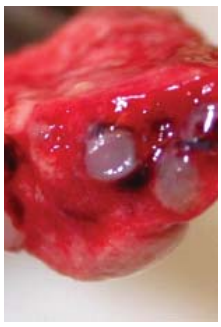

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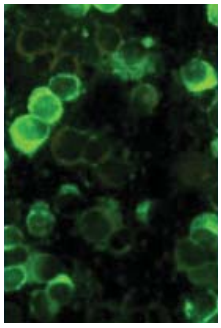

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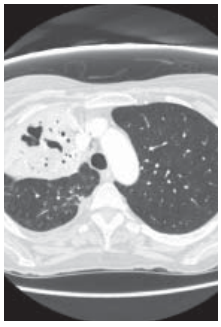

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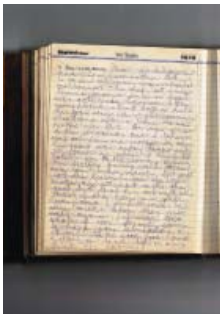

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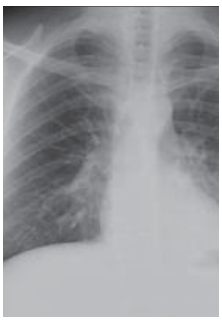

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Investigation of Outbreaks Complicated by Universal Exposure

Alma Tostmann, Teun Bousema, and Isabel Oliver

Outbreaks in which most or all persons were exposed to the same suspected source of infection, so-called universal exposure, are common. They represent a challenge for public health specialists because conducting analytical studies in such investigations is complicated by the absence of a nonexposed group. We describe different strategies that can support investigations of outbreaks with universal exposure. The value of descriptive epidemiology, extensive environmental investigation, and the hypothesis-generation phase cannot be overemphasized. An exposure that seems universal may in fact not be universal when additional aspects of the exposure are taken into account. Each exposure has unique characteristics that may not be captured when investigators rely on the tools readily at hand, such as standard questionnaires. We therefore encourage field epidemiologists to be creative and consider the use of alternative data sources or original techniques in their investigations of outbreaks with universal exposure.

Infectious disease outbreaks in which most or all persons are exposed to the same potential source of infection, so-called universal exposure, are common. They can occur in a range of settings (e.g., healthcare, community) and involve various organisms and vehicles. When outbreaks with universal exposure are investigated, it is difficult to explore the possible association between illness and that specific exposure by using conventional epidemiologic and environmental approaches.

Universal exposure complicates the analysis of epidemiologic studies because of the absence of a nonexposed group, which makes it difficult to test the hypotheses under investigation. This article provides an overview of options for outbreak investigations in which the exposure to the suspected source of infection is universal. We conducted a thorough literature search to collect articles on outbreak investigations in which analytical techniques were used that could be helpful in a situation of universal exposure. In addition, the European Programme for Intervention Epidemiology Training (EPIET) network and the European Programme for Intervention Epidemiology Training Alumni Network were asked to provide published or unpublished outbreak reports of outbreaks with universal exposure

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Outbreaks with Universal Exposure

Biological plausibility of the suspected source is an essential link in the chain of evidence. In a recent outbreak of campylobacteriosis after a wedding dinner in southwestern England, all guests had eaten the same appetizer, chicken liver parfait. This item was the suspected source of the outbreak. The increase in campylobacter outbreaks linked to poultry liver parfait in the United Kingdom appears to be associated with intentional undercooking of the poultry livers (*1*). In this outbreak investigation, we were unable to compare the consumption of this appetizer between case-patients and non–case-patients because exposure was universal. Apparently, almost all guests had completely finished the single portion they were given, and we could not investigate a dose-response association. No food sample was available for microbiologic testing, which further reduced the options to provide evidence for this biologically plausible source of the campylobacter outbreak.

In a salmonellosis outbreak in southwestern England in 2011, illness was linked to the consumption of pork meat served at a hog roast (barbecued pig) in a small town. Eight laboratory-confirmed cases of infection with *Salmonella enterica* serotype Typhimurium of the same genetic subtype were identified through routine surveillance. All case-patients had visited the town on the same day, and an epidemiologic study was conducted to test several hy-

potheses about the potential source of the outbreak. However, almost all persons interviewed had consumed pork from the hog roast, making it impossible to compare pork consumption of case-patients and noninfected persons. The circumstantial evidence pointed toward pork meat being the source of the outbreak because pork can be a source of *S. enterica* ser. Typhimurium (*2*). It was the only common exposure among the case-patients with confirmed salmonellosis, none of the other potential risk factors (including other foods or drinks consumed in the village on that day) were linked to illness, and no other common exposures were identified. Unfortunately, no leftover pork was available for microbiologic testing.

Options for Investigations Involving Universal Exposure

Many public health specialists will recognize outbreak situations such as those described above: a hypothesis based on biological plausibility cannot be confirmed because of the absence of microbiologic evidence and the difficulties of obtaining strong epidemiologic evidence as a result of universal exposure. These situations require creativity to maximize the information that is available from the exposure.

A key step in any outbreak investigation is the hypothesis-generation phase (*3*). In-depth interviews with the first case-patients and other stakeholders involved are essential to collect sufficient background information to generate the hypotheses. In this phase, whether universal exposure will potentially be an issue may become evident. This gives the investigators the opportunity to decide what additional aspects of the exposure could be explored in the analytical study. It is tempting, but limiting, to rely only on standard techniques and trawling questionnaires (series of open questions covering activities before onset of illness) because each outbreak is characterized by unique exposure aspects that may not be captured when one relies exclusively on the tools that are readily at hand.

Possible approaches to investigating outbreaks complicated by universal exposure include robust descriptive epidemiology, optimization of the dose-response analysis, and in-depth exposure analysis to determine whether the alleged universal exposure is indeed uniform exposure. Exposures that seem universal initially may in fact not be universal on in-depth analysis of additional aspects of the exposure. Most techniques described in this article were used in outbreaks not complicated by universal exposure, but they can be applied to outbreaks with universal exposure.

Strong, Descriptive Epidemiology

Well-presented descriptive epidemiologic characteristics can provide sufficient evidence for a suspected source in an outbreak investigation. Outbreak-associated cases are usually described by time, place, and person. Maximizing the information that is available by analyzing different aspects of time, investigating strong geographic clustering, or focusing on the odd, but highly informative, outliers can help build evidence toward the suspected source of infection.

Time: Exposure at Peak Times

The time of eating may be a relevant aspect of exposure in outbreak investigations in which cooking procedures differ over time. For example, at a barbecue, the meat that was served in the beginning, at the end, or at the peak time of preparation may have been undercooked. This variation can also occur at a dinner in which several servings of the same dishes were served consecutively. The data analysis can be stratified by comparing those who ate early with those who ate later or by time of the servings. A difference in attack rates between these subgroups can be an indication for the exposure as source of the outbreak.

In addition, high sales pressure at peak times can potentially result in the sale of undercooked meat products. In 2009, an outbreak of *S. enterica* ser. Enteritidis was linked to a kebab shop in West London (*4*). Because dishes were consumed simultaneously, associations between various food items and illness could not be disentangled. The investigators used till receipts to identify the peak times at the food outlet and found that customers who had consumed chicken kebab during peak times were more likely to becomet ill. A plausible explanation is that during peak times the rotisserie chicken was undercooked.

Place: Strong Geographic Clustering

Geographic clustering can be informative in identifying the source of an outbreak and can be established by mapping case-patients by place of residence. The cholera outbreak investigations of Dr John Snow hold some practical examples of field epidemiology that are still being used today. The strong geographic clustering of cholera cases in central London in 1854 implicated the Broad Street pump as a suspected source of infection (*5*).

In waterborne outbreaks, the pathogen may not always be isolated from the water, because contamination may have ended when the investigation starts. There may still be ways to collect water samples from the time of the outbreak (e.g., ice cubes, looking for water dead ends such as fire hydrants), but generally such investigations rely heavily on epidemiologic evidence rather than confirmation by microbiologic testing.

Tap water is a typical source of universal exposure. However, drinking water is usually distributed in different supply zones, with the result that the exposure is not necessarily universal. After a large outbreak of *Giardia* infections with 1,300 laboratory-confirmed cases in Norway in

2004, geographic clustering of cases in 1 water distribution area pointed toward the water supply serving Bergen city center as the potential source. The attack rate was »18 times higher in this supply zone than the rate in the other supply zones combined (*6*). In addition, within a supply zone, the distance from the suspected point of pathogen introduction can be associated with decreasing attack rates. Thus, even if all study participants reside in the same water supply zone, geographic location within the zone could be used to calculate distance attack rates.

Distance attack rates have been applied to an outbreak of *Coxiella burnetii* infections in the Netherlands. Living near ruminant farms is a risk factor for infection with *C. burnetii*, the causative agent of Q fever. In May 2008, 96 cases of Q fever occurred in a small urban area in the Netherlands, and a common source was suspected (*7*). Several ruminant farms were located in the area, and a geographic information system was used for the investigation, taking into account the postal codes of the patients and the farms. Distance-related attack rates and relative risks for increasingly larger ring buffers were calculated for each of the large farms in that area, highlighting the usefulness of spatial analysis in analyzing a cluster of cases.

Another example is that if an outbreak is confined to a single geographic location and 2 products are suspected as sources of the outbreak, one with a national and one with a local distribution, the product with the local distribution could be considered the most likely source. Also, when nearly universal exposure to a common food item (such as chicken) is presumed, more heterogeneous exposures may be defined if exposure is defined on the basis of the origin of the food item, e.g., the different suppliers or processors.

Person: Focusing on Outliers

The historical example of the cholera outbreak in London not only illustrates the value of spatial analysis, but also the value of outliers in an outbreak investigation. Dr Snow found that nearly all persons who died of cholera lived within a short distance of the Broad Street pump, except for 2 patients who lived at the other end of town, an elderly woman who had not been near Broad Street for many months and her niece. It appeared that the woman had a bottle of Broad Street pump water delivered to her doorstep each day, and this had occurred on the day before she fell ill. The niece drank from the same water when visiting her aunt (*5*).

Early in 2011, 14 persons from southern France, with laboratory-confirmed cases of *S. enterica* ser. Enteritidis infection, had eaten from a common lunch at a hunting party. A cohort study among party attendees showed that the only food item that could explain all cases was wild boar meat. However, almost all of the 50 study participants had consumed wild boar meat. The outliers in this study were 5 participants who had only consumed leftovers: 4 became ill and they all had eaten boar meat. The person who did not fall ill ate only blood pudding. Despite of the absence of a nonexposed group in the analysis of wild boar meat consumption, the results strongly suggested that the wild boar meat was the source of infection (*8*).

Another example of the potential role of the outlier is illustrated by the investigation of an unusual and large outbreak of hemolytic uremic syndrome (HUS) and diarrhea caused by Shiga toxin–producing *Escherichia coli* (STEC) O104:H4, which occurred in Germany in 2011 (*9*,*10*). A satellite outbreak in France, which could be considered as an outlier of the large outbreak in Germany, provided the investigation with central clues that led to the identification of fenugreek sprouts as the vehicle of this outbreak. In June 2011, an outbreak of HUS associated with STEC O104:H4 occurred in Bordeaux, France, and the *E. coli* isolates were genetically related to the strain found in the German outbreak (*11*,*12*). This was the first outbreak outside Germany in which infection was not related to travel to Germany. Preliminary investigations determined that case-patients had attended an open day at a community center where a cold buffet with vegetables was served. Because sprouts were already suspected as a vehicle in the German outbreak, the French investigators examined sprout consumption in detail. Three types of sprouts were served at the buffet: mustard sprouts, rocket sprouts, and fenugreek sprouts. In the analysis, sprouts were identified as a risk factor for illness, and multivariate analysis showed that the fenugreek sprouts were the most likely vehicle for transmission.

Optimizing Dose-Response Analysis

Assessing a dose-response relationship is a well-known analytical tool for identifying the source of an outbreak. An increased risk for disease or increased severity of symptoms with increased exposure dose indicates such a dose-response relation between the suspected source and the illness. Normally, the reference category in a dose-response analysis is the unexposed group, but in outbreaks with universal exposure, the lowest exposure category can be used as a reference group instead.

Exposure levels should be considered in the design phase of the analytical study to ensure that dose response can be evaluated in the analysis stage. Questions about quantities can aim at either the exact quantity (e.g., the number of glasses tap water per day, the number of canapés eaten at a wedding reception) or at more qualitative levels of exposure (e.g., a bite, half a portion, 1 portion, >1 portion). How best to assess dose depends on the type of food investigated and the circumstances under which it was consumed.

In cohort studies, attack rates and relative risks can be calculated for each level of exposure. In case-control stud-

ies, the proportion of persons exposed to each level of exposure can be calculated for case-patients and controls and odds ratios calculated for each level of exposure. The group with the lowest exposure will serve as reference group. An increase in attack rate (cohort study) and effect size (risk ratio or odds ratio) with increasing dose, supported by a statistical test for trend, implicates a dose-response relation.

Two examples illustrate investigations of outbreaks linked to tap water in which the reference group consisted of those with the lowest exposure rather than an unexposed group. An outbreak of campylobacteriosis in Norway was associated with consumption of tap water (*13*). A cohort study showed that the attack rate increased from 40% for those who had drunk 1–3 glasses per day (the reference group) to ≈65% for those who had drunk >6 glasses of tap water per day. In the previously mentioned outbreak of giardiasis in Bergen, Norway, a case–control study showed that persons who drank the largest quantity of water had the highest risk for illness; the strength of the association (odds ratio) increased with increased water consumption (*6*). Assessing a dose-response relationship may be more complicated if the exposure cannot be readily quantified or if the vehicle is heavily contaminated.

Exploring Individual Components of Exposure: Ingredient-based Analysis

The recent large outbreak of STEC in Germany demonstrated that hidden or marginal ingredients such as bean sprouts, sometimes solely used for decoration of a dish, can cause large outbreaks (*9*,*10*). Even though this outbreak was not complicated by universal exposure, it demonstrates valuable lessons for field epidemiologists confronted with universal exposure. It can be highly informative to explore the individual components of an exposure, for example, the actual ingredients of a specific dish as reported by those cooking it rather than the dish or products consumed as reported by the consumer, who may be unaware of the less obvious ingredients.

Although investigation of a foodborne outbreak may reveal that all persons have eaten the same dish, they may not have eaten all ingredients from their plates. An appetizer may include some edible decoration, such as bean sprouts, that some will put aside, a salad may contain different ingredients that not all like to eat, or a seafood cocktail may hold different species from which a person may select only a few. A thorough environmental investigation is therefore essential.

The investigation of the STEC outbreak in Germany consisted of several studies that followed up on each of the findings. Since the earlier studies had not identified a single source of infection, the outbreak control team conducted a cohort study at a restaurant because several case-patients had dined at that restaurant during May 12–16, 2011, as part of a group (*9*,*10*). A review of the booking notes identified 10 groups (total 177 persons) that had eaten in the restaurant during that period. Thirty-one of the 168 customers who were interviewed had become ill, and HUS developed in 8 persons. When the role of the separate ingredients used for each of the dishes was investigated, sprouts were the only food item independently associated with illness.

An outbreak of gastrointestinal illness at a conference in Wales, United Kingdom, in 1991 was associated with the seafood cocktail, which contained precooked mussels, prawns, and cockles (*14*). Although consumption of the cocktail was nearly universal, only three fourths of those who ate the cocktail had eaten the mussels, the only component that was independently associated with illness.

A combination of ingredient based and dose-response analysis established the relation between exposure of methomyl-contaminated salt, and gastrointestinal illness in a Thai restaurant in the United States in 1999; an ultimate example of universal exposure because all restaurant clients had consumed some salt as a consequence of the preparation process (*15*). Because no specific dish could account for most cases, the association with specific ingredients was investigated. The exposure to salt was estimated by using the amount of salt in the recipe for each dish, the quantity that was consumed in each dish, and the quantity of salt that was added by respondents after the meal had been served. When the ingredient was quantified is this manner, the risk for illness was convincingly associated with the total quantify of salt consumed.

In a massive multistate salmonellosis outbreak (≈1,500 cases), the most likely source was red salsa made from tomatoes and raw jalapeño peppers (*16*). Two clusters of outbreak-associated cases (patrons from 2 different restaurants) were investigated. To produce the salsa served during the outbreak period, 1 restaurant used ≈25 boxes of tomatoes of various brands, whereas only 1 box of jalapeño peppers was used. When exposure to ingredients (tomatoes, jalapeño peppers), rather than exposure to the food product (salsa), was considered, the jalapeño peppers were the only common exposure between the 2 restaurants; contaminated peppers were eventually traced back to a single farm in Mexico. In this example, the combination of ingredient-based analysis and geographic aspects of the suspected source (e.g., the origin of the product, distribution area, packing facilities) provided key information in the source-finding process.

These outbreak investigations show that individual components of a mixed dish can be identified as the vehicle of infection. Scrutinizing associations with individual ingredients may reveal that exposure that appears universal is, in fact, heterogeneous. Caveats of ingredient-based analysis include the possibility of cross-contamination between different ingredients and that persons may have

difficulties in recalling what ingredients they have consumed. These possibilities can be overcome by talking to the cook or food producers.

Use of Different Data Sources to Analyze Exposure

Alternative sources of information can be used to support or refute possible hypothesis generated by using standard outbreak investigation methods. Examples include the use of till receipts (*4*) or meteorologic data (*7*,*17*).

In the Q fever outbreak investigation in the Netherlands described previously, the team used meteorological data to further support their hypothesis (*7*). The geographic and veterinary data pointed toward a large dairy goat farm that was located northeast from the town. Meteorologic data gave further support because it showed that there were many days with predominant easterly winds that could have taken contaminated dust particles to the persons living southwest of the farm.

In an outbreak of Q fever in a town in southeastern England, atmospheric dispersion modeling (using meteorologic data, including ground wind speeds and direction) was used to investigate potential airborne transport of *C. burnetii* between several suspected sources and the Q fever case-patients in that town (*17*). Three high-risk farms were identified by the veterinary investigation. Dispersion modeling showed that air from each of these suspected farms may have exposed the town to the bacteria at some point over the study period and none of the suspected farms could be ruled out as a potential source. Nevertheless, this approach shows the potential of using nonconventional data sources in outbreak epidemiology.

Discussion

Outbreaks with universal exposure represent a challenge for public health specialists because conventional investigational approaches may be insufficient to reach informative conclusions. Robust descriptive epidemiology, a thorough environmental investigation, and the hypothesis-generation phase become even more essential. In-depth interviews with the initial patients can be highly informative in determining whether a risk for universal exposure exists. If universal exposure is suspected, additional aspects of that exposure should be explored because exposures that initially seem universal may in fact not be universal after all when studied in more detail.

Although a balance must be found between the quality and quantity of questions in a questionnaire, once a hypothesis is generated on the basis of initial interviews, more detailed information about the exposure to likely sources, such as time and quantity of exposure, can provide useful additional information to support or refute the hypothesis. Universal exposures are often ignored if another item is significantly associated with illness. Investigators need to at least consider the universal exposures before accepting a statistically significant association. It remains, however, essential to rule out other potential exposures before accepting that the universal exposure was the cause of the outbreak. Because of the difficulty in obtaining conclusive findings, studies of outbreak investigations complicated by universal exposure are less likely to be published and less likely to result in recommendations for public health measures to prevent continuation or recurrence of exposure. We hope that this article provides readers with several tools to overcome this problem and maximize the effect of outbreak investigations on public health.

Acknowledgments

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Coccidioidomycosis-associated Deaths, United States, 1990–2008

Jennifer Y. Huang,[1] Benjamin Bristow, Shira Shafir, and Frank Sorvillo

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Learning Objectives

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

- Assess the epidemiology of coccidioidomycosis
- Evaluate the transmission and clinical course of coccidioidomycosis
- Analyze demographic risk factors for mortality in cases of coccidioidomycosis
- Distinguish comorbid conditions associated with a higher risk of mortality in cases of coccidioidomycosis

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Coccidioidomycosis is endemic to the Americas; however, data on deaths caused by this disease are limited. To determine the rate of coccidioidomycosis-associated deaths in the United States, we examined multiple cause–coded death records for 1990–2008 for demographics, secular trends, and geographic distribution. Deaths were identified by International Classification of Diseases, 9th and 10th Revision, codes, and mortality rates were calculated. Associations of deaths among persons with selected concurrent conditions were examined and compared with deaths among a control group who did not have coccidioidomycosis. During the 18-year period, 3,089 coccidioidomycosis-associated deaths occurred among US residents. The overall age-adjusted mortality rate was 0.59 per 1 million person-years; 55,264 potential life-years were lost. Those at highest risk for death were men, persons >65 years, Hispanics, Native Americans, and residents of California or Arizona. Common concurrent conditions were HIV and other immunosuppressive conditions. The number of deaths from coccidioidomycosis might be greater than currently appreciated.

Coccidioidomycosis is a reemerging infectious disease caused by inhalation of airborne spores of the soil fungus *Coccidioides immitis* or *C. posadasii* (*1*). *Coccidioides* spp. are native to arid and desert areas in North America (California, Arizona, Texas, Utah, Nevada,

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New Mexico, and northern parts of Mexico), Central America, and South America (*2*). The manifestations of infection with either organism are assumed to be identical. *Coccidioides* spp. are found in lower elevation areas that receive <20 inches of rain per year and have warm, sandy soil (*3*). They are usually found 4–12 inches below the surface. Organism growth is enhanced in areas of animal droppings, burial sites, and animal burrows (*4*). Among persons living in coccidioidomycosis-endemic areas, ≈10%–50% have been exposed to *Coccidioides* spp. Each year in the United States, an estimated 150,000 new cases of coccidioidomycosis occur (*5*). The trend of incidence varies by state because of differences in epidemiology, reporting standards, and case definitions. For 2010, the 2 states most affected by coccidioidomycosis, Arizona and California, reported incidence of 186.0 and 11.5 cases per 100,000 population, respectively (*6*–*8*).

The epidemiology of coccidioidomycosis varies by area; environmental factors play a major role (*9*). Exposure to *Coccidioides* spp. varies by season, geographic location (*3*), and condition of the air. Exposure to spores is more common in dusty conditions, e.g., after earthquakes, dust storms, droughts, and other natural disasters that increase the amount of dust in the air *(10)*. Persons in certain occupations are at higher risk for exposure to spores, e.g., archeologists (*11*), military personnel, construction workers (*12*), and farmers. Prisons in coccidioidomycosis-endemic areas might place inmates at risk for exposure (*13*).

Pulmonary infection can result from inhalation of 1 spore; however, high numbers of spores are more likely to result in symptomatic disease. With rare exception, animal-to-person or person-to-person transmission does not occur. The incubation period is 1–4 weeks. Most patients are asymptomatic, but others might have an acute or chronic disease that initially resembles a protracted respiratory or pneumonia-like febrile illness primarily involving the bronchopulmonary system. Dissemination to multiple organ systems can occur. Illness is typically characterized by ≥1 of the following: influenza-like signs and symptoms; pulmonary lesion diagnosed by chest radiograph; erythema nodosum or erythema multiforme rash; meningitis; or involvement of bones, joints, skin, viscera, and lymph nodes (*14*,*15*). Extrapulmonary manifestations occur in 0.6% of the general population, most commonly secondary to hematogenous spread; meningitis carries an especially grave prognosis (*16*). The risk for disseminated disease is significantly higher among men (*17*), those with compromised or suppressed immune systems (e.g., persons with HIV), those receiving corticosteroids, and pregnant women. Risk for disseminated disease also seems to be higher for African Americans and Filipino Americans (*18*).

Despite the potential for coccidioidomycosis to be severe and fatal, studies of deaths associated with coccidioidomycosis in the United States are limited (*19*,*20*). To determine possible risk factors for coccidioidomycosis-associated death, we used US multiple-cause-of-death data to assess demographics, secular trends, geographic distribution, and concurrent conditions.

Methods

Data Sources

We analyzed de-identified, publicly available multiple-cause-of-death data from US death certificates from the National Center for Health Statistics for 1990–2008 (*21*,*22*). These death records contained demographic information for each decedent (including age, sex, and race/ethnicity) and geographic information (state of residence and place of death). In addition to designating underlying causes, the physician or coroner can list conditions that are believed to have contributed to the death. These conditions were coded according to the International Classification of Diseases, 9th Revision (ICD-9), for 1990–1998 and International Classification of Diseases, 10th Revision (ICD-10), for 1999–2008 (*23*,*24*). A coccidioidomycosis-associated death was defined as death of a US resident with an ICD-9 code of 114.0–114.9 or an ICD-10 code B38.0–B38.9 listed as an underlying or contributing cause on the death record (*21*,*25*).

Mortality Rates and Trends

We calculated mortality rates and 95% CIs by using bridged-race population estimates derived from US census data, and we subsequently age-adjusted these rates with weights from the 2000 US standard population data. Mortality rates and rate ratios were calculated by a decedent's race/ethnicity (white, black, Hispanic, Asian, Native American), year of death, and state of residence by using aggregated data from all years of study to ensure stable rates. Years of potential life lost were calculated by subtracting age at death from 75 for all who died before 75 years of age (*26*). This method was used for consistency with the Centers for Disease Control and Prevention Web-based Injury and Statistics Query and Reporting System (*27*).

Analysis of Concurrent Illnesses

To identify possible risk factors for coccidioidomycosis-associated death, concurrent conditions that were noted on the death records were compared with those noted on the records of a control group whose deaths were not associated with coccidioidomycosis. Five control decedents were randomly selected and matched to each coccidioidomycosis decedent by 5-year age group, sex, and race. Matched odds ratios and 95% CIs were computed. Concurrent conditions were chosen

on the basis of biological plausibility and known or suspected risk factors for death from coccidioidomycosis. Concurrent conditions were identified on the death record as either an underlying or contributing cause (*21*,*25*). All analyses were conducted by using SAS 9.2 software (SAS Institute, Inc, Cary, NC, USA).

Results

Demographics

During 1990–2008, a total of 3,089 coccidioidomycosis-associated deaths among US residents were identified; these deaths represent 55,264 years of potential life lost. The overall crude mortality rate was 0.58 per 1 million person-years (95% CI 0.56–0.61); after age adjustment, the mortality rate was 0.59 deaths per 1 million person-years (95% CI 0.57–0.61). Age-adjusted mortality rates, by year, are shown in Figure 1. A total of 2,202 (>70%) decedents were men, and 887 (28.7%) were women; age-adjusted mortality rates were 0.94 per 1 million person-years and 0.32 per 1 million person-years, respectively. Death associated with coccidioidomycosis was 2.04× (95% CI 2.84–3.26) more likely for men than for women.

Mortality rates were higher for decedents >65 years of age than for those in other age groups (Table 1). Most (603 [19.5%]) deaths occurred at 65–74 years of age; age-specific mortality rate was 1.70 per 1 million person-years. Although the 206 decedents >85 years of age represented only 6.7% of the total deaths, the mortality rate was highest for this group (2.56 deaths/1 million person-years).

Most decedents whose death was associated with coccidioidomycosis were white (1,693 [54.8%]), 747 (24.2%) were Hispanic, 392 (12.7%) were black, 178 (5.8%) were Asian, and 79 (2.6%) were Native American (Table 2) Age-adjusted mortality rate was highest among Native Americans (2.56 deaths/1 million person-years), followed by Hispanics (1.77 deaths/1 million person-years) and lowest among whites (0.40 deaths/1 million person-years). Age-adjusted race-specific rates were elevated for all nonwhite groups. The likelihood of dying with coccidioidomycosis listed on the death record was 6.34× (95% CI 6.04–6.65) greater for Native Americans, 4.38× (95% CI 4.17–4.60) greater for Hispanics, 2.82× (95% CI 2.69–2.97) greater for Asians, and 1.70× (95% CI 1.61–1.80) greater for blacks than for whites.

Geographic Associations

All states reported coccidioidomycosis-associated deaths; however, most deaths occurred in California (1,451 [47.0%]) and Arizona (1,010 [32.7%]); age-adjusted mortality rates were 2.47 (95% CI 2.35–2.60) and 10.60 (95% CI 9.94–11.25) deaths per 1 million person-years, respectively (Figure 2). No notable temporal or seasonal trends were observed.

Disease Associations

Several conditions were more commonly represented on the death records of those whose death was associated with coccidioidomycosis than on the death records of matched controls whose death was not associated (Table 2.) These conditions are vasculitis (matched odds ratio [MOR] 6.55, 95% CI 3.85–11.12), rheumatoid arthritis (MOR 6.51, 95% CI 4.05–10.45), systemic lupus erythematosus (MOR 4.17, 95% CI 2.52–6.90), HIV infection (MOR 3.92, 95% CI 3.24–4.75), tuberculosis (MOR 2.82, 95%CI 1.66–4.79), diabetes mellitus (MOR 2.12, 95% CI 1.86–2.42), chronic obstructive pulmonary disease (MOR 1.45, 95% CI 1.25–1.68), and non-Hodgkin lymphoma (MOR 1.44, 95% CI 1.03–2.01).

Discussion

The number of coccidioidomycosis-associated deaths in the United States is appreciable. Mortality rates were highest in persons >65 years of age, men, Native Americans, and Hispanics. Since 1997, however, coccidioidomycosis-related mortality rates have been relatively stable.

The increased risk for coccidioidomycosis-associated death among older persons might reflect decreasing immune function and increased prevalence of concurrent diseases. Increasing age has been identified as a potential risk factor for infection with *Coccidioides* spp. (*28*). The increased rate of coccidioidomycosis-associated deaths observed among men might reflect their higher risk for severe pulmonary and disseminated coccidioidomycosis (*17*). The occupations associated with coccidioidomycosis (agricultural work, construction work, military service, and work at archeological sites) might also play an additional role (*10*–*12*) in the high numbers of coccidioidomycosis-associated deaths among men.

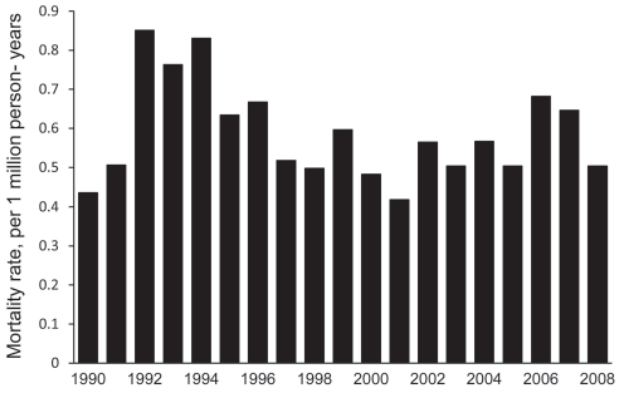

Figure 1. Age-adjusted mortality rates of coccidioidomycosis, per 1 million persons in the United States, by year, 1990–2008.

Table 1. Demographic characteristics, mortality rates, and rate ratios for coccidioidomycosis-associated deaths, United States, 1990–2008*

Characteristic	No. (%)	Age-adjusted mortality rate (95% CI)†	Age-adjusted rate ratio (95% CI)
Overall	3,089 (100)	0.59 (0.57–0.61)	NA
Sex			
F	887 (28.7)	0.31 (0.29–0.33)	Referent
M	2,202 (71.3)	0.93 (0.90–0.98)	3.04 (2.84–3.26)
Race/ethnicity			
White	1,693 (54.8)	0.40 (0.38–0.42)	Referent
Hispanic	747 (24.2)	1.77 (1.63–1.90)	4.38 (4.17–4.60)
Black	392 (12.7)	0.69 (0.62–0.75)	1.70 (1.61–1.80)
Asian	178 (5.8)	1.14 (0.96–1.32)	2.82 (2.69–2.97)
Native American	79 (2.6)	2.56 (1.96–3.15)	6.34 (6.04–6.65)
Age, y			
0–4	8 (0.3)	0.11 (0.03–0.18)	NA
5–14	12 (0.4)	0.02 (0.01–0.03)	NA
15–24	94 (3.0)	0.13 (0.10–0.15)	NA
25–34	302 (9.8)	0.39 (0.34–0.43)	NA
35–44	368 (11.9)	0.45 (0.40–0.50)	NA
45–54	453 (14.7)	0.66 (0.60–0.73)	NA
55–64	490 (15.9)	1.02 (0.93–1.11)	NA
65–74	603 (19.5)	1.70 (1.56–1.84)	NA
75–84	553 (17.9)	2.43 (2.23–2.64)	NA
>85	206 (6.7)	2.56 (2.21–2.91)	NA

*NA, not applicable.
†Crude rates are used for the age category.

Age-adjusted, race-specific, coccidioidomycosis-associated mortality rates were highest for Native Americans and Hispanics; these rates probably reflect the higher density of American Indian and Hispanic populations living in areas that are arid and where coccidioidomycosis is endemic. All the coccidioidomycosis-associated deaths of Native Americans occurred in the western region of the United States. Some literature sources have suggested that Native Americans are at increased risk for exposure to *Coccidioides* spp. because of cultural practices and exposure to contaminated dust (*11*). Poor access to health care services might delay diagnosis, resulting in more severe disease. The high rates observed among Native Americans must be interpreted with caution, given the relatively small number of deaths.

Coccidioidomycosis-associated mortality rates were also higher among blacks and Asians than among whites but lower than rates among Native Americans and Hispanics. Black race and Filipino ancestry are recognized risk factors for disseminated disease (*2*). We were unable to ascertain coccidioidomycosis-associated mortality rates for Filipino Americans. These higher mortality rates might reflect an increased risk for severe disease, greater risk for exposure, or both.

That coccidioidomycosis-associated mortality rates are highest in Arizona and California is expected, given that *Coccidioides* spp. are endemic to these regions. These 2 regions are also classic retirement magnets; they attract elderly persons to migrate and settle down (*29*), thereby introducing new, unexposed populations to *Coccidioides* spp. Every state recorded coccidioidomycosis-associated deaths, which probably reflects population mobility and movement in and out of coccidioidomycosis-endemic areas after exposure.

Chronic illnesses have changed the way opportunistic mycoses affect the population. The conditions that were associated with coccidioidomycosis were all inherently associated with immunosuppression: HIV, tuberculosis, diabetes mellitus, autoimmune diseases, organ transplant, and cancers of lymphatic cells (*30–38*). Despite relatively low numbers of cases, an association was found between coccidioidomycosis-associated deaths and lupus erythematosus, vasculitis, and rheumatoid arthritis.

Table 2. Concurrent conditions listed with coccidioidomycosis on death records, United States, 1990–2008*

Disease	ICD 9 code	ICD 10 code	No. deaths	Matched odds ratio
Vasculitis	446, 447.6	I776, I80, L95, M39, M31	36	6.55 (3.85–11.12)
Rheumatoid arthritis	714	M05-M06	45	6.51 (4.05–10.45)
Systemic lupus erythematosus	710	M32.0-M32.9	31	4.17(2.52–6.90)
HIV infection	042–0449	B20-B24	253	3.92 (3.24–4.75)
Tuberculosis	010.0–018.9	A16-A19	24	2.82 (1.66–4.79)
Diabetes mellitus	250.0–250.9	E11-E14	384	2.12 (1.86–2.42)
Chronic obstructive pulmonary disease	490–492, 494–496	J40-J44, J47	287	1.45(1.25–1.68)
Non-Hodgkin lymphoma	202, 200	C82-C85	47	1.44(1.03–2.01)

*ICD, International Classification of Diseases.

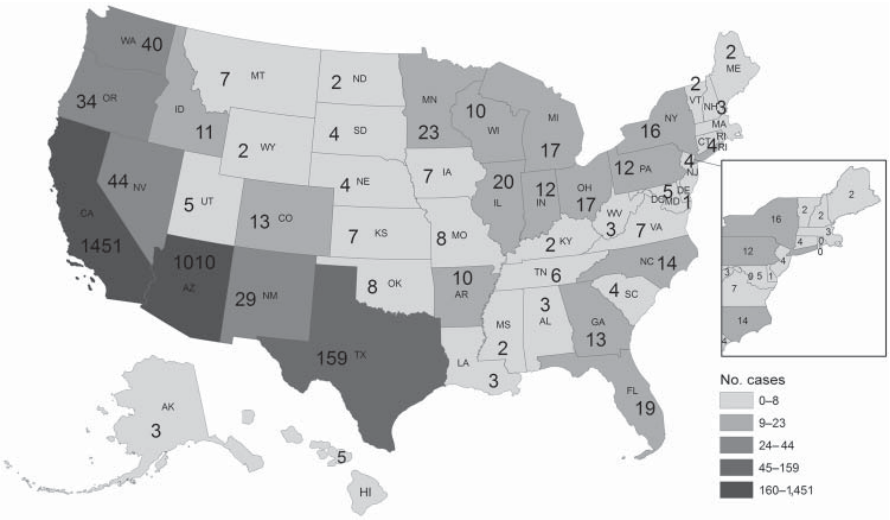

Figure 2. Number of coccidioidomycosis-associated deaths and age-adjusted rates per 1 million persons in the United States, 1990–2008.

Several limitations are inherent when multiple-cause-of-death data are used. Although these data are population based and contain large numbers of observations, death certificates probably underreport causes of death and can contain errors, which have been attributed to a variety of factors (*39*). Mortality rates can be distorted because of errors in population estimates, particularly for race/ethnicity. Because estimates of the at-risk population factor into the denominator for rate calculations, such errors can lead to biased estimates. Although inferential statistics are not designed for use with population-based data, 95% CIs demonstrate that error does exist in the mortality rates and rate ratios reported here. We urge caution in the strict interpretation of our values.

Coccidioidomycosis remains a major cause of death in the United States. Given the growing US population of elderly and immunosuppressed persons, the number of coccidioidomycosis-related deaths will probably increase, resulting in higher costs to the health care system (*38*). Effects of increasing health care costs associated with coccidioidomycosis have been observed in coccidioidomycosis-endemic states; almost half of the reported case-patients are hospitalized and make multiple visits to emergency rooms and outpatient facilities during the course of the illness (*15*). Physicians should be aware of the increased risk for coccidioidomycosis-associated death among those who are immunosuppressed, elderly, male, Hispanic, and/or Native American. For identifying suspected cases, an accurate travel exposure and occupational history are crucial, especially in persons from non–coccidioidomycosis-endemic areas. Further investigation into measures that will effectively decrease coccidioidomycosis exposure risk to the general public is needed, as are more studies of health disparities that surround coccidioidomycosis-associated deaths.

During the study, Ms Huang was a Master's of Public Health student at the University of Southern California. Her research interests include fungal disease epidemiology and association of comorbid chronic conditions with infectious diseases.

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Invasive Pneumococcal Disease and 7-Valent Pneumococcal Conjugate Vaccine, the Netherlands

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Learning Objectives

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

- Analyze previous research into the effects of 7-valent pneumococcal conjugate vaccine (PCV7)
- Compare the effects of PCV7 on different continents
- Distinguish age groups most affected by PCV7
- Evaluate the clinical presentation and outcomes of IPD after introduction of PCV7.

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In the Netherlands, the national immunization program includes 7-valent pneumococcal conjugate vaccine (PCV7) for all newborns born after April 1, 2006. We compared the incidence of invasive pneumococcal disease (IPD) and patient and disease characteristics before PCV7 introduction (June 2004–June 2006) with those after PCV7 introduction (June 2008–June 2010). Culture-confirmed IPD cases were identified by 9 sentinel laboratories covering ≈25% of the Dutch population. Significant declines in overall IPD incidence were observed in children <2 (60%) and in persons

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≥65 (13%) years of age. A trend toward gradual increases in non–PCV7 serotype IPD infections was observed in all age groups; the largest increases were among persons 50–64 (37%) and ≥65 (25%) years of age. In adults, the proportion of immunocompromised persons increased among IPD patients. Overall, deaths from IPD decreased from 16% to 12% because of a lower case-fatality rate for persons with non–PCV7 serotype IPD.

Streptococcus pneumoniae is a major cause of severe invasive infections, such as meningitis, invasive pneumonia, and other bloodstream infections. The highest incidence rates for such infections are for infants and elderly persons (*1*).

Since 2001, many high-income countries included the 7-valent pneumococcal conjugate vaccine (PCV7; Prevenar; Pfizer Pharmaceuticals, Pearl River, NY, USA) in their national immunization programs for newborns (*2*). In general, within a few years after the introduction of PCV7, the age group targeted for vaccination and unvaccinated adults showed a dramatic decrease in invasive pneumococcal disease (IPD) caused by the 7 vaccine serotypes (*2*–*5*). However, at the same time, the incidence of non-PCV7 serotype IPD increased (*3*,*4*,*6*,*7*).

The overall benefit of PCV7 varies by country, perhaps as a result of differences in surveillance methods and the maturity of vaccination programs (*8*). For all age groups, the overall reduction in IPD incidence is greater in the United States than in European countries; the great reduction in the United States is a result of a decrease in PCV7-serotype IPD in adults and less replacement of PCV7-serotype by non–PCV7 serotype IPD in children and older adults (*3*,*4*,*7*). The United States began using PCV7 in 2000, but many European countries did not begin using the vaccine until after 2005–2006, and they have experienced less protection from indirect herd protection (herd immunity). Furthermore, not all European countries implemented a catch-up program for children <5 years of age; catch-up programs speed up eradication of vaccine serotypes. Geographic variations in circulation of PCV7 serotypes before the implementation of routine vaccination also caused differences in the relative proportion of IPD covered by the vaccine (*7*,*8*).

In addition, the benefits of vaccination with PCV7 may have been biased, for example, by changes in the directive for blood culture after 2000, as in the United States (*9*,*10*), and by enhanced surveillance, as reported for England and Wales (*4*). Unlike studies in the United States, studies in Europe, particularly Dutch surveillance studies, have focused almost exclusively on patients requiring hospitalization for severe IPD and who often had other underlying illnesses (*11*,*12*). This difference in reporting leads to different baseline incidence rates and may affect the observed net benefit of vaccination (*13*). For example, compared with healthy persons of the same age, US adults with comorbid conditions benefited less from the indirect effects of PCV7 because of an increase in non–PCV7 serotype IPD after introduction of the vaccine (*14*). Differences in the directive for blood culture and patient populations under surveillance can partly explain the differences in results from use of PCV7.

The invasive disease potential of *S. pneumoniae* and the population at risk for IPD differs by serotype (*12*,*13*,*15*). Therefore, shifts in circulating serotypes may change the clinical manifestations of IPD, the population segment most at risk for infection, and the disease course and outcome. We investigated these issues and changes in IPD incidence in the Netherlands 4 years after a PCV7 vaccine program was implemented and compared our findings with those from the years just before introduction of the vaccine.

Methods

Pneumococcal Vaccination in the Netherlands

PCV7 was introduced into the Dutch national immunization program in June 2006 and was recommended for children born after April 1, 2006, at 2, 3, 4, and 11 months of age (*16*). Vaccination uptake is 94%–95% among Dutch infants (*17*). Use of the 23-valent pneumococcal polysaccharide vaccine is restricted to persons at high-risk for IPD (e.g., persons with asplenia or Hodgkin lymphoma); uptake in elderly persons is negligible (<1%) (*18*).

Surveillance Data

For this study, we registered all persons with a diagnosis of culture-confirmed IPD during June 1, 2008–May 31, 2010 (late post-implementation period) and all case-patients from previous Dutch IPD surveillance studies during June 1, 2004–May 31, 2006 (pre-implementation period) (*1*) and June 1, 2006–May 31, 2008 (post-implementation period) (*11*). All study procedures were the same as those used in the previous studies (*11*).

Nine sentinel laboratories identified IPD case-patients, which were defined as patients for whom *S. pneumoniae* was isolated from blood or cerebrospinal fluid (CSF) samples. The laboratories submitted all invasive pneumococcal isolates to the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM, Academic Medical Center, Amsterdam, the Netherlands) for typing and characterization. We selected the laboratories on the basis of their geographic distribution throughout the country and their reliability for submitting isolates (*1*,*11*). The laboratories were estimated to cover a representative cohort of ≈25% of the Dutch population (≈4.1 million inhabitants, including ≈0.6 million adults ≥65 years of age). In addition, ≈25% of the other meningitis-causing bacterial isolates that were sub-

mitted to NRLBM during the study period were submitted by the 9 sentinel laboratories.

At the NRLBM, co-agglutination was used to type the pneumococcal isolates and the capsular swelling method (Quellung reaction), using antisera (Statens Serum Institute, Copenhagen, Denmark), including serotype 6C, was used for serotyping. For isolates collected before June 2008, serotype 6C was determined by using PCR and antisera. The serotypes were grouped in either PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) or non–PCV7 serotypes (all other serotypes, including 6A).

Clinical Characteristics

Trained medical students, using a standardized data collection form, retrospectively extracted the following information for all case-patients from hospital records, as described (*1*,*11*): patient characteristics, clinical syndrome, comorbidity, and disease course and outcome. We subdivided comorbid conditions as immunocompromising or nonimmunocompromising and categorized clinical syndromes as meningitis, invasive pneumonia, bacteremia with other focus, and bacteremia without focus, as described (*1*). Information on disease course and outcome included the length of hospital stay, admission to an intensive care unit, and death (i.e., in-hospital death and/or death within 30 days after first reported blood/CSF culture positive for *S. pneumoniae*). Cases without clinical information were excluded from all analyses.

Statistical Analyses

National population coverage was ≈25% by the sentinel laboratories; thus, we estimated annual IPD incidence rates per 100,000 inhabitants by dividing the total number of IPD cases in a specific epidemiologic year by 25% of the total Dutch population. Epidemiologic years were defined as June 1st–May 31 of the succeeding year. We used the population on January 1 of each consecutive year as the population at risk for infection (StatLine, www.cbs.nl/en-GB/menu/cijfers/statline/zelf-tabellen-maken/default.htm), assuming a stable population throughout the year.

We assessed the effect of vaccination by determining the incidence rate ratio. The assessment was done by comparing incidences in the late post-implementation period (2008–2010) with those in the pre-implementation period (2004–2006); we also determined 95% CIs.

To evaluate any changes in population at risk, we compared the proportion of patients with comorbid conditions in the pre- and late post-implementation periods. We also determined changes in disease course (intensive care unit admission, median length of hospital stay, and death). Differences in percentages were compared by using the χ^2 test, and differences in median length of hospital stay were compared by using the Mann-Whitney U test.

All analyses were stratified by age group (<2, 2–4, 5–17, 18–50, 50–64, and >65 years) and by serotype group (PCV7/non–PCV7). All p values <0.05 were considered statistically significant.

Results

Overview

In the late post-implementation period (June 1, 2008–May 31, 2010), a total of 1,196 pneumococcal isolates from CSF and blood samples were submitted to the NRLBM by the 9 sentinel laboratories; this number compares with 1,297 and 1,352 isolates submitted during the pre- and early post-implementation periods, respectively. In the late post-implementation period, clinical characteristics were available for 1,144 (96%) case-patients, compared with 1,216 (94%) in the pre-implementation period and 1,304 (96%) in the early post-implementation period (Table 1).

IPD Incidence and Serotype Distribution

The overall incidence of IPD declined from 14.9 to 13.8 cases/100,000 persons during the pre- and late post-implementation periods, respectively (Table 1). A 60% decline in overall IPD incidence (from 35.0 to 14.1 cases/100,000 persons) was observed in children <2 years of age (i.e., children age-eligible for PCV7 vaccination). A similar but nonsignificant decline was seen in children 2–4 years of age. In the age group with the highest incidence rate, i.e., persons ≥65 years of age, the overall IPD incidence had a significant decline of 13% (from 57.7 to 49.9 cases/100,000 persons). IPD incidence rates remained unchanged in persons 5–64 years of age.

The overall decline of IPD incidences seen among persons <2 and ≥65 years of age from the pre- to the late post-implementation period resulted from declines in the incidence of PCV7-serotype IPD of 100% and 55%, respectively (Table 1); in children <2 years of age, no PCV7-serotype IPD cases were reported after June 1, 2008. Of 3 children (2–4 years of age) with PCV7-serotype IPD after June 1, 2008, 2 were born before April 1, 2006 and had not received PCV7. The third patient (2 years of age) experienced a vaccine failure; PCV7–serotype 19F IPD developed even though the child was fully vaccinated with 4 doses of PCV7. The child was previously healthy, without any comorbidity. Overall, infections with all PCV7 serotypes declined significantly, except for infection with serotype 18C, which was already low (Figure).

However, from the pre- to the late post-implementation period, the overall incidence of non–PCV7 serotype IPD increased by 33% (from 8.0 to 10.6 cases/100,000 persons) (Table 1). IPD incidence due to non–PCV7 serotypes showed an increasing trend in all age groups, and the increase was significant in patients 50–64 and ≥65 years of

Table 1. Incidence of invasive pneumococcal disease before and after implementation of a PCV7 vaccination program, the Netherlands, June 2004–May 2010*

Age group, y	Vaccination period†						Late post- vs. pre-implementation period		
	Pre-implementation		Early post-implementation		Late post-implementation				
	No. cases	Incidence	No. cases	Incidence	No. cases	Incidence	IRR	95% CI	p value
All serotypes									
All ages	1,216	14.9	1,304	15.9	1,144	13.8	0.93	0.86–1.01	NS
<2	68	35.0	42	22.8	26	14.1	0.40	0.26–0.63	**<0.001**
2–4	25	8.2	26	8.9	12	4.3	0.52	0.26–1.04	NS
5–17	23	1.8	22	1.7	23	1.8	1.00	0.56–1.78	NS
18–49	181	4.9	209	5.8	197	5.5	1.11	0.91–1.36	NS
50–64	253	16.4	292	18.3	261	15.8	0.96	0.81–1.14	NS
≥65	666	57.7	713	59.6	625	49.9	0.87	0.78–0.97	**0.009**
PCV7 serotypes									
All ages	565	6.9	561	6.9	268	3.2	0.47	0.40–0.54	**<0.001**
<2	48	24.7	15	8.1	0	0.0	0	NA	**<0.001**
2–4	17	5.6	17	5.8	3	1.1	0.19	0.06–0.66	**0.003**
5–17	11	0.9	4	0.3	4	0.3	0.36	0.12–1.14	NS
18–49	56	1.5	66	1.8	48	1.3	0.87	0.59–1.29	NS
50–64	114	7.4	129	8.1	56	3.4	0.46	0.33–0.63	**<0.001**
≥65	319	27.6	330	27.6	157	12.5	0.45	0.37–0.55	**<0.001**
Non–PCV7 serotypes									
All ages	650	8.0	741	9.1	876	10.6	1.33	1.20–1.47	**<0.001**
<2	20	10.3	27	14.7	26	14.1	1.37	0.77–2.46	NS
2–4	8	2.6	9	3.1	9	3.2	1.22	0.47–3.18	NS
5–17	12	0.9	18	1.4	19	1.5	1.58	0.77–3.26	NS
18–49	125	3.4	142	3.9	149	4.1	1.22	0.96–1.54	NS
50–64	139	9.0	163	10.2	205	12.4	1.37	1.11–1.70	**0.004**
≥65	346	30.0	382	32.0	468	37.4	1.25	1.09–1.43	**0.002**

*Cases are number of patients included in a study covering ≈25% of the Dutch population; incidence is number of cases/100,000 persons. Three pneumococcal isolates (1 in the pre- and 2 in the early post-implementation period) were either not typeable or typed as a rough strain and, therefore, could not be classified as 7-valent pneumococcal conjugate vaccine (PCV7) or non–PCV7 serotypes. IRR, incidence rate ratio; NS, not significant (p>0.05); NA, not applicable; **boldface**, significant difference (p<0.05).
†Vaccination periods: pre-implementation period, June 2004–May 2006; early post-implementation, June 2006–May 2008; late post-implementation period, June 2008–May 2010.

age. Non–PCV7 serotypes 1, 19A, 22F, and 23B increased significantly (Figure), although absolute numbers remained relatively small.

Clinical Characteristics

During all 3 study periods, surveillance data were primarily (97%–98%) for hospitalized IPD patients; the few exceptions were data for patients who visited a hospital emergency department and went home the same day. The distribution of clinical IPD manifestations among patients in different age groups did not change between the pre- and late post-implementation period (Table 2). In children <5 years of age, there was no decline in the incidence of meningitis because of an increase in non–PCV7 serotype meningitis in the late post-implementation period. In older children and adults, invasive pneumonia remained the most prevalent manifestation. The incidence of invasive pneumonia declined in the late post-implementation period in persons >65 years of age despite a significant increase in invasive pneumonia caused by non–PCV7 serotypes (Table 2).

Although the overall number of IPD cases declined from 1,216 in the pre-implementation period to 1,144 in the late post-implementation period, the number of IPD patients (all ages) with an immunocompromising condition increased from 216 to 255 (Table 3). This increase mainly occurred among persons >5 years of age, particularly among those ≥65 years of age. The number of PCV7-serotype IPD cases declined from 565 in the pre-implementation period to 268 in the late post-implementation period (all ages), and the number of patients with any comorbidity also showed a clear reduction. However, the number of immunocompromised persons with PCV7-serotype IPD declined only marginally (Table 3), indicating that persons with immunocompromising conditions may benefit less than others from herd immunity against PCV7-serotype IPD. This relatively marginal decline was seen for all PCV7 serotypes (data not shown). For non–PCV7 serotype IPD cases, there were similar increases in the number of infected immunocompromised patients and patients with any comorbidity. Moreover, at baseline a smaller proportion of immunocompromised (41%) than nonimmunocompromised (47%) persons had PCV7-serotype IPD (Table 4). Before and after introduction of PCV7, few children <5 years of age had a comorbid condition along with IPD (online Technical Appendix Table, wwwnc.cdc.gov/EID/pdfs/12-0329-Techapp.pdf).

Despite the relative increase in immunocompromised patients with IPD, the overall death rate for IPD decreased

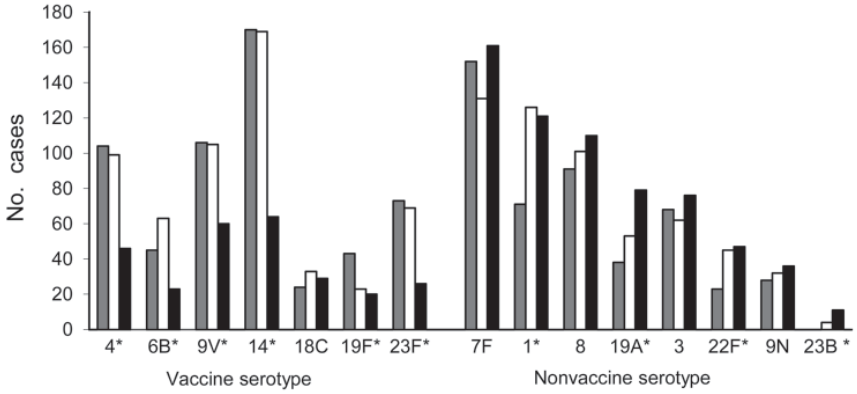

Figure. Serotype distribution of invasive pneumococcal disease in the Netherlands before and after (early and late) introduction of the 7-valent pneumococcal conjugate vaccine (PCV7). The 7 vaccine serotypes and the most prevalent nonvaccine serotypes are shown. The cases represent case-patients included in the study (covering ≈25% of the Dutch population). Gray, pre-implementation period (June 2004–May 2006); white, early post-implementation period (June 2006–May 2008); black, late post-implementation period (June 2008–May 2010); *Significant difference (p<0.05) between pre- and post-implementation periods, calculated by the incidence rate ratio.

significantly from 2.4 to 1.6 cases/100,000 persons. This decline in IPD-related deaths appears to be the result of 1) an overall decrease in the incidence of PCV7-serotype IPD and 2) a lower case-fatality rate among persons with non–PCV7 serotype IPD (Table 3). The lower death rate was seen in all age groups, but the decrease was significant only for patients ≥65 years of age. Moreover, a decrease in the case-fatality rate for non–PCV7 serotype cases was seen not only among otherwise healthy persons (decrease from 10% to 4%; p = 0.02), but also among immunocompromised persons (from 27% to 16%; p = 0.03) and/or persons with other comorbidities (from 19% to 14%; p = 0.03). Likewise, the median length of hospital stay for children >5 years of age and adults was significantly lower during the post-implementation period than in the pre-implementation period (online Technical Appendix Table).

Discussion

Our findings show that 4 years after introduction of PCV7 in the Netherlands, the overall annual incidence of IPD decreased by 60% (from 35.0 to 14.1 cases/100,000 persons) among children <2 years of age, the age group targeted for vaccination; the decrease was a result of virtually complete eradication of PCV7 serotypes. In children 2–4 years of age, a 48% reduction was seen in IPD cases overall. A significant decline of 13% was also observed in persons >65 years of age. No significant decline in overall IPD was seen in persons 5–64 years of age because the

Table 2. Incidence of invasive pneumococcal disease manifestations before and after implementation of a PCV7 vaccination program, the Netherlands, June 2004–May 2010*

	Incidence (%) by infecting serotype and vaccination period†					
	All serotypes		PCV7 serotypes		Non–PCV7 serotypes	
Age group, y, manifestation	Pre-implementation	Late post-implementation	Pre-implementation	Late post-implementation	Pre-implementation	Late post-implementation
<5						
Meningitis	6.80	3.88	4.80	0.43	2.00	3.45
Invasive pneumonia	4.40	1.72	3.00	0	1.40	1.72
Bacteremia other focus	3.60	1.29	2.80	0	0.80	1.29
Bacteremia without focus	3.80	1.29	2.40	0.22	1.40	1.08
5–64						
Meningitis	1.05	1.07	0.48	0.24	0.57	0.82
Invasive pneumonia	4.92	5.36	1.94	1.18	2.98	4.18
Bacteremia other focus	0.45	0.41	0.15	0.14	0.29	0.27
Bacteremia without focus	0.55	0.47	0.20	0.09	0.35	0.38
≥65						
Meningitis	3.38	2.24	1.39	0.40	1.99	1.84
Invasive pneumonia	47.80	40.80	23.21	10.14	24.51	30.66
Bacteremia other focus	1.73	2.63	0.78	0.64	0.95	2.00
Bacteremia without focus	4.42	3.99	2.16	1.12	2.25	2.87

*Incidence is per 100,000 inhabitants. PCV7, 7-valent pneumococcal conjugate vaccine.
†Vaccination periods: pre-implementation period, June 2004–May 2006; late post-implementation period, June 2008–May 2010.

Table 3. Characteristics for persons with invasive pneumococcal disease before and after implementation of a PCV7 vaccination program, the Netherlands, June 2004–May 2010*

Characteristic	No. (%) by infecting serotype and vaccination period†								
	All serotypes			PCV7 serotypes			Non–PCV7 serotypes		
	Before (n = 1,216)	After (n = 1,144)	p value	Before (n = 565)	After (n = 268)	p value	Before (n = 650)	After (n = 876)	p value
Comorbidity									
Immunocompromising condition‡	216 (18)	255 (22)	**0.013**	88 (16)	73 (27)	**0.001**	128 (20)	182 (21)	NS
Any comorbidity§	817 (67)	788 (69)	NS	376 (67)	190 (71)	NS	441 (68)	598 (68)	NS
Disease course/outcome									
ICU admission	258 (21)	243 (21)	NS	115 (20)	60 (22)	NS	143 (22)	183 (21)	NS
Length of hospital stay, median (IQR)	11.0 (7.0–18.0)	9.0 (5.0–16.0)	**<0.001**	11.0 (7.0–18.0)	9.0 (5.0–15.0)	**<0.001**	11.0 (7.0–19.0)	10.0 (5.0–16.0)	**<0.001**
Died	194 (16)	135 (12)	**0.003**	92 (16)	44 (16)	NS	102 (16)	91 (10)	**0.002**
Deaths/100,000 persons	2.4	1.6	**0.001**	1.1	0.5	**0.000**	1.3	1.1	NS

*Cases are number of patients included in a study covering ≈25% of the Dutch population. **Boldface,** significant difference ($p \leq 0.05$) between pre- and post-implementation period as calculated by χ^2 test (% of cases), Mann-Whitney U test (median days of hospitalization), or incidence rate ratio (mortality rate). PCV7, 7-valent pneumococcal conjugate vaccine; NS, not significant ($p > 0.05$).
†Data are no. (%) except as indicated in first column. Vaccination periods: before, pre-implementation period (June 2004–May 2006); after, late post-implementation period (June 2008–May 2010).
‡Immunocompromising condition: primary immunodeficiency, HIV/AIDS, lymphoma, leukemia, myeloma, solid organ or stem cell transplant, current immunosuppressive therapy for malignancy or autoimmune disease, asplenia/splenectomy, sickle cell disease, and renal insufficiency (dialysis required and nephrotic syndrome).
§Any comorbidity: malignancies (within previous 5 y) not considered to be immunocompromising; chronic obstructive pulmonary disease; asthma; diabetes mellitus; myocardial infarction; coronary artery condition; stroke/transient ischemic attack; cardiomyopathy; heart failure; heart valve disease; presence of cerebral/abdominal/thoracic aneurysms; thyroid disease; liver disease; intravenous drug use; long-term alcohol abuse; cerebrospinal fluid leak; recent physical trauma/skull fracture; and, for children, premature birth (<37 weeks for children 0–1 y old and <32 weeks for children 0–4 y old).

decline in PCV7-serotype IPD was offset by a similar increase in non–PCV7 serotype IPD. The proportion of immunocompromised patients within PCV7-serotype IPD also increased. Despite these findings, the length of hospital stay and case-fatality rates declined over the last years. Our findings indicate that use of PCV7 in the Netherlands resulted in a major decrease in PCV7-serotype IPD among all age groups.

Our results for children are in line with those in England and Wales (*4*). However, among persons 5–65 years of age, the effect of herd immunity was less pronounced in the Netherlands than in England and Wales (*4*), where PCV7 was introduced around the same time as in the Netherlands (summer 2006), or in the United States 4 years after the introduction of PCV7 in 2000 (*14*). This difference can be partly explained by the absence of a catch-up campaign for children <2 years of age in the Netherlands. Young children are a primary reservoir for carriage and transmission of pneumococci because of prolonged colonization episodes related to their immature immune systems. Vaccination of toddlers in addition to newborns has a major effect on the speed of onset of herd immunity in the population. Therefore, by continuing surveillance in the Netherlands, we will likely see more reduction of PCV7-serotype IPD in

Table 4. Proportion of vaccine-type and nonvaccine-type invasive pneumococcal disease cases before and after implementation of a PCV7 vaccination program, the Netherlands, June 2004–May 2010*

Vaccination period and infecting serotype(s)	No. (%) patients, by health status at time of infection				
	Otherwise healthy	Immunocompromising condition†	p value	Any comorbidity‡	p value
Pre-implementation period					
Total no. cases	399	216	NA	817	NA
PCV7 cases	189 (47)	88 (41)	NS	376 (46)	NS
Non–PCV7 cases	209 (52)	128 (59)	NS	441 (54)	NS
Post-implementation period					
Total no. cases	356	255	NA	788	NA
PCV7 cases	78 (22)	73 (29)	NS	190 (24)	NS
Non–PCV7 cases	278 (78)	182 (71)	**0.050**	598 (76)	NS

*Cases are number of patients included in a study covering ≈25% of the Dutch population. Pre-implementations period, June 2004–May 2006; post-implementation period, June 2008–May 2010. **Boldface,** significant difference ($p \leq 0.05$, calculated by $\chi 2$ test) compared with otherwise healthy patients. PCV7, 7-valent pneumococcal conjugate vaccine; NA, not applicable; NS, not significant ($p > 0.05$).
†Immunocompromising condition: primary immunodeficiency, HIV/AIDS, lymphoma, leukemia, myeloma, solid organ or stem cell transplant, current immunosuppressive therapy for malignancy or autoimmune disease, asplenia/splenectomy, sickle cell disease, and renal insufficiency (dialysis required and nephrotic syndrome).
‡Any comorbidity: malignancies (within previous 5 y) not considered to be immunocompromising; chronic pulmonary disease (chronic obstructive pulmonary disease and asthma); diabetes mellitus; cardiovascular disease (myocardial infarction, coronary artery condition, stroke/transient ischemic attack, cardiomyopathy, heart failure, heart valve disease, and presence of cerebral/abdominal/thoracic aneurysms); thyroid disease; liver disease; intravenous drug use; long-term alcohol abuse; cerebrospinal fluid leak; recent physical trauma/skull fracture; and, for children, premature birth (<37 weeks for children 0–1 y old and <32 weeks for children 0–4 y old).

the years after 2010. A major issue will be the rise in non–PCV7 serotypes, which is estimated by Choi et al. (*19*) to be ≈90% in England and Wales. Despite this large increase in non–PCV7 serotype IPD, it is expected that this will not offset the decrease in PCV7-serotype IPD in infants and elderly persons.

The decline of IPD cases among persons with immunocompromising conditions was limited compared with the decline among nonimmunocompromised persons. This result may be biased because the number of PCV7-serotype IPD cases in this group was relatively small before introduction of PCV7. However, the case-fatality rate for non–PCV7 serotype IPD in the post-implementation period declined among otherwise healthy persons and among those with comorbid conditions, suggesting a less severe course of disease, even in patients with serious immunocompromising conditions. Thus, even if the incidence of IPD decreased less in immunocompromised persons than in the general population, persons with immunocompromising conditions still appear to benefit from the vaccination program because of a reduction in case-fatality rates.

The reduced case-fatality rate for non–PCV7 serotype IPD since the introduction of PCV7 can be partly explained by a large increase in serotype 1 IPD. This invasive serotype is associated with a low case-fatality rate (*12*,*15*,*20*), which remained low (6%–8%) in the Netherlands during the study period. Case-fatality rates for the other individual serotypes also did not change significantly after introduction of PCV7. In line with a lower case-fatality rate, we also found a reduced length of hospital stay for patients with PCV7-serotype IPD and those with non–PCV7 serotype IPD. However, in the Netherlands, there has been a tendency toward shorter hospital stays, which along with other factors (e.g., improved hospital efficiency) may affect the finding of a reduced length of hospital stay for patients with IPD (*21*). For example, in 2006 a new financial system was introduced in the Netherlands that encourages shortening of the length of hospital stay.

In children, the increase in non–PCV7 serotype disease was most pronounced among patients with meningitis. Although the numbers were too small to yield significant differences, these data indicate that surveillance should be continued and special attention should be paid to patient characteristics and the evolution of serotype circulation over time.

The incidence of IPD caused by nonvaccine– *S. pneumoniae* serotypes 1, 19A, 22F, and 23B increased significantly after introduction of PCV7 in the Netherlands. The increase in serotype 19A has been consistently reported worldwide, especially increased carriage among children (*22*,*23*) and increased cases of serotype 19A–associated invasive disease (*24*) and otitis media (*25*–*27*). The role of PCV7 in promoting serotype 19A carriage in vaccinated children compared with unvaccinated controls has been shown (*22*,*28*). In many countries, the increase in serotype 19A disease is associated with high levels of penicillin resistance (*24*). In the Netherlands, only 1.8% of pneumococcal strains are reported to be resistant (*29*). The increase in serotype 22F was also seen in the United States and in England and Wales (*3*,*4*). The occurrence of serotype 1 was also shown to fluctuate and decline in presence of PCV7 (*4*). We did not see an increase in IPD caused by serotypes 6C and 15B/C, although increases have been reported elsewhere (*3*,*4*). On May 1, 2011, the Dutch government switched from the 7-valent to the 10-valent pneumococcal conjugate vaccine, which includes serotypes 1, 5, and 7F in addition to those in PCV7 (*30*). The 13-valent pneumococcal conjugate vaccine, which has not been introduced in the Netherlands, adds protection against serotypes 3, 6A, and 19A.

Surveillance artifacts resulting from enhanced surveillance and increased awareness after the introduction of the vaccine should be considered when evaluating the effects of the PCV7 vaccination program (*4*). However, adjustments for these artifacts can introduce new biases leading to over- and underestimation of the true effects of the vaccine. We believe there are no indications for enhanced surveillance and increased awareness in our study. The laboratory-based surveillance system remained unchanged during the study period, 2004–2010. Unlike the situation in England and Wales (*4*), the number of pneumococcal isolates obtained from CSF samples in the Netherlands remained stable during the years before PCV7 was introduced (online Technical Appendix Figure 1). Moreover, the incidences of IPD caused by a great majority of non–PCV7 serotypes remained stable during the entire study period; the exceptions were for IPD caused by serotypes 1, 19A, 22F, and 23B (online Technical Appendix Figure 2). If enhanced surveillance had taken place, one would expect an increase in the reported number of IPD cases caused by any of these serotypes. Thus, we made no corrections for increased case ascertainment or awareness in this study.

Our study does have limitations. First, the study periods before and after implementation of the vaccine program were relatively short; this may have caused an overestimation or underestimation of our results. To account for a proper transition period, we did not include June 2006–May 2008 in our comparisons because no clear conclusions could be drawn from this period. Second, changes in IPD epidemiology could have been influenced by variations in the seasonal influenza and the influenza A(H1N1)pdm09 virus epidemics in 2009 (*31*,*32*). Last, no data were available on the national prevalence of comorbidities/diseases. Thus, we could not evaluate IPD incidence rate ratios for the 3 patient groups in our study: otherwise healthy per-

sons, persons with any comorbidity, and persons with immunocompromising conditions.

The results of this study show that PCV7 use has reduced the number of IPD cases and deaths in children <2 years of age (the age group targeted for vaccination) and in persons ≥65 years of age. However, after introduction of PCV7, cases of IPD caused by non–PCV7 serotypes increased significantly among elderly persons, and the proportion of immunocompromised persons with IPD increased. Despite these increases, the overall IPD case-fatality rate among patients ≥65 years of age decreased, which seems to be a positive consequence of shifts in circulating serotypes after introduction of a pneumococcal conjugate vaccine for infants.

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Ms van Deursen is a doctoral candidate at Utrecht University; this manuscript was part of her doctoral research project. Her research interests include the effectiveness of pneumococcal conjugate vaccinations on invasive pneumococcal disease and more common respiratory infections in vaccinated and unvaccinated populations.

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Nasopharyngeal Bacterial Interactions in Children

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Learning Objectives

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

- Describe patterns of nasopharyngeal bacterial colonization and interaction in healthy young children, based on an observational study
- Describe the role of *Haemophilus influenzae* in acute otitis media (AOM) affecting young children, based on an observational study
- Describe the role of other bacteria in AOM affecting young children, based on an observational study

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Antimicrobial treatments and vaccines can alter bacterial interactions in the nasopharynx, thereby altering disease processes. To better understand these interactions, we examined colonization rates of 3 respiratory bacterial pathogens among 320 children when healthy and at onset of acute otitis media (AOM). Bacterial interactions were analyzed with a repeated measures logistic regression model. Among healthy children, *Streptococcus pneumoniae* and *Moraxella catarrhalis* were synergistically (positively) associated. Colonization with *S. pneumoniae* when healthy, but not at onset of AOM, was competitively (negatively) associated with *Staphylococcus aureus.* Among children with AOM, competitive associations were found between *Haemophilus influenzae* and *S. pneumoniae* and between *H. influenzae* and *M. catarrhalis*; rates of colonization with *H. influenzae* were higher. Bacterial interactions result in differing pathogen prevalence during periods of health and at onset of AOM. *H. influenzae* might become a more common cause of AOM among children who receive pneumococcal conjugate vaccine.

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Respiratory bacterial infections, including pneumonia, acute exacerbations of bronchitis, acute sinusitis, and acute otitis media (AOM) among children and adults create major clinical concerns (*1*,*2*). The most common bacteria that cause upper respiratory tract infections are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and

Moraxella catarrhalis (*2*). The human nasopharynx is an ecologic reservoir of these and other bacteria. A broad variety of commensal bacteria and potential bacterial pathogens colonize the nasopharynx (*3*,*4*). Colonization of the nasopharynx is a first, and essential, step toward development of respiratory bacterial infections (*3*). Viruses can join the microbial mix as a prelude to secondary bacterial infections of the respiratory tract (*5*–*7*).

More information about microbial interactions in the nasopharynx is needed (*8*). These interactions can be altered by therapeutic (e.g, antimicrobial drug) and vaccine (e.g.; pneumococcal conjugate vaccination) interventions, resulting in synergistic or competitive outcomes. Information about interactions of the major bacterial respiratory pathogens in the nasopharynx and the conditions conducive to progression to infection (e.g., concurrent viral upper respiratory infections) is limited.

Microbial species can interact synergistically to promote persistence of colonization (positive, or synergistic, association) or they can compete (negative, or competitive, association) (*4*,*9*). Interactions between bacteria can alter the composition of a microbial community and affect incidence of disease (*4*). Several studies have reported competitive associations between colonized *S. pneumoniae* and *S. aureus* in the nasopharynx of children, raising concerns that eradication of *S. pneumoniae* from the nasopharynx by the heptavalent pneumococcal conjugate vaccine (PCV7) might lead to increased *S. aureus* colonization and subsequent infections (*10*–*13*). The introduction of the 13-valent pneumococcal conjugate vaccine will probably exacerbate this effect. Other variables that alter nasopharynx colonization patterns in children include age, gender, daycare attendance, history of having been breast-fed, environmental exposure to tobacco smoke, and otitis-prone condition (*14*,*15*).

Several recent reports have described interactions among the 3 major pathogens—*S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*—in young children (*8*,*10*,*11*,*16*), but the results were contradictory (*9*). We investigated the interactions of these 3 pathogens in the nasopharynx of young children while healthy (healthy visits) and at onset of AOM (AOM visits). Our aims were to understand differences in nasopharynx colonization rates and bacterial interactions according to the child's health status.

Materials and Methods

Study Design and Participants

We analyzed data collected during June 2006–May 2011 from children enrolled in a 5-year prospective study supported by the National Institute of Deafness and Other Communication Disorders. In that study, healthy children with no previous episodes of pneumonia, sinusitis, or AOM were enrolled at 6 months of age from 5 middle-class, suburban pediatric practices in Rochester, New York, USA. Nasopharyngeal and oropharyngeal samples were obtained from healthy children at 6, 9, 12, 15, 18, and 24 months of age and examined for *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus*. If symptoms compatible with an AOM infection developed, a tympanocentesis was performed, as described, to confirm the diagnosis (*17*). At the time of diagnosis, nasopharyngeal and oropharyngeal samples were obtained for bacterial pathogen cultures. All children received age-appropriate standard vaccinations, including pneumococcal conjugate vaccine (PCV7) (Prevnar; Wyeth Pharmaceuticals, Collegeville, PA, USA).

We analyzed culture data from nasopharyngeal samples collected during 1,183 healthy visits and 334 AOM visits among 320 children 6–24 months of age. All samples included in this study were from children who had not received antimicrobial therapy for at least 3 weeks. Nasopharynx colonization at healthy versus AOM visits was compared among children at 6, 9, 12, 15, 18, and 24 months of age. This time frame includes peak incidence of AOM infection caused by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis.*

Nasopharyngeal and oropharyngeal samples were obtained for culture as described (*18*). The pathogens *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* were isolated and identified according to the Manual of Clinical Microbiology (*19*).

The study was approved by the Institutional Review Board of the University of Rochester and the Rochester General Hospital. Written informed consent was obtained from parents or guardians before the children were enrolled.

Statistical Analyses

The rates of nasopharynx **c**olonization among children of the same age at healthy and AOM visits were compared by using the Fisher exact test and GraphPad Prism software (www.graphpad.com). Bacterial interactions were analyzed by using repeated measures logistic regression models. Predicted outcomes of colonization with *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were examined by using multivariate logistic regression. Generalized estimating equations were used to model exchangeable correlation within participants (*20*). Two logistic regression models (1 for healthy visits and 1 for AOM visits) were calculated by using R version 2.13.2 (www.r-project.org/). To examine the effects of covariates on each of the 3 pathogens, we modeled colonization of each pathogen separately by using the remaining 2 pathogens as predictors and including the interaction term (*8*). Because few *S. aureus* were isolated, we did not separately model colonization outcome for *S. aureus* (*8*). For each model, we estimated odds ratios

(ORs) for the response pathogen given the presence of each predictor pathogen alone, then jointly; synergistic associations between bacteria are indicated by OR>1; competitive associations, by OR<1. The absence of both predictor pathogens was used as the reference condition. $p \leq 0.05$ was considered significant; $p \leq 0.01$, strongly significant; $p \leq 0.1$, weakly significant. The model was also used to estimate the OR for each pathogen relative to the risk factors of sex, age, daycare attendance, history of having been breast-fed, environmental exposure to tobacco smoke, and otitis-prone condition.

Results

Polymicrobial Nasopharynx Colonization

Differences in nasopharynx colonization rates between healthy and AOM visits remained generally similar across the longitudinal samplings (Table 1). The rate of nasopharynx colonization by *S. pneumoniae* for children of all ages at healthy visits, when neither *H. influenzae* nor *M. catarrhalis* was present, was 14.2%; this rate did not differ statistically from that at AOM visits (14.4%; p = 0.93) (Table 1). In contrast, the rate of nasopharynx colonization by *H. influenzae* for children of all ages at AOM visits, when neither *S. pneumoniae* nor *M. catarrhalis* was present, was 19.5%; this rate was >4-fold higher than the rate at healthy visits (4.5%; p<0.0001) (Table 1). The rate of nasopharynx colonization by *M. catarrhalis* for children of all ages at healthy visits, when neither *H. influenzae* or *S. pneumoniae* was present, was 20.6%; this rate was 2-fold higher than that for AOM visits (10.5%; p<0.0001) (Table 1).

Polymicrobial colonization of the nasopharynx was significantly less common at healthy visits than at AOM visits. At the onset of an infection, polymicrobial colonization increased by 1.5–2.8-fold overall for children of all ages (p<0.05 for all). At healthy visits, the proportion of children with polymicrobial nasopharynx colonization was 18.1% (214/1,183); whereas at AOM visits, the proportion was 45.5% (152/334) (p<0.0001). A comparison of single-pathogen colonization and polymicrobial colonization at ages 6, 9, 12, 15, 18, and 24 months and differences between healthy visits and AOM visits are shown in the Figure.

The overall colonization rates for any of the 3 pathogens were 57.4% (679/1,183) at healthy visits and 89.8% (300/334) at AOM visits (p<0.0001). The culture-positive rates at healthy visits were 30.3% (358/1183) for *S. pneumoniae,* 11.7% (138/1,183) for *H. influenzae,* and 36.3% (429/1,183) for *M. catarrhalis*; whereas at AOM visits, rates were 52.7% (176/334), 47.9% (160/334), and 43.4% (145/334), respectively (p<0.0001 for all) (Table 1).

Bacterial Interactions

The predicted outcome of nasopharynx colonization with *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* is

Table 1. Nasopharyngeal colonization of children of when healthy and at onset of acute otitis media, Rochester, NY, USA, June 2006–May 2010*

			% Total visits									
			Single			Multiple				Overall		
Group	Age, mean mo ± SD	No. visits	*Spn*	*Hflu*	*Mcat*	*Spn+ Hflu*	*Spn+ Mcat*	*Hflu+ Mcat*	*Spn+Hflu+ Mcat*	*Spn*	*Hflu*	*Mcat*
6 mo												
Healthy	6.4 ± 0.6	304	13.2	3.3	19.1	3.0	9.2	1.6	1.3	26.6	9.2	31.3
AOM	6.5 ± 1.2	66	22.7	19.7	9.1	10.6	13.6	4.5	13.6	60.6	48.5	40.9
9 mo												
Healthy	9.3 ± 0.4	237	12.7	3.0	21.1	2.5	10.5	1.3	2.5	28.3	9.3	35.4
AOM	9.0 ± 0.5	63	14.3	6.3	9.5	15.9	22.2	11.1	7.9	60.3	41.3	50.8
12 mo												
Healthy	12.3 ± 0.4	205	15.1	3.9	22.4	1.0	11.2	0.5	2.4	29.8	7.8	36.6
AOM	12.1 ± 1.1	90	15.6	24.4	8.9	10.0	15.6	5.6	5.6	46.7	45.6	35.6
15 mo												
Healthy	15.3 ± 0.5	170	17.1	5.3	19.4	2.9	13.5	1.8	2.4	35.9	12.4	37.1
AOM	15.3 ± 0.5	30	6.7	23.3	10.0	20.0	20.0	6.7	6.7	53.3	56.7	43.3
18 mo												
Healthy	18.4 ± 0.6	155	14.2	5.2	21.9	3.2	8.4	1.9	3.9	29.7	14.2	36.1
AOM	19.0 ± 1.2	42	4.8	28.6	16.7	9.5	16.7	7.1	9.5	40.5	54.8	50.0
24 mo												
Healthy	24.4 ± 0.5	112	14.3	9.8	20.5	1.8	15.2	7.1	7.1	38.4	25.9	50.0
AOM	24.9 ± 2.4	43	14.0	16.3	11.6	14.0	16.3	9.3	9.3	53.5	48.8	46.5
All ages												
Healthy	12.5 ± 5.6	1183	14.2	4.5	20.6	2.5	10.9	1.9	2.8	30.3	11.7	36.3
AOM	14.1 ± 7.0	334	14.4	19.5	10.5	12.6	17.1	7.2	8.7	52.7	47.9	43.4

**Spn*, *Streptococcus pneumoniae*; *Hflu, Haemophilus influenzae*; *Mcat*, *Moraxella catarrhailis;* AOM, acute otitis media.

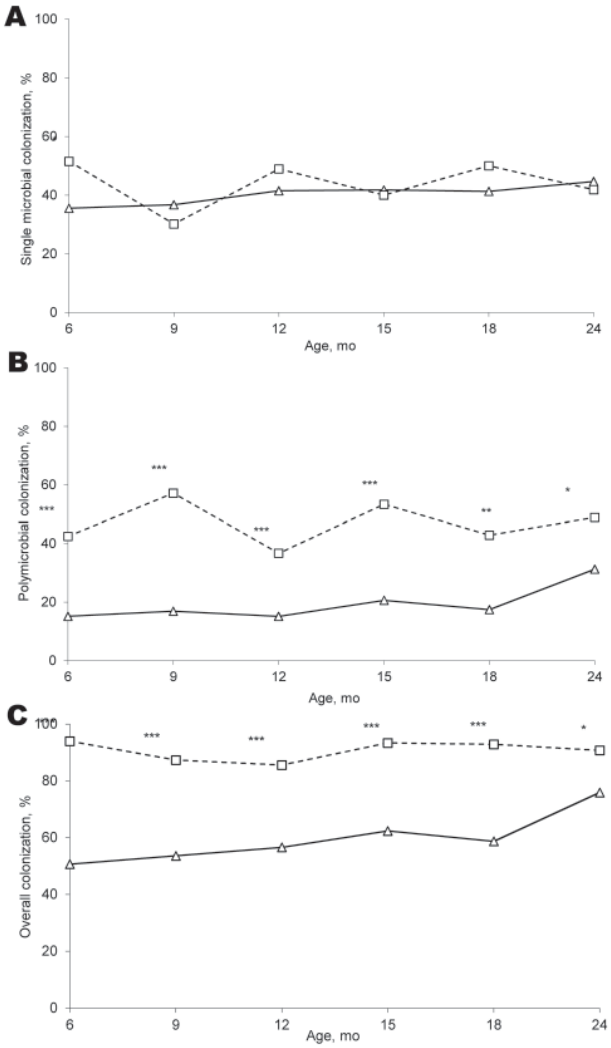

Figure. Nasopharyngeal colonization of children with *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* during routine doctor visits when healthy (triangles) and during visits for onset of acute otitis media (squares), Rochester, NY, USA, June 2006–May 2011. A) Single microbial colonization; B) polymicrobial colonization; C) overall colonization (single and polymicriobial). *p<0.05; **p<0.01, ***p<0.001, compared with healthy visits, by Fisher exact test.

shown in Table 2. At healthy visits, when colonization with *S. pneumoniae* was the predicted outcome, *S. pneumoniae* was synergistically associated with *M. catarrhalis* colonization (OR 1.42, p = 0.015) but not with *H. influenzae* colonization (OR 1.33, p = 0.28). When colonization with *H. influenzae* was the predicted outcome, no significant associations were found between *H. influenzae* and *S. pneumoniae* (OR 1.43, p = 0.19) or between *H. influenzae* and *M. catarrhalis* (OR 0.81, p = 0.43). When colonization with *M. catarrhalis* was the predicted outcome, *M. catarrhalis* colonization was synergistically associated with *S. pneumoniae* colonization (OR 1.51, p = 0.0059) but not with *H. influenzae* colonization (OR 0.83, p = 0.49).

At onset of AOM infection, the predictions differed greatly. When colonization with *S. pneumoniae* was the predicted outcome, *S. pneumoniae* colonization was competitively associated with *H. influenzae* colonization (OR 0.40, p = 0.0014) but not with *M. catarrhalis* colonization (OR 1.02, p = 0.94). When colonization with *H. influenzae* was the predicted outcome, *H. influenzae* colonization was competitively associated with *S. pneumoniae* colonization (OR 0.41, p = 0.0021) and *M. catarrhalis* colonization (OR 0.37, p = 0.0022). When colonization with *M. catarrhalis* was the predicted outcome, *M. catarrhalis* colonization was competitively associated with *H. influenzae* colonization (OR 0.35, p = 0.0015) but not with *S. pneumoniae* colonization (OR 1.02, p = 0.95).

The higher prevalence of a potential otopathogen in the nasopharynx need not imply greater association. For example, the marginal rates for the pathogens in the healthy group (all ages) were 0.303, 0.117, and 0.363, the product of which is 0.0129 (Table 1). The actual rate for the occurrence of all 3 is 0.028, indicating an increase in association compared with chance. In contrast, the corresponding rates for the AOM group are 0.527, 0.479, 0.434, the product of which is 0.1096, compared with an actual rate of occurrence of 0.087 for all 3, indicating a decrease in association compared with chance. These totals are therefore compatible with the ORs reported in Table 2.

Effects of Other Risk Factors on Bacterial Colonization

Analysis of the effects of *S. aureus* colonization and host factors on nasopharynx colonization by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in children when healthy and at onset of AOM indicated that nasopharyngeal cultures were positive for *S. aureus* at 7.7% (91/1,183) of healthy visits and 5.7% (19/334) of AOM visits (p = 0.23). *S. aureus* was competitively associated with *S. pneumoniae* at healthy visits (OR 0.55, p = 0.011) but not at AOM visits (OR 0.95, p = 0.89). No significant associations were identified between *S. aureus* and *H. influenzae* or between *S. aureus* and *M. catarrhalis* at either type of visit (Table 2). At the healthy visits, daycare attendance was significantly positively associated with *S. pneumoniae* (OR 1.87, p = 0.0001) and *H. influenzae* colonization (OR 1.71, p = 0.015) but not with *M. catarrhalis* colonization (OR 1.28, p = 0.11). At onset of AOM, daycare attendance was significantly positively associated with *H. influenzae* colonization (OR 2.06, p = 0.0032) but not with *S. pneumoniae* (OR 1.29, p = 0.28) or *M. catarrhalis* colonization (OR 0.91, p = 0.70). The otitis-prone condition (defined as 3 episodes of AOM infection within 6 months or 4 AOM infections within 12 months) was significantly positively associated with

Table 2. Predicted outcome of nasopharyngeal colonization among children when healthy and at onset of acute otitis media, Rochester, NY, USA, June 2006–May 2010*

	Outcome, OR (95% CI)					
	Spn		*Hflu*		*Mcat*	
Characteristics	Healthy	AOM	Healthy	AOM	Healthy	AOM
Hflu x *Mcat*						
Neither§	1.0	1.0	NA	NA	NA	NA
Hflu	1.33 (0.78–2.27)	**0.40† (0.22–0.71)**	NA	NA	NA	NA
Mcat	**1.42‡ (1.06–1.99)**	1.02 (0.58–1.79)	NA	NA	NA	NA
Both	3.40 (1.99–5.81)	0.79 (0.41–1.51)	NA	NA	NA	NA
Spn x *Mcat*						
Neither§	NA	NA	1.0	1.0	NA	NA
Spn	NA	NA	1.43 (0.83–2.45)	**0.41† (0.23–0.73)**	NA	NA
Mcat	NA	NA	0.81 (0.48–1.38)	**0.37† (0.19–0.71)**	NA	NA
Both	NA	NA	2.10 (1.30–3.40)	0.28 (0.15–0.52)	NA	NA
Spn x *Hflu*						
Neither§	NA	NA	NA	NA	1.0	1.0
Spn	NA	NA	NA	NA	**1.51† (1.12–2.03)**	1.02 (0.58–1.80)
Hflu	NA	NA	NA	NA	0.83 (0.49–1.41)	**0.35† (0.18–0.68)**
Both	NA	NA	NA	NA	2.17 (1.29–3.64)	0.68 (0.36–1.32)
Saur						
Absent§	1.0	1.0	1.0	1.0	1.0	1.0
Present	**0.55‡ (0.34–0.88)**	0.95 (0.43–2.08)	0.72 (0.33–1.54)	0.62 (0.19–2.00)	1.07 (0.69–1.66)	1.19 (0.52–2.70)
Male	0.0.84 (0.61–1.17)	0.85 (0.54–1.35)	0.83 (0.53–1.28)	0.75 (0.46–1.23)	1.07 (0.80–1.42)	**1.94† (1.21–3.11)**
Daycare	**1.87† (1.34–2.59)**	1.29 (0.81–2.05)	**1.71‡ (1.1–2.67)**	**2.06† (1.26–3.38)**	1.28 (0.94–1.73)	0.91 (0.58–1.45)
Breast-fed	1.29 (0.92–1.83)	1.07 (0.65–1.77)	0.75 (0.48–1.16)	0.81 (0.48–1.38)	1.00 (0.74–1.35)	1.02 (0.63–1.63)
Smoke	0.77 (0.48–1.22)	0.65 (0.31–1.34)	0.74 (0.38—1.43)	0.61 (0.29–1.30)	0.80 (0.53–1.20)	0.98 (0.41–2.30)
Otitis prone	**2.34‡ (1.19–4.59)**	0.98 (0.63–1.53)	**3.10† (1.51–6.36)**	1.42 (0.89–2.27)	1.16 (0.51–2.66)	0.98 (0.63–1.52)

*__Boldface__ indicates significance. OR, odds ratio; *Spn*, *Streptococcus pneumoniae*; *Hflu, Haemophilus influenzae*; *Mcat*, *Moraxella catarrhailis*; AOM, acute otitis media; NA, not applicable; *Saur*, *Staphylococcus aureus*. Bacterial interactions were analyzed by using repeated measures logistic regression models.
†p≤0.01.
‡p≤0.05.
§Reference.

S. pneumoniae (OR 2.34, p = 0.016) and *H. influenzae* colonization (OR 3.1, p = 0.0017) at healthy visits but not at AOM visits. We found no significant association between smoking exposures and colonization or between a history of breastfeeding and colonization at either type of visit (Table 2). Being male was positively associated with *M. catarrhalis* colonization (OR 1.94, p = 0.005) at AOM visits but not at healthy visits.

Discussion

We found that patterns of nasopharynx colonization associations in children differed at onset of AOM and when healthy. *H. influenzae* colonization was competitively associated with *S. pneumoniae* and *M. catarrhalis* colonization at AOM visits but not at healthy visits; the rates of nasopharynx colonization by *H. influenzae* were 4-fold higher during AOM visits than during healthy visits.

Our findings suggest that during the PCV era, *H. influenzae* might increase as a bacterial pathogen of AOM. Our data among young children show that nasopharynx colonization studies of healthy children might not reflect the polymicrobial mix at the time of onset of AOM; the nasopharyngeal environment during onset of AOM favors *H. influenzae* colonization. It is the mix of bacteria at time of infection that determines which organisms are most likely to cause infection (*12*). We have previously shown that among children, the changes in nasopharynx colonization by *S. pneumoniae* and *H. influenzae* caused by PCV7 resulted in a remarkable proportionate decrease in AOM infection caused by *S. pneumoniae* and a proportionate increase in

AOM infection caused by *H. influenzae* (*17*,*21*,*22*). At onset of AOM, when *H. influenzae* co-colonizes with *S. pneumoniae* or *M. catarrhalis, H. influenzae* predominates over these 2 bacteria to cause AOM (*18*). The elimination of *S. pneumoniae* strains expressing PCV7 serotypes has resulted in the remaining *S. pneumoniae* strains, except serotype 19A, competing less effectively with *H. influenzae* in the nasopharynx (*18*). Therefore, with a further reduction in nasopharynx colonization by *S. pneumoniae,* including strains expressing serotype 19A (as will probably result from the recent introduction of PCV13 in some countries), our data suggest that *H. influenzae* might fill the nasopharyngeal niche at the onset of AOM, and consequently, *H. influenzae* might become a more prominent cause of AOM.

S. aureus also can cause respiratory infections; several reports have suggested that *S. aureus* might be replacing *S. pneumoniae* as a dominant nasopharynx colonizer as a consequence of the introduction of PCV. Concern has been expressed that *S. aureus* might emerge as a more prominent respiratory pathogen (*10*–*13*,*23*). However, our results do not support that *S. aureus* has emerged as a frequent pathogen of AOM after introduction PCV7.

Knowledge regarding interactions among *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* is limited, appears contradictory, and is confined to studies among children. Zemlickova et al. found no significant association between *S. pneumoniae* and *H. influenzae* or between *S. pneumoniae* and *M. catarrhalis* in the nasopharynx of 425 healthy children, 3–6 years of age, in the Czech Republic (*11*). Madhi et al. found synergistic associations between *S. pneumoniae* and *H. influenzae* in PCV9-vaccinated healthy children, 5 years of age, in South Africa (*10*). Jacoby et al. found synergistic associations between nasopharynx colonization by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in healthy children, 1–24 months of age, but no significant association between *S. pneumoniae* and *S. aureus* or between *H. influenzae* and *S. aureus* (*16*). At onset of respiratory viral infections and in association with AOM infection, Pettigrew et al. (*8*) found competitive associations between colonization by *S. pneumoniae* and *H. influenzae*, *H. influenzae* and *M. catarrhalis, S. pneumoniae* and *S. aureus,* and *H. influenzae* and *S. aureus* in children 6–36 months of age. Thus, our results comparing nasopharynx colonization patterns during times of health with patterns at onset of AOM help explain the prior contradictory results.

Although we found a synergistic association between *S. pneumoniae and M. catarrhalis* at healthy visits and a significant increase of polymicrobial colonization at AOM visits, the tendency toward polymicrobial colonization did not result in synergistic associations at AOM visits. On the contrary, competitive associations between *H. influenzae* and *S. pneumoniae and* between *H. influenzae* and *M. catarrhalis* were found at AOM visits. The increases in colonization of individual bacterial pathogens during AOM might randomly result in an increase of polymicrobial colonization and might not result from synergistic associations among the potential pathogens. Further study on the mechanism is needed.

The mechanisms to explain competitive and synergistic interactions among *S. pneumoniae, H. influenzae, M. catarrhalis*, and *S. aureus* have been explored. In a mouse model, Lysenkoet al. found that when *H. influenzae* colonized with *S. pneumoniae* in the nasopharynx, *S. pneumoniae* was rapidly cleared (*24*). The competitive interaction was dependent on cellular components of *H. influenzae* activating the host innate immune response involving complement and neutrophils; the end result was the killing of *S. pneumoniae* (*24*). The mouse model results are consistent with our observations among children but differ from results observed during in vitro experiments that predict that *S. pneumoniae* should inhibit the growth of *H. influenzae* (*25*,*26*). A competitive association between *S. pneumoniae* and *S. aureus* might be mediated by *S. pneumoniae* production of hydrogen peroxide (*25*,*27*). Our results and those of others suggest that this mechanism might be active during times of health but not, on the basis of our observation, at onset of AOM (*10*,*11*,*13*,*28*).

We did evaluate the effects of age, sex, daycare attendance, breast-feeding history, exposure to tobacco smoke, and an immunologically driven increased susceptibility to AOM (*15*,*29*) in our study population. The results differed at healthy versus AOM visits except for the effect of daycare attendance on *H. influenzae* colonization; daycare attendance had a positive association with *H. influenzae* colonization at both healthy and AOM visits. Others have shown daycare attendance to be consistently associated with increased colonization by *S. pneumoniae, H. influenzae,* and *M. catarrhalis* in healthy children and in children with viral upper respiratory infections (*8*). Our previous studies have shown that children who are prone to otitis have much weaker immune responses to *S. pneumoniae* surface antigens than those who are not prone (*15*,*30*). We therefore assessed the otitis-prone condition as a predictor in the models in this study. We found that the otitis-prone condition was positively associated with *S. pneumoniae* and *H. influenzae* colonization at healthy visits but not at AOM visits.

The rates of nasopharynx colonization with *S. pneumoniae, H. influenzae*, and *M. catarrhalis* and the mix at onset of AOM that we observed occurred mostly in the context of a concurrent viral upper respiratory tract infection. A limitation of our study is that we did not evaluate bacterial–viral interactions. Revai et al. (*31*) and Chonmaitree et al. (*32*) found that among young children, viral upper respiratory tract infections preceded >90% of

AOM infections. At onset of AOM, 93% of the children in our study had clinical signs of a viral upper respiratory tract infection; the predominant viruses detected were influenza, parainfluenza, respiratory syncytial, and adenovirus (A. Chang, unpub. data). The culture and PCR methods used in that study did not identify rhinovirus, metapneumovirus, or bocavirus. Previous studies have shown that viral upper respiratory tract infections affect bacterial nasopharynx colonization (*5*,*7*). Respiratory viruses up-regulate epithelial cell receptors for some species of respiratory bacteria (*7*); they cause inflammation and down-regulate innate and adaptive host defenses (*7*,*33*). Different viruses have varying effects on different bacteria (*34*–*36*). The comparison of healthy visits with AOM visits suggests that viral infections alter the host nasopharynx environment by facilitating a shift in the polymicrobial mix and enhancing polymicrobial colonization.

We did not study the additional effect of antimicrobial drugs on nasopharynx colonization. Such treatment modifies nasopharynx colonization patterns. Pettigrew et al. (*8*) showed that antimicrobial drug therapy was associated with a lower prevalence of colonization with *S. pneumoniae* and *M. catarrhalis* but not with *H. influenzae*. Varon et al. (*37*) showed that colonization by *S. pneumoniae, H. influenzae*, and *M. catarrhalis* decreased after antimicrobial drug thereapy; the reduction in colonization was less for *H. influenzae* than for *S. pneumoniae* or *M. catarrhalis*. Current national guidelines endorse the use of amoxicillin for first-line treatment of AOM, sinusitis, and community-acquired pneumonia in children (*38*–*40*). Amoxicillin is ineffective for eradicating β-lactamase–producing *H. influenzae* and *M. catarrhalis*. We have previously shown that ≈65% of *H. influenzae* and 100% of *M. catarrhalis* colonizing the nasopharynx of children in our study population elaborate β-lactamase (*17*,*21*,*22*). Therefore, empiric treatment with amoxicillin would probably increase *H. influenzae* and *M. catarrhalis* nasopharynx colonization in children.

Our results led us to 2 conclusions. First, nasopharyngeal bacterial interactions among *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* differ during health and at onset of AOM in young children. Second, at the onset of AOM, the nasopharynx environment among children vaccinated with PCV7 is favorable for *H. influenzae* colonization. Consequently, our results predict that *H. influenzae* might be become a more prominent bacterial pathogen of AOM in the era of PCV. Further studies of virus–bacterium–host interactions in the nasopharynx and additional studies of the mechanisms driving the observed shifts in bacterial species and polymicrobial makeup are needed.

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Lack of Evidence for Zoonotic Transmission of Schmallenberg Virus

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The emergence of Schmallenberg virus (SBV), a novel orthobunyavirus, in ruminants in Europe triggered a joint veterinary and public health response to address the possible consequences to human health. Use of a risk profiling algorithm enabled the conclusion that the risk for zoonotic transmission of SBV could not be excluded completely. Self-reported health problems were monitored, and a serologic study was initiated among persons living and/or working on SBV-affected farms. In the study set-up, we addressed the vector and direct transmission routes for putative zoonotic transfer. In total, 69 sheep farms, 4 goat farms, and 50 cattle farms were included. No evidence for SBV-neutralizing antibodies was found in serum of 301 participants. The lack of evidence for zoonotic transmission from either syndromic illness monitoring or serologic testing of presumably highly exposed persons suggests that the public health risk for SBV, given the current situation, is absent or extremely low.

In November 2011, scientists in Germany identified novel viral sequences in serum from cattle affected by a febrile syndrome that was reported during August–September 2011 in Germany and the Netherlands. Clinical signs included decreased milk production and diarrhea. The virus, named Schmallenberg virus (SBV), was isolated from blood of affected cattle, and similar clinical manifestations were observed in experimentally infected calves (*1*). In the Netherlands, SBV was detected retrospectively in serum from affected cattle in December 2011 (*2*).

Since the end of November 2011, an unusually high number of ovine and bovine congenital malformations were reported in the Netherlands. The main macroscopic findings included arthrogryposis; torticollis; scoliosis; brachygnathia inferior; hydranencephaly; and hypoplasia of cerebrum, cerebellum, and spinal cord. SBV genome was detected in the brain of malformed lambs and calves (*3*–*5*). These findings, together with detection of SBV RNA in multiple types of samples, e.g., amniotic fluid, meconium, and placenta remains from diseased lambs and calves, strongly pointed to SBV as the causative agent of the clinical manifestations (*6*). The teratogenic effects in ruminants are hypothesized to reflect virus circulation in late summer/early autumn 2011, leading to intrauterine infection with SBV during a specific period of gestation (*4*).

In June 2012, seven additional European countries (Belgium, Denmark, France, Italy, Luxemburg, Spain, and the United Kingdom) confirmed SBV in ruminants, accumulating to a total of 3,745 PCR-confirmed infected animal holdings (*4*,*7*). In the Netherlands 1,670 holdings were suspected to be affected by SBV on the basis of births of animals with malformations typical of SBV infection, of which 350 were confirmed by PCR as of June 12, 2012. The holdings with confirmed SBV comprise 237 cattle, 107 sheep, and 6 goat farms (*8*).

SBV has been identified as most related to Sathuperi virus, and for the small and large segments, Shamonda

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virus segments show the highest sequence identity. All those viruses are members of the Simbu serogroup, family *Bunyaviridae*, genus *Orthobunyavirus*, and known as arthropod-borne viruses that can cause illness in ruminants (*9*). The orthobunyaviruses comprise ≈170 virus isolates, assigned to 48 distinct species, arranged in 18 serogroups, including the Simbu serogroup. Serogroups within the genus are based on cross–hemagglutination-inhibition and antibody neutralization relationships. Phylogenetic relationships are consistent with the results of serologic relationships (*10*–*12*).

Because the family *Bunyaviridae* contains several medically relevant zoonotic viruses, of which Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, Sin Nombre virus, and sandfly fever Naples virus are examples, the emergence of SBV triggered a joint veterinary and public health response in the Netherlands to address the possible consequences to human health. We present the public health risk ascertainment of the emergence of SBV in ruminants in the Netherlands and most likely other European countries were SBV has emerged.

Methods

Profiling Risks to Humans

We used a standard in-house checklist for profiling the risk to human health of novel emerging viruses to assess the public health risks for SBV. This checklist comprised 10 items: 1) situation assessment; 2) review of taxonomic position of the newly identified virus; 3) review of human health risks associated with closely related viruses; 4) review of epidemiology of related viruses (transmission cycle, reservoirs, and vectors); 5) review of clinical manifestations in humans of related viruses (including kinetics of immune response and shedding); 6) assessment of potential for human exposure and identification of related risk factors; 7) assessment of human diagnostics; 8) design of a literature/evidence-based testing algorithm; and 10) conclusions and recommendations.

Virus and Validation Serum

An SBV strain, isolated from SBV reverse transcription PCR–positive, homogenized brain tissue of a malformed lamb in the Netherlands, was obtained from the Central Veterinary Institute (Lelystad, the Netherlands). Putative cross-reacting orthobunyaviruses circulating in Europe, Batai virus (*13*), Tahyna virus (*14*), and Inkoo virus (*15*), were obtained from the Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany). All viruses were propagated and titrated (50% tissue culture infectious dose [$TCID_{50}$]) in continuous African green monkey kidney cells (Vero E6, ATCC CRL-1586). SBV-positive control serum from a ewe that had given birth to an SBV PCR-positive lamb was obtained from the Animal Health Service (AHS), and positive serum sample from an experimentally infected ewe was obtained from the Central Veterinary Institute.

Well-defined negative and positive human serum cohorts were not available because SBV is a novel emerging virus with unknown zoonotic potential. Therefore, we validated the virus neutralization test (VNT) using presumed seronegative serum from 1) 56 patients without travel history submitted to the National Institute for Public Health and the Environment during February 28, 2007–February 25, 2008, for routine diagnostic testing for *Bordetella pertussis*; 2) 73 inhabitants of municipalities with known SBV activity in 2011 that had been collected during August 15, 2010–October 15, 2010, for routine screening; and 3) 93 veterinary students collected in 2006 and 2008. Serum from 92 veterinary students sampled during 2011 and from 73 inhabitants of municipalities with known SBV activity collected during August 15, 2011–October 15, 2011, for routine screening were considered to represent community samples from possibly exposed populations and were added to the validation panel. Anonymized use of serum from the National Institute for Public Health and the Environment was covered by the rules of the code of conduct for proper use of human tissue of the Dutch Federation of Medical Scientific Associations. The cohort study of the veterinary students included screening for zoonotic infections and was approved by the Medical Ethical Committee of the University Medical Centre Utrecht.

VNT

For VNT, Vero E6 cells were seeded in 96-well plates and incubated overnight at 37°C with 5% CO_2 until the cells were ≈80%–90% confluent. Serum was heated for 30 min at 56°C to inactivate complement before use. Serum was serially diluted in 2-fold steps in minimum essential medium (GIBCO/Life Technologies, Bleiswijk, the Netherlands). We added 100 $TCID_{50}$ of virus to the diluted serum (volume of 60 μL each). To rule out the presence of other cytopathic effect–inducing factors, serum dilutions also were added to control wells to which no virus was added. After incubation at 37°C in 5% CO_2 for 1 h, 100 μL of the virus-plus-serum mixture, no virus-serum controls, and a virus dilution control were added to the Vero E6 cells and incubated for 3 d at 37°C. Assays were performed in duplicate. Cells were monitored for cytopathic effect after 3 days.

Monitoring of Health Symptoms

Persons in close contact with affected animals or their birth materials in whom fever developed (>38°C) within 2 weeks after exposure were asked to contact the regional public health service (PHS) for evaluation and assessment of the need for follow-up. This request was made through

an email-based alert system hosted by the AHS and farmers association to veterinarians. The alert system prompted veterinarians to inform farmers on SBV-affected holdings. When a relation between reported fever and SBV was considered possible, a short questionnaire was filled in by study participants, and serum was tested by real-time PCR (as described in [*6*]) and VNT to diagnose a possible SBV infection.

Design of Serologic Study in Persons with High Probability of Exposure

A serologic survey was designed to determine the presence of SBV antibodies in serum from persons living and working on farms where SBV had been highly suspected on the basis of pathologic findings consistent with typical SBV-induced malformations in calves or lambs, most confirmed by PCR and/or serology. The target cohort, consisting of adult (≥18 years of age) farmers, farm residents, farm employees, and veterinarians who had been exposed to affected herds, were invited to participate by donating a serum sample and filling in a questionnaire. A total of 240 affected animal holdings were approached through direct mailing by the AHS. Employees of the regional PHS visited the affected farms and collected serum samples and questionnaires. The veterinarians were collectively contacted to be sampled at a national conference after a preannouncement of the purpose of the study.

The questionnaire addressed demographics, the animal species involved, the type and level of exposure (birth materials, feces, milk or other products, insects), protective equipment used during work, general health, (recent) health complaints, and presence of wounds on hands. The study protocol, information material, and questionnaires were assessed by the Medical Ethical Committee of the University Medical Centre Utrecht and approved (METC no. 12–106).

On the basis of a literature review of seroprevalence studies in regions with known orthobunyavirus outbreaks, a seroprevalence of 2% was established as the lower bound in an affected human population (N. Cleton, unpub. data; *16–19*). In this scenario with 2% seroprevalence, testing of, for example, 200 exposed persons would give a probability of 98.24% to detect ≥1 seropositive persons (Table 1).

Table 1. Probability of detecting at least 1 seropositive sample among different sample sizes and hypothetical seroprevalences in study to determine whether Schmallenberg virus can be zoonotically transmitted, the Netherlands

Sample size	Hypothetical seroprevalence, %	Probability* of detecting at least 1 seropositive, %
50	2.00	63.58
100	2.00	86.74
150	2.00	95.17
192	2.00	97.93
200	2.00	98.24
301	2.00	99.77
301	1.00	95.14
301	0.50	77.88
301	0.25	52.93
301	3.00	99.99

*The probability was calculated as 1 – (1 – seroprevalence) ^ sample size, so for a seroprevalence of 2% and a sample size of 200, the probability of detecting at least 1 seropositive = 1 – (1 – 0.02) ^ 200.

Results

Profiling the Human Risks for SBV

Human Disease in Related Viruses

The literature indicates that zoonotic transmission of SBV could not be completely ruled out. The taxonomic position of SBV had been identified as family *Bunyaviridae*, genus *Orthobunyavirus*, Simbu serogroup (*1*). At least 30 orthobunyaviruses have been associated with human disease. Virologic or serologic evidence for zoonotic infection has been found for several viruses within the Simbu serogroup, including viruses considered to be primarily livestock pathogens (Aino and Shuni virus; Table 2). Among the many reasons for vigilance was the lack of full characterization of SBV. Genetic reassortment between orthobunyaviruses within the same serogroups has led to emergence of new viruses, occasionally with increased pathogenicity and potentially with changes in host range (*21*,*36–40*).

Modes of Transmission

The related Shamonda, Sathuperi, Aino, and Akabane viruses are transmitted mainly by biting midges (*23*; *41* in online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0560-Techapp.pdf), and the epidemiology of the infection in animals and the first detections of SBV genome in *Culicoides* spp. midges in Belgium, Denmark, and Italy suggested vector-borne spread as a mode of transmission for SBV as well (*1*,*2*; *42–44* in online Technical Appendix). In addition, the birth defects in lambs and calves increased the need for assistance from veterinarians during parturition, and high loads of viral RNA were detected in birth materials of sheep and cattle (*6*). Therefore, if SBV is zoonotic, transmission could have occurred to persons who could have been exposed to infected vectors (residents, farmers, veterinarians) and/or through direct contact with animals that had congenital malformations or with birth material, e.g., during assistance at deliveries (farmers, veterinarians). A testing algorithm was designed (Figure). Professionals were advised to respect common hygiene measures for veterinarian-assisted deliveries and handling of affected newborn ruminants. Pregnant women were advised not to assist at ruminant deliveries.

Table 2. Evidence for zoonotic infection within the family *Bunyaviridae*, *Orthobunyavirus* genus, the Netherlands*

Serogroup, species	Geographic distribution	Reservoir	Human infection	Evidence	Reported symptoms in humans	Congenital disease in humans	References
Simbu							
Oropouche virus	South America	Humans, sloths, marmosets	Yes	Outbreaks	Febrile illness, arthralgia, diarrhea	NR	(*18*,*20*)
Iquitos virus	South America	Humans, unknown	Yes	Unexplained fever surveillance	Febrile illness, arthralgia, diarrhea	NR	(*21*)
Akabane virus	Asia, Israel, Kenya, Australia	Cattle, horses, sheep	No	No serology	NR	NR	(*22*,*23*)
Shamonda virus	Africa	Cattle	No	No serology	NR	NR	(*24*,*25*)
Aino virus	Asia	Cattle	Possibly	Serology	Unknown	Unknown	(*22*,*23*,*26*)
Shuni virus	Africa	Cattle, horses, sheep	Possibly	Virus isolation (one case)	Fever, no hospital submission	NR	(*27*–*29*)
Sathuperi virus	Asia, Africa	Cattle	Unknown	Unknown	Unknown	Unknown	(*30*)
California encephalitis							
California encephalitis virus	North America	Rodents, lagomorphs	Yes	Endemic, common	Febrile illness, encephalitis	NR	(*31*)
La Crosse virus	North America	Rodents, lagomorphs	Yes	Endemic common	Febrile illness, encephalitis	NR	(*31*)
Tahyna virus	Europe, Asia, Africa	Lagomorphs, rodents, hedgehogs	Yes	Serology, cases	Febrile illness, respiratory symptoms meningitis (mild)	NR	(*32*)
Inkoo virus	Northern Europe	Lagomorphs	Yes	Surveillance febrile illness, CNS illness,	Febrile illness, Meningitis (mild)	NR	(*32*)
Snowshoe hare virus	North America, Far-Eastern Europe	Lagomorphs, rodents	Yes	Serology, case reports	Febrile illness	NR	(*32*) (*31*)
Bunyamwera							
Batai virus	Europe, Asia, Africa	Birds,pigs, horses, ruminants	Yes	Serology (rare)	Febrile illness, affection	NR	(*32*)
Cache Valley virus	North America	Deer, sheep, horses, cattle	Yes	2 Case reports, serology	Febrile illness, encephalitis	Under discussion	(*33*–*35*)
Ngari virus	Africa	Humans, unknown	Yes	Outbreaks, virus isolation	Febrile illness, hemorragic fever	NR	(*36*–*38*)

*NR, not reported; CNS, central nervous system.

Validation of VNT

Because the viremic phase in orthobunyavirus infections typically is short, we chose to use serologic testing by VNT to evaluate an immunologic response in exposed persons (*1*,*21*). For assay validation, possible cross-reacting zoonotic viruses circulating in Europe were identified. Zoonotic viruses in the Simbu serogroup are not known to circulate in Europe, but related orthobunyaviruses that may infect humans are Batai virus (BATV), Tahyna virus (TAHV), and Inkoo virus (INKV) (Table 2). No cross-neutralization was observed when the SBV-positive control serum was tested against 100 $TCID_{50}$ of BATV, INKV, and TAHV, whereas the homologous titer was 512 (data not shown). The reverse experiment could not be conducted because of a lack of reference reagents. A control cohort of 222 serum samples, presumed negative on the basis of collection data before 2011, were all negative in the VNT (data not shown). Another validation cohort of 165 serum samples, possibly positive on the basis of collection data in 2011 and putative exposure through residence and professional activities, were all negative as well (data not shown).

Monitoring of Symptoms

Symptoms that could be attributed to a putative infection with SBV were determined on the basis of an inventory made of syndromes related to human infection with closely related viruses of the Simbu group, i.e., Oropouche virus and Iquitos virus (Table 2). These viruses typically cause a febrile illness accompanied by chills, general malaise, headache, anorexia, muscle and joint pain, muscle weakness, and vomiting. Symptoms of meningitis or a rash occasionally develop. The reported diseases generally are self-limiting (*20*,*21*).

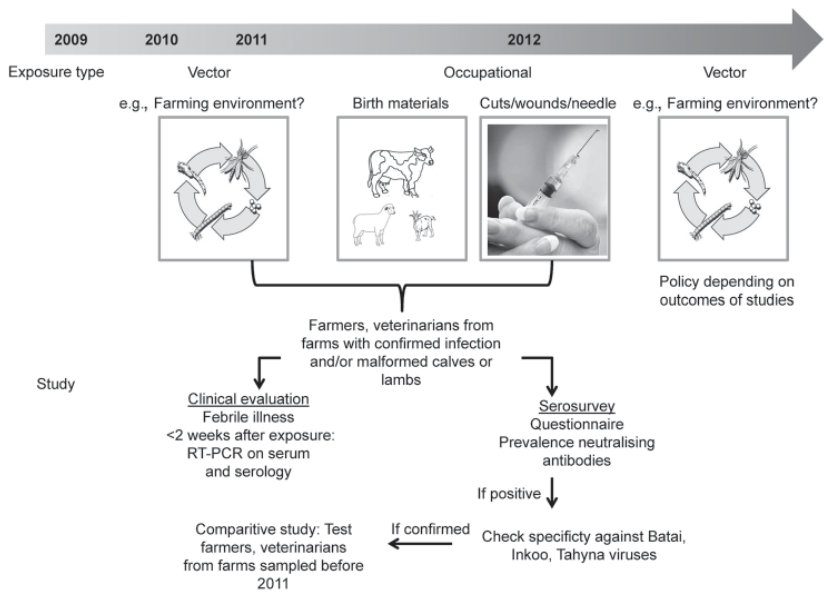

Figure. Testing algorithm to determine whether Schmallenberg virus can be zoonotically transmitted, the Netherlands. RT-PCR, reverse transcription PCR.

Because the range of symptoms described was diverse, we decided to monitor patients who suited our case definition: febrile disease >38°C within 2 weeks after contact with malformed calves or lambs or their birthing materials (in the absence of the supposed vector during the winter season). The 2-week period was based on the known incubation period for Oropouche virus in humans, typically 4–8 days (*20*). Eight cases were reported by the PHS during January 1–April 15, 2012. Four of these were excluded because they did not meet the case definition. The remaining 4 cases were tested by PCR and VNT (for 3 cases only because only vesicle fluid was available for 1 study participant). None of the tested suspected case-persons showed evidence of an SBV infection.

In addition, no unusual trends were noted during or since summer 2011 in the existing routine surveillances for neurologic illness, gastroenteritis, and influenza-like illness at the Netherlands Centre for Infectious Disease Control (H. van der Avoort, E. Duizer, and A. Meijer, pers. comm.).

Serology in High-Exposure Groups

To enable evidence-based risk profiling, serologic surveillance was initiated in persons residing at locations with proven SBV circulation and professionals in close contact with infected animals and their birth materials. In this study set-up, we addressed the vector and the direct transmission routes for putative zoonotic transfer.

The study comprised 301 participants. Of these, 192 worked or lived on farms with laboratory-confirmed SBV circulation in animals, 42 persons worked or lived on farms where animals were being raised and where SBV infection was highly suspected, and 67 were veterinarians who had been in contact with malformed animals (Tables 3, 4). These 123 farms consisted of 69 sheep, 4 goat, and 50 cattle farms that had animals with typical SBV malformations (no other pathogens were circulating in the Netherlands that cause congenital malformations, including arthrogryposis), of which most were PCR and/or VNT confirmed (83%; Table 4). SBV-specific antibodies were detected in livestock serum at 97.7% (83/85) of the farms for which serum was available (Table 4). Overall, 229 participants specifically reported direct exposure to newborn calves, lambs, and/or birth materials from SBV-infected herds; these participants comprised 179 farmers, and 50 veterinarians (39 of whom were exposed while assisting with deliveries at farms and 11 during postmortem examination of malformed newborns at the AHS). A total of 150 participants reported insect bites on SBV-infected farm(s), exposing them potentially to SBV during the vector season (Table 3).

None of the 301 participants showed serologic evidence of SBV infection in the VNT, whereas a titer of neutralizing antibodies was high in the ovine control serum. In a scenario of 2% seroprevalence, testing of 301 persons would have led to a probability of 99.77% to detect ≥1 seropositive persons (97.93% on the basis of 192 persons with laboratory-confirmed exposure; Table 1). Nevertheless, sporadic infections cannot be excluded entirely.

Discussion

The Netherlands has an integrated structure for human–animal risk analysis and response to zoonoses, established after the massive Q fever outbreak in 2007–2010. The continuous emergence of zoonotic viruses from livestock reservoirs, with examples of Nipah virus, Japanese encephalitis virus, highly pathogenic avian influenza

Table 3. Main characteristics of study participants* in study to determine whether SBV can be zoonotically transmitted, the Netherlands*

Exposure group, risk factor	Exposed	Participants, no. (%)/additional information
All		
Total		301/age 18–88 y; mean 47 y; 25th–75th percentile 36–58 y; 62% male
Exposure to biting insects on SBV-infected farm(s)	Yes	150 (50)
	No	71 (24)
	Unknown	80 (26)
Farmers, total		234
Working and living on SBV-infected farm		191 (82)
Working on SBV-infected farm		26 (11)
Living on SBV-infected farm		15 (6)
Unknown		2 (1)
Exposure to animals		
Sheep		
Regular contact with lambs and/or birth products on SBV-infected farm	Yes	110, of whom 88 reported hand (skin) injuries during work
	No	31
Total		141
Goats		
Regular contact with kids and/or birth products on SBV-infected farm	Yes	3, of whom 3 reported regular hand (skin) injuries during work
	No	10
Total		13
Cattle		
Regular contact with calves and/or birth products on SBV-infected farm	Yes	90, of whom 72 reported regular (hand) skin injuries during work
	No	38
Total		128
Veterinarians		
Total		67/1–50 SBV-infected farms visited per veterinarian; median 4
Exposure to animals		
Sheep		
Contact with malformed lambs and/or birth products	Yes†	19, of whom 18 reported regular hand (skin) injuries during work
	No	29
Total		48
Goats		
Contact with malformed lambs and/or birth products	Yes†	1 who reported regular hand (skin) injuries during work
	No	29
Total		30
Cattle		
Contact with malformed calves and/or birth products	Yes†	33, of whom 28 reported regular hand (skin) injuries during work
	No	20
Total		53
Contact with malformed lambs/calves during section at Animal Health Service	Yes	11, of whom 6 reported regular hand (skin) injuries during work.

*Overall, 50 (75%) of 67 veterinarians reported contact with malformed lambs/calves and/or birth products of which 40 reported regular hand (skin) injuries during work. Overall, 179 (76%) of 234 farmers, farm residents and farm employees reported regular contact with newborn lambs/calves and/or birth products on SBV-infected farms, of which 140 reported regular hand (skin) injuries during work. SBV, Schmallenburg virus.
†All tested seronegative.

A (H7N7) and A (H5N1) viruses, and coronaviruses, underscores the relevance of the One Health approach in assessing the risks for novel emerging pathogens, such as SBV (*45–49* in online Technical Appendix). The emergence of SBV in 2011 was a test case for this collaborative approach to risk assessment. Information, protocols, and samples were shared rapidly, facilitating a quick public health response.

On the basis of the findings of an in-house risk-assessment algorithm, we concluded that zoonotic transmission of the virus could not be excluded, triggering the study described here. We found no evidence for infection by serology, but ruling out zoonotic infections with high certainty is not simple, particularly in a complex situation with >1 possible mode of transmission.

If zoonotic, transmission of SBV could have occurred through vector-borne transmission during the period of high vector density in summer and fall 2011. The level of exposure to SBV by arthropods depends on the vector capacity of the residing vectors. Vector capacity is a measure

Table 4. Characteristics of participating farms and number of human participants in study to determine whether SBV can be zoonotically transmitted, the Netherlands*

Animal species†	No. farms (no. animals per farm [median])	Laboratory-confirmed SBV infection in animals, no. (%)			No. human participants	
		PCR	VNT	PCR and/or VNT	Total (no. per farm)	No. (%) from farms with laboratory-confirmed SBV infection
Sheep	69 (5–1,676 [83])	48 (70)	44 (64)	61 (88)	130 (1–6)	112 (86)
Goat	4 (4–1144 [759])	1 (25)	2 (50)	2 (50)	8 (1–4)	2 (25)
Cattle	50 (23–468 [136])	4 (8)	37 (74)	39 (78)	96 (1–5)	78 (81)
Total	123	53 (43)‡	83 (68)§	102 (83)	234	192 (82)

*A total of 240 farms were invited to participate in the study (113 sheep, 7 goat, and 120 cattle farms; all were highly suspected to be SBV infected on the basis of pathologic findings consistent with typical malformations in calves or lambs). 123 (51%) farms responded. SBV, Schmallenburg virus; VNT, virus neutralization test.
†Mixed farms were classified according to the main animal species.
‡For 9 farms, PCR results not available.
§For 38 farms, VNT results not available.

of the efficiency of vector-borne disease transmission comprising vector competence, susceptible host density, vector host feeding preferences, vector survival rate, vector density, and vector feeding rates (*50* in online Technical Appendix). In this study, we found no evidence for human SBV infection, despite the high infection rate of sheep and cattle in the same localities (up to 100% within-herd seroprevalence (*51* in online Technical Appendix) and the high level of reported insect bites during work on SBV-infected farms. From the high infection rates in ruminants, we conclude that the capacity of residing vectors to transmit SBV to cattle and sheep was high, indicating that vector-competence, vector densities, and vector survival rates were sufficient for SBV transmission. Therefore, the absence of SBV antibodies in humans implies that humans are not susceptible to SBV infection but only under the assumption that the vectors of SBV have host feeding preference for humans. Research into the host preferences of identified SBV vector species and, if proven anthropophilic, their feeding rates could clarify this issue.

If vector transmission would have been a route for zoonotic transmission leading to 2% seroprevalence in exposed persons, i.e., persons reporting insect bites on SBV-infected farms, in this study the probability of detecting at least 1 of such seropositive persons would have been 99.77%. However, this calculation is based on an assumed test specificity and sensitivity of 100%. A high specificity was justified on the basis of the negative results with the 387 control serum and the absence of neutralizing capacity of an SBV-positive ovine serum sample to INKV, BATV, and TAHV. Because SBV is a novel pathogen, no well-defined seropositive human serum cohorts were available to assay the analytical sensitivity of our test. However, even with sensitivity as low as 90%, the probability of detecting at least 1 seropositive person still would have been 99.69% (data not shown).

The second possible exposure could occur through contact with affected animals and/or birth materials. The congenital malformations in lambs and calves with SBV infection are such that increased assistance during delivery was needed from farmers and veterinarians. Direct exposure to newborn ruminants and/or birth materials was reported in 76% of the study participants. If contact during delivery would have been an active route for zoonotic transmission, leading to 2% seroprevalence in exposed persons, the probability of detecting at least 1 of such seropositive persons would have been 99.02%.

A third option is that exposure to newborns and their birth materials has a higher risk for infection if exposed persons had blood contact with the affected materials (e.g., by hand wounds). Sixty percent of participants reported small wounds on hands; thus, the probability of detecting such seropositives would have been high (i.e., 97.37% with 2% seroprevalence). In addition, 2 persons in the syndromic monitoring reported needlestick incidents, again without any evidence for infection through antibody testing.

The absence of evidence for direct transmission of SBV from ruminants to humans is in line with observations for other Simbu serogroup viruses (Akabane and Shamonda) infecting livestock (Table 2). Moreover, a serologic survey of 60 sheep farmers with sheep husbandry in the SBV epizootic area in Germany yielded no evidence for human SBV infection. However, of these farmers, only 48 had contact with lambs with SBV characteristic malformations, whereas SBV was laboratory confirmed in the livestock of only 36 participants (*52* in online Technical Appendix), but the level of exposure through contact with affected animals and/or birth material is difficult to quantify (*4*). In the Netherlands, SBV RNA has been detected in the brains of malformed animals on 18.6% of reported cattle farms and on 30.6% of reported sheep farms (*8*), and high loads of viral RNA have been detected in some placentas and in birth fluids.

Current data suggest that infections might have been cleared by the time of delivery, particularly in cattle, which have longer gestations. Furthermore, finding RNA in birth materials does not give any information about the actual presence of infectious virus particles in these materials. Attempts to isolate viruses from such specimens have met with little success, and further research is needed to address

the issue of infectivity of birth materials. This lack of virus isolation implies that the number of persons in this study directly exposed to infectious virus particles from affected animals and/or birth material might be lower than assumed on the basis of the number of participants reporting this exposure. Nevertheless, the lack of seropositive samples indicates that the risk for infection through contact with contaminated materials, regardless of whether they contain infectious virus particles, is minimal. Therefore, given the high seroprevalence of SBV in affected herds (*51* in online Technical Appendix), the lack of any evidence for zoonotic transmission from either the syndromic monitoring or this serologic study suggests that the public health risk for SBV given the current situation is absent or extremely low.

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Unchanged Severity of Influenza A(H1N1)pdm09 Infection in Children during First Postpandemic Season

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We conducted a nationwide hospital-based prospective study in Germany of influenza A(H1N1)pdm09 cases among children <15 years of age admitted to pediatric intensive care units and related deaths during the 2009–10 pandemic and the 2010–11 postpandemic influenza seasons. We identified 156 eligible patients: 112 in 2009–10 and 44 in 2010–11. Although a shift to younger patients occurred in 2010–11 (median age 3.2 vs. 5.3 years), infants <1 year of age remained the most affected. Underlying immunosuppression was a risk factor for hospital-acquired infections (p = 0.013), which accounted for 14% of cases. Myocarditis was predictive of death (p = 0.006). Of the 156 case-patients, 17% died; the difference between seasons was not significant (p = 0.473). Our findings stress the challenge of preventing severe postpandemic influenza infection in children and the need to prevent nosocomial transmission of influenza virus, especially in immunosuppressed children.

In Germany during the influenza A(H1N1)pdm09 pandemic, there were ≈1,070,000 influenza-related medical consultations and ≈1,800 hospitalizations for children 0–14 years of age during October 12, 2009–January 15, 2010, as determined using data provided by the German syndromic surveillance system for acute respiratory infections (*1*). Moreover, 29 laboratory-confirmed A(H1N1)pdm09 infection–related deaths in children were notified through the mandatory German surveillance system for infectious diseases (*2*). The highest number of notified hospitalizations and deaths were among children 10–14 years of age (*3*,*4*). In a nationwide hospital-based observational study investigating severely ill children who had been admitted to pediatric intensive care units (PICUs) or had died with laboratory-confirmed A(H1N1)pdm09, we reported a high proportion (75%) of case-patients with underlying risk factors, of which neurodevelopmental disorders were most prevalent (*5*). In addition, we found that in 10% of the cases, children had acquired their infection while hospitalized and that few had been vaccinated, revealing a need for improving preventive measures to reduce severe disease and adverse outcomes (*5*).

On August 10, 2010, the general director of the World Health Organization declared the world was no longer in phase 6 of influenza pandemic alert; we were moving into the postpandemic phase (*6*). Experience from past pandemics suggested that the pandemic virus would gradually take on the behavior of a seasonal influenza virus and circulate for several years. However, in view of the potential for transformation of the virus into a more virulent form (*7*), as suggested by higher rates of mortality during second pandemic waves in Copenhagen (1918), the United States (1957), and Eurasia (1968–1970) (*8*,*9*), the World Health Organization acknowledged the unpredictability of pandemic viruses; recommended continued vigilance; and issued advice on surveillance, vaccination, and prompt clinical management of cases during the postpandemic phase (*6*).

Little is known about the severity of A(H1N1)pdm09 in children during the first postpandemic season (*10*). To obtain information on critically ill A(H1N1)pdm09-infected children and to compare risk factors and disease course, outcome, and severity for patients during the pandemic and first postpandemic season, we prospectively and continuously performed a nationwide study in Germany during August 3, 2009–July 29, 2011.

Methods

Study Design

We conducted a nationwide prospective observational study in Germany by using the German Survey Center for

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Rare Pediatric Diseases (ESPED; an established children's hospitals network comprising all 375 pediatric hospitals in Germany) to identify children <15 years of age admitted to PICUs with confirmed A(H1N1)pdm09 infection and related deaths. Cases and related deaths during August 3, 2009–July 29, 2011 were reported by using a standardized form. In pandemic season 2009–10 (August 3, 2009–August 9, 2010), the case definition included only A(H1N1)pdm09 infection; in postpandemic season 2010–11 (August 10, 2010–July 29, 2011), the case definition included all influenza virus infections.

Data Collection

On notification by treating physicians of patients with A(H1N1)pdm09 infection, the ESPED study center distributed and subsequently collected a structured questionnaire, which had been adapted by the authors from an earlier study on seasonal influenza (*11*). Of 284 distributed questionnaires requested by 186 hospitals, 95% (271/284) were returned to the study center (Figure 1). After excluding 3 questionnaires that had been notified twice and 101 questionnaires for patients who did not meet the case definition, 62% (167/271) of the questionnaires from 83 hospitals remained. Reasons for not meeting the case definition included patient age >15 years and patient not admitted to PICU. In accordance with the case definition, only cases of A(H1N1)pdm09 infection were reported during the pandemic season (2009–10), and 9 cases of influenza A (not further subtyped) and 2 cases of influenza B infection were reported in the postpandemic season (2010–11). Therefore, further analyses were restricted to A(H1N1)pdm09 cases.

The structured questionnaire covered anonymous patient information and information regarding the hospital stay, clinical signs and symptoms, clinical and laboratory findings, specific treatments, status of influenza vaccination, disease complications, and underlying chronic medical conditions (chronic respiratory diseases; cardiac diseases; immunodeficiency; and neurodevelopmental disorders, including developmental delay, cerebral palsy, epilepsy, and other cognitive disorders). Answer categories were predetermined, but free space was designated for respondents to provide information about other diagnoses and coexisting illnesses/medical conditions. Hospital-acquired infection was defined by a date of symptom onset being >2 days after the date of hospital admission; 2 days corresponds with the median incubation time for A(H1N1)pdm09 according to Cao et al. (*12*). Data were double entered by using EpiData 3.1 software (www.epidata.dk/) in an electronic database.

Data Analysis

Reported values are those for children with available information. Descriptive statistics comprised the calculation of median and interquartile ranges (IQRs) for continuous variables and absolute numbers and proportions (together with 95% binomial exact CIs, when appropriate) for categorical variables. Comparative analyses were based on the Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables. Odds ratios (ORs) and 95% CIs were calculated. Multivariable analysis was performed by using a logistic regression with a stepwise approach to compare cases of hospital-acquired infection with cases of community-acquired infection and survivors with nonsurvivors in PICUs. In doing so, risk factors with a p value <0.2 were considered for multivariable analysis, with the exception of age, sex, and season, which were included in all models. Reported p values are 2-sided, and $p<0.05$ was considered significant. Statistical analyses were performed by using Stata 11.0 (StataCorp LP, College Station, TX, USA).

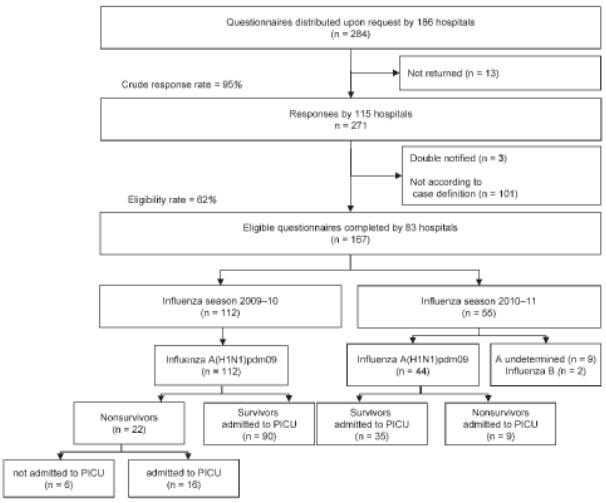

Figure 1. Overview of study participation and participant groups among severe pediatric cases with A(H1N1)pdm09, Germany, 2009–2011. PCIU, pediatric intensive care unit.

Data Protection and Ethics Clearance

Adherence to national data protection laws was approved by the Federal Commissioner for Data Protection and Freedom of Information of Germany. Ethical approval was granted by the Ethics Committee, Charité-Universitätsmedizin, Berlin, Germany.

Results

Comparison of 2009–10 and 2010–11 Seasons

We identified 156 critically ill children with confirmed A(H1N1)pdm09 infection: 112 in 2009–10 and 44 in 2010–11 (Figure 1). Dates of symptom onset ranged from September 21, 2009, to March 20, 2010, for 2009–10

and from December 20, 2010, to February 22, 2011, for 2010–11 (Figure 2). Cases were reported from 15 of the 16 federal states in Germany during 2009–10 and from 10 during 2010–11.

The proportion of boys among case-patients was higher in 2009–10 than 2010–11 (59% vs. 37%, p = 0.02) (Table 1). The median age of case-patients was 5.3 and 3.2 years in 2009–10 and 2010–11, respectively, and differed statistically (p = 0.007) between the 2 seasons. The age distribution in 2010–11 compared with that in 2009–10 was characterized by a markedly higher proportion of children < 2 years of age and a lower proportion of children 10–14 years of age (Figure 3). In both seasons, infants <1 year of age represented the age group with the highest number of cases (Figure 4).

Of the 146 children with available information, 114 (78%) had >1 chronic underlying medical condition; the difference between seasons for these conditions was not statistically significant (Table 1). In both seasons, neurodevelopmental disorders were the most prevalent underlying medical condition. Of the 156 critically ill case-patients, 130 were >6 months age and thus eligible for vaccination against A(H1N1)pdm09 virus; however, for children with available information on vaccination status, only 5 (7%) of 67 vaccine-eligible case-patients had been vaccinated in the 2009–10 season, and none had been vaccinated in the 2010–11 season. Of the 69 total children in both seasons with underlying chronic medical conditions, 64 (93%) had not been vaccinated against A(H1N1) pdm09 virus.

More cases of sepsis were reported during the postpandemic season than during the pandemic season (21% vs. 8%; p = 0.048) (Table 1). Treatment with oseltamivir was used equally (in ≈62% of children) during both seasons. The time to oseltamivir administration after symptom onset (median 4 days) was similar throughout both seasons. The use of catecholamine and mechanical ventilation was more frequent in 2010–11 than in 2009–10, but the difference was not statistically different.

Hospital-acquired Infections

Hospital-acquired infections accounted for 11% (11 of 101) of the cases in 2009–10 and for 23% (8/35) in 2010–11 (p = 0.0931) (Table 1). Of the total study cohort, 14% (19/136) of the patients (9 in a general ward and 10 in a PICU) most likely had hospital-acquired infection. For these case-patients, the median time from hospital admission to symptom onset was 29 days (IQR 12–73 days). The median age for patients with hospital-acquired infection was 1.1 years, and 56% (10/18) were boys (difference not statistically significant between seasons).

The overall case-fatality ratios were 26% (5/19) among patients with hospital-acquired infection and 20% (23/117) among those with community-acquired infection (p = 0.543). Compared with patients with community-acquired infection, those with hospital-acquired infection had more complications, including acute respiratory distress syndrome (ARDS) (OR 2.7, p = 0.054) and sepsis (OR 3.1, p = 0.064), but the differences were not statistically significant (Table 2). In the multivariable model, immunodeficiency (OR 5.9, 95% CI 1.5–23.9; p = 0.013) and mechanical ventilation (OR 8.9, 95% CI 1.1–74.7; p = 0.043) were significantly associated with hospital-acquired infection after adjusting for age, sex, and season.

Case Fatalities Ratios

The case fatality ratio in PICUs did not differ between seasons: 15% (16/106) and 21% (9/44) of PICU case-patients died in 2009–10 and 2010–11, respectively (p = 0.473) (Table 1). For the 2 seasons, 25 of 150 PICU case-patients died, corresponding to a case-fatality ratio of 17% (95% CI 11%–24%). On hospital discharge, 26% (27/104) of the survivors were reported to have possible sequelae or worsening of a pre-existing medical condition.

No statistical differences were found between survivors and nonsurvivors in underlying chronic medical conditions and vaccination status. ARDS (OR 3.2, 95% CI 1.1–9.2, p = 0.029), myocarditis (OR 30.9, 95% CI 2.6–360.7,; p = 0.006), and mechanical ventilation (OR 18.3; 95% CI 1.3–251.6, p = 0.030) were independently associ-

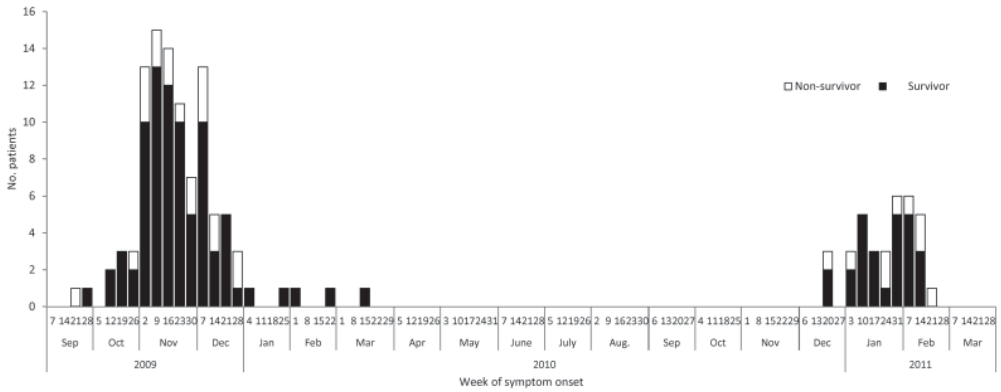

Figure 2. Distribution of 136 critically ill children with confirmed A(H1N1) pdm09, by date of disease onset, September 21, 2009–February 22, 2011, Germany. Only cases with available date of symptom onset are represented.

Table 1. Comparison of severe cases of influenza A(H1N1)pdm09 virus infection in children during the pandemic and the first postpandemic seasons, Germany, 2009–2011*

Variable	No. patients/no. total (%)	Influenza season		p value
		2009–10	2010–11	
Male sex	81/154 (53)	65/111 (59)	16/43 (37)	0.020
Median age, y (IQR)	4.2 (1.2–9.2)	5.3 (1.7–10.1)	3.2 (0.5–6.5)	0.007
Hospital-acquired infection	19/136 (14)	11/101 (11)	8/35 (23)	0.093
Clinical diagnosis				
Pneumonia	108/156 (69)	79/112 (71)	29/44 (66)	0.569
Secondary pneumonia	30/156 (19)	22/112 (20)	8/44 (18)	1.000
Encephalopathy	11/156 (7)	7/112 (6)	4/44 (9)	0.506
ARDS	43/156 (28)	29/112 (26)	14/44 (32)	0.551
Sepsis	18/156 (12)	9/112 (8)	9/44 (21)	0.048
Myocarditis	8/156 (5)	4/112 (4)	4/44 (9)	0.223
Febrile seizure	7/156 (5)	3/112 (3)	4/44 (9)	0.099
Underlying chronic medical conditions				
Any	114/146 (78)	82/107 (77)	32/39 (82)	0.652
Neurodevelopmental disorders	84/151 (56)	61/110 (56)	23/41 (56)	1.000
Respiratory disease	44/141 (31)	35/104 (34)	9/37 (24)	0.409
Immunodeficiency	17/137 (12)	15/97 (16)	2/40 (5)	0.152
Cardiac disease	20/143 (14)	12/102 (12)	8/41 (20)	0.286
Treatment				
Oseltamivir	90/145 (62)	65/105 (62)	25/40 (63)	1.000
Catecholamine	52/138 (38)	35/101 (35)	17/37 (46)	0.240
Mechanical ventilation	98/145 (68)	68/107 (64)	30/38 (79)	0.107
Vaccination†	5/88 (6)	5/67 (8)	0/21 (0)	0.332
Outcome				
All deaths	31/156 (20)	22/112 (20)	9/44 (21)	1.000
Death in PICU	25/150 (17)	16/106 (15)	9/44 (21)	0.473

*Values are no. positive/no. with available information (%), except as indicated. Pandemic season, 2009–10; postpandemic season, 2010–11; IQR, interquartile range; ARDS, acute respiratory distress syndrome; PICU, pediatric intensive care unit.
†Influenza A(H1N1)pdm09 vaccination of patients >6 mo of age.

ated with a fatal outcome in the multivariable model after adjusting for age, sex, and season (Table 3).

Compared with survivors, nonsurvivors more frequently required mechanical ventilation (p = 0.001) and treatment with catecholamine (p = 0.002); no differences were found in oseltamivir administration (65% vs. 62%, p = 0.8185). Time from symptom onset to oseltamivir uptake did not differ between survivors (median 4 days, IQR 1–6 days) and nonsurvivors (median 4 days, IQR 2–8 days).

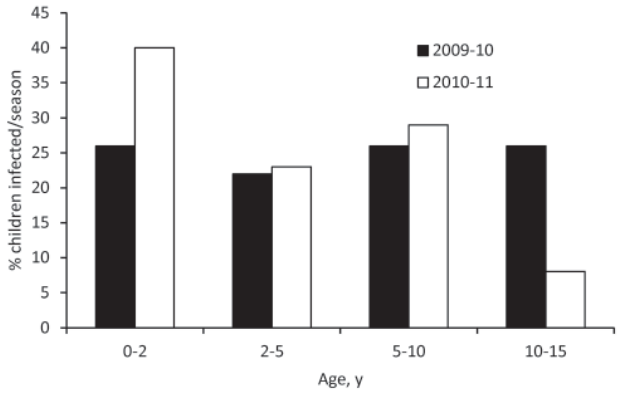

Figure 3. Proportion of critically ill children with A(H1N1)pdm09 by age group and season, Germany.

Discussion

During the first postpandemic season, fewer cases of A(H1N1) pdm09 infection were reported, but the severity and outcome of cases did not differ between the pandemic and postpandemic seasons. We further analyzed data from the 2 seasons as 2 outbreak waves of 1 virus and identified a high number of hospital-acquired infections and ARDS and myocarditis as 2 predictors for a fatal outcome.

Compared with the 2009–10 pandemic season, the 2010–11 postpandemic season started later in the winter and had less than half the number of cases. High disease awareness during the pandemic season may have enhanced testing and reporting during 2009–10; thus the reduced case number for 2010–11 should be interpreted with caution. However in the United States, where reporting of influenza-related deaths in children is mandatory, a similar decline in the number of fatal A(H1N1)pdm09-associated cases was noted between the 2009–10 and 2010–11 influenza seasons (282 and 71 deaths among children, respectively) (*13*). Before the 2009–10 pandemic and similar to the postpandemic season, an average of 82 (range 46–153) children in the United States died each year from seasonal influenza–related illnesses (*14*). However, in the postpandemic 2010–11 season, different proportions of all circulating influenza subtypes might have led to different numbers of persons exposed to A(H1N1)pdm09, which makes comparisons between seasons and across countries difficult.

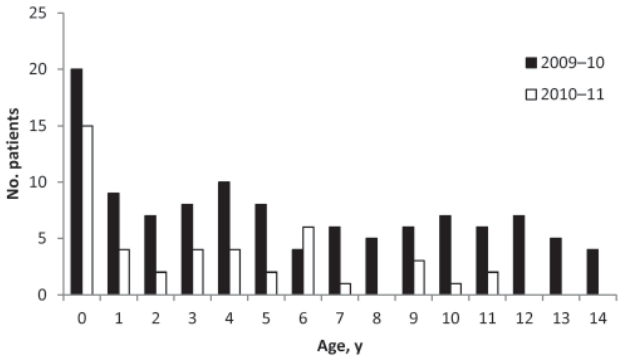

Figure 4. Age distribution of the 156 critically ill children with confirmed A(H1N1)pdm09, by season, Germany.

For the 2010–11 season, we assumed a more limited number of susceptible persons because exposure to influenza virus during the pandemic might have provided immunologic protection (*15–17*). This hypothesis is supported by our results showing a shift toward infection in younger age groups in 2010–11. A similar finding was reported in a prospective study of children with A(H1N1)pdm09 infection in a Spanish hospital (median age 7.0 and 0.8 years in 2009–10 and 2010–11, respectively) (*10*). During both seasons, children <1 year of age were more affected than those in other age groups, and the numbers infected in the 2 seasons were similar; therefore, influenza infection in this immunologically naive age group might always reflect a pandemic-like situation. It remains unexplained why more boys in 2009–10 (59%, 65/111) than in 2010–11 (37%, 16/43) had serious A(H1N1)pdm09 infection, although it has been suggested that the difference in age distribution between the 2 seasons could have influenced the sex distribution (*18*).

Our results show that case-fatality ratios for the 2 seasons were similar. In Greece (*19*) and New-Zealand (*20*), according to the respective national surveillance systems in intensive care unit settings, case-fatality ratios among all age groups were also similar for the 2 seasons. This result is reassuring, in view of concerns of a possible transformation of the strain into a more severe form (*7*), and is in agreement with the antigenic and genetic homogeneity of the virus since its emergence (*21*).

In both seasons, we identified a large number of probable hospital-acquired A(H1N1)pdm09 infections. Immunodeficiency, most often reported as acute lymphoblastic leukemia, was associated with hospital-acquired infection, and this underlying chronic medical condition, has also been identified as a risk factor for community-acquired A(H1N1)pdm09 (*22*,*23*). Findings from a retrospective hospital-based study investigating the prevalence of respiratory virus infections among children with cancer or HIV

Table 2. Comparison of hospital- and community-acquired cases of severe influenza A(H1N1)pdm09 infection in children during the pandemic and first postpandemic influenza seasons, Germany, 2009–2011*

Variable	No. patients/ total (%)	Hospital-acquired cases	Community-acquired cases	Univariable analysis		Multivariable analysis	
				OR (95% CI)	p value	OR (95% CI)	p value
Male sex	72/134 (54)	10/18 (56)	62/116 (54)	1.1 (0.4–3.4)	1.000	1.4 (0.4–4.5)	0.622
Median age, years (IQR)	4.3 (1.0–9.2)	1.1 (0.3–10.1)	4.8 (1.6–11.9)	NA	0.168	1.0 (0.8–1.1)	0.583
2010–11 season	35/136 (26)	8/19 (42)	27/117 (23)	2.4 (0.8–7.4)	0.093	1.5 (0.4–5.7)	0.517
Clinical diagnosis							
Pneumonia	96/136 (71)	15/19 (79)	81/117 (69)	1.7 (0.5–7.4)	0.588	NA	NA
Secondary pneumonia	27/136 (20)	4/19 (21)	23/117 (20)	1.1 (0.2–3.8)	1.000	NA	NA
Encephalopathy	9/136 (7)	0/19 (0)	9/117 (8)	0.0 (0.0–2.5)	0.360	NA	NA
ARDS	38/136 (28)	9/19 (47)	29/117 (25)	2.7 (0.9–8.2)	0.054	NA	NA
Sepsis	17/136 (13)	5/19 (26)	12/117 (10)	3.1 (0.7–11.4)	0.064	NA	NA
Myocarditis	8/136 (6)	0/19 (0)	8/117 (7)	0.0 (0.0–2.9)	0.600	NA	NA
Febrile seizure	6/136 (4)	0/19 (0)	6/117 (5)	0.0 (0.0–3.9)	0.595	NA	NA
Underlying chronic medical conditions							
Any	101/129 (78)	19/19 (100)	82/110 (75)	NA (1.7–NA)	0.013	NA	NA
Neurodevelopmental disorders	75/133 (56)	13/19 (68)	62/114 (54)	1.8 (0.6–6.2)	0.321	NA	NA
Respiratory disease	17/126 (14)	3/18 (17)	14/108 (13)	1.3 (0.2–5.7)	0.710	NA	NA
Immunodeficiency	15/121 (12)	5/17 (29)	10/104 (10)	3.9 (0.9–15.2)	0.037	5.9 (1.5–23.9)	0.013
Cardiac disease	17/126 (14)	3/18 (17)	14/108 (13)	1.3 (0.2–5.7)	0.710	NA	NA
Treatment							
Oseltamivir	80/128 (63)	13/18 (72)	67/110 (61)	1.7 (0.5–6.4)	0.4378	NA	NA
Catecholamine	47/124 (38)	11/16 (69)	36/108 (33)	4.4 (1.3–17.2)	0.011	NA	NA
Mechanical ventilation	85/129 (66)	17/18 (94)	68/111 (61)	10.8 (1.6–459.4)	0.006	8.9 (1.1–74.7)	0.043
Vaccination†	5/80 (6)	0/10 (0)	5/70 (7)	0.0 (0.0–5.5)	1.000	NA	NA
Outcome							
All death	28/136 (21)	5/19 (26)	23/117 (20)	1.5 (0.4–4.9)	0.543	NA	NA
Death in PICU	22/130 (17)	4/18 (22)	18/112 (16)	1.5 (0.3–5.5)	0.507	NA	NA

*Values are no. positive/no. with available information (%), except as indicated. Pandemic season, 2009–10; postpandemic season, 2010–11; OR, odds ratio; IQR, interquartile range; NA, not applicable; ARDS, acute respiratory distress syndrome; PICU, pediatric intensive care unit.
†Influenza A(H1N1)pdm09 vaccination of patients >6 mo of age.

infection reported that 40% of the respiratory infections were acquired during the hospital stay, and influenza A virus was the second most prevalent respiratory infection (*24*). In our study, patients with hospital-acquired infection had more complications, including ARDS and sepsis, than patients with community-acquired infections. However, a significant association between hospital-acquired infection and death was not found, possibly because of the small number of cases, as found by Spaeder et al. (*25*) in a retrospective cohort study in PICU setting. In this study, hospital acquisition of viral respiratory infection was shown to be associated with an increased risk for death, even after adjusting for chronic medical conditions that predispose to an increased risk for complications from viral illness. Our findings emphasize the need for isolation of and preventive measures for children with immunodeficiency, as reported (*26*). Preventive measures should include the vaccination of health care workers. Indeed, a survey in Germany showed that only 35% (n = 3,900) of the health care workers in a university hospital setting were vaccinated during the 2010–11 influenza season (*27*). Studies in earlier seasons showed even lower influenza vaccination rates among health care workers (*28*).

We identified 25 A(H1N1)pdm09-associated deaths among children admitted to PICUs during the pandemic and postpandemic seasons. ARDS was the most prevalent complication among case-patients who died (60% of cases) and was highly associated with death. Myocarditis was also highly associated with death in children; this finding supports those among adults (*29*,*30*) and other findings among A(H1N1)pdm09-infected children (*31*).

During both seasons, 62% of the children received oseltamivir treatment. This proportion is lower than described in other studies in PICU settings, e.g., 81% in an inception-cohort study in Australia and New Zealand (*32*), 88% in a US cohort (*31*), 96% in a retrospective observational multicenter study in Turkey (*33*), and 100% of children in a retrospective Dutch cohort (*34*). Observational and random clinical trials have shown the potential of oseltamivir to reduce the length of hospitalization when started <24 hours of illness onset (*35*,*36*). The Infectious Diseases Society of America recommends that any person with confirmed or suspected influenza who requires hospitalization receive influenza antiviral therapy, even if the patient enters care >48 hours after illness onset (*37*). The German Society for Pediatric Infectious Diseases recommends that immunocompetent children without underlying chronic medical conditions should not receive influenza antiviral therapy >48 hours after onset of influenza symptoms (*38*). Most A(H1N1)pdm09 virus isolates tested worldwide remain sensitive to oseltamivir; thus, strategies to optimize the use of oseltamivir should be considered, and additional evidence should be collected with respect to reduction of nosocomial spread of A(H1N1)pdm09 virus and to potential benefits from late treatment in severely ill children.

Table 3. Comparison of severe cases of influenza A(H1N1)pdm09 infection among children in PICUs during the pandemic and first postpandemic influenza seasons, Germany, 2009–2011*

Variable	No. patients/ total (%)	Nonsurvivors	Survivors	Univariable analysis		Multivariable analysis	
				OR (95% CI)	p value	OR (95% CI)	p value
Male sex	81/148 (55)	15/25 (60)	66/123 (54)	1.3 (0.5–3.5)	0.661	2.5 (0.8–7.4)	0.098
Median age, years (IQR)	4.2(1.0–8-6)	5.7 (1.6–9.8)	4.1 (1–7.8)	NA	0.091	1.1 (1.0–1.2)	0.232
2010–11 season	44/150 (29)	9/25 (36)	35/125 (28)	1.4 (0.5–3.9)	0.4728	1.6 (0.5–5.1)	0.435
Hospital-acquired infection	18/130 (14)	4/22 (18)	14/108 (13)	1.5 (0.3–5.5)	0.507	NA	NA
Clinical diagnosis							
Pneumonia	103/150 (69)	13/25 (52)	90/125 (72)	0.4 (0.2–1.1)	0.060	NA	NA
Secondary pneumonia	30/150 (20)	8/25 (32)	22/125 (18)	2.2 (0.7–6.2)	0.108	NA	NA
Encephalopathy	11/150 (7)	1/25 (4)	10/125 (8)	0.5 (0.01–3.7)	0.692	NA	NA
ARDS	43/150 (29)	15/25 (60)	28/125 (22)	5.2 (1.9–14.3)	<0.001	3.2 (1.1–9.2)	0.029
Sepsis	17/150 (11)	6/25 (24)	11/125 (9)	3.3 (0.9–11.0)	0.040	NA	NA
Myocarditis	8/150 (5)	4/25 (16)	4/125 (3)	5.8 (1.0–32.9)	0.027	30.9 (2.6–360.7)	0.006
Febrile seizure	7/150 (5)	0/25 (0)	7/125 (6)	0.0 (0.01–2.7)	0.601	NA	NA
Underlying chronic medical condition							
Any	108/140 (77)	19/23 (83)	89/117 (76)	1.5 (0.4–6.5)	0.596	NA	NA
Neurodevelopmental disorder	78/145 (54)	16/24 (67)	62/121 (51)	1.9 (0.7–5.5)	0.186	NA	NA
Respiratory disease	42/135 (31)	8/22 (36)	34/113 (30)	1.3 (0.4–3.8)	0.617	NA	NA
Immunodeficiency	16/131 (12)	1/21 (5)	15/110 (14)	0.3 (0.0–2.3)	0.467	NA	NA
Cardiac disease	20/137 (15)	4/23 (17)	16/114 (14)	1.3 (0.3–4.6)	0.746	NA	NA
Treatment							
Oseltamivir	87/139 (63)	15/23 (65)	72/116 (62)	1.1 (0.4–3.4)	0.8185	NA	NA
Catecholamine	52/133 (39)	16/23 (70)	36/110 (33)	4.7 (1.6–14.6)	0.002	NA	NA
Mechanical ventilation	98/140 (70)	23/24 (96)	75/116 (65)	12.6 (1.9–530.3)	0.001	18.3 (1.3–251.6)	0.030
Vaccination†	5/82 (6)	0/14 (0)	5/68 (7)	0.0 (0.0–3.7)	0.582	NA	NA

*Values are no. positive/no. with available information (%), except as indicated. PICUs, pediatric intensive care units; pandemic season, 2009–10; postpandemic season, 2010–11; OR, odds ratio; IQR, interquartile range; NA, not applicable; ARDS, acute respiratory distress syndrome.
†Influenza A(H1N1)pdm09 vaccination of patients >6 mo of age.

We showed that 93% of the children with underlying chronic medical conditions who were eligible for vaccination had not been vaccinated. This finding highlights a need to improve vaccine coverage among this population, for which influenza vaccination is recommended in Germany (*39*). Children who did not survive received more intensive treatment (mechanical ventilation and catecholamine) than those who survived, and nearly all influenza A viruses tested continue to be antigenically similar to those found in the current trivalent vaccine (*40*); thus, enhanced prevention in children through vaccination, especially among those with underlying chronic medical conditions, remains a high priority.

Our study is subject to several limitations. Factors such as physicians' awareness, diagnostic testing, and reporting behavior, which may have had different influences in the 2 seasons, were not assessed. Only children hospitalized in pediatric hospitals were included in the study; however, it can be assumed that critically ill children hospitalized in general hospitals were transferred to pediatric hospitals covered by the ESPED network. In addition, our knowledge of the clinical features of patients was based only on information provided in the structured questionnaires. Ascertainment of underlying chronic medical conditions was not standardized and, thus, may have differed among treating physicians.

Conclusions

During the first postpandemic A(H1N1)pdm09 season, the situation for children with severe A(H1N1)pdm09 disease did not differ from that for children with severe disease during the pandemic. Signs of pulmonary failure or suspected myocarditis in such children should alert health care providers to immediately initiate maximum care, and prevention of nosocomial transmission of influenza virus should be reinforced, especially in immunosuppressed children. The unchanged severity of influenza A(H1N1)pdm09 virus infections in the first postpandemic season (2010–11) and the constant high proportion of possibly hospital-acquired infections stress the challenge of preventing severe cases in children beyond the pandemic situation.

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Dr Altmann is epidemiologist at the Robert Koch Institute. His research interests include infectious disease epidemiology and international health.

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Mycoplasmosis in Ferrets

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We report an outbreak of severe respiratory disease associated with a novel *Mycoplasma* species in ferrets. During 2009–2012, a respiratory disease characterized by nonproductive coughing affected ≈8,000 ferrets, 6–8 weeks of age, which had been imported from a breeding facility in Canada. Almost 95% became ill, but almost none died. Treatments temporarily decreased all clinical signs except cough. Postmortem examinations of euthanized ferrets revealed bronchointerstitial pneumonia with prominent hyperplasia of bronchiole-associated lymphoid tissue. Immunohistochemical analysis with polyclonal antibody against *Mycoplasma bovis* demonstrated intense staining along the bronchiolar brush border. Bronchoalveolar lavage samples from 12 affected ferrets yielded fast-growing, glucose-fermenting mycoplasmas. Nucleic acid sequence analysis of PCR-derived amplicons from portions of the 16S rDNA and RNA polymerase B genes failed to identify the mycoplasmas but showed that they were most similar to *M. molare* and *M. lagogenitalium.* These findings indicate a causal association between the novel *Mycoplasma* species and the newly recognized pulmonary disease.

The number of pet ferrets in the United States has grown rapidly, from an estimated 800,000 in 1996 (*1*) to an estimated 7–10 million in 2007 (*2*). Also in the United States, ferrets have become the third most common household pet; their popularity as a pet in Europe is similar (*3*). The common respiratory diseases in pet ferrets are caused by viruses; canine distemper is probably the most virulent (*4*). Ferrets also are highly susceptible to human influenza virus, but disease is rarely severe (*5*,*6*). Bacteria rarely cause disease outbreaks in ferret populations, but they do cause disease in individual ferrets (*7*–*9*).

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In 2007, in the state of Washington, USA, an outbreak of respiratory disease characterized by a dry, nonproductive cough was observed in 6- to 8-week-old ferrets at a US distribution center of a commercial pet vendor (video of a coughing ferret available at wwwnc.cdc.gov/EID/article/18/11/12-0072-V1.htm). Over a 4-year period, ≈8,000 ferrets, equal numbers of both sexes, were affected. Every 2–3 weeks, kits had been shipped in groups of 150–200 from a commercial breeding facility in Canada to the distribution center. At 5 weeks of age, before shipment to the distribution center, each kit received a single vaccination for distemper (DISTEM R-TC; Schering Plough, Kenilworth, NJ, USA).

Some ferrets exhibited hemoptysis, labored breathing, sneezing, and conjunctivitis. Almost 95% of the ferrets were affected, but almost none died. Symptomatic ferrets were selected from each shipment for testing; results of heartworm screening, PCR and serologic testing for distemper, and serologic testing for influenza virus were negative. Cytologic examination of bronchioalveolar lavage (BAL) samples yielded few inflammatory cells. Thoracic ultrasonography found no abnormalities. Thoracic radiographs showed a mild bronchointerstitial pattern with peribronchial cuffing (Figure 1). Complete blood counts and chemistry results were within reference ranges (*10*,*11*).

Affected ferrets received broad spectrum antimicrobial drugs, bronchodilators, expectorants, nonsteroidal anti-inflammatory drugs, and nebulization; all clinical signs except the dry cough temporarily decreased. Numerous ferrets from the distribution center were later surrendered to a ferret rescue and shelter operation, where their cough continued for as long as 4 years.

Materials and Methods

Affected Ferrets

In April 2009, a 2-year-old, spayed female ferret at the ferret rescue and shelter, which had originated from the breeding facility in Canada and passed through the US distribution center, became acutely dyspneic and died within

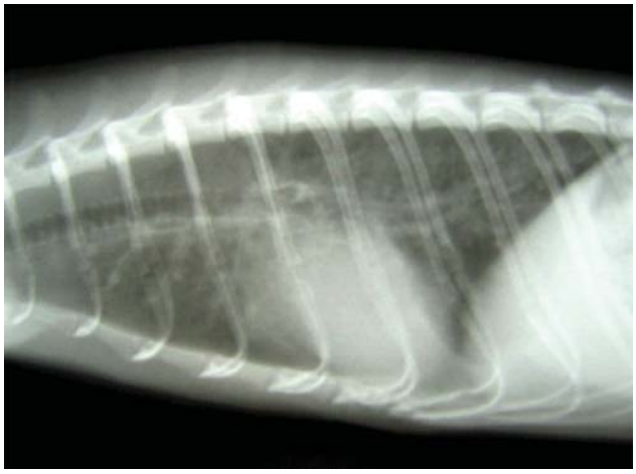

Figure 1. Lateral radiographic view of the thorax from a 2-year-old ferret with cough and labored breathing, showing a bronchointerstitial pattern with peribronchial cuffing.

15 minutes. The ferret had shown signs of respiratory disease since arrival at the shelter. Previously at the shelter, 2 ferrets, 4.5 years of age, had shown chronic cough; 1 died of dyspnea in October 2010, and the other was euthanized for humane purposes in November 2010. Both had originated from the breeding facility and passed through the distribution center. Postmortem examinations were performed on all 3 ferrets. After the 2-year-old ferret died in April 2009, BAL samples and ocular swabs were obtained in July 2009 from 3 other ferrets with a history of respiratory disease since their arrival at the ferret shelter. For further diagnostic investigation, BAL samples and ocular swabs were collected from 9 additional affected ferrets in January 2010; one of these was the ferret that died in October 2010.

Survey of Healthy Ferrets

At a large commercial breeding facility in which signs of respiratory disease had not been observed, BAL samples were obtained from 10 euthanized healthy male ferrets, 5 weeks to 5 years of age. Before postmortem examination, samples were collected from the euthanized ferrets by BAL through an incision in the caudal trachea. Nonbacteriostatic saline (10 mL/kg) was flushed into the caudal trachea and lungs and then recovered by aspiration into the syringe. That process was repeated 2× and the final flush fluid was submitted for bacterial culture. Complete postmortem examinations were performed, and sections of lung were collected for bacterial and mycoplasma culture. Additional tissue samples were collected from the lungs, trachea, nasal turbinates, brain, liver, kidneys, spleen, stomach, small and large intestine, thoracic and mesenteric lymph nodes, pancreas, and adrenal glands for routine histopathologic examination.

Histologic and Immunohistochemical Analyses and Confocal Microscopy

From the 3 ferrets that died April 2009–November 2010, postmortem tissue samples (lungs, trachea, nasal turbinates, brain, liver, kidneys, spleen, stomach, small and large intestine, thoracic and mesenteric lymph nodes, pancreas, and adrenal glands) were collected. They were fixed in neutral-buffered, 10% formalin solution and processed by standard methods for histopathologic examination.

For immunohistochemical examination, paraffin-embedded samples of lung from the 3 ferrets that died were cut into 5-μm sections. An Enhanced Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) and bulk buffers specifically designed for use on the BenchMark Automated Staining System (Ventana Medical Systems, Inc.) were used for immunolabeling. Slides were baked in a drying oven at 60°C for 20 min, barcode labeled, and placed in the BenchMark for deparaffinization and heat-induced epitope retrieval. Slides were then incubated with a mouse monoclonal antibody against mycoplasma (primary antibody) (Chemicon, Billerica, MA, USA) at a concentration of 1:100 for 30 min. The monoclonal antibody was raised against *M. bovis* strain M23, but it is known to cross-react with numerous other mycoplasma species.

The slides were counterstained by using hematoxylin (Ventana Medical Systems, Inc.), then dehydrated, cleared, and mounted. For a positive control, we used formalin-fixed, paraffin-embedded sections of lung from an *M. bovis*–positive cow (tested by bacterial culture). For negative controls, we replaced the primary antibody with homologous nonimmune serum.

A Zeiss 510 microscope (Jena, Germany) was used for confocal imaging to acquire fluorescent images, and the Zeiss LSM image analysis software was used for characterizations. The images represented a differential interference contrast/Nomarski image with green (488 nm, argon laser excitation, fluorescein isothiocyanate [FITC]) and red (543 nm, rhodamine, helium–neon excitation, tetramethylrhodamine-5- [and 6-] isothiocyanate [TRITC])–labeled overlay to demonstrate localization of labels, as described, with slight modified according to Ubels et al. (*12*).

Transmission and Scanning Electron Microscopy

For transmission electron microscopy, lung tissue samples that had been fixed in neutral-buffered, 10% formalin solution were trimmed into 2-mm pieces and postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 2 h. Tissues were serially dehydrated in acetone and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA, USA) in flat molds. Sections were obtained with a Power Tome XL ultramicrotome (Boeckeler Instruments, Tucson, AZ, USA). To identify areas of interest, we

stained semithin (0.5-μm) sections with epoxy tissue stain and examined them under a light microscope. Then we cut ultrathin (70-nm) sections, mounted them onto 200-mesh copper grids, stained them with uranyl acetate and lead citrate, and examined them under a 100 CXII transmission electron microscope (JEOL, Peabody, MA, USA).

For scanning electron microscopy, formalin-fixed lung tissues were trimmed into 2–4-mm pieces, postfixed for 1 h in 1% osmium tetroxide, and rinsed for 30 min in 0.1 M sodium phosphate buffer. Tissues were serially dehydrated in ethanol and dried in a critical point dryer (Model 010; Balzers, Witten, Germany) with liquid carbon dioxide as the transitional fluid. Samples were mounted on aluminum studs by using carbon suspension cement (SPI Supplies, West Chester, PA, USA). Samples were then coated with an osmium coater (NEOC-AT; Meiwa Shoji Co., Tokyo, Japan) and examined in a JSM-7500F (cold field emission electron emitter) scanning electron microscope (JEOL).

Bacterial Cultures

We submitted 12 BAL samples and 12 ocular swab samples for bacterial and mycoplasma culture by standard microbiologic techniques. The samples were from live ferrets that originated from the distribution center and showed clinical signs of respiratory disease, including coughing. We also submitted 10 BAL samples from 10 healthy ferrets from a different commercial breeding facility not affected by respiratory disease.

PCR and Sequence Analysis

Only mycoplasmas obtained from BAL samples were analyzed by PCR and nucleic acid sequencing. A plug of agar containing *Mycoplasma* spp. colonies was gouged from the surface of a mycoplasma agar plate by using a 10-μL disposable inoculation loop and transferred to a microcentrifuge tube. The agar plug was digested by addition of 200 μL of Buffer ATL (QIAGEN, Valencia, CA, USA) and 20 μL of proteinase K solution (QIAGEN), followed by overnight incubation at 56°C. DNA was extracted from the digest by using a DNeasy Blood and Tissue kit (QIAGEN) according to manufacturer's instructions.

For PCR, we used 2 sets of primers selective for the bacterial 16S rDNA or the mycoplasma RNA polymerase B (*rpoB*) gene. The nucleic acid sequences for the 16s rDNA gene were 5′-AGAGTTTGATCMTGGCTCAG-3′ for the forward primer and 5′-GGGTTGCGCTCGTTR-3′ for the reverse primer; this primer set produced an amplicon of ≈1,058 bp. The nucleic acid sequences for the mycoplasma *rpoB* gene were 5′- GGAAGAATTTGTCCWATTGAAAC-3′ for the forward primer and 5′- GAATAAGGMCCAACACTACG-3′ for the reverse primer; this primer set produced an amplicon of ≈1,613 bp. The PCRs were performed by using Platinum Taq DNA Polymerase High Fidelity (Invitrogen Corp., Carlsbad, CA, USA). The reaction mixture consisted of 3 μL DNA; 1 unit of Platinum Taq DNA Polymerase High Fidelity; 60 mmol/L Tris-SO4 (pH 8.9); 18 mmol/L ammonium sulfate; 2 mmol/L magnesium sulfate; 0.2 mmol/L each of dATP, dCTP, dGTP and dTTP; 16.9 μL molecular biology grade water; and 0.5 μmol/L each of the PCR primer. The reaction conditions for the 16s rDNA gene were 1 cycle at 94°C for 4 min; 35 cycles at 94°C for 30 s, 58°C for 45 s, 68°C for 75 s; followed by a final extension step at 68°C for 5 min. The reaction conditions for the *rpoB* gene were 1 cycle at 94°C for 4 min; 40 cycles at 94°C for 45 s, 55°C for 45 s, 68°C for 90 s; followed by a final extension step at 68°C for 5 min.

The PCR products were stained with ethidium bromide and examined after electrophoresis through a 1.5% agarose gel. The PCR amplicons were excised from gels, purified by using the QIAquick Gel Extraction Kit (QIAGEN), and submitted to the Research Technology Support Facility at Michigan State University for nucleic acid sequencing. Several internal primers were designed to derive the complete sequences of the PCR amplicons. The derived sequences were edited by using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA) and analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The nucleic acid sequences of the mycoplasma isolates and sequences from other *Mycoplasma* spp. obtained from GenBank were imported into the MEGA4 program (www.megasoftware.net), aligned by using ClustalW in the MEGA4 program, and subjected to phylogenetic analyses. For each isolate analyzed, 933 bp of the 16S rDNA gene sequence and 733 bp of the *rpoB* gene sequence were available. Phylogenetic trees were constructed by using the neighbor-joining method; data were resampled 1,000× to generate bootstrap percentage values.

Results

Gross and Histologic Lesions

Gross and histologic lesions from the 3 ferrets that died or were euthanized because of respiratory disease were similar and restricted to the lungs. The lungs were characterized by multifocal, tan to gray, somewhat firm nodules centered on airways randomly distributed throughout the pulmonary parenchyma (Figure 2). Hematoxylin and eosin–stained lung sections revealed a moderate bronchointerstitial pneumonia with severe bronchiole-associated lymphoid tissue (BALT) hyperplasia (Figure 3, panel A). BALT hyperplasia was commonly associated with marked narrowing of airway lumina. Additional findings included moderate perivascular lymphoid cuffing and diffuse pulmonary congestion. The lumina of some bronchi contained large amounts of mucus admixed with few sloughed epithelial cells and lymphocytes (catarrhal bronchitis).

Figure 2. Lungs from a 2-year-old ferret that died of acute dyspnea, showing multifocal, tan to gray semifirm nodules centered on airways and severely narrowed lumina of affected airways. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/12-0072-F2.htm).

Immunohistochemical examination (with antibodies against mycoplasmas) of affected lung tissue from all 3 ferrets that died exhibited strong labeling along the brush border of terminal respiratory epithelial cells (Figure 3, panel B). There was no penetration of organisms into the adjacent pulmonary parenchyma. With the same antibodies against mycoplasmas labeled with a fluorescent chromogen, confocal laser microscopy showed positive labeling along the apical border of the lining epithelium of terminal airways (Figure 3, panel C). Additional immunohistochemical examination and reverse transcription PCR for canine distemper and influenza A viruses, performed on samples of lung from all 3 ferrets that died, detected no virus.

Transmission electron microscopy showed bronchial epithelial cells with loss of cilia and cellular degeneration characterized by swelling of endoplasmic reticulum, vacuolization of mitochondria with loss of christae, and intranuclear chromatin dispersement. Attached to the apical surface of a ciliated cell were pleomorphic, round to ovoid, »0.8-µm mycoplasma-like organisms (Figure 4).

Electron microscopy showed severe denudation of bronchial epithelial cells. Cilia were commonly lost or had undergone degenerative changes characterized by bulbous swelling (Figure 5, panel A). Many necrotic bronchial epithelial cells were adhered to the luminal surface, and many pleomorphic mycoplasma-like organisms were diffusely attached to the mucosal surface of bronchi and bronchioles (Figure 5, panel B). In some areas, focal loss of cilia and cell membrane damage and mycoplasma-like organisms were observed along the periphery of such lesions (Figure 5, panel C). In other areas, the mucosal surface was covered by many mycoplasma-like organisms that completely obscured the cilia (Figure 5, panel D). Among the 10 healthy ferrets, no gross or histologic lesions suggestive of mycoplasma infection were identified.

Bacteria

The 12 BAL samples from affected ferrets were all positive for fast-growing, glucose-fermenting mycoplasmas but negative for other bacteria. Ocular swabs from these ferrets were negative for bacteria. No bacteria or mycoplasmas were isolated from the 10 healthy ferrets.

PCR and Sequences

Analyses of nucleic acid sequences from the 16S rDNA gene (GenBank accession nos. JQ910955– JQ910966) for each of the 12 mycoplasma isolates showed that the isolates were 99% similar to each other and segregated the isolates into 2 groups defined by nucleotide differences at 3 positions. Phylogenetic analysis with partial 16S rDNA gene sequences showed that the isolates were 96% to 97% similar to *M. molare* (isolated from a canid). Other closely related *Mycoplasma* spp. included *M. lagogenitalium* (isolated from Afghan pika), *M. neurolyticum* (isolated from mice and rats), *M.* sp. LR5794 (isolated from raccoons), *M. collis* (isolated from mice and rats), *M. cricetuli* (isolated from Chi-

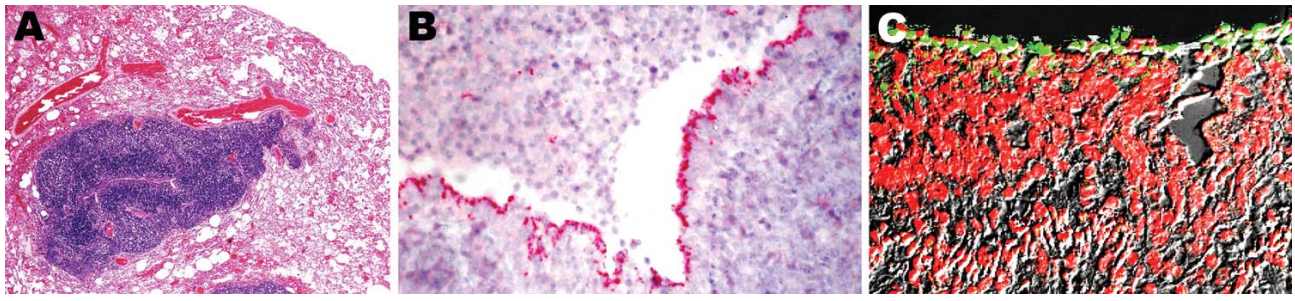

Figure 3. Micrographs of a section of lung from a 2-year-old ferret that died of acute dyspnea. A) Image shows moderate bronchointerstitial pneumonia with severe hyperplasia of bronchiole-associated lymphoid tissue around a narrowed airway lumen; original magnification ×40. B) Immunohistochemical analysis conducted with antibodies against mycoplasmas demonstrates intense labeling along the apical border of the ciliated respiratory epithelium; original magnification ×40. C) Confocal scanning laser microscopy conducted with antibodies against mycoplasmas demonstrates intense fluorescent labeling along the brush border of the bronchial epithelial cells; original magnification ×40. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/12-0072-F3.htm).

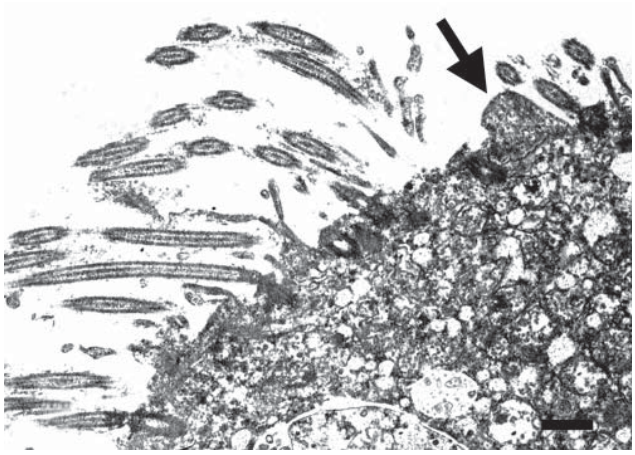

Figure 4. Transmission electron micrograph of the lung from a 2-year-old ferret that died of acute dyspnea, showing loss of cilia in bronchial epithelial cells and cellular degeneration characterized by swelling of endoplasmatic reticulum, vacuolization of mitochondria with loss of christae, and intranuclear chromatin dispersion. Attached to the apical surface of a ciliated cell is a 0.8-μm pleomorphic mycoplasma-like organism (arrow). Scale bar = 0.5 μm.

nese hamsters), and *M.* sp. EDS (isolated from house musk shrews) (Figure 6, panel A). On the basis of the 16S rDNA gene sequences, these mycoplasmas isolated from ferrets, along with the aforementioned closely related *Mycoplasma* spp., are in the hominis group of mycoplasmas.

Analyses of nucleic acid sequences from the *rpoB* gene (GenBank accession nos.. JQ910967–JQ910978) for each of the mycoplasma isolates from ferrets segregated the isolates into 2 groups of genetic variants (groups 1 and 2), which were 90% –91% similar to each other. Within a group, the isolates were 99%–100% or 98%–100% similar to each other. Although nucleotide differences were identified in as many as 12 positions within a group and 65 positions between groups, the corresponding amino acid sequences were 100% similar within a group and differed at only 2 aa positions between groups. Phylogenetic analysis showed that the partial *rpoB* gene sequences of the isolates were only 85%–86% similar with *M. molare* and 84%–86% similar to *M. lagogenitalium,* the most closely related *Mycoplasma* species. (Figure 6, panel B). Grouping of the isolates according to sequences of 16S rDNA and *rpoB* gene were in agreement for all but 1 isolate. Phylogenetic relatedness of these newly identified mycoplasmas to other *Mycoplasma* spp. was similar for the 16s rDNA and the *rpoB* genes.

Discussion

Mycoplasma spp. are the smallest free-living prokaryotic microorganisms of the class Mollicutes (*16*). They lack a cell wall and are thought to have developed from genome reduction of gram-positive bacteria (*17*). Most species are host-specific facultative anaerobes and do not usually replicate in the environment (*18*). Their complex growth requirements include cholesterol, fatty acids, and amino acids (*19*). In the respiratory tract, mycoplasmas attach to ciliated epithelial cells by surface-exposed adhesions (*20*). Although the pathogenesis of host cell injury remains largely unknown, proposed virulence mechanisms include induction of proinflammatory cytokines by phagocytes (*21*), oxidative damage to host cells by production of toxic by-products (*22*), and cleavage of host DNA through nucleases (*23*). Many mycoplasmas cause B lymphocytes and/or T lymphocytes to commence dividing in a nonspecific manner (*24*). This mitogenic effect probably explains the characteristic BALT hyperplasia observed in infected host tissues. A commonly described strategy for immune evasion is phenotype plasticity, whereby reversible switching or modification of membrane protein antigens results in altered surface antigens (*25*). This mechanism might support the persistent, chronic nature of mycoplasmosis often observed. The precise role of mycoplasmas in various host

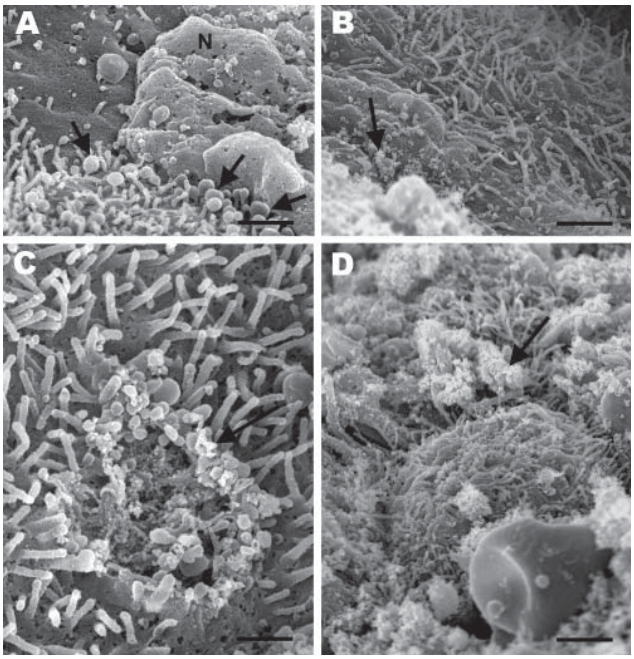

Figure 5. Scanning electron micrographs of the lung from a 2-year-old ferret that died of acute dyspnea, showing A) marked loss of cilia with multifocal degenerative changes characterized by bulbous swelling of cilia (arrows) and necrosis of bronchial epithelial cells (N) (scale bar = 1 μm); B) marked loss of cilia and numerous pleomorphic mycoplasma-like organisms diffusely attached to the mucosal surface (arrow) (scale bar = 1.25 μm); C) focal area of cilia loss and cell membrane damage with mycoplasma-like organisms (arrow) at the periphery of the lesion (scale bar = 400 nm); and D) many mycoplasma-like organisms (arrow) covering ciliated bronchial epithelial cells (scale bar = 2 μm).

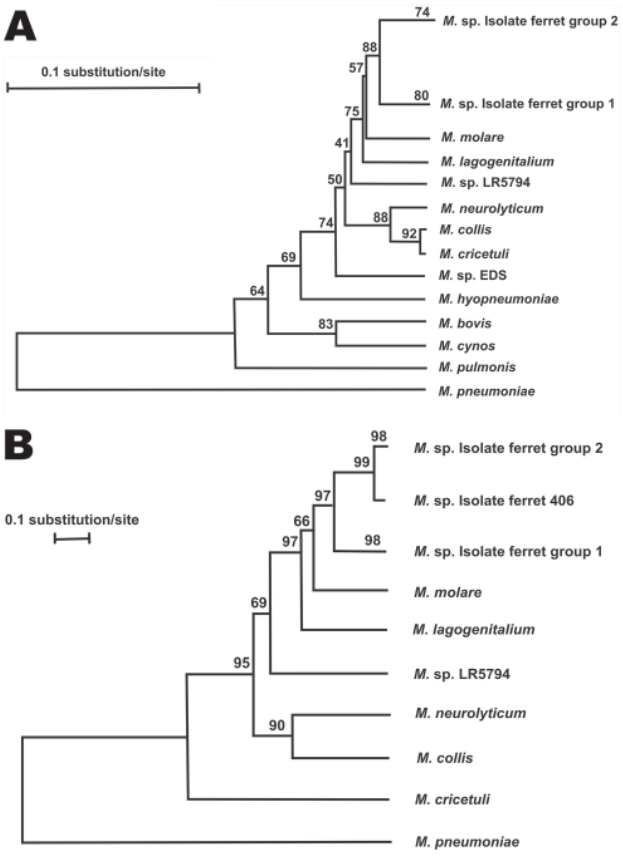

Figure 6. Phylogenetic analysis of A) partial 16S rDNA gene (933 bp) and B) partial RNA polymerase B gene (733 bp) for the new mycoplasma isolates and other closely related *Mycoplasma* species as conducted in MEGA4 (*13*). The bootstrap consensus phylogenetic trees were constructed by using the neighbor-joining method (*14*). The bootstrap values as shown above the branches were inferred from 1,000 replicates of data resampling to represent the evolutionary distances of the species analyzed (*15*). The tree is drawn to scale; branch lengths are in the same units as those of the evolutionary distances used to infer the phylogenetic tree (i.e., the units of the number of base substitutions per site).

species is often difficult to interpret because certain mycoplasmas can be isolated from apparently healthy animals.

The data presented here describe a recently emerging respiratory disease of ferrets, characterized especially by high morbidity rates and a dry, nonproductive cough, associated with an infection by a novel *Mycoplasma* species. To our knowledge, no *Mycoplasma* species have been associated with clinical disease in ferrets or other mustelids. On the basis of limited sequence data, the isolated mycoplasmas most likely represent a novel *Mycoplasma* specie or species.

In 1982, a study from Japan reported isolation of a glucose-fermenting mycoplasma from the oral cavities of 81% of clinically healthy ferrets kept in a laboratory setting (*26*). This mycoplasma isolate was not antigenically related to any reference strains from dogs, cats, sheep, cattle, mice, raccoon dogs, or a Japanese badger. In 1983, similarly fast-growing, glucose-fermenting mycoplasmas were isolated from the lungs of healthy mink kits (1–2 months of age) in Denmark (*27*). This species was named *M. mustelae*. Because the *Mycoplasma* spp. isolates from healthy ferrets or mink were not genetically characterized, comparison with the isolates from ferrets with respiratory disease in this study was not possible. The *Mycoplasma* species isolated from affected ferrets showed the highest sequence similarity to *M. molare* and *M. lagogenitalium*. *M. molare* was first isolated in 1974 from the pharynx of dogs with mild respiratory disease (*28*). However, the pathogenicity of *M. molare* in dogs or other species remains speculative. *M. lagogenitalium* was first isolated in 1997 from prepucial samples from apparently healthy Afghan pikas (*29*).

The 3 mycoplasma isolates obtained from BAL samples from 3 ferrets in July 2009 were highly homogenous according to limited sequence data. All 3 isolates were included in the mycoplasma isolate group 2. Of note, only 3 of the 9 isolates obtained from BAL samples from 9 ferrets in January 2010 had the same partial *rpoB* amino acid sequence data as the previous isolates and were also included in the mycoplasma isolate group 2. In contrast, the partial *rpoB* sequence of 5 of the more recent isolates differed by 9%–10% from that of the previous isolates, and the isolates were identified as belonging to mycoplasma isolate group 1. Whether these differences represent multiple *Mycoplasma* species circulating through the ferret population or a genetic change of the original mycoplasma over time is uncertain, as is the virulence of each of the potential strains. Only 1 isolate was identified in the bronchoalveolar lavage sample from a ferret for which postmortem examination confirmed lesions consistent with a mycoplasma infection. Experimental reproduction with the different isolates is required to further elucidate the virulence of each putative novel mycoplasma.

Respiratory disease attributed to mycoplasma infections in cattle (*30*), pigs (*31*), poultry (*32*), mice, and rats has been well described (*33*). The clinical signs and microscopic lesions in ferrets with the emerging respiratory disease described here closely resembled signs and lesions described for pigs infected with *M. hyopneumoniae* (*34*), rats infected with *M. pulmonis* (*35*), and cattle infected with *M. bovis* (*36*). For all of these species, chronic pulmonary mycoplasmosis is characterized by lymphoplasmacytic perivascular cuffing and extensive BALT hyperplasia, as was observed in ferrets in this study. Furthermore, *M. cynos* (*37*) and an untyped *Mycoplasma* species (*38*) reportedly cause pulmonary lesions similar to those in dogs and cats, respectively.

The similarity between the pathologic changes in the ferrets and those in other species with mycoplasmal pneumonia highly supports a causal relationship between the pulmonary disease and the identified novel mycoplasma in these ferrets. In addition, mycoplasmas were the only bacterial pathogens recovered from the respiratory tract of diseased ferrets, there was no microscopic evidence of a viral disease, and immunohistochemical and reverse transcription PCR results for canine distemper and influenza A were negative. Furthermore, mycoplasmas were not detected in the sampled population of healthy domestic ferrets 5 weeks to 5 years of age.

Because mycoplasmas have been recovered from the respiratory tract of apparently healthy mustelids (*26*,*27*), other unknown factors might have predisposed the lungs of these ferrets to colonization. The severity of the clinical signs might have been exacerbated by infections with secondary bacteria, as commonly occurs in other species (*30*,*31*,*33*), and antimicrobial drug therapy might have prevented isolation of such bacteria. A concurrent viral disease seems unlikely because characteristic microscopic lesions were absent and common respiratory viral pathogens in ferrets were not identified. We speculate that the stress of shipment from the breeding facility to the distribution center might have resulted in the disease manifestation. To more fully elucidate pathogenicity and disease dynamics in this species, experimental reproduction of the respiratory disease in ferrets is necessary.

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Lack of Cross-protection against *Bordetella holmesii* after Pertussis Vaccination

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Bordetella holmesii, a species closely related to *B. pertussis,* has been reported sporadically as a cause of whooping cough–like symptoms. To investigate whether *B. pertussis*–induced immunity is protective against infection with *B. holmesii*, we conducted an analysis using 11 human respiratory *B. holmesii* isolates collected during 2005–2009 from a highly *B. pertussis*–vaccinated population in Massachusetts. Neither whole-cell (wP) nor acellular (aP) *B. pertussis* vaccination conferred protection against these *B. holmesii* isolates in mice. Although T-cell responses induced by wP or aP cross-reacted with *B. holmesii*, vaccine-induced antibodies failed to efficiently bind *B. holmesii*. *B. holmesii*–specific antibodies provided in addition to wP were sufficient to rapidly reduce *B. holmesii* numbers in mouse lungs. Our findings suggest the established presence of *B. holmesii* in Massachusetts and that failure to induce cross-reactive antibodies may explain poor vaccine-induced cross-protection.

Bordetella pertussis and *B. parapertussis* commonly cause whooping cough, a highly contagious, acute coughing illness, in humans (*1*,*2*). Licensed in the mid-1940s, the first whooping cough vaccines consisted of whole-cell inactivated *B. pertussis* (wP), and their use led to a dramatic decrease in disease incidence by the mid-1960s (*2*,*3*). The potential for health risks related to wP vaccine, however, led to adaptation of acellular (aP) vaccines, which contain combinations of purified *B. pertussis* proteins. Despite high vaccine coverage, reported whooping cough incidence in industrialized countries has been increasing during the past 20 years, although this could be the result of greater awareness and improved analytical tools (*4*–*9*).

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In November 1983, the Centers for Disease Control and Prevention (CDC) received a gram-negative bacterium isolated from an asplenic patient (*10*). During the following decade, additional clinical isolates with the same microbiological characteristics (slow-growing, gram-negative, small coccoid, asaccharolytic, oxidase negative, nonmotile and brown-soluble-pigment-producing) were submitted to the CDC for identification (*10*). Subsequent biochemical analysis, 16S rRNA sequencing, and DNA relatedness studies revealed that these strains were new *Bordetella* species, which was named *Bordetella holmesii* to honor Barry Holmes (*10*). Since then, this bacterium has been isolated from numerous countries, including Australia, Canada, Chile, France, Germany, Japan, Netherlands, Switzerland, the United Kingdom, and the United States (*6*–*15*). These findings indicate that *B. holmesii* is a widespread pathogen among populations that are highly vaccinated against *B. pertussis*.

Comparative analysis of *B. holmesii* and *B. pertussis* by using 16S rRNA suggests that *B. holmesii* is closely related to *B. pertussis*, but further analysis of cellular fatty acid composition, housekeeping genes, and the BvgAS locus suggests that *B. holmesii* may not share many of the highly conserved virulence factors of *B. pertussis* (*16*). Antibodies against *B. pertussis* pertactin, pertussis toxin, fimbriae, adenylate cyclase toxin, and filamentous hemagglutinin recognize few, if any, proteins from multiple *B. holmesii* isolates (*14*), results that suggest *B. holmesii* may be antigenically distinct from *B. pertussis*.

Although *B. holmesii* has been isolated primarily from immunocompromised hosts (asplenic or sickle cell disease patients and transplant recipients) (*14*,*17*–*20*) and was first isolated from blood, the bacterium has also been found to cause respiratory diseases (*11*,*12*,*21*–*23*). *B. holmesii* was isolated from pleural fluid and lung biopsy specimens from

an immunocompetent adolescent who had fever and pulmonary fibrosis (*12*) and from sputum of patients with respiratory failure (*22*). Moreover, *B. holmesii* was isolated from nasopharyngeal specimens of previously healthy persons who had whooping cough–like symptoms, including paroxysms, whooping, or post-tussive vomiting (*11*,*21*,*23*). Therefore, *B. holmesii* appears to be able to colonize the respiratory tract in the same manner as other *Bordetella* species. A case study in Japan also found epidemiologic links between 5 persons colonized with *B. holmesii*, which indicates the ability of this pathogen to transmit from person to person (*6*).

In collaboration with the Massachusetts Department of Public Health (MDPH), we reviewed *B. holmesii* surveillance data collected in Massachusetts during 2005–2009. *B. holmesii* was isolated from several patients experiencing whooping cough–like symptoms. By using a murine infection model, we examined the effects of *B. pertussis* vaccination on *B. holmesii* infection susceptibility.

Materials and Methods

Identification of *B. holmesii* Cases in Massachusetts

Culture-confirmed *B. holmesii* cases identified during 2005–2009 by the State Laboratory Institute at the MDPH were included in our analysis. According to MDPH guidelines, a nasopharyngeal swab was cultured if the patient was <11 years of age or had a cough for <14 days. For all other patients (≥11 years of age and >14 days of cough), a serum test was performed. Details on culturing methods and *Bordetella* spp. identification tests performed have been described (*21*). A total of 41 *B. holmesii* infections were reported; the case records, including symptomology for 26 of these, are maintained in the Massachusetts Virtual Epidemiologic Network.

Bacterial Strains and Growth

B. pertussis strain 536 (*24*) and *B. parapertussis* strain CN2591 (*25*) have been described. *B. holmesii* strain P3421 was isolated in Massachusetts and used for animal experiments. Bacteria were maintained on Bordet-Gengou agar (Difco, Sparks, MD, USA) supplemented with 10% sheep's blood (Hema Resources, Aurora, OR, USA) without antimicrobial drugs (*B. holmesii*) or with 20 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) (*B. pertussis* or *B. parapertussis*). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (*26*,*27*).

Phylogenetic Analysis

Phylogenetic analyses were performed on the basis of *atpD, rpoB, tuf,* and *rnpB* gene sequences as described (*16*). Gene amplifications were completed on 30 *B. holmesii* isolates obtained from MDPH or the CDC, *B. pertussis* strain 536, *B. parapertussis* strain 2591, *B. bronchiseptica* strain RB50, *B. avium* strain 197N, and *B. hinzii* strain BC304. Concatenated sequences were aligned and used to construct unweighted pair group method using average linkages trees in MEGA4 software (www.megasoftware.net/mega4/mega.html).

Animal Experiments

All protocols were approved by Institutional Animal Care and Use Committee (IACUC). All animals were C57BL/6 mice and were handled in accordance with institutional guidelines (IACUC approval no. 31297). Animal experiments were performed as described, with 4 mice per group, and were performed in replicate (*28*–*33*). For vaccinations, sedated 4–6-week-old mice were vaccinated by intraperitoneal injection of 10^8 CFU of heat-inactivated (65°C for 30 min) bacteria in 200 μL of phosphate-buffered saline (PBS; Omnipur, Gibbstown, NJ, USA) for whole-cell *B. holmesii* vaccine (wH) or wP *B. pertussis* vaccine; one fifth human dose of Adacel (Sanofi-Pasteur, Swiftwater, PA, USA) (0.5 μg PT, 1 μg FHA, 0.6 μg pertactin, 5 μg fimbriae 2 and 3 per mouse) with Imject Alum (Thermo Scientific) (aP); or only Imject Alum in 200 μL PBS on days 14 and 28 before challenge (*28*,*33*).

For challenge, 50 μL PBS containing 5×10^5 CFU of *B. pertussis* or *B. parapertussis* or 10^7 CFU of *B. holmesii* was added by pipetting onto the external nares of sedated mice (*29*). A larger inoculum of *B. holmesii* was used to achieve reproducibility and detect *B. holmesii* from the respiratory tract at later time points, because it is cleared more rapidly from the lower respiratory tract than are *B. pertussis* or *B. parapertussis*. For adoptive transfer of serum antibodies, mice were vaccinated with the indicated bacteria on days 0 and 14, and serum samples were collected on day 28 from vaccinated or naive animals. Serum samples of 200 μL were intraperitoneally injected immediately before mice were inoculated with 5×10^6 CFU of *B. holmesii* (*30*,*32*). Bacterial numbers were quantified as described (*30*).

Splenocyte Restimulations

Splenocytes were isolated from vaccinated mice as described (*31*,*33*) and stimulated with either media alone or media containing 10^7 CFU (multiplicities of infection of 5) of the indicated heat-killed bacteria (*28*,*31*). After 3 days, the supernatants were collected and analyzed for interferon (IFN) γ and interleukin-10 (IL-10) production by using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Titer ELISAs

Antibody titers were determined as described (*34*–*36*). In brief, wP- or wH-induced/naive serum samples (1:200

dilution) or aP/adjuvant-induced serum samples (1:50 dilution) from each individual mouse were added to and serially diluted 1:2 across plates coated with heat-inactivated exponential-phase bacteria. After incubation, samples were probed with 1:4,000 dilution of goat anti-mouse Ig horseradish peroxidase–conjugated antibodies (Southern Biotech, Birmingham, AL, USA). Titers were determined by using the endpoint method (*33*).

Western Blot Analysis

Lysates containing 10^7 CFU of indicated heat-killed bacteria were subjected to 10 % sodium dodecyl sulfate–polyacrylamide gel under denaturing conditions. Polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) were probed overnight with either naive serum (1:100 dilution) or wH- (1:500 dilution), wP- (1:500 dilution), aP- (1:100 dilution) induced serum. A 1:10,000 dilution of goat anti-mouse Ig horseradish peroxidase–conjugated antibodies was used as the detector antibody (*35*,*37*). The membrane was visualized with ECL Western Blotting Detection Reagent (Pierce Biotechnology, Rockford, IL, USA).

Statistical Analysis

Mean ± SE values were determined for all appropriate data. Two-tailed, unpaired Student *t* tests, analysis of variance and Tukey's simultaneous test in Minitab (www.minitab.com) with similar significance were used to determine statistical significance between groups.

Results

B. holmesii Endemicity in Massachusetts

In 1999, Yih et al. reported an increase in culture-positive *B. holmesii* cases from 1995 to 1998 (0.2% to 0.6%) (*23*). Here, collaborating with the same MDPH research team, we report the numbers of *B. holmesii* culture-positive nasopharyngeal specimens submitted to the MDPH during 2005–2009. Over these 5 years, *B. holmesii* was isolated from the nasopharyngeal swabs of 41 patients who had similar respiratory symptoms, which is 8 more total cases than observed by Yih et al. during 1994–1998 (33 total cases) (*23*). At least 2 isolates were recovered each year, and 17 cases were identified in 2006, the highest number observed (Figure 1, panel A). The rate of *B. holmesii*–positive nasopharyngeal swabs ranged from 0.1% to 0.4%, in line with previous results (Table). Similar to observations made in prior years, 71% of cases occurred in persons 10–19 years of age (Figure 1, panel B), compared with >80% of cases during 1994–1998.

Symptom documentation was obtained for 26 of the 41 cases. All 26 of these patients had a cough; 17 (65%) had a paroxysmal cough, 6 (23%) had post-tussive vomiting, and 4 (15%) had an inspiratory whoop. Nineteen patients (73%) exhibited >1 of these classic symptoms of whooping cough and met the World Health Organization clinical case definition for pertussis (www.who.int/immunization_monitoring/diseases/pertussis_surveillance/en/index.html). No data were collected regarding any previous underlying diseases or potential co-infections among these patients. Although we have no evidence that *B. holmesii* is the causative agent of these pertussis-like illness, these data suggest that *B. holmesii* is consistently present in the nasopharynx of a small number of patients who have respiratory infections in Massachusetts.

Phylogenetic Relationships among *B. holmesii* Isolates

To evaluate the phylogenetic relationships among *B. holmesii* isolates, an unweighted pair group method using average linkages tree was constructed on the basis of concatenated nucleotide sequences amplified from regions of *atpD*, *rpoB*, *tuf*, and *rnpB* genes (*16*). Twelve isolates were obtained from CDC (designated with the letter 'G'), while the remaining isolates were obtained from the MDPH. Consistent with previous findings (*16*), all the *B. holmesii* isolates tested were more closely related to *B. hinzii* and *B. avium* than to the classical bordetellae (Figure 2). Al-

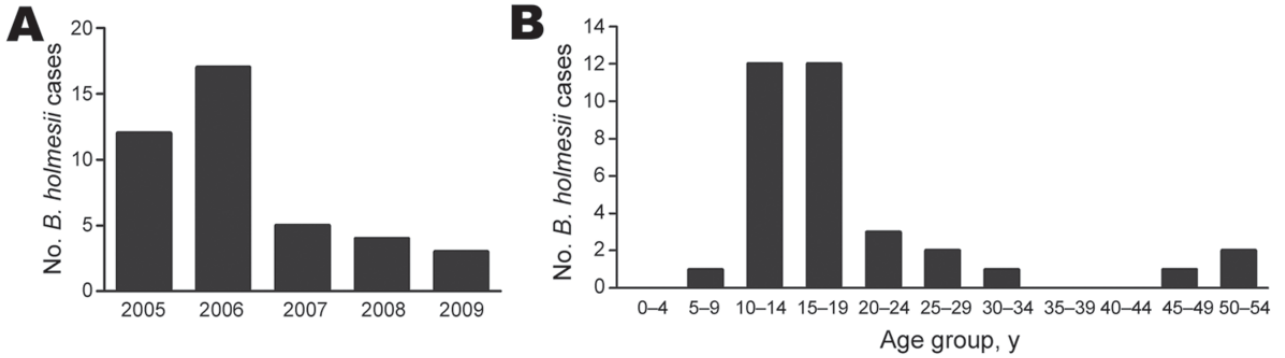

Figure 1. *Bordetella holmesii* cases in Massachusetts, USA. A) Nasopharyngeal specimens culture-positive for *B. holmesii* infection as confirmed by the Massachusetts Department of Public Health, by year, 2005–2009. B) Age distribution of case-patients with *B. holmesii* infection during 2005–2007 (cases shown in the Table).

Table. Results of testing of nasopharyngeal swabs for each *Bordatella* species at Massachusetts State Laboratory Institute, 2005–2007*

Test results and species detected	No. (%) swabs		
	2005	2006	2007
Positive			
B. holmesii	12 (0.37)	17 (0.35)	5 (0.14)
B. parapertussis	23 (0.71)	14 (0.29)	17 (0.49)
B. pertussis	196 (6.05)	188 (3.87)	204 (5.87)
Negative	3,007	4,644	3,248
Total tested	3,238	4,863	3,474

*Years for which full data on all laboratory specimens tested were available.

though single-nucleotide polymorphisms exist among *B. holmesii* isolates, pairwise comparisons among the 10 nasopharyngeal isolates from Massachusetts showed >99% sequence identity among 2,958 aligned bases, indicating their close relatedness. By comparison, the CDC isolates were more diverse, with the lowest percent sequence identity 97.5% between CDC strains G7851 and G4363. Moreover, *B. holmesii* isolates from blood and nasopharyngeal specimens do not cluster separately, which suggests that evolutionary relationship among *B. holmesii* isolates is not associated with the anatomic site of isolation.

B. holmesii Susceptibility to *B. pertussis* Vaccine-induced Immunity

Most records for the identified *B. holmesii* culture-positive cases did not include information regarding vaccination history; however, 16 patients that were culture positive for *B. holmesii* received ≥3 doses of pertussis vaccine. The 2010 coverage among children in Massachusetts for DTaP (diphtheria toxoid–tetanus toxoid–acellular pertussis vaccine) 4-dose vaccine was estimated to be >91%, ranking third among the 50 United States (www.cdc.gov/vaccines/stats-surv/imz-coverage.htm). However, adolescents and adults have lower *B. pertussis* vaccine coverage and waning immunity against *B. pertussis*. More adolescents and adults compared with children were culture-positive for *B. holmesii* in Massachusetts, which suggests that *B. pertussis* vaccine may confer some level of protection against *B. holmesii* in recently vaccinated persons, similar to the cross-protection against *B. bronchiseptica* in a murine model of infection (*38*). Although DTaP was not designed to prevent *B. holmesii* infections, it is critical to evaluate whether *B. pertussis* vaccines confer cross-protection against *B. holmesii*.

To test whether *B. pertussis* vaccines provide cross-protection against *B. holmesii*, we vaccinated C57BL/6 mice with wP or aP. These mice or vaccine-naive mice were then challenged with *B. holmesii* or *B. pertussis* and euthanized 3 days later. Because *B. holmesii* colonization efficiency in the murine respiratory tract is low compared with the classical bordetellae, possibly because of the decreased attachment to mouse respiratory epithelium (A.T. Karanikas and E. T. Harvill, unpub. data), a higher challenge dose was used than for *B. pertussis*. wP vaccination reduced *B. pertussis* numbers in the lungs by >99.99% compared with naive mice; however, wP failed to reduce *B. holmesii* numbers (Figure 3, panel A). In fact, wP vac-

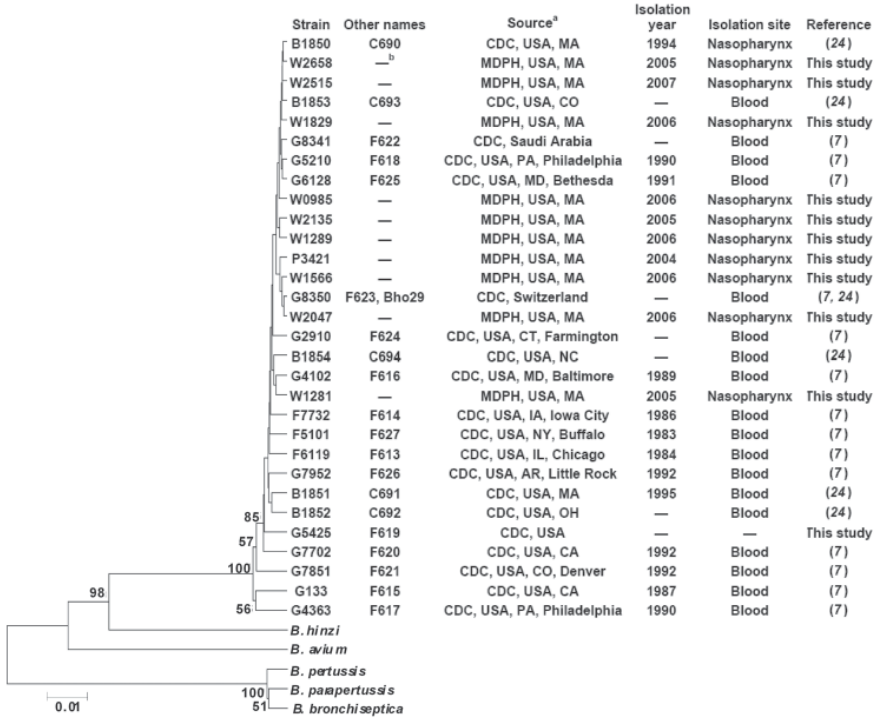

Figure 2. Phylogenetic tree showing 30 *Bordatella holmesii* isolates, *B. pertussis* 536, *B. parapertussis* 2591, *B. bronchiseptica* RB50, *B. avium* 197N, and *B. hinzii* BC304. Tree was constructed on the basis of concatenated nucleotide sequences of *atpD*, *rpoB*, *tuf* and *rnpB* genes. Bootstrap values >50% in 1,000 replicates are indicated. Scale bar indicates substitutions per site. CDC, Centers for Disease Control and Prevention; MDPH, Massachusetts Department of Public Health; –, unknown. Scale bar indicates nucleotide substitutions per site.

cination appeared to increase *B. holmesii* numbers in the lungs compared with naive mice. Although aP-vaccinated mice also reduced *B. pertussis* numbers in their lungs by »98% (Figure 3, panel B), they were not capable of reducing *B. holmesii* numbers. Together, these data indicate that wP- or aP-induced immunity does not protect against *B. holmesii* infections.

To determine whether *B. holmesii* immunization induces protection against itself or cross-protection against *B. pertussis* and/or *B. parapertussis*, we vaccinated C57BL/6 mice with heat-killed *B. holmesii* (wH) and challenged these or naive mice with *B. pertussis*, *B. parapertussis*, or *B. holmesii*; the mice were then euthanized on day 3 postinoculation for bacterial quantification. Similar numbers of *B. pertussis* were recovered from the lungs of naive and wH-vaccinated mice (Figure 3, panel C), an indication that *B. holmesii* vaccination failed to reduce *B. pertussis* numbers within 3 days. However, *B. holmesii* vaccination reduced the *B. parapertussis* load by ≈70% in the lungs, which indicates a modest cross-protection provided by *B. holmesii* against *B. parapertussis*. Compared with naive mice, mice who received *B. holmesii* vaccination had a ≈97% reduction of *B. holmesii* in the lungs (Figure 3, panel A). This experiment indicates that *B. holmesii* antigens induce efficient protective immunity against *B. holmesii* but have little effect against *B. parapertussis* and are even less protective against *B. pertussis*.

T-cell Responses to wH and wP/aP for *B. holmesii*

Because wP and aP fail to reduce *B. holmesii* numbers, we hypothesized that the pertussis vaccines may induce a different T-cell response than the wH vaccine does. To determine whether T-cell responses after vaccination are cross-reactive, splenocytes from naive or wH-, wP-, or aP-vaccinated mice were stimulated with media or heat-killed *B. pertussis*, *B. holmesii*, or *B. parapertussis* for 72 hours. Cell culture supernatant cytokine concentrations of IFN-γ and IL-10, representing Th1 and Treg cytokines, respectively, were determined. wH vaccination did not induce IFN-γ and IL-10 production by splenocytes at greater levels than those produced by naive splenocytes (Figure 4). However, splenocytes from wP-vaccinated mice produced high levels of IFN-γ and IL-10 on stimulation with heat-killed *B. pertussis*, *B. parapertussis*, or *B. holmesii*, which indicates that wP induces a strong cross-reactive T-cell response to *B. holmesii*. Although splenocytes from aP-vaccinated mice produced little IFN-γ, they produced ≈2,500 pg/mL IL-10 on stimulation with heat-killed *B. pertussis*, *B. holmesii*, or *B. parapertussis*, which indicates cross-reactive T-cell responses following aP vaccination. These data indicate that *B. pertussis* vaccines can induce cross-reactive T-cell responses to *B. holmesii* and thus do not explain the lack of cross-protection between these species.

wP/aP-induced Antibodies for *B. holmesii*

Njamkepo et al. observed that proteins in *B. holmesii* cell lysates are not recognized by antibodies specific for *B. pertussis* vaccine antigens (*14*). To determine whether *B. holmesii* and *B. pertussis* vaccine–induced antibodies are cross-reactive, Ig titers of wH-, wP-, or aP-induced serum samples were examined by using ELISA with heat-

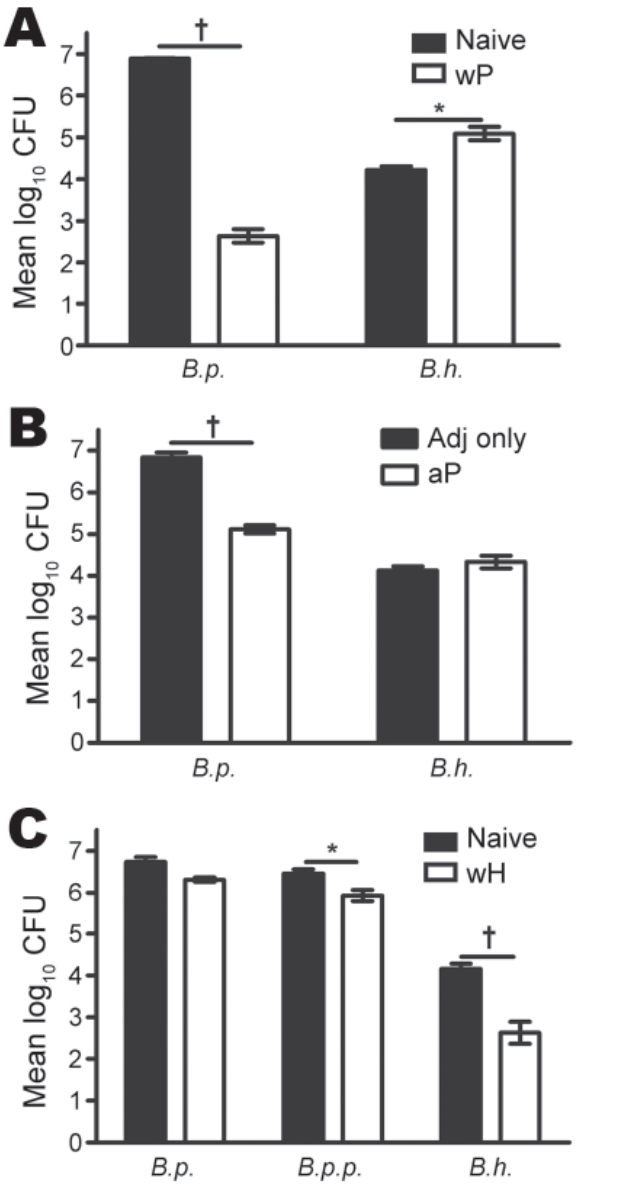

Figure 3. Results of testing of *Bordatella pertussis* and *B. holmesii* vaccines and protection against *B. holmesii* in mice. A) Mice vaccinated with whole-cell pertussis vaccine (wP) versus naive mice; B) mice vaccinated with aceullar pertussis vaccine (aP) versus adjuvant (adj) only vaccinated; C) mice vaccinated with whole-cell *B. holmesii* vaccine (wH) versus naive mice. All mice were challenged with *B. pertussis* (*B.p.*), *B. parapertussis* (*B.p.p.*) or *B. holmesii* (*B.h.*), and euthanized on day 3 postinoculation. Error bars indicate SE. *p£0.05; †p ≤ 0.01.

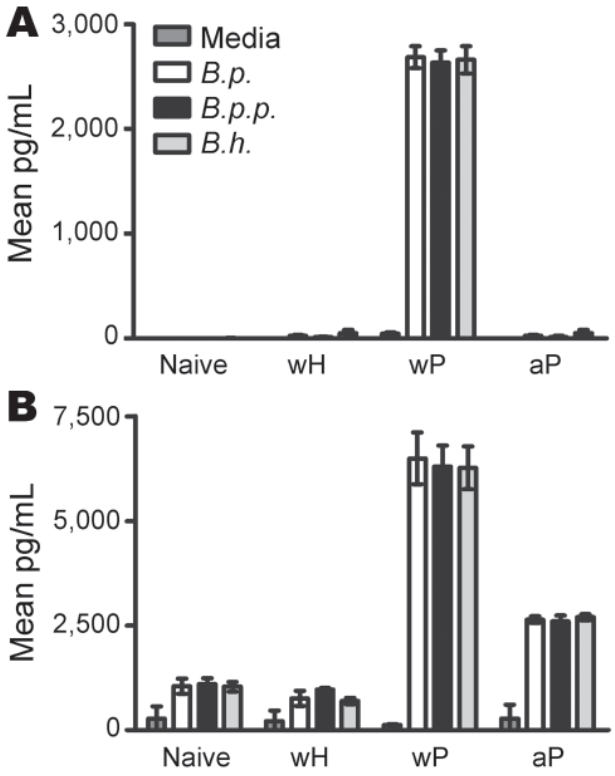

Figure 4. Comparison of splenic interferon (IFN)–γ (A) and interleukin (IL)–10 (B) responses in naive mice versus mice vaccinated with whole-cell *Bordetella holmesii* vaccine (wH), hole-cell pertussis vaccine (wP), and aceullar pertussis vaccine (aP). Splenocytes from naive mice or wH-, wP-, or aP-vaccinated mice were stimulated with media only or media containing heat-killed *B. pertussis* (*B.p.*), *B. parapertussis* (*B.p.p.*), or *B. holmesii* (*B.h.*). Error bars indicate SE.

inactivated bacteria as antigens. The *B. holmesii*–specific Ig titer of wH-induced serum was >450,000, which is ≈10-fold and 25-fold higher than *B. parapertussis*– and *B. pertussis*–specific serum titers, respectively (Figure 5, panel A); this result indicates partial cross-reactivity. *B. pertussis*–specific Ig titers of wP- and aP-induced serum antibodies were 290,000 and 2,500, respectively wP- and aP-induced antibodies bind less well to *B. parapertussis* (Figure 5, panel A) and confer little protection against *B. parapertussis* in vivo (*33*). wP- or aP-induced antibodies bound even less well to *B. holmesii*. A similar trend was observed when live bacteria were used to coat the ELISA plates (data not shown), which rules out the possibility that heat inactivation selectively destroys cross-reactive antigens.

To compare the antigens recognized by serum samples from different groups, Western blot analyses were performed on *B. pertussis*, *B. parapertussis*, or *B. holmesii* lysates probed with naive serum or wH-, wP-, or aP-induced serum. wH-induced serum antibodies recognized *B. holmesii* antigens of various molecular masses but lacked cross-recognition of some higher molecular mass *B. pertussis* and *B. parapertussis* antigens (Figure 5, panel B). The wP- and aP-induced serum antibodies bound less efficiently to *B. parapertussis* antigens than to *B. pertussis* antigens, consistent with published data (*33*). Although wP-induced antibodies recognized some *B. holmesii* antigens, they lacked recognition of higher molecular mass (>60 kDa) *B. holmesii* antigens. aP-induced antibodies only poorly recognized a single *B. holmesii* antigen. Together, these data suggest that antibodies generated following *B. pertussis* vaccination do not efficiently recognize *B. holmesii* antigens.

B. holmesii–specific Antibodies and wP-induced Immunity against *B. holmesii*.

On the basis of our data, we discerned that wP induces sufficient T-cell responses but that antibody responses are not sufficient to confer cross-protection against *B. holmesii*. If the lack of antibody cross-recognition is the only reason wP and aP vaccines are ineffective against *B. holmesii* infection, then adding *B. holmesii*–specific antibodies to wP should render the vaccination effective against *B. holmesii*.

To test this hypothesis, C57BL/6 mice were wP vaccinated or left untreated and later received either naive serum or wP- or wH-vaccinated mouse serum before *B. holmesii* challenge. *B. holmesii* lung colonization was determined 3 days postinoculation. Neither naive serum nor wP-induced serum reduced *B. holmesii* numbers, but vaccinated mice that received *B. holmesii*–immune serum had substantially lowered *B. holmesii* numbers in the lungs (Figure 6). The finding indicates that the addition of *B. holmesii*–specific antibodies to wP increases its efficacy against *B. holmesii*.

Discussion

The epidemiologic data collected by the MDPH suggest that *B. holmesii* is endemic in Massachusetts and is associated with classic whooping cough–like symptoms (Figure 1). Since the establishment of *B. holmesii* as a species in 1995 (*10*), infections have been sporadically reported worldwide (*6*–*9*,*12*,*13*,*15*,*17*,*18*,*20*,*21*,*23*). Increasing awareness of pertussis and improved analytical tools in industrialized countries may have contributed to the increased numbers of reported *B. holmesii* cases. However, nasopharyngeal *B. holmesii* specimens submitted to the MDPH each year during 2005–2009 likely represent a small fraction of *B. holmesii* infections in Massachusetts. Serologic testing and PCR are the dominant diagnostic *Bordetella* identification assays because of their high sensitivity and time efficiency, but no serologic test or PCR specific for *B. holmesii* is widely accepted or used by the MDPH. Less than 25% of *B. pertussis* cases reported in Massachusetts during 1990–2008 were identified by cul-

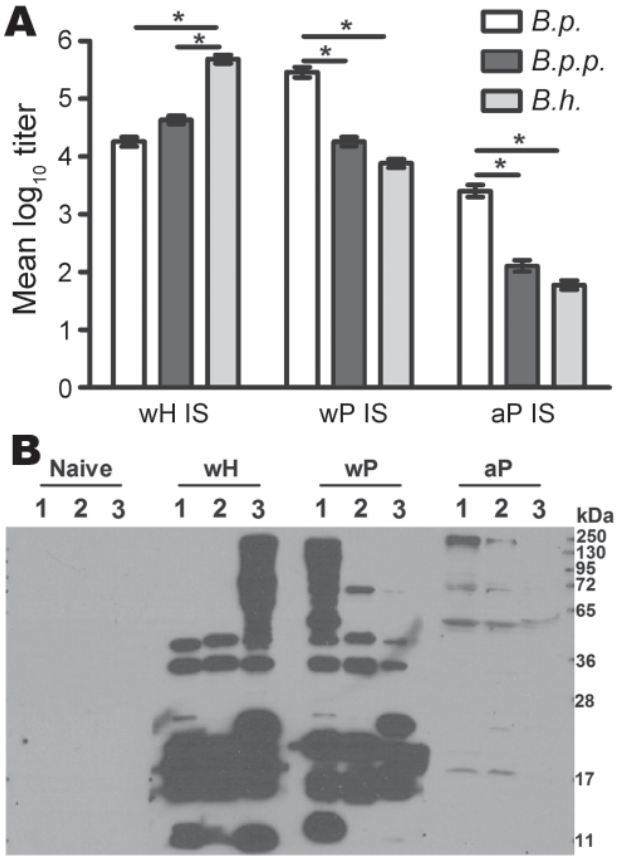

Figure 5. Antibody responses to whole-cell pertussis vaccine (wP), aceullar pertussis vaccine (aP), and whole-cell *Bordetella holmesii* vaccine (wH). A) Specific Ig titers of serum antibodies for *B. pertussis* (*B.p.*), *B. parapertussis* (*B.p.p.*), or *B. holmesii* (*B.h.*) for wH-, wP- or aP- vaccinated mice. Error bars indicate SE. *p<0.01. B) Western blots of *B. pertussis* (1), *B. parapertussis* (2), and *B. holmesii* (3) lysates probed with naive serum or wH-, wP-, or aP-induced serum (IS).

ture, the only test that currently detects *B. holmesii*. Therefore, larger numbers of *B. holmesii* cases might be identified if additional *B. holmesii*–specific serologic or PCR diagnostic tests are used.

When *B. holmesii* has been clinically identified, several reports have demonstrated little heterogeneity among isolates on the basis of pulsed-field gel electrophoresis banding patterns (*21*) and 16S rRNA heterogeneity (*10*,*17*). By using a sequence-based approach, Diavatopoulos et al. analyzed 7 *B. holmesii* isolates and observed only 1 nonsynonymous polymorphism among 3,666 bases (*16*). Using a similar method, we further analyzed 20 isolates from CDC and 10 from MDPH and identified 174 variable sites among the 2,958 aligned bases; this finding suggests more genetic variation among *B. holmesii* than previously recognized. Similar analyses on a wider range of *B. pertussis*, *B. parapertussis*, and *B. holmesii* isolates could better elucidate the evolutionary history among these human-adapted bordetellae.

Although vaccine studies are better completed in a natural host of the pathogen, the murine model of human-adapted bordetellae infection is well-established. *B. pertussis* and *B. parapertussis* murine infections mimic the course of infection and the immune responses in humans (*30*,*39*,*40*), although an animal model of *B. holmesii* infection has not been previously described. Unlike the classic *Bordetella* species, *B. holmesii* colonizes the murine respiratory tract only when relatively large intranasal inoculums are delivered. This model may be improved, for example, by administering antimicrobial drugs or creating transgenic mice. In this study, reproducible colonization of the respiratory tract was achieved, and statistically significant differences were observed after wH vaccination, which suggests that the mechanisms guiding protective immunity in this model can be used to investigate rapid immune-mediated interactions within the respiratory tract.

We determined that *B. pertussis* vaccines confer little, if any, protection against *B. holmesii*, even though 16S rRNA comparative analysis of *B. holmesii* and *B. pertussis* suggested that *B. holmesii* is closely related to *B. pertussis*. Furthermore, antibodies against *B. pertussis* pertactin, pertussis toxin, fimbriae 2 and 3, adenylate cyclase toxin, and filamentous hemagglutinin recognized few, if any, proteins from multiple *B. holmesii* isolates (*14*), which suggests that these proteins are absent from *B. holmesii* or are antigenically distinct from *B. pertussis*. Although vaccine-induced T-cell responses are cross-reactive to *B. holmesii* (Figure 4), *B. pertussis* vaccine-induced antibodies poorly bind *B.*

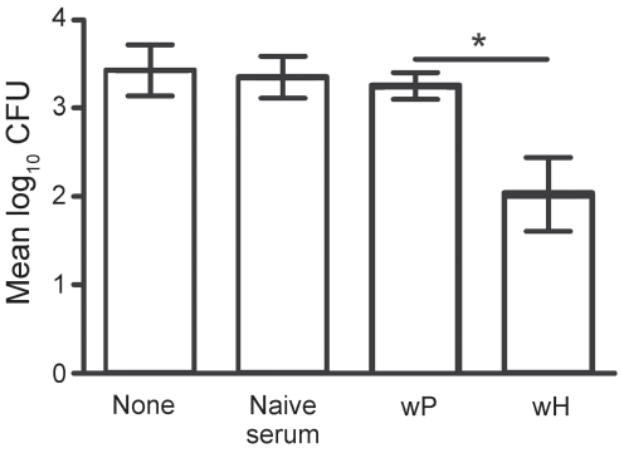

Figure 6. Supplementation of whole-cell pertussis vaccine (wP) with *B. holmesii*- but not *B. pertussis*- specific antibodies. Groups of four wP-vaccinated C57BL/6 mice were left untreated (none) or treated with naive serum, wP-induced sereum, or whole-cell *Bordetella holmesii* vaccine (wH)–induced serum, and challenged with *B. holmesii*. Bacterial numbers in the lungs on day 3 postinoculation are shown. Error bars indicate SE. *p<0.05.

holmesii (Figure 5). Furthermore, *B. holmesii*–specific, but not *B. pertussis*–specific, antibody administration efficiently decreased *B. holmesii* numbers in the lungs of vaccinated mice (Figure 6), which suggests that the lack of cross-reactive antibody responses may result in poor cross-protection of *B. pertussis* vaccines against *B. holmesii*.

Our data indicate that *B. holmesii* is circulating in Massachusetts and that *B. pertussis* vaccination confers little protection against *B. holmesii*. Careful *B. holmesii* surveillance is required to better evaluate its prevalence and transmission. *B. holmesii* genome sequencing may identify novel virulence determinants to explain its emergence in the human population and guide effective vaccine design.

Acknowledgments

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Seroprevalence of Antibodies against Chikungunya, Dengue, and Rift Valley Fever Viruses after Febrile Illness Outbreak, Madagascar

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In October 2009, two–3 months after an outbreak of a febrile disease with joint pain on the eastern coast of Madagascar, we assessed serologic markers for chikungunya virus (CHIKV), dengue virus (DENV), and Rift Valley fever virus (RVFV) in 1,244 pregnant women at 6 locations. In 2 eastern coast towns, IgG seroprevalence against CHIKV was 45% and 23%; IgM seroprevalence was 28% and 5%. IgG seroprevalence against DENV was 17% and 11%. No anti-DENV IgM was detected. At 4 locations, 450–1,300 m high, IgG seroprevalence against CHIKV was 0%–3%, suggesting CHIKV had not spread to higher inland-altitudes. Four women had IgG against RVFV, probably antibodies from a 2008 epidemic. Most (78%) women from coastal locations with CHIKV-specific IgG reported joint pain and stiffness; 21% reported no symptoms. CHIKV infection was significantly associated with high bodyweight. The outbreak was an isolated CHIKV epidemic without relevant DENV co-transmission.

In October 2009, the sentinel surveillance system for early outbreak detection in Madagascar (*1*) reported an increase of cases of fever with joint pain on the eastern coast. At the beginning of February 2010, chikungunya virus (CHIKV) infection was diagnosed in a patient from the Mananjary district. The International Federation of Red Cross and Red Crescent Societies reported 702 clinically diagnosed cases of chikungunya during February 9–February 15, 2010. Six hundred occurred in the coastal city of Mananjary, and 96 occurred in the small village of Irondro at the crossroads between the towns of Mananjary, Manakara, and Ifanadiana, indicating that the area was a focal point of the epidemic (Figure 1) (*2*).

Arthropod-borne viruses (arboviruses) such as CHIKV (*3*), dengue virus (DENV), and Rift Valley fever virus (RVFV) (*4*,*5*) are emerging pathogens in the southwestern Indian Ocean region. In 2005–2006, CHIKV caused outbreaks and epidemics on La Réunion, Mauritius, Mayotte, and the Seychelles (*6*), which caused considerable illness and death (*7*). Chikungunya appears to occur as an epidemic and an endemic disease in this region. The endemic disease affects mainly populations with high levels of IgG against CHIKV who live in rural areas in Africa (*8*). The epidemic disease occurs in Asia and the Indian Ocean region in populations in which herd immunity is weak, often in urban areas where *Aedes aegypti* and *Ae. albopictus* mosquitoes are the main transmission vectors. During epidemics, humans are the primary reservoirs. Monkeys, rodents, birds, and cattle have been identified as animal reservoirs (*9*–*11*). The onset of chikungunya epidemics is acute with high attack rates as seen in 2005–2006 on La Réunion (*12*). Concurrent epidemics of dengue and chikungunya have been reported from Asia (*13*) and Africa (*14*). In 2006, a combined outbreak of dengue fever and chikungunya fever occurred near the Madagascan city

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of Toamasina (*15*), but the rest of the country remained unaffected by the epidemic. An increased number of RVFV infections was noticed in Madagascar during the rainy seasons of 2008 and 2009 with 476 (19 fatal) and 236 (7 fatal) suspected cases, respectively (*4*).

The sudden emergence of chikungunya in the Mananjary area indicated a lack of herd immunity in the affected population. The previous outbreak of CHIKV infection in Madagascar in 2006 in the Toamasina region occurred in conjunction with DENV infection. Because of the recent reports of RVFV infections in animals and humans, our investigation of the recent outbreak in Madagascar included assessment of serologic parameters against CHIKV, DENV, and RVFV.

Approximately 2–3 months after the peak and 1–2 months after the decline of the outbreak of chikungunya, we retrospectively assessed the serologic markers and reported clinical features of women who came for routine pregnancy follow-up visits at 6 geographic locations. By focusing on pregnant women, we could reduce the need to stratify for age and sex and thus minimize the fragmentation of data. The focus could then to be placed on 1) assessing a possible inward spread of the epidemic, 2) evaluating whether the epidemic was limited to CHIKV or due to a simultaneous occurrence of DENV or RVFV infections, and 3) detecting factors associated with an increased risk of CHIKV infection.

Methods

The cooperative project of the University of Antananarivo and the Bernhard Nocht Institute for Tropical Medicine was carried out during May–July 2010. Overall, 1,244 pregnant women were included from 6 different sites (Figure 1). Investigations were conducted in 2 coastal cities: Mananjary, the suspected epicenter of the chikungunya epidemic in February, and Manakara. Inland study sites included Ifanadiana, located at the ascending road from the above-mentioned cities to the highlands, the 2 highland cities of Tsiroanomandidy and Ambositra, and Moramanga, a highland city between Antananarivo and Toamasina. The study sites were chosen to include the coastal cities where the suspected chikungunya outbreak was reported (Manajary and Manakara), a city lying at the main road leading to the outbreak region on a moderate elevation level of 466 m (Ifanadiana), and 3 arbitrarily chosen cities in the highlands (Moramanga, Tsiroanomandidy, and Ambositra). Pregnancy follow-up services were chosen for a population comprehensive enough to allow the collection of ≈200 samples within a week. All women attending the routine pregnancy follow-up services were included.

The study was approved by the "Comité d'ethique de la Vice Primature Chargée de la Santé Publique" and discussed with representatives of the World Health Organization (WHO) during a meeting at the WHO office in Antananarivo on April 23, 2010. The study was explained to every participant, and informed consent was either signed (or, in the case of illiteracy, a fingerprint was obtained), and signature of a witness was acquired. Data on the course of pregnancy were obtained from interviews and review of the pregnancy follow-up booklet. To assess symptoms of CHIKV infections, researchers questioned the participants regarding current symptoms, symptoms that occurred since the time of their last menorrhea, or symptoms they recalled from a recent confirmed or suspected CHIKV infection.

Figure 1. Madagascar (gray shading in inset), showing the main roads, the capital of Antananarivo (square), the harbor city of Toamasina (white circle), and the locations of the 6 study sites (black circles) from which serologic samples from pregnant women were screened for IgG against chikungunya virus, dengue virus, and Rift Valley fever virus. The altitudes of the locations are as follows: Mananjary and Manakar, coastal; Ifanadiana, 466 m; Moramanga, 920 m; Tsiroanomandidy, 860 m; Ambositra, 1,280 m; Toamasina, coastal; Irondro, 40 m; Antananarivo, 1,300 m.

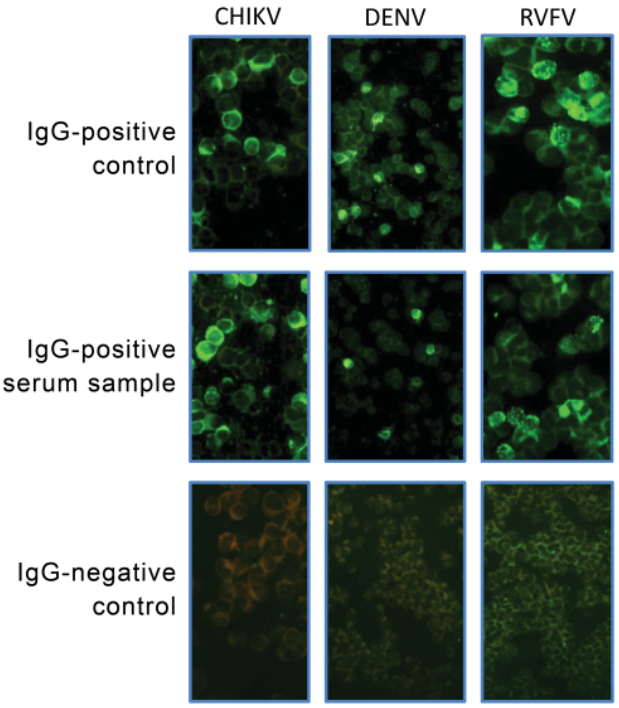

Figure 2. Images from immunofluorescence assays in Vero E6 cells for IgG against chikungunya virus (CHIKV), dengue virus (DENV), and Rift Valley fever virus (RVFV). Original magnification ×100 and 200. A color version of this figure is available online (wwnc.cdc.gov/EID/article/18/11/11-1036-F2.htm).

A venous blood sample collected into EDTA was taken for measurement of IgG against CHIKV, DENV, and RVFV. To detect acute CHIKV or DENV infections, we measured levels of IgM against CHIKV and DENV. Immunofluorescence assays (IFAs) for CHIKV, DENV, and RVFV were performed with virus-infected Vero E6 cells as described (*16*). In brief, Vero cells were spread onto slides, air dried, and fixed in acetone. Plasma samples were serially diluted in phosphate-buffered saline, starting with an initial dilution of 1:10, added to the cells, and incubated for 90 minutes at 37°C. After slides were washed with phosphate-buffered saline, they were incubated with fluorescein isothiocyanate–labeled rabbit antihuman IgG and IgM (SIFIN, Berlin, Germany) at 37°C for 25 minutes (Figures 2,3). IgG or IgM titers >100 were considered positive.

The serologic controls used for the IFA were the standard routine controls of the WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research in Hamburg, Germany. Moreover, the controls used in this serologic survey were used as external quality assessment samples by the WHO Collaborating Centre for Quality Assurance and Standardization in Laboratory Medicine, Berlin, Germany. The sensitivity and specificity of the IFAs were demonstrated to be 100%, according to results of external quality assessments organized by the European Network for Diagnostics of Imported Viral Diseases (*17*). The DENV nonstructural protein 1 (NS1) antigen ELISA was performed according to the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). The DENV NS1 antigen is a marker for DENV antigen circulation and thus detects acute infections up to day 21 after onset of symptoms.

The following retrospectively self-reported symptoms were assessed: fever, joint pain, and stiffness, skin symptoms and rashes, hip vibrations and pain, conjunctivitis, and stooped posture. For the 2 coastal locations where seroprevalence of CHIKV was highest, we analyzed potential risk factors for CHIKV infection, taking into account patient's age, mosquito protective measures (bednet, fan, repellent, air conditioner), and because our study population consisted of pregnant women, we also considered pregnancy-related factors such as parity, weight, and trimester of pregnancy.

Statistical analysis was performed by using STATA version 10 (www.stata.com). The risk factor analysis was done by using univariable and multivariable logistic regressions. If the odds ratios suggested a trend over

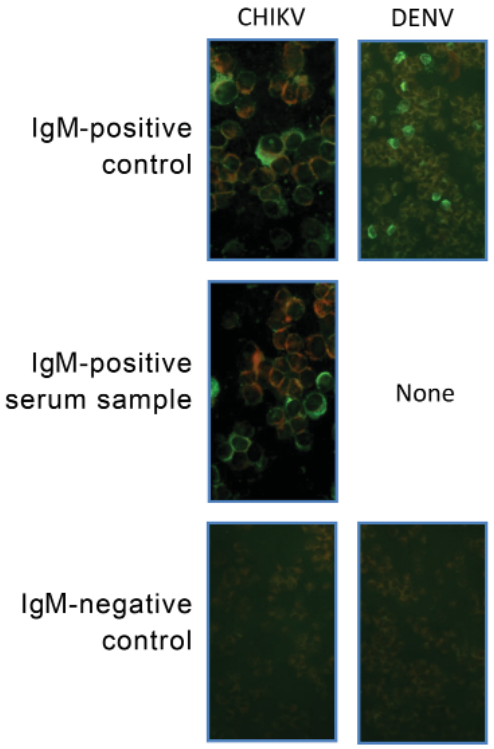

Figure 3. Images of immunofluorescence assays in Vero E6 cells for IgM against chikungunya virus (CHIKV) and dengue virus (DENV). For each of the viruses, a positive control, an example of a positive serum sample (if available), and a negative control are shown. Original magnification ×100 and 200. A color version of this figure is available online (wwnc.cdc.gov/EID/article/18/11/11-1036-F3.htm).

several categories, a nonparametric test for trend was conducted in the univariable analysis. For multivariable models, a Mantel-Haenszel test for trend, adjusted for the other variables in the model, was performed.

Results

A total of 1,244 women from 6 locations in Madagascar were included in the study. All locations, when data were presented graphically, showed a right-skewed age distribution. The median age was 25 years (range 12–50 years). The median age of women from the 2 coastal locations (Mananjary and Manakara) was not significantly different from that of women from higher altitudes ($p = 0.95$, by Wilcoxon rank sum test). No relevant difference was found regarding the proportion of primiparas by location (28.4% in the highlands compared with 27.4% at the coast, $p = 0.70$, by χ^2 test). None of the participants had an air conditioner at home; 3 women who lived on the coast reported using a fan. Two women who lived in the highlands reported using mosquito repellents. The only noteworthy mosquito protective measure used frequently was bednets (70.3%). Bednet use was significantly higher for women from the coastal cities of Mananjary (88.2%) and Manakara (90.8%) than for those living in the highland cities of Moramanga (65.7%), Ambositra (21.5%), and Tsiroanomandidy (56.7%). Of those from Ifanadiana, a coastal city at 450-m altitude, 94% used bednets.

Table 1 shows the levels of IgG against CHIKV, DENV, and RVFV, and the results of the IFAs that measured IgM against CHIKV and DENV. IgM against CHIKV was only detected in samples from Mananjary and Manakara taken ≈2–4 months after the peak of the epidemic. Although 27.5% of the samples from Mananjary and 5.2% of the samples from Manakara were positive for IgM against CHIKV, IgM against CHIKV was not detected in samples from other noncoastal locations. IgM against DENV and DENV NS1 antigen, indicators of DENV viremia, were not detected in any location.

A total of 154 of the 1,244 pregnant women (12.4%) were positive for IgG against CHIKV, and 116 of them (75.3%) had reported a history of symptoms of CHIKV infection since their last menorrhea. The highest rates of IgG against CHIKV (Mananjary 44.6%, Manakara 22.7%) and IgM against DENV (Mananjary 17.4%, Manakara 10.8%) were found in the 2 coastal cities (Figure 1; Table 1). IgG against CHIKV and DENV were found in 7.2% and 2.4% in Mananjary and Manakara, respectively. Differences in seroprevalences at the other locations, all at higher altitudes, were negligible, apart from the high frequency of IgG against DENV in Moramanga (11.7%) and Tsiroanomandidy (3.9%). Among persons with IgG against CHIKV, the proportion of women who reported a history of symptoms related to CHIKV infection during the recent outbreak was the same in Mananjary and Manakara (79%). None of the women interviewed from Moramanga reported a history of recent symptoms, even if they had IgG against CHIKV or DENV.

According to the results, only persons with IgG against CHIKV from Mananjary and Manakara could be confidently assigned to the 2009–2010 outbreak. Therefore, reported symptoms and analysis of risk factors for CHIKV infection was confined to these 2 locations (Tables 2, 3). Risk for previous CHIKV infection increased with body weight (Table 2). This association persisted after adjusting for parity, bednet use, and age (Table 3) and after additionally adjusting for the trimester of pregnancy (Table 3).

Discussion

In contrast to the outbreak in the Toamasina area of Madagascar in 2006, the outbreak investigated here appears to be exclusively caused by CHIKV infections without concomitant DENV infection. Although one third of the participants in coastal cities at the epicenter of the outbreak were infected with CHIKV, the epidemic did not spread to higher altitudes and further inland. The chikungunya epidemic curve reached its peak in Mananjary in February and abated in March 2010; in Manakara, the epidemic occurred ≈1 month later. The duration of the epidemic roughly corresponds to the rainy season in Madagascar from November to April. Anti-CHIKV IgG was detected in all samples positive for anti-CHIKV IgM, and we assumed that the outbreak had ended before the investigation

Table 1. Measurement of antibodies against CHIKV, DENV, and RVFV in samples from pregnant women in Madagascar, May–July 2010*

Location	Altitude, m	Month	Total no. women	No. (%) women with IgG against CHIKV	IgG against DENV	IgG against RVFV	IgG against CHIKV/DENV†	IgM against CHIKV‡
Mananjary	0 (coastal)	May	195	87 (44.6)	34 (17.4)	2 (1)	14 (7.2)	53 (27.5)
Manakara	0 (coastal)	Jun	251	57 (22.7)	27(10.8)	1 (0.4)	6 (2.4)	13 (5.2)
Ifanadiana	466	Jun	197	2 (1.0)	4(2.0)	0	0	0
Moramanga	920	Jul	198	6 (3.1)	23(11.7)	0	1 (0.5)	0
Tsiroanomandidy	860	Jul	203	0	8 (3.9)	1 (0.5)	0	0
Ambositra	1,280	May	175‡	2 (1.1)	1(0.6)	0	1 (0.6)	0

*CHIKV, chikungunya virus; DENV, dengue virus; RVFV, Rift Valley fever virus.
†Samples that were positive for IgG against both CHIKV and DENV.
‡For 25 women from Ambositra, no serologic samples were taken. No samples were positive for IgM against DENV.

Table 2. Signs and symptoms of pregnant women with IgG against CHIKV from Mananjary and Manakara, Madagascar, May–July 2010*

Symptom/sign	No. (%) women with IgG against CHIKV, n = 144	No. (%) women with IgG against DENV, n = 61
Fever	107 (74)	21 (34)
Joint pain and stiffness	113 (78)	22 (36)
Skin symptoms or rashes	87 (60)	19 (31)
Hip vibrations and pain	84 (58)	20 (33)
Stooped posture†	77 (53)	18 (30)
Conjunctivitis	27 (18)	9 (15)
Asymptomatic	30 (21)	39 (64)

*CHIKV, chikungunya virus; DENV, dengue virus.
†Stooped posture refers to the posture that persons affected by strong joint and muscle pains adopt to relieve pain. The word "chikungunya" comes from the Makonde language spoken in southeast Tanzania and means "the one who bends up and curls like a drying leaf."

described here was started. In the noncoastal locations, all samples were negative for IgM against CHIKV, which suggests that the epidemic was constrained to the coast.

By focusing on pregnant women, we achieved a relatively homogenous study population, which facilitated the comparison of differences between study sites by reducing differences between persons (e.g., age or sex) and overfragmentation of the data. In addition to DENV, the presence of RVFV was assessed because this virus has been recently reported in Madagascar (*4*,*18*). In 22 persons, IgG against CHIKV and IgG against DENV were detected. The major mosquito vectors of both viruses are equally susceptible to CHIKV and DENV, with simultaneous transmission being confirmed in experimental settings (*19*,*20*). Although a considerable number of the samples from Mananjary (17.4%) and from Manakara (10.8%) were positive for IgG against DENV, none was positive for DENV IgM or DENV NS1 antigen, which suggests past DENV infections, independent of the recent chikungunya epidemic.

A limitation of the study is the possibility that some women may have had prior CHIKV infections before the outbreak investigated in this study. A CHIKV outbreak was reported in the Toamasina region in 2006 (*15*). However, it seems unlikely that a relevant proportion of the resident population of the Mananjary region acquired CHIKV immunity during the Toamasina outbreak because this region is 400 km distant from Mananjary, and no tarred road exists along the coast. The elevated seroprevalance of IgG and IgM against CHIKV in the coastal city of Manakara, which lies 100 km south of Mananjary, suggests that the outbreak spread southwards along the coast.

The International Federation of Red Cross and Red Crescent Societies reported that the town of Irondo, where the inbound roads from Mananjary and Manakara meet, was also affected by the outbreak (*2*).The town is situated 30 km from the coastline at an altitude of ≈40 m. Although an altitude of 400–500 m does not avert mosquito survival, the low seroprevalence in Ifanadiana (altitude 466 m, 70 km inland) provides evidence against an upward and inbound spread of the epidemic. The long travel distance between Irondro and Ifanadiana, with serpentine roads and long uninhabited stretches between small settlements, may have interrupted transmission chains. Samples taken from the highland cities of Ambositra (1,280 m), Tsiroanomandidy (860 m), and Moramanga (920 m) indicate that the highland population of Madagascar remained unaffected by the

Table 3. Protective or risk factors for CHIKV infection in pregnant women from 2 coastal locations Mananjary (N = 195) and Manakara (N = 251), Madagascar, May–July 2010*

Factor	No. women	Bivariable analysis, n = 446 OR (95% CI)†	p value	Multivariable model 1, n = 433 OR (95% CI)	p value	Multivariable model 2, n = 376 OR (95% CI)	p value
Bednet use							
No	40	Reference	0.70	Reference	0.98	Reference	0.95
Yes	400	0.87 (0.44–1.73)		0.99 (0.47–2.06)		1.02 (0.47–2.22)	
Parity							
Multipara	324	Reference	0.93	Reference	0.44	Reference	0.65
Primipara	122	0.98 (0.63–1.53)		0.79 (0.43–1.44)		0.86 (0.44–1.67)	
Age, y							
<20	94	Reference	0.67†	Reference	0.17	Reference	0.37
20–30	225	0.82 (0.49–1.36)		0.70 (0.36–1.34)		0.81 (0.39–1.69)	
>30	125	0.87 (0.49–1.53)		0.57 (0.26–1.23)		0.68 (0.29–1.60)	
Weight, kg							
<40	17	Reference	0.001†	Reference	0.0006	Reference	0.001
40–49	242	1.22 (0.38–3.87)		1.22 (0.38–3.92)		1.45 (0.39–5.45)	
50–59	142	1.82 (0.56–5.88)		1.86 (0.57–6.08)		2.36 (0.61–9.09)	
60–70	32	2.53 (0.67–9.47)		2.84 0.74–10.8		3.82 (0.85–17.2)	
>70	8	9.75 (1.38–68.8)		10.8 (1.52–77.2)		11.4 (1.39–93.0)	
Trimester							
1st	9	Reference	0.45			Reference	0.39‡
2nd	142	0.37 (0.10–1.45)				0.44 (0.10–1.90)	
3rd	236	0.36 (0.10–1.40)				0.40 (0.10–1.71)	

*CHIKV, chikungunya virus; OR, odds ratio.
†Nonparametric test of trend.
‡Mantel-Haenszel test for trend, adjusted for the other variables.

recent chikungunya outbreak.

According to our data, 21% of the women from the coastal cities who were positive for IgG against CHIKV did not report symptoms corresponding to those of chikungunya during the outbreak. Similarly, the proportion of asymptomatic infections during a CHIKV outbreak in a naïve population from northeastern Italy in 2007 was 18% (*21*). In contrast, during the 2005–2006 chikungunya outbreak on La Réunion, only 5% of infections remained asymptomatic (*22*,*23*).

Entomologic data were not collected during the chikungunya outbreak on Madagascar. The main vectors for CHIKV are *Ae. albopictus* and *Ae. aegypti* mosquitoes. In a study from Gabon, *Ae. albopictus* mosquitoes outnumbered *Ae. aegypti* mosquitoes in most suburban areas, and in urban areas, where *Ae. aegypti* mosquitoes were more commonly found, CHIKV and DENV were only found in *Ae. albopictus* (*19*) mosquitoes. *Ae. albopictus* mosquitoes were the main vectors on La Réunion during the 2005–2006 epidemic.

Recent entomologic data for Madagascar were scarce in 2010, when the chikungunya outbreak took place. In a report from 1989, *Ae. albopictus* was identified as the predominant *Aedes* species on the eastern coast and on the central highland plateau, where all our study sites were located (*24*). A recent study provides evidence for an expansion of an invasive lineage of *Ae. albopictus* that has spread throughout the island and possibly caused a decline of *Ae. aegypti* at least in urban areas (*25*). More research on the vector dynamics of *Ae. albopictus* and *Ae. aegypti* mosquitoes in Madagascar is needed.

The analysis of risk and protective factors for infection was confined to the epidemic coastal cities. The finding that bednet use had no influence on the risk of CHIKV infection is in accordance with the fact that the chikungunya vectors *Ae. albopictus* and *Ae. aegypti* mosquitoes bite during the daytime. The results indicate a positive association between body weight and the risk for CHIKV infection but the causality could not be assessed in this cross-sectional study. Notably, a study from India found evidence of a higher risk for chronic sequelae among obese persons who acquired chikungunya (*26*). Future studies on risk factors for chikungunya should include body weight as a possible influence.

The 2009–2010 arboviral outbreak in coastal eastern Madagascar was an isolated CHIKV epidemic without relevant DENV co-transmission. With more than one third of all women affected in the epicenter, the infection rate in the population was high. Data from other locations suggest that the epidemic did not spread to higher altitudes and inland.

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Epidemic Myalgia in Adults Associated with Human Parechovirus Type 3 Infection, Yamagata, Japan, 2008

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Human parechovirus has rarely been shown to cause clinical disease in adults. During June–August 2008, a total of 22 adults sought treatment at Yonezawa City Hospital in Yamagata, Japan, for muscle pain and weakness of all limbs; most also had fever and sore throat. All patients received a clinical diagnosis of epidemic myalgia; clinical laboratory findings suggested an acute inflammatory process. Laboratory confirmation of infection with human parechovirus type 3 (HPeV3) was made for 14 patients; we isolated HPeV3 from 7 patients, detected HPeV3 genome in 11, and observed serologic confirmation of infection in 11. Although HPeV3 is typically associated with disease in young children, our results suggest that this outbreak of myalgia among adults was associated with HPeV3 infection. Clinical consideration should be given to HPeV3 not only in young children but also in adults when an outbreak occurs in the community.

Human parechovirus (HPeV) is a positive-sense, single-stranded RNA virus belonging to the family *Picornaviridae* and the genus *Parechovirus* (*1*,*2*). HPeV type 1 (HPeV1) and HPeV2 were discovered in the United States in children with diarrhea in 1956; initially designated echovirus types 22 and 23, respectively, these viruses were recently reclassified and renamed (*1*,*2*). In 1999, HPeV3 was identified from a 1-year-old child with transient paralysis, fever, and diarrhea in Japan (*3*). Complete genome sequences are available for HPeV1–8, and viral protein (VP) 1 coding region sequences have recently been reported for HPeV9–16 (*1*,*4*).

HPeV1 and HPeV2 mainly cause mild gastrointestinal or respiratory illness, but more serious diseases have been occasionally reported, including myocarditis, encephalitis, pneumonia, meningitis, flaccid paralysis, Reye syndrome, and fatal neonatal infection (*2*,*5*,*6*). HPeV3 also causes not only mild gastrointestinal and respiratory tract illness but also severe illness in young children, including sepsis and conditions involving the central nervous system (*1*,*5*,*7*–*13*).

Although the seroprevalence of the recently discovered HPeV4–8 are unknown, HPeV1–3 infections usually occur in early infancy (*1*,*3*). Because all children have antibodies against HPeV1 after 1 year of age, HPeV1 seroconversion during the early months of life has been clearly established (*2*). HPeV3 is reported to infect younger children more often than HPeV1; HPeV3 infections occur most commonly among infants <3 months of age (*1*,*10*,*11*). In contrast, reports in the literature that describe HPeV3 infection in persons >10 years of age are rare (*1*,*14*).

An unusual outbreak of epidemic myalgia among adults occurred during June–August 2008 in Yonezawa,

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Yamagata, Japan, and epidemiologic investigation found that it was associated with HPeV3 infection. We describe this outbreak and provide expanded information about the clinical spectrum of HPeV3 infection.

Methods

Case-Patients

During June 17–August 6, 2008, an outbreak of an unknown disease was observed among adults in Yonezawa, Yamagata, Japan. A total of 22 patients who lived in the city of Yonezawa who had myalgia, muscular weakness, sore throat, and orchiodynia (among men) sought treatment at Yonezawa City Hospital. Because all had symptoms of severe myalgia, these patients were given a diagnosis of with acute myalgia syndrome of unknown cause. The patients consisted of 15 men and 7 women ages 25–66 years (mean 37 years). Because several patients had contact with other persons who had similar symptoms, the outbreak was considered to be associated with an infectious agent. Virologic and serologic analyses were carried out to find the associated agent. This study was approved by the Ethics Committee of the Yonezawa City Hospital.

Screening for Pathogens

Throat swab and stool specimens were collected from 14 patients (Table 1). Throat swab specimens were placed immediately in tubes containing 3 mL of transport medium, and stool specimens were put in a stool container and transported to the Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata, to undergo virus isolation and reverse transcription PCR (RT-PCR) for enteroviruses, HPeV1, and HPeV2.

Virus isolation was carried out using a described microplate method (*15*). In brief,, HEF, HEp-2, Vero E6, MDCK, RD-18S, and GMK cell lines were prepared in the wells of a 96-well microplate (Greiner Bio-One, Frickenhausen, Germany). After a medium change, throat swabs and 10% stool suspension specimens were centrifuged at 1,500 × *g* for 20 min, and 75 μL of the supernatant was added to 2 wells of each of the cell lines. The inoculated plates were centrifuged for 20 min at 450 × *g*, incubated at 33°C in a 5% CO_2 incubator, and assessed for cytopathic effect.

RNA was extracted from 200 μL of each throat swab specimen or 10% stool suspension using a High Pure Viral RNA Kit (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions and then transcribed into complementary DNA (cDNA) as described (*16*). PCR was performed as described (*17*,*18*), except that we used a mixture of 224 and 222 primers for the first PCR and a mixture of AN89 and AN88 primers for the nested PCR to detect enteroviruses and K28, K29, and K30 primers to detect HPeV1 and HPeV2. The remainder of each specimen and the cDNA specimens were stored at –80°C.

Ultra-high Throughput Direct Sequencing Analysis

Ultra-high throughput direct sequencing analysis was carried out as described (*19*). Total DNA or RNA was prepared from the specimens of case-patient 11 by using a viral nucleic acid purification kit (Roche Diagnostics). Double-stranded cDNA (ds-cDNA) was prepared from 1 μg of total RNA using the random priming method with the SuperScript Choice System for cDNA synthesis (Invitrogen, Carlsbad, CA, USA). A mixture of DNA and ds-cDNA was purified by using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany).

An ≈300-bp length DNA library was prepared from a mixture of 2 μg of total DNA and ds-cDNA by using a genomic DNA sample prep kit (Illumina, San Diego, CA, USA), and DNA clusters were generated on a slide using a Single Read Cluster Generation kit version 4 on an Illumina cluster station (Illumina) according to the manufacturer's instructions. To obtain ≈1.0×10^7 clusters for 1 lane, the general procedure as described in the manufacturer's standard recipe was performed. All sequencing runs for 83-mers were performed with GA II using the Illumina Sequencing Kit version 5. Fluorescent images were analyzed using the Illumina SCS2.8/RTA1.8 to obtain FASTQ formatted sequence data. The obtained DNA sequence reads were investigated by using a MEGABLAST search (*20*) with an e^{-5} e-value cutoff against the nonredundant nt database nt, followed by taxonomic classification using MEGAN version 3.9.0 (*21*) with the following parameters: minimum support 1; minimum score 35.0.

RT-PCR was performed by using ≈100 ng of total RNA, the appropriate primer pair, and the PrimeScript II High Fidelity One-Step RT-PCR Kit (TaKaRa, Shiga, Japan). The following quantitative RT-PCR program was used: reverse transcription reaction 45°C for 10 min; initial denaturation 94°C for 2 min; and 3 steps of amplification (× 35 cycles) at 98°C for 10 s, 55°C for 15 s, and 68°C for 1 min. PCR products were resolved and purified by agarose gel electrophoresis and then sequenced by Sanger sequencing by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Repeat of Virus Isolation and Molecular Detection for HPeV3

After HPeV3 was detected in the specimens from case-patient 11 by ultra-high throughput direct sequencing analysis, to investigate whether HPeV3 was associated with the myalgia epidemic, we repeated the virus isolation focusing on HPeV3, using GMK, Vero, and LLC-MK2 cell lines using the 28 stocked throat swab and stool samples

shown in Table 1. We used 2 LLC-MK2 cell lines, one provided by the National Institute of Infectious Diseases Japan and the other by the Niigata Prefectural Institute of Public Health and Environmental Sciences. We also attempted to specifically amplify the HPeV3 genome via RT-PCR using frozen cDNA and our original primers (Parecho3-VP1F1Y and Parecho3-VP1R1Y for the first PCR and Parecho3-VP1F2Y and Parecho3-VP1R2Y for the nested PCR [Table 2, Appendix, wwwnc.cdc.gov/EID/article/18/11/11-1570-T2.htm]), using the same conditions as in the preliminary screening procedure. When the RT-PCR result was positive, PCR products were purified and sequenced on a Sanger sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers listed in Table 2, Appendix. We also attempted to amplify the HPeV3 genome using RT-PCR from all serum specimens that were initially collected for serologic analysis.

Serologic Study

To further evaluate the role of HPeV3 in this outbreak of myalgia, we measured the neutralizing antibody response against HPeV3 in 1–5 serum samples from 20 of the patients (Table 1). To observe seroconversion and changes in antibody titers, we measured neutralization antibodies using a microneutralization test on a 96-well microplate. Sample serum samples were inactivated at 56°C for 30 min and then diluted from 1:8 to 1:4,096 by serial 2-fold dilution. One HPeV3 isolate in this outbreak in Yamagata (1356-Yamagata-2008) was used as a challenge virus antigen. We mixed and incubated (37°C, 60 min) 60 μL of each diluted serum sample with 60 μL of virus fluid containing ≈10^2 50% tissue culture infectious dose. We prepared confluent monolayers of the LLC-MK2 cell line provided by the Niigata Prefectural Institute of Public Health and Environmental Sciences, washed the cells with phosphate-buffered saline without calcium or magnesium, added 50 μL of maintenance media, and injected 50 μL of each incubated virus–serum mixture into each of 2 cultures. The plates were then incubated in a 5% CO_2 incubator at 33°C for cytopathic effect observation. The reciprocal value of the highest dilution of serum neutralizing the virus compared to the control was taken to be the titer. Seropositivity was defined as titer ≥8. Virus infection was

Table 2. Primers used to detect and sequence analysis for human parechovirus 3 among patients with epidemic myalgia, Yonezawa, Yamagata, Japan, June–August 2008

Primer	Nucleotide sequence, 5′ → 3′	Nucleotide position*
Parecho3-F2K	AACAAGTGACACTATGGATCTGATC	576–601
Parecho3-F3K	CAAAGTAGCAGATGATGCTTCCAA	824–849
Parecho3-F4K	CAAGCCAAATATTTTGCTGCAGTAA	1165–1190
Parecho3-F5K	TGCATTGGTGGTTTATGAGCCTAA	1244–1268
Parecho3-F21K	TCAGACAACACCACACCTTCAAG	1822–1844
Parecho3-VP1F1Y	GGGCCTTTGGGTAATGAGAAA	2452–2472
Parecho3-VP1F2Y	TGACAACATATTTGGTAGAGCTTGGT	2538–2563
Parecho3-F6K	AGGAGATAATGTATATCAATTGGAT	3095–3120
Parecho3-F7K	TGACGGCTGGTTTAATGTCAACTAT	3368–3392
Parecho3-F8K	CTGAATCAATGTCCAACACAGACGA	3737–3761
Parecho3-F9K	TGGACTATGCCTCTGATATTATTGT	3994–4019
Parecho3-F10K	AATAATGGCCATTTGCTTTAGGAGT	4098–4122
Parecho3-F11K	CTTGTAAATTAAATGGTGTGTACAC	4304–4328
Parecho3-F12K	ACTAGGAAGGAGAAAGATATTGAAA	4402–4426
Parecho3-F13K	CAGCCAAAGCATATAGTAGGGCTG	4609–4633
Parecho3-F14K	TTGAACAAATGGAAGCCTTCATTGA	4916–4940
Parecho3-F15K	GTTGTAGACTGGTTCAGTAGTAAG	5011–5034
Parecho3-F16K	AAAGGAACTTTCCCAGTCACGCAGA	5201–5225
Parecho3-F17K	CAGAGAGTATGTTGATTTGGATGAC	5553–5576
Parecho3-F18K	GTGGCTATTCCTTTCAATTTTCTT	5778–5802
Parecho3-F19K	GGCCCAGCAGTTTTAAGCAAATCAG	5863–5947
Parecho3-F20K	TTGTCATGATTCACCTGATCTTGTC	6608–6633
Parecho3-R13K	AGTTTGTGGTATTTACAGTGGTTGT	912–938
Parecho3-R11K	TTTTAACGTAGTTTGTGTCTGCA	1380–1402
Parecho3-R15	CATGTATAGAATATGAATGTTTATT	2673–2697
Parecho3-VP1R2Y	ACCCCTGCTCTGCCATGTATA	2693–2710
Parecho3-VP1R1Y	TCCCGTGCATCATTGGTCTA	2883–2902
Parecho3-R10K	TGACAGATGATTCAAGATACTTCAC	3238–3253
Parecho3-R9K	AGTGGTACACTTCTGCACAAGTAAG	3468–3492
Parecho3-R8K	ATCCACCAATCAATATGTCTGAATG	3859–3884
Parecho3-R7K	TGGATAGTGTGTGTGTTAGGAAAGA	4264–4288
Parecho3-R5K	CCACTTTAGAAATAAGCAGACCACC	5701–5725
Parecho3-R4K	CCATTTTGACTTCCACTGCTCCA	5901–5923
Parecho3-R3K	CCTAAATTTGGACTTGACACAGG	5993–6015
HPeV3whole-RT-RK	TTTTGGTATGTCCAATATTCCAAATTAGTG	7296–7321

*Relative to human parechovirus 3 strain A308/99 (GenBank accession no. AB084913).

considered confirmed if seroconversion from negative to positive could be documented. Alternatively, if all serum samples were positive, a 4-fold increase in antibody titer was considered serologic evidence of infection with HPeV3.

Results

Case-Patients

The 22 case-patients in this outbreak had similar symptoms (Table 1); all had severe myalgia involving mainly the proximal muscles of the upper and lower extremities. The next most common symptom was muscular weakness in the arms and legs (21, 95.5%), followed by fever (19, 86.4%), pharyngitis (15, 68.2%), orchiodynia (4, 18.2%), and seizures (1, 4.5%). Symptoms worsened rapidly within 1–2 days after onset. Eight patients were hospitalized on a visit to Yonezawa City Hospital and remained hospitalized for 5 (4 patients), 6 (1 patient), 8 (2 patients), or 10 days (1 patient); mean hospitalization was 6.5 days. Case-patient 14 had seizures and subsequent disturbance of consciousness. For all patients, muscular weakness improved as muscle pain decreased.

Laboratory Findings

Creatinine phosphokinase (CPK), C-reactive protein, and myoglobin levels were higher than reference ranges in 12, 19, and 16 patients, respectively (Table 1). Elevated CPK levels returned to normal within several days. An examination of cerebrospinal fluid showed no sign of inflammation.

Magnetic resonance imaging (MRI) of the muscles of case-patients 4, 13, and 15 showed increased signal intensity on T2-weighted images. We suspected that case-patient 14 had encephalitis on the basis of clinical symptom, but results of MRI of the brain and cerebrospinal fluid examination did not support this diagnosis. Clinical symptoms and laboratory findings for all patients were consistent with inflammatory myositis.

Detection of Potential Pathogens

No viruses were detected by screening steps at the Yamagata Prefectural Institute of Public Health using virus isolation and RT-PCR targeting enteroviruses, HPeV1, and HPeV2. Thus, we investigated possible uncharacterized pathogens by using direct DNA sequencing. To determine potential pathogens for the patients, we performed direct sequencing of a mixture of purified DNA and ds-cDNA from the total RNA extracted from either the throat swab or stool specimens of case-patient 11. No other possible viral sequences were detected by the high-throughput sequencing for case-patient 11.

Next-generation DNA sequencer GA II produced ≈1.5×10^7 83-mer short reads from the mixed DNA library. To exclude the human-derived read sequences, all obtained reads were initially aligned to a reference sequence of human genomic DNA, followed by quality trimming to remove low-quality reads and exclude reads with similarities to ambiguous human sequences. All remaining possible pathogen reads were further analyzed using a MegaBLAST search against nonredundant databases.

One type of HPeV was found in the analyzed specimens. Three HPeV reads were identified from the throat swab specimen and 1,505 HPeV reads from the stool specimen of case-patient 11. To further characterize the type of HPeVs detected, de novo assembly was performed by using Euler-SR version 1.0 (*22*); the resulting partial contigs showed higher similarity to HPeV3 than to other HPeVs.

To determine the whole HPeV sequence for the isolates we obtained, RT-PCR was performed (Figure 1), and the products were sequenced. The whole coding nucleotide sequence of the polyprotein in the HPeV detected in the stool sample from case-patient 11 was aligned against all available HPeV complete genomes, including HPeV1–8. A phylogenetic tree was generated for the VP1 region (Figure 2), and the HPeV in the stool specimen of case-patient 11 was identified as HPeV3. All detected HPeV3 VP1 sequences among the 2008 myalgia cases were identical (Table 1), except that we found a single nucleotide substitution in sequences from the serum samples from case-patients 4 and 22.

Repeat of Virus Isolation and Molecular Detection Targeting HPeV3

We passaged isolates 6 times using GMK and LLC-MK2 cell lines, but all strains except 1 (isolated after 5 passages) were recovered within 3–4 passages. We could not isolate HPeV3 using the LLC-MK2 cell line provided by the National Institute of Infectious Diseases Japan, but we were successful when using LLC-MK2 cell line from the Niigata Prefectural Institute of Public Health and Environmental Sciences. In total, we isolated HPeV3 strains from either the throat swab or stool specimen of 7/14 patients analyzed (Table 1).

RT-PCR was successful in detecting the HPeV3 genome in the throat swabs or stool specimens from 9/14 patients (Table 1). We also detected the HPeV3 genome in 3 serum specimens collected within 3 days after the onset of illness (Table 1). Sequence data were registered under GenBank accession nos. AB668029–AB668045.

Serologic Study

Of 20 analyzed patients, seroconversion was observed in 6 patients. A 4-fold increase in neutralization antibodies against HPeV3 was confirmed in 5 patients (Table 1).

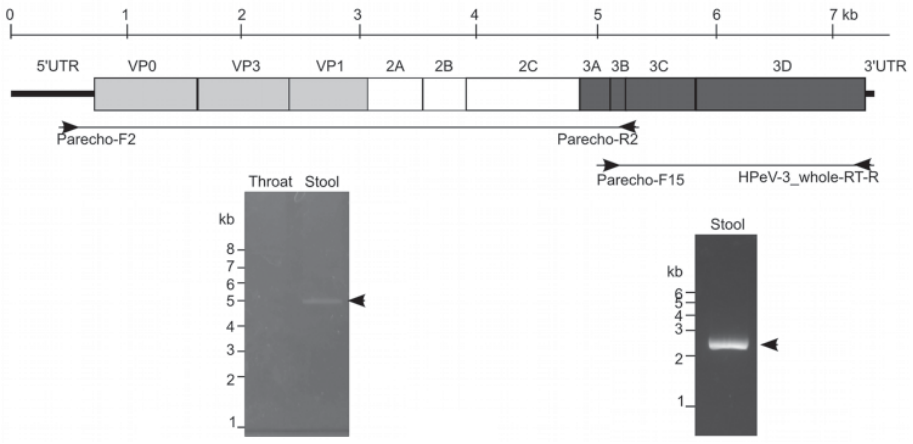

Figure 1. Schematic representation of the human parechovirus 3 (HPeV3) genome sequence and coding polyprotein. Reverse transcription PCR results are shown below the sequence. VP, viral protein; UTR, untranslated region.

Discussion

By conducting virus isolation, RT-PCR, and serologic examination, we confirmed an outbreak of epidemic myalgia among adults in Yonezawa, Yamagata, Japan, during June–August 2008 was associated with HPeV3 infection. Although we did not detect any virus in our first screening, direct sequencing analysis suggested that these patients were infected with HPeV3. We next tried to detect this virus specifically and isolated HPeV3 strains from the throat swabs or stool specimens of 7 patients; detected the virus genome in the throat swabs or stool or serum specimens of 11 patients; and observed seroconversion or 4-fold increases in antibodies against HPeV3 in 11 patients. Altogether, we confirmed HPeV3 infection in 14 of the 22 patients in this outbreak. All patients, except 1 who experienced complications related to epilepsy, recovered completely within 1 week after the onset of illness through treatment with antiinflammatory drugs only.

Enteroviruses have been implicated in the pathogenesis of human neuromuscular diseases because of their association with certain acute and chronic acquired myopathies and paralytic motor neuron syndromes (*23*). Epidemic pleurodynia (Bornholm disease), an acute febrile illness with myalgia caused by picornaviruses such as group B coxsackieviruses, is perhaps best known (*24*–*26*). However, in this outbreak, none of the patients showed the chest and abdominal pain typical of pleurodynia; they instead showed muscular weakness, which is not generally observed in epidemic pleurodynia (*23*,*25*). Conversely, it has been reported that patients with acute enterovirus myositis experience fever, chills, myalgia, and generalized weakness and that thigh muscle or generalized muscle involvement may occur (*25*,*26*). Among these patients, laboratory studies may demonstrate myoglobinemia, myoglobinuria, and an elevated levels of muscle enzymes such as CPK (*25*,*26*). The HPeV3 outbreak in Yonezawa resembles these descriptions of acute myositis outbreaks; patients commonly had fever, myalgia, and muscle weakness mainly in the arms and legs, and laboratory findings and MRI studies suggested the presence acute inflammation around the peripheral muscles. Thus, we concluded that the disease in this outbreak was an acute inflammatory muscle disease associated with HPeV3 infection in adults.

In enterovirus viremia, viruses enter through the oral or respiratory route, replicate in the pharynx and alimentary tract, spread to multiple organs such as the central nervous system, heart, and skin, and then diminish and disappear after neutralizing serum antibodies are produced (*24*). HPeV3 has been reported to cause severe systemic diseases, especially in neonates and infants (*1*,*7*,*11*,*13*). HPeV3 has been isolated using the Vero cell line or detected by real-time PCR in not only nasopharyngeal swabs but also in the lungs, colons, and spleens of dead infants (*27*).

In this study, we showed HPeV3 viremia in 3 patients without neutralizing antibodies within a few days after the onset of illness. Thus, we conclude that HPeV3 viremia affects many organs, including the peripheral muscles as well as the organs normally targeted by enteroviruses. Several male patients in the Yonezawa outbreak also had orchitis, which is described as a symptom of epidemic pleurodynia (*24*) but may also be associated with systemic infection, although we have no evidence to support this hypothesis.

HPeV often grows poorly in culture, and typing reagents are not widely available for newly discovered types (HPeV3–14) (*1*,*28*). In particular, HPeV3 is difficult to culture in standard diagnostic cell lines, and its isolation is largely determined by the cell lines used (*14*). So far, Vero, LLC-MK2, Caco2, and BSC-1 cell lines have been used (*3*,*7*,*8*,*10*,*14*,*27*). In the first screening in our study, we could not isolate HPeV3 using 6 cell lines that we use routinely (*15*). However, we succeeded in whole-sequence analysis using a stool specimen from case-patient 11, which

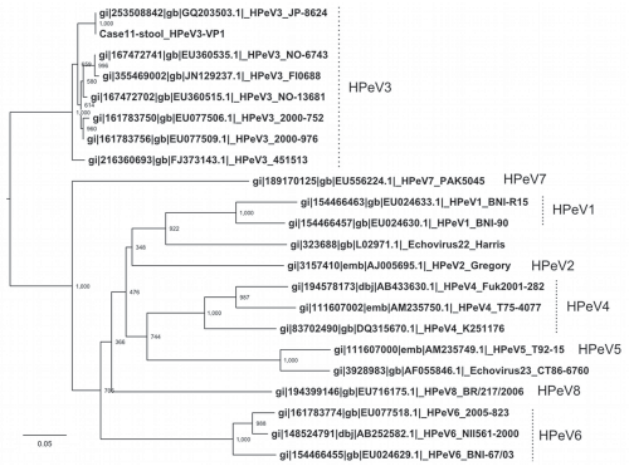

Figure 2. Phylogenetic tree of the viral protein 1 region sequence in the available human parechovirus (HPeV) genomes, including HPeV1–8. The tree was constructed by the neighbor-joining method with 1,000× bootstrapping. Scale bar indicates nucleotide substitutions per site.

suggests the failure to isolate HPeV3 was not a result of low viral load in the specimens from the patients with epidemic myalgia. In our second attempt at isolation using several monkey kidney cell lines, such as GMK and LLC-MK2, we were able to isolate HPeV3 after only several passages. Although 2 LLC-MK2 cell lines were used for HPeV3 isolation, the susceptibility of the lines to HPeV3 infection differed, further demonstrating the difficulties in culturing HPeV3. Because we analyzed a range of respiratory viruses, which are isolated from nasopharyngeal specimens, we routinely culture specimen-inoculated plates at 33°C; however, to isolate HPeV3, plates should be cultured at 37°C.

Although we used a new direct-sequence analysis method and a slightly older RT-PCR method to amplify the HPeV3 genome in this study, new laboratory diagnostic procedures for HPeVs have been developed during the past few years. HPeV RT-PCRs now exist to detect all known HPeV types with great sensitivity (*29*), and HPeV3 and all the other HPeVs can be typed by nested or semi-nested PCR coupled with sequencing of the VP1 gene (*30*).

Our finding of HPeV3 infection in adults conflicts with most of the literature, which suggests that HPeV3 infections occur in early infancy (*1*,*3*,*7*,*10*,*11*,*13*). Harvala et al. observed that HPeV3 infections were seen exclusively in children <3 months of age (*1*,*11*,*13*). Watanabe et al. reported that HPeV3 was isolated from a 35-year-old woman with influenza-like illness, while 12/16 HPeV3-isolated cases were from patients <3 years of age (*14*). Our findings show that epidemic myalgia associated with HPeV3 infection might occur among adults, particular among those ≈30–40 years of age.

It remains unclear why these HPeV3 infections occurred among adults in 2008. HPeV3 infections occur in the spring and summer seasons, whereas HPeV1 infections are observed in small numbers throughout the year but predominantly during the fall and winter (*9*,*10*,*12*,*31*). National surveillance data for infectious diseases in Japan show HPeV3 yearly detection numbers was 1, 2, 52, 1, and 81 cases each year for 2004–2008; 117 cases (85.4%) were detected during June–September (*32*). The higher number of cases in 2008, coupled with a reported epidemic of HPeV3 among children, most <3 months of age, in Hiroshima Prefecture, Japan (*33*), indicates a possible summer outbreak of HPeV3 in 2008 in Japan. We postulate that an outbreak of HPeV3 among children was a necessary background condition for the outbreak of epidemic myalgia among adults in 2008.

In conclusion, we document detection of HPeV3 infection among adult patients with epidemic myalgia in Yamagata, Japan, during 2008. Clinical consideration should be given to HPeV3 not only in young children but also in adults when an HPeV3 outbreak occurs in the community. Continued research on adults with HPeV3 infection is needed to further understand the etiology and epidemiology of HPeV3.

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Infectious Disease Mortality Rates, Thailand, 1958–2009

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To better define infectious diseases of concern in Thailand, trends in the mortality rate during 1958–2009 were analyzed by using data from public health statistics reports. From 1958 to the mid-1990s, the rate of infectious disease–associated deaths declined 5-fold (from 163.4 deaths/100,000 population in 1958 to 29.5/100,000 in 1997). This average annual reduction of 3.2 deaths/100,000 population was largely attributed to declines in deaths related to malaria, tuberculosis, pneumonia, and gastrointestinal infections. However, during 1998–2003, the mortality rate increased (peak of 70.0 deaths/100,000 population in 2003), coinciding with increases in mortality rate from AIDS, tuberculosis, and pneumonia. During 2004–2009, the rate declined to 41.0 deaths/100,000 population, coinciding with a decrease in AIDS-related deaths. The emergence of AIDS and the increase in tuberculosis- and pneumonia-related deaths in the late twentieth century emphasize the need to direct resources and efforts to the control of emerging and re-emerging infectious diseases.

Infectious diseases were responsible for a considerable number of deaths in Thailand during the mid–twentieth century (*1*). During 1948–1955, as Thailand experienced substantial economic and social development and transitioned from an agricultural to an urban and industrial society (*2*), the mortality rate began to decline (*3*). In 1968, infectious diseases such as tuberculosis (TB) and pneumonia were the main cause of death in Thailand; fewer deaths were caused by noninfectious diseases (e.g., diseases of the heart, malignancies). However, in the early 1980s, an epidemiologic transition was taking place, and non-communicable diseases became of greater public health concern (*4*). In contrast with the low mortality rates in many industrialized countries, where communicable diseases are well controlled (*5*,*6*), mortality rates in Thailand remained relatively high. However, the emergence of HIV/AIDS contributed to an increase in deaths in the 1990s and interrupted the epidemiologic transition (*7*,*8*).

Communicable diseases are still responsible for a considerable number of illnesses (10% of total diseases in 2009) and deaths in Thailand (*9*). Because of the increasing threat from emerging and reemerging infectious diseases, it is vital to understand the patterns of infectious disease–related deaths in a country that has undergone economic development and concurrent improvements in health, sanitation, and access to healthcare. We used publicly available vital statistics for deaths in Thailand to analyze trends in infectious disease mortality rates during 1958–2009. This assessment helps us better understand past trends and inform policy on current infectious diseases of public health concern.

Methods

Source of Data

Death-related data were obtained from a published series called the Report of Public Health Statistics (Bureau of Policy and Strategy, Ministry of Public Health [MOPH], Nonthaburi, Thailand). The reports summarize data, by year, from death certificates provided by the MOPH in collaboration with the Ministry of Interior (MOI) as part of the Vital Registration System. The MOI is responsible for registering deaths at the local administrative level; the MOPH is responsible for processing vital statistics data for

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the whole country and for disseminating the information on an annual basis through publication of the Report of Public Health Statistics. Death certificates record only 1 cause of death, which is the underlying cause of death.

Using data from 1958–2009, we created an electronic database from the series of Report of Public Health Statistics. The database provided aggregated information on the total number and rate of deaths by age group, sex, year of death, and cause of death. Cause of death was coded according to the International Classification of Disease (ICD). During 1958–2009, the following ICD revisions were used: 1958–1967, ICD version 7 (ICD-7); 1968-1976, ICD-8; 1977–1993, ICD-8; and 1994–2009, ICD-10. For each ICD revision, death data were grouped differently, resulting in different group numbers for the ICD versions: ICD-7, 50 groups; ICD-8, 150; ICD-9, 56; and ICD-10, 103. During 1958–1983, mortality rates were calculated by using population denominator data from the Population and Housing Censuses conducted in 1960, 1970, and 1980. The estimated population between census years was adopted from the Report of the Working Group on Population Projection. After 1984, the annual mid-year estimated population was obtained from the Bureau of Registration Administration, MOI.

Determination of Infectious versus Noninfectious Disease

To assess trends in deaths caused by infectious diseases, we applied a classification scheme developed at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) (*6*). This coding system used ICD-9 codes to classify infection-associated diseases as 1) an infectious disease (e.g., pneumonia), 2) possibly an infectious disease (e.g., pityriasis rosea), and 3) result of an infectious disease (e.g., rheumatic fever). The system was developed to address the exclusion of some infectious diseases from the infectious disease category of the ICD system, e.g., influenza, which was placed in the respiratory disease category. For the purposes of this study only, those diseases classified as an infectious disease or as the result of an infectious disease (*1*,*3*) were used to classify deaths from infectious disease.

Several adjustments were made to the original coding system. First, we had to account for deaths that were reported by using grouped ICD codes without distinction for infectious versus noninfectious disease (e.g., bronchitis, emphysema, and asthma are grouped in ICD-7). Deaths were reported by using only the shorter, less detailed 3-digit codes rather than the 4-digit subcodes used in the CDC classification system; thus, we re-coded as infectious any 3-digit codes for which ≥80% of the subcodes were for infectious diseases, and we re-coded as noninfectious any 3-digit codes for which <80% of the subcodes were for infectious diseases.

Second, we classified deaths during 1994–2009 (reported with ICD-10 codes) by using the CDC system and assigning the infectious determination of their correlated ICD-9 code. These classifications facilitated an analysis of overall trends in deaths caused by infectious versus noninfectious diseases.

Third, we specifically excluded deaths from septicemia, which were first reported in the series of Report of Public Health Statistics in 1994, when use of ICD-10 coding began. Septicemia accounted for ≈15% of in-hospital deaths in vital registration data, but after verbal autopsy was used to validate cause of death data, septicemia was determined to cause <1% of deaths; thus, it was decided that deaths due of septicemia should be largely reassigned to cerebrovascular disease (*10*). To avoid misclassification, deaths from septicemia from 1994 forward were removed from the infectious disease category.

To address the problem posed by the frequent revisions of the ICD coding system, we used ICD-9 as the standard and recoded deaths that were reported by using other ICD versions. The codes used for all deaths reported by using ICD-7 and -8 were converted to ICD-9 codes by using proper disease names as defined, respectively, in each version of the ICD system. The conversion from ICD-10 to ICD-9 codes was done using a published tool developed by the American Academy of Professional Coders (*9*). A complication in the conversion backward from ICD-10 to ICD-9 was the presence of several many-to-one and one-to-many coding relationships, caused by a significant change in the detail of diagnostic codes; this change resulted in a near doubling of the number of diagnostic codes (*11*). Once uniform coding was established for all deaths, the infectious disease coding system described above was applied to these deaths.

Determination of Specific Infectious Categories

The cause-of-death data from the series of Public Health Statistics were reported according to the ICD tabulation list, which differed among the ICD revisions. These lists provide a short set of aggregate codes intended to facilitate cause-of-death reporting in countries with more limited capacity. We consistently identified 8 categories of infectious diseases for analysis as separate categories: TB, gastrointestinal infection, HIV/AIDS, pneumonia, sexually transmitted diseases, diphtheria, polio, and malaria (Table).

Analysis of Trends

Multiple period regression was used to estimate the difference of the magnitude of trend in mortality rate. Average annual rate of change and 95% CIs are presented. All-cause deaths were age-adjusted by 5 age groups (0–4, 5–24, 25–44, 45–64, and ≥65 years); because of the struc-

Table. ICD codes for 8 groups of infectious disease groups consistently found in a study of trends in infectious disease mortality rates, Thailand, 1958–2009*

Disease	ICD-7, 1958–1967	ICD-8, 1968–1976	ICD-9, 1977–1993	ICD-10, 1994–2009
Malaria	110–117	84	080–088	B50–B54
Tuberculosis	001–008, 010–019	010–019	010–018	A15–A19
HIV/AIDS	NA	NA	NA	B20–B24
Pneumonia	490–493	480–486	480–486	J12–J18
Gastrointestinal infection	040, 043, 045–048	000–004, 006, 008, 009	001–009	A00–A09
Sexually transmitted infection	020–029	090–098	090–099	A50–A64
Diphtheria	055	032	032	A36
Polio	080	040, 043, 044	040, 043, 044	A80

*ICD, International Classification of Disease; NA, not applicable.

ture (aggregate data) of the database, it was not possible to age-adjust other rates.

Results

Overall Mortality Rate Trends

The all-cause mortality rate during 1958–2009 was characterized by a decrease during 1958–1986 and an increase during 1987–2009; however, in the early 2000s, the rate leveled (Figure 1). The average annual decrease for 1958–1986 was 2.8 deaths/100,000 population (95% CI 14.1%–11.5%) and average increase for 1987–2009 was 10.8 deaths/100,000 population (95% CI 9.3%–12.4%). The pattern was similar for both sexes, but the annual rate for males consistently exceeded that for females (Figure 1).

The all-cause mortality rate varied by age group, but it was among persons ≥65 years of age (Figure 2). The highest average annual decline in the mortality rate was among children <5 years of age; the decline (34.3 deaths/100,000 population; 95% CI 26.2% –42.4%) represented a 10-fold reduction from 1,820.1 deaths/100,000 population in 1958 to 188.1/100,000 in 2009. From the 1990s through 2009, there was almost no difference between the mortality rate for boys and that for girls (Figure 2). During this same period, all-cause mortality rates among persons 5–24 years of age declined 3-fold from 250.0 deaths/100,000 population in 1958 to 83.5/100,000 in 2009. For persons 5–64 years of age, the mortality rate was higher for males than females. The difference in the mortality rate between sexes was most pronounced for persons 25–44 years of age, especially during 1996, when there was a 3-fold difference for males (605.9 deaths/100,000 population) versus females (178.4 deaths/100,000 population). All-cause mortality rates in persons 5–24 and 25–44 years of age increased during 1990–2000.

Infectious Disease Mortality Rate Trends

From 1958 through the late 1990s, the infectious disease mortality rate in Thailand declined 5-fold, from 163.4 deaths/100,000 population in 1958 to 29.5/100,000 in 1997 (average annual reduction 3.2 deaths/100,000 population; 95% CI 2.8%–3.7%) (Figure 3). This decline paralleled the decline in overall deaths from 1958 to the late 1990s. In 1998, infectious disease–related mortality rates started to increase and the trend continued through 2003 (average annual increase 7.6 deaths/100,000 population; 95% CI 5.9%–9.4%). In 2004, infectious disease–related deaths began to decline again; during 2004–2009, the average annual reduction was 3.5 deaths/100,000 population.

Mortality rates for several specific infectious diseases declined during 1958–2009 (Figure 4). For example, malaria deaths declined from 36.0/100,000 population in 1958 to 0.1/100,000 population in 2009. Diphtheria-related deaths decreased throughout the study period. In contrast, deaths from polio showed much year-to year variability, with a surge in 1975; however, polio-related deaths remained low (<20 deaths/year) during 2002–2009. Mortality rates for gastrointestinal infection fluctuated through the 1960s but then declined rapidly.

Three phases characterized TB-related mortality rates: 1958–1994, 1995–2003, and 2004–2009 (Figure 4). During the first phase, rates sharply declined from 36.7 deaths/100,000 population in 1958 to 5.9/100,000 in 1994. During the second phase, the trend deaths reversed, increasing from 7.0 deaths/100,000 population in 1995 to 11.0/100,000 in 2003. During the third phase, TB-related mortality rates again declined, decreasing from 9.7 deaths/100,000 population in 2004 to 7.2/100,000 in 2009. Deaths from HIV/AIDS, first reported in the series of Report of Public Health Statistics in 1994, peaked at 26.8/100,000 population in 2003 but declined to 6.4/100,000 in 2009 (Figure 4).

Pneumonia-related mortality rates, similar to those for TB, decreased from 37.0 deaths/100,000 population in 1958 to 7.0/100,000 in 1991 and then increased sharply (Figure 4). The increasing trend in pneumonia-related deaths, beginning in 1993, was more pronounced among persons 25–44 and ≥60 years of age (data not shown).

During 1959–1968, mortality rates for sexually transmitted diseases dropped sharply. However, starting in the early 1970s, the rates increased for a decade before declining again (Figure 4).

Discussion

Our findings demonstrate a substantial decrease in deaths overall in Thailand from 1958 through 1986, fol-

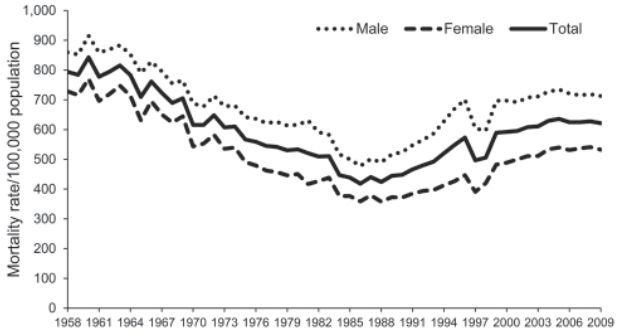

Figure 1. All-cause mortality rates, Thailand, 1958–2009.

lowed by an increase beginning in 1987 and then a leveling-off beginning in the early 2000s. All-cause mortality rate trends were similar for males and females, but the rate was consistently higher for males. The gender gap in all-cause mortality rates among persons 25–44 years became more pronounced in recent years, mostly because of fatal traffic accidents and HIV/AIDS-related deaths among men, a group that was more affected by HIV/AIDS in the first phase of the epidemic (*12*,*13*). Age-specific mortality rates for children 0–4 years of age showed the greatest decline; the development of and equitable access to maternal and child health care services and vaccination may have contributed to this decline (*14*,*15*). The all-cause mortality rate for children 0–4 years of age declined over the study period; however, after the introduction of the Expanded Program on Immunization (EPI), the rate declined 28% (from 715 to 514 deaths /100,000 population). The extensive geographic coverage of primary health care services contributed significantly to maternal and child health outcomes (*16*).

From 1958 through the mid-1990s, the infectious disease mortality rate in Thailand declined substantially, largely because of declines in malaria, TB, pneumonia, and gastrointestinal infection. Several factors contributed to this trend, including general improvements in sanitation, improved access to medical care (a result of health infrastructure expansions at the district level), and financial risk protection (*16*), and the introduction of routine childhood vaccination through EPI, which was officially launched in 1977 (*14*). Since 1987, coverage with TB, diphtheria-tetanus-pertussis, and tetanus toxoid vaccines has been 96%, 75%, and 60%, respectively (*17*). Deaths related to vaccine-preventable infectious disease declined sharply in association with >90% EPI coverage in the 1990s (*18*). In the United States, studies have shown a decline in infectious disease–related deaths during the twentieth century (*5*,*6*).

In the late 1990s, the decreasing trend for the infectious disease–related deaths reversed. Disease categories that contributed most to this reversal were HIV/AIDS, TB, and pneumonia; all of which had sharply elevated mortality rates during 1997–2003 and decreasing rates in 2004. HIV/AIDS emerged in Thailand in the mid-1980s and spread rapidly with devastating effects (*19*). In 1999 in Thailand, HIV/AIDS had become the leading cause of death in men 25–44 years of age, resulting in a widening gap in the mortality rate between men and women (*8*,*13*). Co-infection with TB and HIV is common; thus; the rising number of TB-related deaths during 1995–2003 coincided with the explosive epidemic of HIV infection and gradually led to the emergence of multidrug-resistant TB (*20*,*21*). Moreover, the reported incidences of pneumonia and pneumonia-related deaths had been increasing in Thailand since the mid-1980s (*22*). It is likely that HIV/AIDS contributed to the pneumonia-related mortality rate. However, this contribution was not readily apparent until later because HIV/AIDS was not a reportable cause of death until 1994 and because stigma was likely a barrier to reporting in the early years

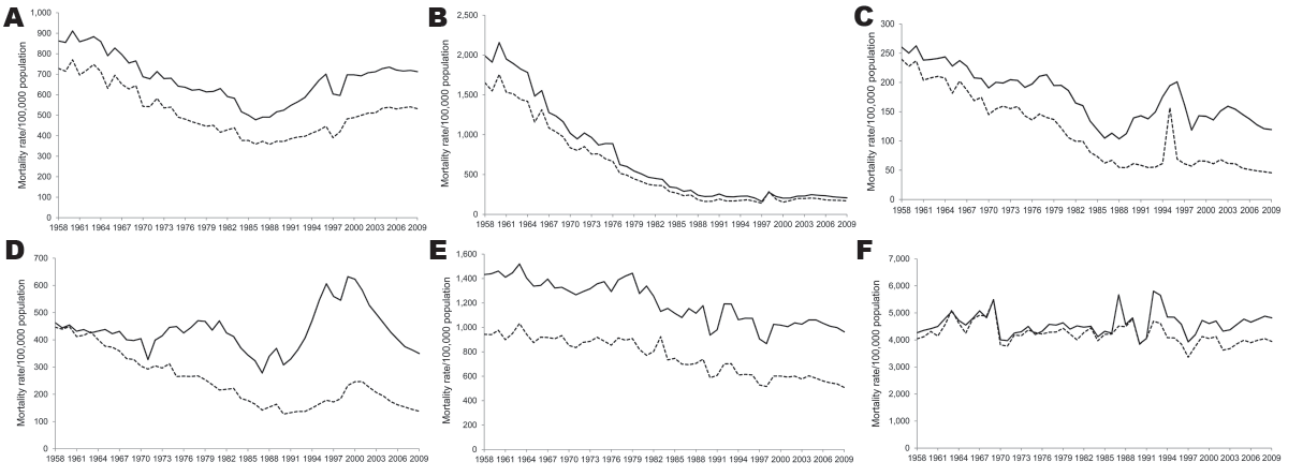

Figure 2. All-cause mortality rates, by age group and sex (solid lines, male; dashed lines, female), Thailand, 1958–2009. A) All ages; B) 0–4 years of age; C) 5–24 years of age; D) 25–44 years of age; E) 45–64 years of age; F) ≥65 years of age.

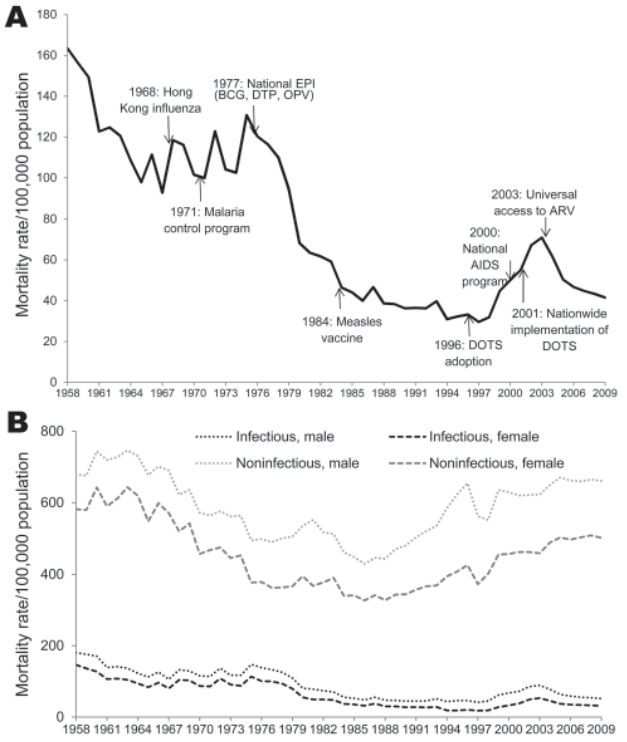

Figure 3. Mortality rates for infectious and noninfectious diseases, Thailand, 1958–2009. A) Infectious disease–related mortality rates, major events, and key public health interventions. B) Comparison of infectious disease–related mortality rates with noninfectious disease–related mortality rates. EPI, Expanded Program on Immunization; BCG, bacillus Calmette–Guérin vaccine; DTP, diphtheria, tetanus, and pertussis vaccine; OPV, oral polio vaccine; ARV, antiretroviral treatment; DOTS, directly observed treatment, short course.

of the HIV/AIDs epidemic (*23*). Furthermore, if the code for opportunistic infectious diseases was used for deaths caused by HIV/AIDS, the number of deaths from HIV/AIDs may have been underestimated (*10*,*24*). In persons with HIV/AIDS in Southeast Asia, the common opportunistic infections were TB, cryptococcosis, and *Pneumocystis carinii* and *Pennicillium marneffei* fungal infections, all of which can cause pneumonia (*25*). The observed increase in pneumonia-related deaths in very elderly persons may also reflect the misclassification of the cause of death because pneumonia is often the immediate, rather than underlying, cause of death. A study to verify cause of death data found that ≈31% of deaths coded as being caused by pneumonia should have been classified as being caused by cerebrovascular disease, chronic obstructive pulmonary disease, diabetes, or genitourinary disease (*10*).

We found that deaths from HIV/AIDS and TB declined during 2004–2009; this decline may account for the concurrent leveling-off of infectious disease–related mortality. Thailand implemented a national AIDS program in 1991 and a national antiretroviral (ARV) treatment program in 2000. The ARV treatment program was designed to increase access to health care and treatment, but it was not until 2004 that the universal ARV treatment program was fully implemented, providing ARV treatment for all eligible patients under the National Access to ARVs for People Living with HIV/AIDS program. As the program scaled up, universal ARV contributed significantly to the reduction in AIDS-related deaths (*26*–*28*). Two parallel prevention programs, vertical transmission prevention and condom promotion, contributed to decreasing the incidence of HIV infection and changed the epidemic in Thailand from one that was generalized to one that was concentrated in certain subpopulations, particularly injection-drug users, homosexual men, and youth. TB-related deaths were also reduced through early initiation of ARV treatment program for persons with HIV, implementation of DOTS (directly observed treatment, short course), and appropriate antimicrobial drug regimens for TB treatment (*29*).

DOTS was implemented nationwide in Thailand in 2001, and since then, the country has achieved the international goal for detecting ≥70% of the estimated cases of infectious TB (i.e., cases in persons with a TB-positive sputum smear); however, Thailand has not met the international goal for successfully treating ≥85% of the detected cases (*30*). The main issues relating to TB control were the fragmented delivery of services; limited capacity of stakeholders and limited coordination between stakeholders; weak referral linkages between hospitals and health centers; high TB/HIV co-infection rates and limited access to ARV treatment, especially among poor persons and those with less education; unsupervised treatment with high default rates; and widespread unregulated use of second-line antimicrobial drugs, which could lead to an outbreak of multidrug-resistant TB and extensively drug-resistant TB (*31*). Certain infectious diseases (e.g., TB) are re-emerging, and emerging HIV/AIDS epidemics in certain subpopulations have considerable implications for the Thai population. Continued monitoring and evaluation of the effect of interventions on disease incidence and mortality rates are critical if the global goal of curing 85% of TB cases is to be achieved.

We reviewed mortality rates during 1958–2009 in Thailand by using a standardized infectious disease classification scheme. Three possible artifacts in year-to-year fluctuations in the mortality rate over the study period should be considered. The first artifact concerns changes that were made to Thailand's data recording system during the study period. Four versions of the ICD systems were used, and ICD code changes can lead to substantial changes in long-term trends in cause-specific mortality rates (*32*). Our results show an increasing trend in deaths from pneumonia and TB since 1994, when the switch was made from ICD-9

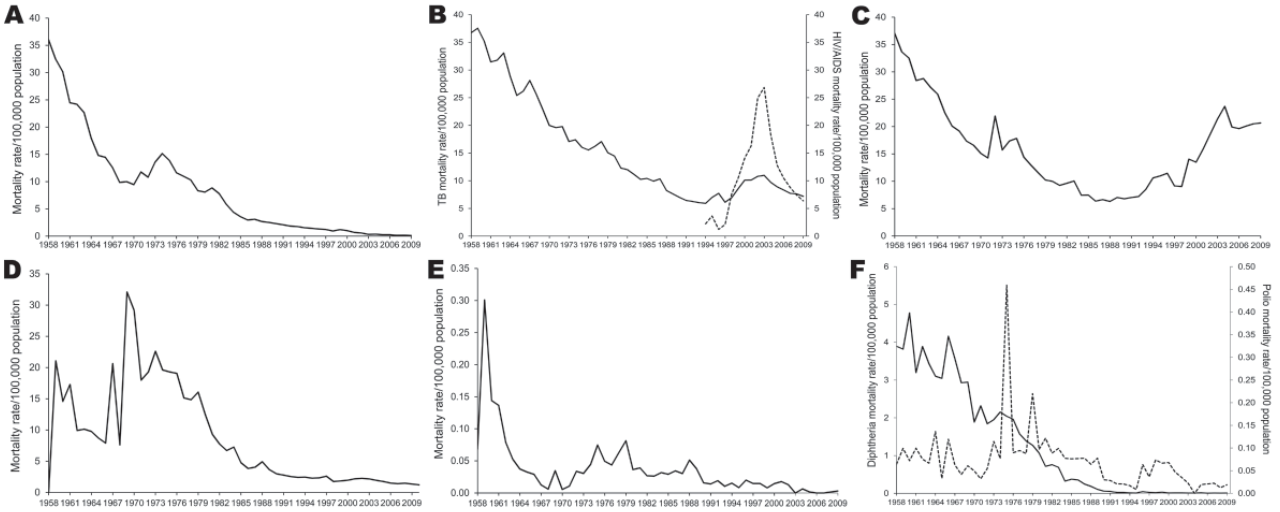

Figure 4. Infectious disease–related mortality rates for select diseases, Thailand, 1958–2009. A) Malaria; B) tuberculosis and HIV/AIDS; C) pneumonia; D) gastrointestinal infection; E) sexually transmitted infections; F) diphtheria and polio.

to ICD-10 coding; we have not accounted for the differences in diagnostic trends concurrent with the changes in coding. When a US National Center for Health Statistics comparability ratio was applied to the US mortality rates for influenza and pneumonia, it appeared that the declines mainly resulted from the introduction of the ICD-10 coding system (*33*). We did not apply comparability ratios in our analysis because such ratios were not developed with the data provided by the Thai MOPH.

The second possible artifact is that deaths sharply declined from 1996 to 1997, and this decline was followed by a sharp increase from 1998 to 1999. We cannot rule out that such sudden changes may have resulted from changes in the process for reporting deaths, which was implemented in 1996. In the new death certification system, each death was entered into the computer database in the Civil Registration Database at the Bureau of Registration Administration of the MOI and then transferred to the vital registration database at the MOPH.

The third possible artifact is the cause of death coding errors. The coding of polio deaths since 1997, is an example of such errors. The Polio Eradication Campaign in Thailand was started in 1990, and the last polio case was reported in April 1997 (*34*). Cases of and deaths from acute flaccid paralysis are aggressively investigated, making it unlikely that a single death could be missed. However, despite the lack of any reported cases of polio since 1997, a total of 274 polio-related deaths were coded during 1997–2009. In general, the quality of death statistics in Thailand is considered poor because many death registrations are incomplete and a large proportion have poorly defined causes of death (*11*,*35*). Furthermore, during the 1960s and 1970s, only ≈60% of deaths were registered. That percentage increased to 76% in the mid-1980s and to 95% in the mid-1990s. The highest proportion of unregistered deaths was for infants; this was a result of death occurring, in many cases, before the birth was registered (*4*,*36*). The proportion of in-hospital deaths increased from 20% during the 1980s to 43% in 2009 (*37*). The validity of the overall cause-of death statistics during that time is questionable because many out-of-hospital deaths were coded by persons not medically qualified to determine the cause of death. All of these factors may have influenced the study findings.

This study has some possible limitations. The analysis was based on death attributed to the underlying cause of death as it was reported on death certificates and published in the Report of Public Health Statistics. Because death records list only one cause of death, we knew only the underlying cause of death and, thus, may have underestimated the effect of infectious diseases as a contributing cause of death. As a result, this study may underestimate the role of infectious diseases on mortality rates. In addition, the aggregate nature of our data prevented additional exploration of the specific cause of death by age group. Our estimate of the extent of infectious disease is conservative, focusing exclusively on deaths. This focus reflects only part of the effects from disease because infectious disease may result in substantial illness or disability, or both, without causing death. Future analyses of other dimensions of the effects of infectious diseases, such as their effect on the economy, the number of hospitalizations, and the number of life-years lost because of disability, will provide information to inform policy-making decisions.

The implementation of a malaria control program and new and effective antimicrobial drugs for treating TB contributed considerably to the reduction in communicable

disease–related illness and deaths in the second half of the twentieth century (*1*,*38*–*40*). The emergence of HIV/AIDS and the increase in TB- and pneumonia-related deaths in the late twentieth century dramatically interrupted the epidemiologic transition in Thailand. A universal ARV treatment program has rapidly scaled up and resulted in a decrease in the number of deaths from HIV/AIDS after 2003; however, this decline remains unstable. Recent trends emphasize the dynamic process of infectious diseases and highlight the need for sustained resources and efforts to combat their emergence or re-emergence. These data also highlight the importance of addressing health disparities between men and women. Reliable, relevant, and timely mortality data are crucial to guide effective policy responses to protect and promote population health.

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HIV Infection and Geographically Bound Transmission of Drug-Resistant Tuberculosis, Argentina

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During 2003–2009, the National Tuberculosis (TB) Laboratory Network in Argentina gave 830 patients a new diagnosis of multidrug-resistant (MDR) TB and 53 a diagnosis of extensively drug- resistant (XDR) TB. HIV co-infection was involved in nearly one third of these cases. Strain genotyping showed that 7 major clusters gathered 56% of patients within restricted geographic areas. The 3 largest clusters corresponded to epidemic MDR TB strains that have been undergoing transmission for >10 years. The indigenous M strain accounted for 29% and 40% of MDR and XDR TB cases, respectively. Drug-resistant TB trends in Argentina are driven by spread of a few strains in hotspots where the rate of HIV infection is high. To curb transmission, the national TB program is focusing stringent interventions in these areas by strengthening infection control in large hospitals and prisons, expediting drug resistance detection, and streamlining information-sharing systems between HIV and TB programs.

During the early 1990s, HIV-associated multidrug-resistant tuberculosis (MDR TB) emerged in Argentina (*1*). In Buenos Aires, the country's most heavily populated city, certain multidrug-resistant *Mycobacterium tuberculosis* strains spread quickly among patients with AIDS (*2*,*3*). Specifically, the so-called M strain caused a major MDR TB outbreak at the Hospital Muñiz, a referral treatment center for infectious diseases (*4*). HIV-infected patients repeatedly seeking assistance at different health centers introduced the M strain into hospitals in nearby districts, where secondary transmission occurred (*5*). This strain was later responsible for the emergence of MDR TB in HIV-negative patients who had not previously undergone TB treatment (*6*). In 2002, the M strain was isolated from 2 patients with extensively drug-resistant TB (XDR TB). Two other MDR TB outbreak strains, Ra and Rb, emerged in Rosario, the third largest city in Argentina, simultaneously with the M strain (*7*).

MDR TB emergence highlighted the need for a MDR/XDR TB surveillance system focused on incidence and transmission. In 2003, the National TB Laboratory Network launched a systematic registry of all incident MDR/XDR TB cases diagnosed throughout the country. The registry includes a genotype database for all MDR/XDR TB patients going back to the initial outbreaks and population studies. We present the findings of a 7-year follow-up study of MDR and XDR TB in Argentina, with emphasis on potential transmission events involving strains responsible for previous outbreaks.

Materials and Methods

Study Group

Isolates from all patients with newly diagnosed MDR or XDR TB from January 2003 through December 2009 were included in the study (1 isolate per patient, collected at time of diagnosis). MDR TB was defined as disease caused by *M. tuberculosis* resistant to at least isoniazid and rifampin and XDR TB as disease caused by MDR *M. tuberculosis* showing further resistance to any fluoroquino-

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lone and any second-line injectable anti-TB drug. A patient with newly diagnosed MDR or XDR TB was defined as a patient with disease first confirmed by drug susceptibility testing (DST) during the study period, regardless of previous treatment history. A hotspot was defined as an area where a MDR TB outbreak had been documented before the study period. Two or more patients were considered to be epidemiologically related when they were in the same place and time or shared similar behavioral risk factors.

Available demographic and clinical data were collected through the national TB laboratory network. A special effort was made to retrieve data from clinical records in special groups, i.e., XDR TB, patients in hotspot areas, and those in clusters with <6 bands in the IS6110 restriction fragment length polymorphism (RFLP). This research was approved by the research review board of the Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud (INEI ANLIS) "Carlos G. Malbran."

Bacteriologic Studies

On the basis of programmatic guidelines, DST was performed on isolates from patients at risk for drug resistance: patients with TB treatment failure and retreatment, HIV or other concomitant conditions, or exposure to drug-resistant TB in household, prison, or hospital. In Buenos Aires and Rosario, culture and DST are available to test virtually all persons with suspected TB who seek assistance at large referral treatment centers. In the rest of the country, persons not included in the high-risk group are highly unlikely to contract MDR TB. In all, ≈10,000 TB cases are reported annually in Argentina, of which »4,500 are diagnosed on the basis of a positive culture. Among cases that are culture-positive, ≈3,000 have isolates submitted for DST; MDR TB is diagnosed for 4% of these patients.

M. tuberculosis DST to first-line drugs (isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide) was performed in 19 TB network laboratories under regular proficiency testing, according to World Health Organization standards (*8*). The supranational reference laboratory at Instituto Nacional de Enfermedades Infecciosas ANLIS conducted external quality control, confirmed multidrug resistance, and tested susceptibility to second-line drugs (kanamycin, amikacin, capreomycin, and ofloxacin), according to World Health Organization recommendations (*9*).

Genotyping

All available isolates underwent standard IS*6110* DNA RFLP fingerprinting and spoligotyping (*10*,*11*). Patterns were compared by using BioNumerics 5.1 software (Applied Maths, St-Martens-Latem, Belgium), using the Dice coefficient with 1% tolerance and the unweighted pair-group method with arithmetic averages (*12*). The RFLP pattern of the reference strain *M. tuberculosis* Mt14323 was used for gel normalization.

For RFLP patterns with ≥6 bands, a cluster was defined as a group of ≥2 isolates whose RFLP patterns and spoligotypes were 100% identical when compared with all other patterns found within the study period. RFLP patterns with <6 bands were included in a cluster when epidemiologic links were established in addition to identical spoligotypes. Similarly, a proven epidemiologic link was required for including in a cluster a variant of a cluster genotype; a genotype variant was defined as a genotype with a 1-band difference in the RFLP or 1-spacer difference in the spoligotype but not both. Because of the unusually large size of some clusters, a major cluster was defined as ≥15 patients and a minor cluster as <15 patients with MDR TB newly diagnosed during the period. Shared International Type (SIT) and genotype family were assigned by consulting the SITVIT database (www.pasteur-guadeloupe.fr:8081/SITVIT) (*13*), except for genotypes H4, T1-Tuscany, and LAM3/S convergent, which were reclassified as Ural, LAM-Tuscany, and S, respectively, according to Abadia et al. (*14*). Orphan genotypes were those lacking a SIT in the SITVIT database.

Statistical Analysis

We used univariate and multivariate logistic regression analyses to determine factors associated with being in a cluster and being in a major cluster. The explanatory variables were patient age, country of birth, place of diagnosis, HIV status, previous TB treatment, and disease localization. Within the subgroup included in major clusters, we used logistic regression analysis to determine factors associated with being infected by the M strain. In this latter model, the explanatory variables were patient age, country of birth, HIV status, previous TB treatment, hospital exposure, and isolate drug resistance.

We divided patients into 3 age groups: ≤15, 16–45, and >45 years of age. Gender was removed from the models because it was associated with particular settings in 2 major clusters; unknown categories were removed from all the variables included in the model. Because of the limited numbers per category, the age category ≤15 years was removed from the multivariate analyses.

We applied 3 tests to assess the performance of the models: overall model fit, Hosmer & Lemeshow test, and receiver operating characteristic area under the curve. We considered a model to be adequate when values were: overall model fit $p<0.2$, Hosmer & Lemeshow test $p>0.5$, and area under the curve >0.70. We used the χ^2 test for linear trends for assessing changes in the annual number of MDR TB patients in cluster M compared with changes in numbers in other major clusters. Statistical analyses were performed by using MedCalc version 12 software (MedCalc, Mariakerke, Belgium).

Results

MDR TB Patients, Genotypes, and Clustering

Genotyping Coverage

Genotyping was available for isolates from 787/830 (94.8%) newly diagnosed MDR TB patients registered during the study period (2003, 93.2%; 2004, 97.7%; 2005, 99.1%; 2006, 86.4%; 2007, 93.0%; 2008, 96.5%; 2009, 97.5%) (Figure 1). Coverage was lower in 2006 because of a technical mishap that resulted in a loss in the isolate collection.

Genotype Family Distribution

The 3 predominant genotype families were LAM (38.8%), Haarlem (36.3%), and T (13.9%). Other genotypes were S (2.8%), U (1.7%), Beijing (1.5%), X (0.9%), and Ural (0.4%); orphan genotypes accounted for 3.8%. Within the 3 predominant genotype families, the most frequent subfamilies were H2 (29.5%), LAM3 (16.4%), T1 (8.9%), LAM5 (6.6%), LAM9 (6.5%), and Tuscany (5.5%). Of 12 patients carrying Beijing genotypes, 1 was born in Indonesia, 7 in Peru, and 4 in South America with no information on country of birth.

Clusters

Of 787 patients for whom isolate genotype was available, 438 (55.7%) fitted into 7 major clusters and 151 (19.2%) into 45 minor clusters; 198 (25.2%) harbored unique genotypes (Table 1). In the multivariate regression analysis, the outcome of being in a cluster was significantly dependent on being 15–44 years of age, born in Argentina, and HIV-infected. Being in a major cluster was significantly dependent on the 2 latter predictors and of having the MDR TB diagnosis occur in a hotspot area (Table 2).

Characteristics of the 7 major clusters are described in Table 3 and DST profiles in Table 4. Genotype patterns are shown in Figure 2 and geographic distribution in Figure 3. Altogether, the 3 largest clusters (M, Ra, and Rb) accounted for 355 (45.1%) patients; isolate patterns matched 3 genotypes previously associated with MDR TB outbreaks (*4*,*7*,*15*). The other 4 major clusters (Pr, At, Ob, and Os) accounted for 83 (10.5%) patients; these genotypes had been reported only sporadically before the study period.

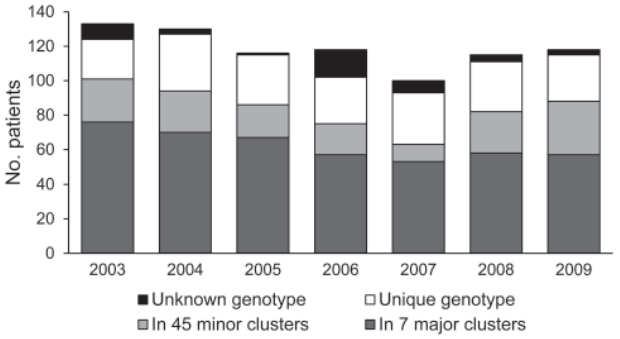

Figure 1. Numbers of patients with newly diagnosed multidrug-resistant tuberculosis reported per year, grouped according to genotype analysis, Argentina, 2003–2009. Major cluster, ≥15 patients; minor cluster, <15 patients.

The predominant cluster, M, was largely confined to the city of Buenos Aires and the surrounding area, with only 5/228 patients having MDR TB diagnosed elsewhere. Twenty patients in this cluster were immigrants from neighboring countries (Bolivia 11, Paraguay 6, Peru 2, Uruguay 1). Most patients had ≥1 commonly acknowledged risk factors for MDR TB (129 patients had 1, 64 had 2, and 16 had 3 risk factors) (Table 3). The cluster included the 2 previously reported outbreak variants of the M strain (*4*), Mm in 180 patients and Mn in 35 patients (Figure 2), and 9 sporadic variants, observed in 13 patients who had proven epidemiologic links with other patients in cluster M. An isolate resistant to 5 drugs was strongly associated with disease produced by the M strain (Table 5). The numbers of patients affected by this strain decreased significantly within the period when compared with the numbers of patients in the other 6 major clusters ($p = 0.002$). In particular, the proportion of HIV-infected patients affected by the M strain decreased significantly during the study period, from 65% in 2003 to 24% in 2009 ($p = 0.02$; Figure 4). No similar trend was observed in the HIV-negative group ($p = 0.77$).

Table 1. Patients with newly diagnosed multidrug-resistant TB, by year and genotype cluster, Argentina, 2003–2009

Genotype	No. patients							Total no. (%) patients
	2003	2004	2005	2006	2007	2008	2009	
Cluster M	46	40	33	29	31	28	21	228 (29.0)
Cluster Ra	13	6	19	12	15	14	10	89 (11.3)
Cluster Rb	7	10	2	1	5	6	7	38 (4.8)
Cluster Pr	4	5	4	4	1	3	5	26 (3.3)
Cluster At	3	4	1	1	1	4	7	21 (2.7)
Cluster Ob	2	1	5	5	0	1	4	18 (2.3)
Cluster Os	1	4	3	5	0	2	3	18 (2.3)
Minor cluster*	25	24	19	18	10	24	31	151 (19.2)
Unique	23	33	29	27	30	29	27	198 (25.2)
Total	124	127	115	102	93	111	115	787 (100.0)

*A total of 45 minor clusters were identified during the study period, each consisting of <15 new patients with multidrug-resistant tuberculosis.

Table 2. Predictors for being in cluster and in major cluster for 787 patients with multidrug-resistant TB, Argentina, 2003–2009*

Characteristic	No. patients	% Patients in cluster	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†	% Patients in major cluster‡	Unadjusted OR (95% CI)	Adjusted OR (95% CI)§
Age, y, n = 640							
<15	24	83.3	2.9 (0.9–8.9)	ND	58.3	1.5 (0.6–3.6)	ND
16–45	495	78.6	**2.1 (1.4–3.2)**	**2.5 (1.3–5.0)**	57.9	1.5 (1.0–2.2)	1.0 (0.5–2.0)
>45	121	63.6	1	1	48.3	1	1
Country of birth, n = 541							
Argentina	412	80.1	**2.7 (1.7–3.9)**	**3.5 (1.9–6.4)**	66.7	**7.6 (4.7–12.1)**	**8.0 (4.3–15.0)**
Other	129	61.2	1	1	20.9	1	1
Place of diagnosis, n = 787							
Hotspot¶	634	77.9	**2.1 (1.5–3.1)**	1.6 (0.7–3.7)	62.3	**4.2 (2.9–6.2)**	**5.9 (2.5–13.8)**
Other	153	62.1	1	1	28.1	1	1
HIV status, n = 604							
Positive	254	86.6	**2.7 (1.7–4.1)**	**2.4 (1.0–5.6)**	76.4	**3.3 (2.-4.7)**	**3.7 (1.8–7.7)**
Negative	350	70.9	1	1	49.4	1	1
Previous TB, n = 557							
Yes	313	71.9	0.7 (0.5–1.0)	0.8 (0.5–1.5)	51.8	0.7 (0.5–1.0)	0.7 (0.4–1.2)
No	244	79.1	1	1	59.4	1	1
Site of disease, n = 775							
Pulmonary only	698	74.1	0.6 (0.3–1.2)	1.4 (0.5–4.2)	55.0	0.8 (0.5–1.3)	2.7 (1.1–7.0)
Other	77	81.8	1	1	59.7	1	1

*__Boldface__ indicates significance. TB, tuberculosis; OR, odds ratio; ND, not done.
†Cluster model overall model fit p < 0.0001, Hosmer & Lemeshow test p = 0.5888, area under the receiver operating characteristic curve 0.723.
‡Cluster including ≥15 patients in the study period.
§Major cluster model overall model fit p < 0.0001, Hosmer & Lemeshow test p = 0.7766, area under the receiver operating characteristic curve 0.793.
¶Area where a multidrug-resistant outbreak was previously documented.

The second major cluster, Ra, was mainly limited to the overpopulated area of Rosario City and surroundings, another MDR TB hotspot (*7*). Only 8 patients in this cluster were found outside that area. Of 38 patients in the third major cluster, Rb, 26 received a diagnosis of MDR TB in Buenos Aires, including 10 transvestite sex workers; 8 patients had the disease diagnosed in Rosario, and 4 elsewhere. Cluster Pr consisted mainly of inmates in several state prisons for men. An association of gender with clustering was only observed in clusters Rb and Pr, in which men predominated (32/38 and 24/26, respectively).

XDR TB Patients and Genotypes

XDR TB was newly diagnosed in 53 patients during 2003–2009. Of these patients, 37 first received a diagnosis of MDR TB during the same period, so these patients were included in the MDR and XDR TB groups. The other 16 XDR TB patients received a diagnosis of MDR TB before this period. The male:female ratio for XDR TB patients was 1.25:1; median age was 37.4 years (SD 11.6, range 21–72 years). Fifty-two patients were born in South America (Argentina 30, Bolivia 4, Peru 6, Paraguay 1, Brazil 1, undetermined 11), and 1 was born in Indonesia.

Table 3. Putative risk factors for case-patients with newly diagnosed multidrug-resistant TB in 7 major genotype clusters, Argentina 2003–2009

Cluster (SIT)*	Area	Total no. case-patients	Risk factor, no. (%) case-patients						
			Previously treated for TB	HIV positive	HCWs	Other hospital exposure†	Prison	Household exposure	Unknown
M H2 (2)	Buenos Aries	228	78 (34.2)	116 (50.9)	21 (9.2)	38 (16.7)	23 (10.1)	29 (12.7)	19 (8.3)
Ra LAM3 (33)	Rosario	89	40 (44.9)	28 (31.5)	2 (2.2)	10 (11.2)	17 (19.1)	16 (18.0)	14 (15.7)
Rb Tuscany (159)	Buenos Aries, Rosario	38	11 (28.9)	22 (57.9)	0 (0.0)	1 (2.6)	0 (0.0)	13‡ (34.2)	6 (15.8)
Pr LAM9 (42)	Buenos Aries	26	10 (38.5)	10 (38.5)	0 (0.0)	2 (7.7)	15 (57.7)	5 (19.2)	2 (7.7)
At T1 (53)	Atlantic Coast	21	9 (42.9)	7 (33.3)	1 (4.8)	3 (14.3)	2 (9.5)	7 (33.3)	2 (9.5)
Ob LAM5 (725)	Buenos Aries	18	7 (38.9)	4 (22.2)	0 (0.0)	2 (11.1)	3 (16.7)	5 (27.8)	2 (11.1)
Os LAM5 (93)	Salta	18	7 (38.9)	6 (33.3)	0 (0.0)	3 (16.7)	1 (5.6)	9 (50.0)	2 (11.1)

*Sum does not equal total because of patients with more than one risk factor. Buenos Aires includes the city and surroundings; MDR, multidrug-resistant; TB, tuberculosis; SIT, Shared International Spoligo Type according to Brudey et al. (*13*); HCW, health care workers.
†Previous hospitalization(s) or concomitant condition.
‡Ten of these case-patients shared a single residence with transvestite sex workers.

Table 4. Resistance of *Mycobacterium tuberculosis* isolates in 7 major clusters to antimicrobial drugs in addition to isoniazid and rifampin, Argentina, 2003–2009*

Cluster	Total no. isolates	No. (%) isolates with additional resistance to			
		0 drugs	1 drug	2 drugs	≥3 drugs
M	228	2 (0.9)	13 (5.7)	30 (13.2)	183 (80.3)
Ra	89	8 (9.0)	61 (68.5)	15 (16.9)	5 (5.6)
Rb	38	26 (68.4)	6 (15.8)	4 (10.5)	2 (5.3)
Pr	26	26 (100)	0	0	0
At	21	6 (28.6)	5 (23.8)	6 (28.6)	4 (19.0)
Ob	18	13 (72.2)	0	3 (16.7)	2 (11.1)
Os	18	0	2 (11.1)	5 (27.8)	11 (61.1)

*Additional drugs tested were streptomycin, ethambutol, pyrazinamide, kanamycin, amikacin, capreomycin, and ofloxacin.

Characteristics of XDR TB patients related to clustering are described in Table 6. Four major clusters (M, Os, At, and Rb) comprised 31 (58%) XDR TB patients; 6/14 HIV-positive patients with XDR TB were in cluster Os, and 5 in cluster M. Five of 11 immigrants from other South American countries to Argentina harbored the M strain, and the patient from Indonesia harbored a Beijing strain. Of 15 patients with no record of previous TB treatment, 12 were included in major clusters. Annual numbers of XDR TB patients by genotype are shown in Table 7.

Discussion

The annual number of newly diagnosed MDR and XDR TB cases decreased slightly, with minor fluctuations, during the study period. HIV infection was associated with almost one third of MDR TB cases; this proportion is 2–4′ higher than that attributed by different studies to all forms of TB in the country (*16*,*17*). As previously observed (*18*), annual fluctuations in the numbers of total MDR/XDR TB cases during the study period paralleled closely annual fluctuations in the numbers of HIV-infected patients.

All 7 major clusters in our study were connected with particular geographic areas, institutional settings, or both. Furthermore, most patients in these clusters underwent TB treatment in health centers that had ongoing MDR TB transmission or had a household or a prison contact with persons who had MDR TB. These findings indicate that these major clusters represent true transmission events.

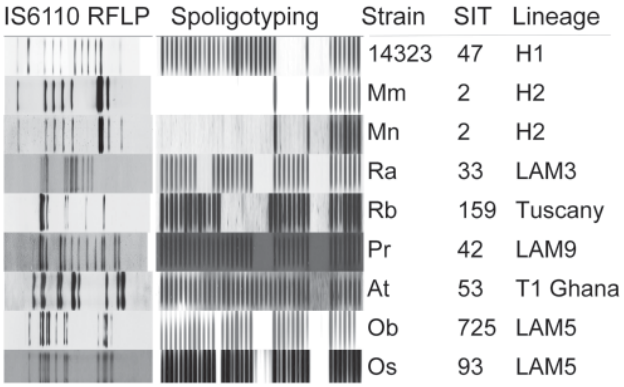

Figure 2. IS*6110* restriction fragment length polymorphism (RFLP) patterns and spoligotypes of 7 major cluster strains, including 2 main variants of M strain, and reference strain Mt 14323. SIT, Shared International Type in SITVIT database (www.pasteur-guadeloupe.fr:8081/SITVIT).

Many cases in this outbreak were caused by the M strain, an apparently autochthonous outbreak genotype. In a countrywide survey performed in 1998, this strain was found to be responsible for 42% of all MDR TB cases but was confined to the metropolitan area of Buenos Aires (National TB Laboratory Network, unpub. data). Since then, the M strain has been the most frequently identified in every MDR TB investigation performed in the country. We found the M strain to be the most prevalent and that its transmission was virtually restricted to the area of the initial outbreak. However, its numbers decreased by more than half within the study period, particularly among HIV-infected patients, which suggests that the epidemic curve of the M strain has entered a declining phase. The other 2 strains associated with previous MDR TB outbreaks, Ra and Rb, were found to persist with lower, fluctuating frequencies.

The expansion of M strain transmission in Argentina was initially fostered by clinical mismanagement. During the early 1990s, patients with advanced AIDS were hospitalized in large, referral treatment centers, where they shared facilities with patients who had MDR TB. At that time, virtually no respiratory protection policy was in force because it was wrongly assumed that MDR TB patients were barely infectious. After genotyping confirmed outbreaks of MDR TB among the patients with AIDS (*4*), hospital infection control interventions were adopted, microbiologic diagnosis and drug-resistance detection were expedited, and second-line TB drugs and highly active antiretroviral therapy became available. As a result of those interventions, hospital transmission was substantially reduced but not completely controlled (*18*). At the time of our study, however, the M strain had long expanded beyond the hospital environment (*6*).

The national *M. tuberculosis* genotype database in Argentina identified very few patients with non–MDR TB harboring H2 genotypes (V. Ritacco, unpub. data). Another study supported this observation (*19*), and the H2 genotype was found to be infrequent in other South American countries (*13*,*20*–*22*). The IS*6110* RFLP pattern of the M strain was absent outside Argentina in the Ibero-American MDR

Figure 3. Locations of 7 major multidrug-resistant tuberculosis clusters, labeled by strain type, Argentina, 2003–2009.

TB genotype database (*23*) and was not present in the *M. tuberculosis* genotype database in the Netherlands (D. van Soolingen, pers. comm.). This pattern was registered in other countries, anecdotally, in 2 MDR TB patients with AIDS: a patient from Argentina who died in San Francisco, California, shortly after his arrival in the United States (*24*); and a patient from Asuncion, Paraguay, who visited the Hospital Muñiz in Buenos Aires each month for antiretroviral therapy (*20*).

Different *M. tuberculosis* genotypes may have affinity with certain geographic areas and ethnic groups (*25*), which could explain why the M strain persists in the Buenos Aires area. In addition, most persons at risk for MDR TB are underprivileged and cannot afford to travel far distances. This mobility limitation might also help to explain why this strain has remained virtually confined to the original hotspot area.

A small number of patients affected by the M strain were immigrants from neighboring countries who had settled in Buenos Aires. Cross-border and domestic migration toward large metropolitan areas is a long-observed demographic and public health concern in Argentina. More than 80% of the patients in this study were assisted in metropolises designated as MDR TB hotspots. Even though immigrants with TB have access to higher quality health care in these areas than in other parts of the country, they are also at higher risk of becoming newly infected with an outbreak MDR TB strain.

The M strain was overrepresented among patients with XDR TB; isolate resistance to ≥5 anti-TB drugs was found to be a strong predictor of disease caused by the M strain (*4*,*26*). The accumulation of drug resistance–conferring mutations would be expected to have reduced the epidemiologic fitness of this strain, but it has prevailed for 15 years. The epidemiologic fitness of a strain can be influenced by a range of factors, e.g., the genetic backgrounds of host and pathogen, host–pathogen interactions, and the environment (*27*–*29*). Compensatory evolution restoring in vivo fitness, as well as social and behavioral factors, might

Table 5. Predictors for being in cluster M among 438 patients with multidrug-resistant TB who were in clusters of >15 patients, Argentina, 2003–2009*

Characteristic	No. patients	% Patients in M cluster	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
Age, y, n = 347				
16–45	288	50.7	0.9 (0.5–1.6)	1.4 (0.5–4.3)
>45	59	52.5	1	1
Country of birth, n = 302				
Argentina	275	51.0	0.7 (0.3–1.6)	0.6 (0.1–2.3)
Other	27	74.1	1	1
HIV status, n = 360				
Positive	194	60.3	**1.6 (1.0–2.4)**	1.4 (0.6–3.3)
Negative	166	49.4	1	1
Previous TB treatment, n = 304				
Yes	160	47.5	1.0 (0.7–1.6)	0.8 (0.4–1.8)
No	144	46.5	1	1
Hospital exposure‡				
Yes	86	72.1	**2.9 (1.7–4.8)**	2.6 (1.0–6.8)
No	352	47.2	1	1
Isolate resistant to				
≥5 drugs	207	88.4	**31.5 (18.4–53.9)**	**22.7 (10.1–50.9)**
<5 drugs	231	19.5	1	1

*__Boldface__ indicates significance. TB, tuberculosis; OR, odds ratio.
†Overall model fit p < 0.0001. Hosmer & Lemeshow test p = 0.899, area under the receiver operating characteristic curve 0.854.
‡Previous hospitalization(s), concomitant condition, or health care worker.

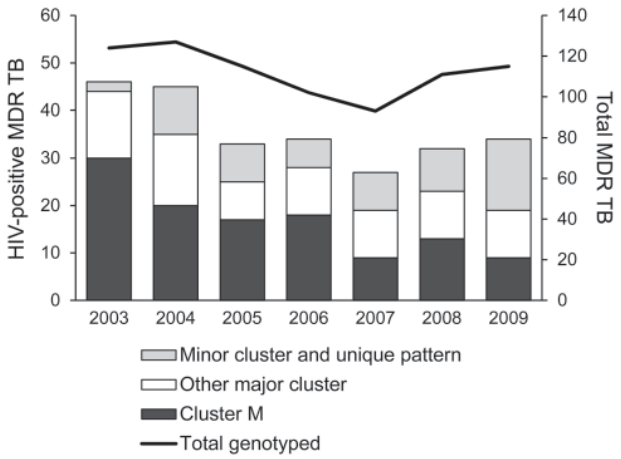

Figure 4. Numbers of HIV-positive patients with multidrug-resistant tuberculosis (MDR TB), classified by genotype cluster, and total number of newly diagnosed MDR TB patients per year with identified genotype, Argentina, 2003–2009.

have played a role in the epidemiologic persistence of the M strain (*30*). These factors might also have preferentially fostered the spread of drug-resistant strains of the H2 genotype in our setting. Further studies are needed to evaluate the most critical risk factors.

Drug-resistance profiles were not uniform within the M strain clusters. The variations in susceptibility to individual drugs reflect the existence of various ongoing chains of transmission, some of which might have started before, or simultaneously with, the first documented outbreak. One limitation of our study is the failure to identify individual chains. Factors that precluded the reliable characterization of subclusters were the long time elapsed since the outbreak onset, the insufficient epidemiologic documentation in many cases, and the unavailability of additional molecular markers.

Our study has another major limitation. Incomplete demographic and clinical data on patients were retrieved, and several observations had missing values. If missing values were systematically associated with a given force or factor, results presented here would be biased. We are not aware of any association of missing values with the dependent variables under study and assume that those data were missing at random. Missing values may have affected the analyses by reducing the number of observations, which may have reduced the power of the model to detect significant associations but without necessarily biasing the associations reported. However, the possibility that bias might have resulted from missing data cannot be ruled out. Therefore, statistical significances of our analyses should be interpreted cautiously.

In the MDR TB hotspots in Argentina, the distinction between primary and acquired MDR TB on the basis of a history of previous TB treatment was not decisive because patients could have been exposed to hospital-associated MDR TB infection while being treated for community-acquired TB. This fact could explain why clustering was not more frequent among patients without previous TB treatment in our study.

The national TB network includes all the laboratories performing bacteriological TB diagnosis in the country; therefore, the patients in this study represent all newly diagnosed MDR TB cases in Argentina. The structure, geographic coverage, and personnel of the TB laboratory network are adequate to provide DST for all patients at risk for MDR TB in Argentina. However, a few MDR TB patients might remain undiagnosed because of operational factors, e.g., inefficient detection of risk factors, insufficient or delayed requests for DST, and disorganized information systems.

The geographically restricted distribution of successful MDR TB genotypes that we found has public health implications. As a result of this study, specific interventions are being reinforced, particularly in the MDR TB hotspots: implementing universal culture and strategies to expedite drug

Table 6. Characteristics of 53 patients with extensively drug-resistant TB, Argentina 2003–2009*

Characteristic	No. patients	% In cluster	% In major cluster
Sex			
M	29	79.3	58.6
F	24	75.0	58.3
Age group, y			
15–29	16	81.3	50.0
30–44	24	75.0	66.7
>45	8	87.5	50.0
Unknown (adult)	5	60.0	60.0
Country of birth			
Argentina	30	76.7	53.3
Other (South America)†	11	81.8	63.6
Unknown (South America)‡	11	81.8	72.7
Indonesia	1	0	0
Place of diagnosis			
Former MDR TB hot spot	31	74.2	71.0
Other	22	81.8	40.9
HIV status			
Positive	14	85.7	78.6
Negative	33	75.8	54.5
Unknown	6	66.7	33.3
Site of disease			
Pulmonary	49	81.6	61.2
Disseminated	3	33.3	33.3
Unknown	1	0	0
Previous TB			
Yes	38	71.1	50.0
No	10	90.0	70.0
Unknown	5	100.0	100.0
AFB smear microscopy			
Positive	41	78.0	56.1
Negative	7	71.4	57.1
Unknown	5	75.0	75.0

*TB, tuberculosis; MDR, multidrug-resistant; AFB, acid-fast bacilli.
†From a country in South America other than Argentina
‡From an unknown country in South America.

Table 7. Number of patients with extensively drug-resistant tuberculosis of different genotypes, by year, Argentina, 2003–2009

Genotype	No. patients in year							Total no. (%) patients
	2003	2004	2005	2006	2007	2008	2009	
Cluster M	5	4	5	2	0	3	2	21 (39.6)
Cluster Rb	0	0	0	0	0	1	0	1 (1.9)
Cluster At	0	1	0	0	0	0	1	2 (3.8)
Cluster Os	0	0	2	1	2	1	1	7 (13.2)
Minor cluster*	2	1	3	3	1	1	0	11 (20.8)
Unique pattern	2	2	2	1	1	0	3	11 (20.8)
Total	9	8	12	7	4	6	7	53 (100.0)

*Cluster of <15 patients with newly diagnosed multidrug-resistant tuberculosis during the study period

resistance detection; decentralizing specialized health care; streamlining information-sharing systems between HIV and TB programs; and strengthening administrative infection control measures in prisons and large hospitals with high TB infection load. A national advisory group on MDR TB clinical management has also been recently created. Control interventions have already started to reduce MDR TB spread in the hospital that was the epicenter of the main outbreak (*17*). Still, centrally coordinated actions are needed in Argentina to curb long-term transmission of MDR TB.

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Risk for Travel-associated Legionnaires' Disease, Europe, 2009

Julien Beauté, Phillip Zucs, and Birgitta de Jong

Legionnaires' disease is underreported in Europe; notification rates differ substantially among countries. Approximately 20% of reported cases are travel-associated. To assess the risk for travel-associated Legionnaires' disease (TALD) associated with travel patterns in European countries, we retrieved TALD surveillance data for 2009 from the European Surveillance System, and tourism denominator data from the Statistical Office of the European Union. Risk (number cases reported/number nights spent) was calculated by travel country. In 2009, the network reported 607 cases among European travelers, possibly associated with 825 accommodation sites in European Union countries. The overall risk associated with travel abroad was 0.3 cases/million nights. We observed an increasing trend in risk from northwestern to southeastern Europe; Greece had the highest risk (1.7). Our findings underscore the need for countries with high TALD risks to improve prevention and control of legionellosis; and for countries with high TALD risks, but low notification rates of Legionnaires' disease to improve diagnostics and reporting.

Legionnaires' disease (LD) is a severe multisystem disease, typically manifesting as pneumonia; it is caused by gram-negative bacteria, *Legionella* spp., which are found in freshwater environments worldwide, and can be can be transmitted by aspirating contaminated drinking water or by inhaling airborne droplets from contaminated sources such as building ventilation units and spas (*1*). A travel-associated Legionnaires' disease (TALD) surveillance system at the European Union (EU) level has been in place since 1987 (*2*); EU surveillance of Legionnaires' disease, regardless of patient travel history, began in 1996 (*3*). The rationale for the surveillance of travel-associated cases of Legionnaires' disease (TALD) is that it enables the timely detection of clusters and the identification of the source of infection, which would otherwise probably remain undiscovered (*4*). Since April 2010, both surveillance systems have been operated by the European Legionnaires' Disease Surveillance Network (ELDSNet) under the coordination of the European Centre for Disease Prevention and Control (ECDC).

Since 2005, 5,000–6,000 cases of Legionnaires' disease have been reported each year by the 27 EU Member States, Iceland, and Norway (*5*,*6*). Annual case-fatality rates of notified Legionnaires' disease have been ≈10%, and ≈20% of reported cases are travel-associated (*5*–*7*), which is similar to the situation described in the United States (*8*). There is evidence that the risk for Legionnaires' disease might be higher under certain environmental conditions; warm and wet weather has been associated with higher incidence rates in the Netherlands and the United Kingdom (*9*,*10*). Using that evidence, Hicks et al. reported that an increased case count would be expected after heavy rains during the warm season (*11*). However, in Europe, an increasing trend from northern to southern countries is not clearly evident in surveillance data.

Because many cases of Legionnaires' disease might go undiagnosed by clinicians, and because some clinicians do not report confirmed cases to national authorities, surveillance systems are likely to miss a sizeable proportion of Legionnaires' disease deaths (*1*). The ECDC estimated that as few as 10% of Legionnaires' disease cases are reported in Europe and reported that notification rates differ substantially across countries and that causes of undernotification are likely to vary (*6*). Because notification rates tend to reflect the quality of national surveillance rather than the local risk for Legionnaires' disease, it is difficult to estimate and compare risk for Legionnaires' disease across countries.

The surveillance of Legionnaires' disease associated with international travel might, at least partly, overcome this limitation. A similar approach has been used in pre-

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vious studies to estimate and compare the risks for foodborne and waterborne diseases in Europe (*12*,*13*). Tourism is a massive industry in Europe, and its summer peak coincides with the peak of Legionnaires' disease. Cases of TALD in travelers who contract the disease in the country they visit are mostly reported by the patient's country of residence independent of the quality of surveillance in the travel destination country. These cases may therefore enable not only a better estimation of local disease risk but also an assessment of the local quality of reporting. Finally, Legionnaires' disease surveillance in Europe has traditionally focused more on TALD, because of the added value of improved prevention and control of international clusters. TALD surveillance in Europe can therefore be assumed to be less prone to underascertainment than the surveillance of Legionnaires' disease not associated with travel.

This analysis is intended to assessing the risks for TALD in European countries on the basis of travel patterns. A secondary objective is to provide an estimate of the extent of underascertainment by country of destination.

Methods

Data and Definitions

In travel medicine, the use of surveillance data is a valid method for calculating absolute risk estimates in travelers (*14*). Notification of Legionnaires' disease cases is mandatory in all 27 EU Member States (notification has been mandatory in the United Kingdom since 2010) and Iceland and Norway, the 2 European Economic Area (EEA) member states that form part of ELDSNet. TALD cases were reported during 2009 to ECDC by most ELDSNet members. Germany did not participate in TALD reporting during this period. Reporting was performed on a daily basis through the web-based European Surveillance System, known widely as TESSy. TALD cases must conform to the official EU case definition of Legionnaires' disease (*15*), and case-patients must have a history of travel, i.e., at least 1 night spent in commercial accommodation within the EU/EEA countries, away from their residence (including travel within their home country) within the incubation period of Legionnaires' disease (2–10 days before disease onset). Date of onset and date of travel are carefully checked.

For the purpose of this analysis, we included TALD cases with disease onset in 2009 and retrieved the patients' places of residence and travel history from TESSy. Cases counted were restricted to European residents traveling in EU/EEA countries. The travel history could include more than 1 travel destination or accommodation site, each of which was considered a potential source of exposure. Therefore, a case would be counted in more than 1 country if the case-patient's travel history involved accommodation sites in various countries. The exact duration of stay was not taken into account in the analysis. Tourism denominator data for 2009 were obtained from the Statistical Office of the European Union (Eurostat) (*16*). We used the number of nights spent in tourist accommodations by Europeans traveling in their home countries and in foreign countries within Europe. The number of nights spent was also categorized by a traveler's country of residence and destination country.

Analysis

Case-patients were described by age, sex, month of illness onset, travel destination, type of travel, and type of accommodation. To estimate the exposure rate, we used the number of nights spent by country of destination. For a given country of destination, risk for TALD was defined as the number of cases divided by the number of nights spent in that country by all travelers (population at risk). To allow comparisons of risk by country of origin, we excluded case-patients for which the reporting country was different from the country of residence. Risk at country level was calculated only for countries with at least 10 cases reported. To estimate underascertainment, a set of reference reporting countries with at least 20 cases reported in nondomestic travelers was chosen. We then compared the risk for Legionnaires' disease of travelers originating from these reference countries to the risk for domestic travelers, calculating incidence ratios with 95% CIs for each destination country. Last, on the basis of the risk for travelers from the reference countries, we estimated the number of cases expected in each travel destination country. These estimates were obtained by multiplying the risk in reference countries with the total number of nights spent in tourist accommodations by all travelers in a given destination country.

Results

Cases and Sites

In 2009, 607 TALD cases were reported among European residents traveling in EU/EEA countries, of which 363 (60%) were related to domestic travel (Table 1). Travel histories involved 825 accommodation sites in 24 countries. The average number of sites per case-patient was 1.4 (range 1–7); a similar distribution was calculated among countries. As travel destinations, France, Italy, and Spain accounted for 72% of cases and sites. Of the 825 sites involved, 447 (54%) had been occupied by domestic travelers. Notification rates increased with age and were higher in male case-patients for all age groups (Figure 1). The male:female ratio for case-patients was 2:7 and the median age at date of onset was 61 years (interquartile range 51–69 years). All case-patients were >15 years of age; 40% were ≥65 years of age. The seasonal distribution of cases peaked in September; the date of onset for

Table 1. Cases of travel-associated Legionnaires' disease by country of residence and destination country, Europe, 2009*

Country of residence	Reported no. cases by destination country				
	Italy	France	Spain	Other countries	Total
Italy	140	5	2	3	149
France	14	106	5	10	133
United Kingdom	12	6	33	80	120
Netherlands	14	11	10	58	72
Spain	4	2	42	8	51
Denmark	9	1	1	15	22
Austria	7	1	0	7	14
Sweden	2	2	2	8	13
Other countries	7	3	3	21	33
Total	209	137	98	210	607

*A case-patient could have a travel history involving >1 country.

48% was during the third quarter of the year. Hotels were the most common accommodation sites associated with TALD, accounting for 578 (70%) of 825 sites of known type. This proportion applied to sites associated with domestic travel and sites associated with foreign travel. Other accommodation sites reported were campsites (8%), private accommodations rented for commercial purposes (6%), apartments (5%), cruise ships (<1%), and other accommodations (10%).

Travel Patterns

In 2009, two billion nights were spent in tourist accommodations in EU/EEA countries by travelers who resided in Europe. Of those nights, 46% were spent in France, Italy, and Spain (Table 2). Overall, travel within the tourists'country of residence represented 66% of all nights spent in tourist accommodations. Among Europeans ≥65 years of age, the proportion of domestic travelers (19%) was higher than the proportion of travelers who visited other countries (15%; $p<0.01$). Conversely, the proportion of travelers in the 45–64 year age group was lower in domestic travelers (31%) than in those traveling to other countries (33%; $p<0.01$). Data at destination country level were not available.

Risk for TALD

Calculating on the basis of 2 billion nights spent in Europe by European residents and 607 cases, the average risk for TALD in Europe in 2009 was 0.30 cases/1 million nights (95% CI 0.27–0.32). The highest risk for domestic travelers was in Italy (0.66 cases/1 million nights), followed by France (0.54) and the Netherlands (0.30) (Table 2). On average, the risk for nondomestic travelers (0.36/1 million nights) was 1.3 × the risk for domestic travelers (0.27 cases/1 million nights). France, Italy, the Netherlands, and the United Kingdom were the only countries with higher risks for domestic travelers than for nondomestic travelers. The average risk in nondomestic travelers ranged from 0.02 cases/1 million nights in the United Kingdom to 0.88 cases/1 million nights in Greece.

Underascertainment of Cases

The United Kingdom, the Netherlands, France, and Denmark reported the highest numbers of cases in nondomestic travelers: 81, 72, 28, and 21 cases, respectively. These countries also reported the highest rates for nondomestic travelers, ranging from 0.62 to 1.24 cases/1 million nights. Sweden's reported rate (0.74 cases/million nights) was within this higher range, but was based on 13 cases reported. The United Kingdom, the Netherlands, France, and Denmark were selected as reference reporting countries with a pooled overall risk of 0.55 cases/million nights and a pooled risk of 1.68 cases/1 million nights when traveling to Greece (Table 3). The risk for travelers from these reference countries becoming infected by *Legionella* was greater when traveling in Greece, Portugal, Germany, Italy, Austria, Spain, and France than when traveling in the United Kingdom (Table 3). Risk was high in southeastern countries (Greece and Italy), moderate in southwestern and central Europe (Austria, Germany, and Portugal), and low in northwestern countries (Figure 2). Based on these figures, 1,127 TALD cases would be expected to be reported in European residents traveling in Europe (Table 3). The highest levels of underascertainment were observed in Greece,

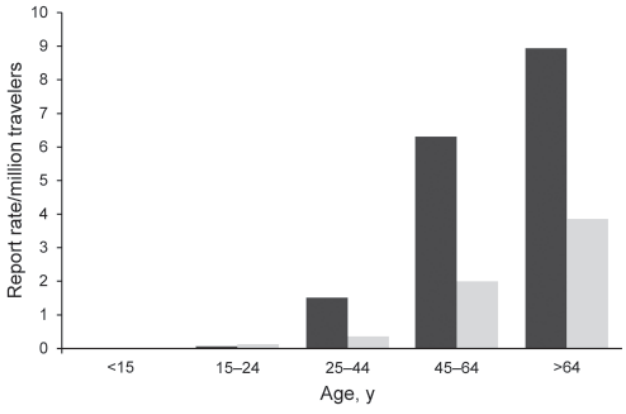

Figure 1. Notification rate of travel-associated Legionnaires' disease (n = 607), by age group and sex, European Union/European Economic Area, 2009. Black bar, male case-patients; light gray bar, female case-patients.

Table 2. Cases of travel-associated Legionnaires' disease and risk per million nights in European domestic and nondomestic travelers, Europe, 2009*

Destination	No. (% cases in domestic travelers)	No. nights spent by travelers, millions (% by domestic travelers)	TALD risk in cases/million nights	
			Domestic travelers	Nondomestic travelers
Italy	209 (67)	330 (64)	0.66	0.58
France	137 (77)	272 (72)	0.54	0.41
Spain	98 (43)	328 (45)	0.28	0.31
United Kingdom	45 (98)	228 (80)	0.24	0.02
Greece	34 (0)	58 (33)	0.00	0.88
Germany	28 (0)	296 (88)	0.00	0.79
Netherlands	21 (86)	79 (76)	0.30	0.16
Portugal	20 (20)	41 (47)	0.21	0.73
Austria	20 (15)	94 (33)	0.10	0.27
Other countries	42 (14)	314 (72)	0.03	0.33
Total	607 (60)	2,039 (66)	0.27	0.36

*A case-patient could have a travel history involving >1 country. TALD, travel-associated Legionnaires' disease.

Portugal, and Austria. Germany was not taken into account because cases in domestic travelers were not reported.

Discussion

We report risk estimates of TALD for domestic and international travelers in Europe. These results are valuable because sentinel studies that rely on travel medicine departments may not report on TALD (*17*,*18*). In addition, most studies focus on travelers who are returning from developing countries (*19*). Logarithmic scales of health risk among travelers, as provided by Steffen et al. (*20*), need regular updates. On the basis of the most recent data, collected during 2003 in Europe (*2*), the TALD risk was estimated to be between 0.0001% and 0.001% per month of stay in developing countries. The highest risk estimate that we found was 1.68 cases/1 million nights (Greece), which corresponds to an incidence rate of 0.005%/month, which is ≥5 × higher than the aforementioned estimate. Our risk estimates ranged from 0.001%/month in the United Kingdom to 0.005%/month in Greece and Italy.

It is highly probable that a similar risk would be found in other Mediterranean countries that share similar climate and tourist facilities. The seemingly higher risk among nondomestic travelers may reflect a better awareness of clinicians when investigating pneumonia in a patient who is returning from a trip abroad. Indeed, clinicians might be more likely to ask for additional tests, including urinary antigen, in patients with a recent history of travel abroad. It could also reflect, to some extent, better reporting in northern European countries, the residents of which tend to spend their vacations in southern Europe. Another hypothesis would be that domestic travelers have a better immunity against local strains. The low TALD risk in nondomestic travelers to the Netherlands and the United Kingdom could be explained by the fact that both countries receive proportionately far fewer and younger tourists than Mediterranean countries.

This study clearly indicates an increasing risk of TALD from northwestern to southeastern Europe. Greece did not report any domestic TALD cases in 2009, but the risk estimate for nondomestic travelers who had visited Greece was the highest. This discrepancy likely reflects inadequate disease diagnosis or reporting in Greece. When geographic latitude was considered, Germany posed a higher TALD risk than expected, and Spain appeared to have a lower risk than expected. The seemingly higher risk in Germany could be associated with travelers from the Netherlands, who accounted for nearly one third of all nights spent by nondomestic travelers to Germany, and whose cases were likely to be reported to their health authorities. Conversely, German travelers ac-

Table 3. Expected risk for Legionnaires' disease in European travelers to nondomestic destinations in Europe, based on reference data reported by the United Kingdom, the Netherlands, France, and Denmark, Europe, 2009*†

Destination	Risk for travelers to destination (cases/million nights)	Incidence ratio (95% CI)	Total no. cases	
			Reported	Estimated
Greece	1.68	7.2 (4.2–12.2)	34	98
Italy	1.40	6.0 (3.9–9.2)	209	463
Germany	1.19	5.1 (2.9–8.7)	22	353
Portugal	1.06	4.6 (2.1–9.0)	20	44
Austria	1.01	4.4 (2.2–8.2)	20	95
Spain	0.57	2.5 (1.6–3.8)	98	188
France	0.53	2.3 (1.6–3.3)	137	145
Netherlands	0.33	1.4 (0.8–2.4)	21	26
United Kingdom	0.23	1.0 (Ref.)	45	53
Other countries	0.90	3.9 (2.3–6.4)	42	282
Total	0.55	NA	607	1,127

*A case-patient could have a travel history involving >1 country. Ref., referent; NA, not applicable.
†In the United Kingdom, reporting of Legionnaires' disease became mandatory in 2010.

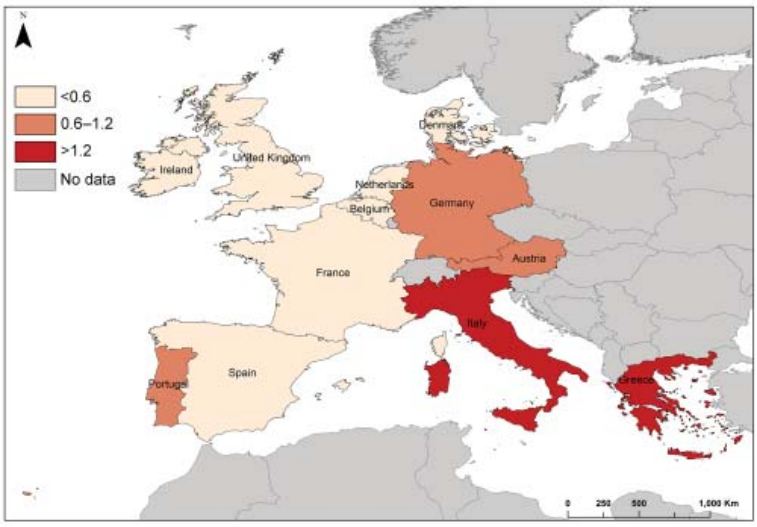

Figure 2. Risk for travel-associated Legionnaires' disease in residents of Denmark, France, the Netherlands, and the United Kingdom who traveled to countries in Europe. Risk is displayed in countries where travelers spent at least 5 million nights in commercial accommodations during 2009, European Union/European Economic Area (in cases per million nights).

counted for nearly one third of all nights spent by nondomestic travelers in Spain; because Germany did not participate in the European TALD surveillance, any cases that may have occurred in German travelers were not reported.

Nevertheless, this relatively high risk observed in nondomestic travelers who visited Germany might confirm a report of an increasing trend in France from west to east in 2010 (*21*). Heavy rains during the warm season in continental climates might have favored the growth of *Legionella* spp. The magnitude of TALD underascertainment in Austria, where German travelers accounted for 60% of all nights spent by nondomestic travelers, demonstrates the effect that the lack of participation in reporting by Germany had on epidemiologic studies of the EU/EEA (As of September 2012, Germany participates in the European TALD surveillance). The use of a pooled risk estimate as reference suggests that underascertainment of TALD remains substantial in Europe and concerns all nonreference countries. This conclusion is consistent with results of capture-recapture studies conducted in some European countries to assess the extent of undernotification (*22*–*24*). According to our study, ≈1,100 TALD cases would be expected annually in the EU/EEA.

This study has some limitations. First, it was assumed that travel habits were similar among all European travelers. Duration of stay could affect the likelihood of exposure while traveling and could vary across countries, age groups, and type of travel. Second, because of the lack of data on age group distribution among travelers, the calculation of risk could not be standardized by age. We know that domestic travelers are older and therefore more prone to be infected by *Legionella* spp. However, the proportions of Europeans ≥65 years of age remained comparable among domestic travelers and those traveling outside their home country. In addition, distribution of travelers by age groups may substantially differ across countries. Third, the study clearly highlights the lack of data for Eastern European countries, probably because tourism is lower than elsewhere in Europe and because TALD is under-identified. Increasing numbers of tourists and improving local clinical and diagnostic awareness might help estimate the TALD risk in this part of Europe in the future.

Further analyses should confirm these results over time. Larger datasets could allow adjustment for age, seasons, and accommodation types. In large countries, regional stratification would also be valuable since risk may differ within a country.

In conclusion, these European TALD risk estimates can provide data for several purposes. First, they may help raise clinicians' awareness and enhance reporting in countries where risk for Legionnaires' disease and TALD is high, but reporting rates are low. Second, they could serve as a basis for monitoring future trends. Considering global warming, increasing use of manmade water systems, and an aging European population, Legionnaires' disease and TALD incidence might also be expected to rise.

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Litchi–associated Acute Encephalitis in Children, Northern Vietnam, 2004–2009

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Since the end of the 1990s, unexplained outbreaks of acute encephalitis in children coinciding with litchi harvesting (May–July) have been documented in the Bac Giang Province in northern Vietnam. A retrospective ecologic analysis of data for 2004–2009 involving environmental, agronomic, and climatic factors was conducted to investigate the suspected association between the outbreaks and litchi harvesting. The clinical, biological, and immunologic characteristics of the patients suggested a viral etiology. The ecologic study revealed an independent association between litchi plantation surface proportion and acute encephalitis incidence: Incidence rate ratios were 1.52 (95% CI 0.90–2.57), 2.94 (95% CI 1.88–4.60), and 2.76 (95% CI 1.76–4.32) for second, third, and fourth quartiles, respectively, compared with the lowest quartile. This ecologic study confirmed the suspected association between incidence of acute encephalitis and litchi plantations and should be followed by other studies to identify the causative agent for this syndrome.

Acute encephalitis syndrome (AES) is a major public health problem in Asia. The main etiologic agent is the Japanese encephalitis virus (JEV), a positive-sense single-stranded flavivirus transmitted by *Culex* spp. mosquitoes. It is responsible for ≈50,000 encephalitis cases every year in the region (*1*). Recently, the Nipah and Chandipura viruses were identified as responsible for acute encephalitis outbreaks in Malaysia and India (*2*,*3*). In addition, many other viral encephalitis cases of unknown etiology exist throughout Asia (*4*).

In Vietnam, according to the National Institute of Hygiene and Epidemiology (NIHE), the annual incidence rate for AES in the general population was 2.24–2.90 cases per 100 000 inhabitants during 1998–2005. This rate corresponds with 1,800–2,300 cases per year, two thirds of which occurred in northern Vietnam. Since the inclusion in 1997 of the JEV vaccine into the Extended Program on Immunization by the World Health Organization (WHO), the relative proportion of non-JE cases has increased substantially among patients hospitalized with AES in Vietnam, from ≈40% in 1996 to ≈90% in 2009 (P.T. Nga, unpub. data).

In northern Vietnam, unexplained outbreaks of non-JE acute encephalitis have been documented since 1999. These outbreaks are unusual because of their specific location (Bac Giang Province), their strict seasonality (92% of unexplained AES occur during May–July), the restricted age group of persons at risk (88% are ≤15 years old), and the clinical features (abrupt febrile onset, rapid progression to coma, and higher case-fatality rate than for JE). Approximately 50–100 children are referred to the provincial hospital each epidemic season, but the actual number of cases could be underreported because some patients might have died at home.

The local population and public health practitioners have anecdotally attributed the emergence of AES to the recent intensification of litchi production in the province: production rose from 870 tons during 1985–1989 to

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400,000 tons during 2000–2005. Bac Giang Province has the highest litchi production in Vietnam, three fourths of which is consumed domestically and the rest is exported mainly to People's Republic of China (*5*,*6*). Vietnamese litchis are mostly of the Thieu variety, which has a short harvest period of ≈1 month during May–July (*7*), which coincides with the epidemic season of the outbreaks in Bac Giang.

Because of the distinct early clinical manifestations (*8*), the syndrome has been locally termed Ac Mong encephalitis (AME), after the Vietnamese word for nightmare. The typical clinical illness starts with headache and fever, followed by seizures (often during the night); approximately one third of cases progress to coma and death.

The causative agent of AME has remained unidentified and may be responsible for unexplained acute encephalitis elsewhere in the world, particularly in regions sharing similar ecology and environment. Litchi is widely distributed throughout subtropical and tropical regions. The 5 leading litchi-producing countries are China, India, Taiwan, Thailand, and Vietnam (*9*).

Our first objective was to describe the epidemiologic and clinical features of this severe encephalitis among children in northern Vietnam. Our second objective was to strengthen or weaken the hypothesis that litchi cultivation is associated with acute outbreaks of AES in Bac Giang Province. We investigated this suspected association using a retrospective ecologic analysis for 2004–2009 in Bac Giang Province that involved various environmental, agronomic, and climatic factors. Confirmation of this association would pave the way for further hypothesis-testing studies investigating the causal mechanisms behind this ecologic correlation.

Materials and Methods

Study Area

Bac Giang Province is located in northeastern Vietnam, 50 km from the capital, Hanoi (Figure 1). This mainly rural area covers 3,827 km^2 and has a population of ≈1.6 million inhabitants. About one third of the land area is devoted to agriculture and one fourth to forestry and timber production. The climate comprises 2 distinct seasons: the cold, dry season from October through March and the hot, rainy season from April through September. The province is divided into 10 districts and 230 communes. The only hospital is in Bac Giang City, which is the capital of the province.

Data Collection and Laboratory Analyses

Records from patients admitted to the provincial hospital with suspected AES from January 2004 through December 2009 were collected from Bac Giang Preventive Medicine Centre and NIHE. Among these patients, criteria for inclusion in the ecologic analyses were clinical diagnosis of suspected acute viral encephalitis, age <15 years, onset date during May 1–July 31, and negative serology for JEV IgM (IgM antibody-capture ELISA) or full immunization against JEV (receipt of 3 doses). Patients meeting these criteria are referred to as suspected AME case-patients.

Detailed clinical and biologic examinations, including blood analyses, cerebrospinal fluid (CSF) analyses, and cranial computed tomography scans, were available for 88 AME patients hospitalized during 2006–2009 that were included in a parallel case–control study (in progress). Alanine aminotransferase and aspartate aminotransferase levels also were tested in 17 patients. The representativeness of this subgroup was evaluated by comparing it with the whole group on all common measured characteristics (age, sex, day of onset, and geographic coordinates) by χ^2 and *t* tests.

To determine the nature of the host response to AME, Multiple Analyte Profiling (Luminex Inc., Austin, TX, USA) was performed on the serum and CSF of a subset of AME patients collected in 2007. Samples were analyzed for 39 separate chemokines and cytokines by using the Luminex 100IS technology (Miliplex MAP kits; Milipore, Billerica, MA, USA). The analyzed group comprised samples from 5 patients who survived, 5 who died of the

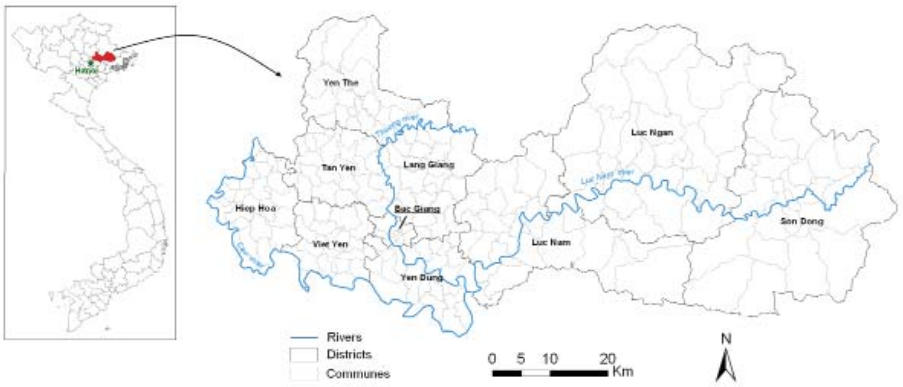

Figure 1. Location and map of Bac Giang province in Vietnam. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/11-1761-F1.htm).

disease, and 4 serum samples from asymptomatic siblings. Data are presented as concentration (pg/mL). We used a Mann-Whitney U test to compare values between groups.

In virology, a collection of 9 supernatants from short-term cell cultures (including Vero and C6/36 cells) and their corresponding CSF and serum samples, taken from patients during 2006–2008 who had severe, including fatal, forms of disease, were all screened for known viruses by using PCR-based assays at Institut Pasteur (Paris, France). Searched viruses included JEV, dengue viruses 1–4, West Nile virus, chikungunya virus, herpes simplex viruses 1 and 2, varicella-zoster virus, Rift Valley fever virus, Crimean-Congo hemorrhagic fever virus, and most viruses of genera *Enterovirus* and *Alphavirus*. All assay results were negative.

Ecologic Data Collection and Mapping

Demographic and agronomic data were collected by the Bac Giang Preventive Medicine Centre from the district statistics offices for each year from 2004 through 2009 for the 230 communes. These data included population count; production of rice and litchi; surface area dedicated to rice cultivation, litchi cultivation, and forestry; and the number of pigs, water buffaloes, cattle, and poultry. Starting and ending months of the rice and litchi harvests were collected at the district level. Daily meteorologic data were collected at the provincial level from the National Centre for Hydro-Meteorological Forecasting, Ministry of Natural Resources and Environment (Hanoi, Vietnam). These data included mean temperature, mean relative humidity, and total rainfall during January 2004–December 2009.

A commune-level map of Bac Giang Province was retrieved from the Map Library of the WHO health mapping program. To improve the accuracy of the WHO map, a 1:50,000 topographic map of Bac Giang Province (Cartography Office of Vietnam National Institute of Geography) was digitized and geo-referenced (WGS84 coordinate system) with SavGIS software (www.savgis.org). From these 2 cartographic sources, a new, more precise, map of Bac Giang communes and rivers (WGS84-UTM48N projection) was created with ArcGIS software (version 10.1, ESRI Inc., Redlands, CA, USA). In addition, mean elevations of the communes were derived from satellite raster data (US Geological Survey, Shuttle Radar Topography Mission), collected from the FTP server of Global Land Cover Facility, University of Maryland (ftp://ftp.glcf.umiacs.umd.edu/glcf/).

Statistical Analyses

To investigate the spatiotemporal association between AME incidence and potential ecologic factors, including litchi cultivation, we conducted a commune-level retrospective ecologic regression analysis in Bac Giang Province for 2004–2009. A 2-level (year and commune) binomial negative regression model with fixed-effect coefficients and random-effect intercepts was conducted by using STATA software (version 11.0; StataCorp LP, College Station, TX, USA). The outcome was the number of AME cases per commune per year. The covariates included in the analysis are listed in Table 1 (Appendix, wwwnc.cdc.gov/EID/article/18/11/11-1761-T1.htm). Continuous variables were modeled as categorical variables (by quartiles for agronomical variables and by terciles for meteorologic variables). Population size was introduced as an offset in the model so that the measure comparing the risk across exposure categories was an incidence rate ratio (IRR). The multivariate analysis included covariates with a p value <0.25 in the univariate analyses, and a backward deletion of variables by Wald test was conducted until all variables in the final model had p values <0.05.

In addition, we investigated the temporal correlation between the epidemics and the litchi harvests to test whether AME cases occurred earlier in areas that harvested litchis earlier in the year. A Mann-Whitney U test was used to compare the distribution of the week numbers (1–52) of cases in the 5 districts that harvested litchis during May–June (group 1) to the distribution of the week numbers of cases in the 5 districts that harvested litchis during June–July (group 2). The test was performed in R (version 2.12.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Ethical Considerations

This study was approved by the Clinical Research Committee of Institut Pasteur, and ethical clearance was obtained from the NIHE Institutional Review Board. Informed consent for participation in the study was obtained from each child's legal representative (parent, guardian, or other legally recognized person as defined by Vietnamese national laws).

Results

Spatial and Temporal Distribution of Cases

During the study period, 239 children met the inclusion criteria. Their median age was 5 years (interquartile range 2–7.5), and the male:female ratio was 1.21:1. Temporally, the number of AME cases in the study decreased from 57 in 2004 to 19 in 2006, then increased to 58 in 2008 and fell to 12 in 2009 (Figure 2, panel A). The annual litchi production in the province is plotted for comparison purposes (Figure 2, panel B). Spatially, we estimated the cumulative incidence of the disease at the commune level during the study period, which ranged from 0 to 205 cases per 100,000 inhabitants (Figure 3, panel A). The highest incidences were mainly found in communes in

Table 2. Clinical and laboratory features of Ac Mong encephalitis patients, Bac Giang Province, Vietnam, 2004–2009*

Feature	No. (%)	Median (IQR)
Sex, n = 88		
F	41 (47)	NA
M	47 (53)	NA
Age, y, n = 88		
<2	16 (18)	NA
2–4	27 (31)	NA
4–6	19 (22)	NA
6–15	26 (30)	NA
Symptoms/signs before and at admission		
Headache, n = 62	36 (58)	NA
Fever,† n = 85	76 (89)	NA
Temperature at admission, °C, n = 87	NA	38.2 (38.0–39.0)
Seizures, n = 87	71 (82)	NA
Altered mental status, n = 75	65 (87)	NA
Coma, n = 70	7 (10)	NA
Meningeal symptoms,‡ n = 67	57 (85)	NA
Limb paralysis, n = 75	4 (5)	NA
Cranial nerve palsy, n = 60	2 (3)	NA
Vomiting, n = 58	37 (64)	NA
Diarrhea, n = 52	4 (8)	NA
Days between onset and admission, n = 88	NA	1 (0–2)
Blood analysis	NA	NA
Leukocytes, $\times 10^9$ cells/L, n = 85	NA	17.5 (10.7–22.0)
Lymphocytes, %, n = 72	NA	19 (13–29)
Platelets, $\times 10^9$/L, n = 68	NA	254.5 (207.5–309.5)
Hemoglobin, g/L, n = 70	NA	114 (100–130)
Glucose, mmol/L, n = 72	NA	5.0 (3.2–6.1)
CSF sample		
Leukocytes, $\times 10^9$ cells/L, n = 16	NA	20 (12.5–85)
Lymphocytes, %, n = 18	NA	57 (40–75)
Protein level >0.5 g/L, n = 66	9 (14)	NA
Transparent appearance of CSF, n = 75	73 (97)	NA
Increased CSF pressure, i.e., >20 cm H_20, n = 70	35 (50)	NA
Cranial CT scan, n = 50		
Normal	29 (58)	NA
Diffuse edema	14 (28)	NA
Evidence of brain herniation	4 (8)	NA
Hemorrhagic stigma	3 (6)	NA
Intraparenchymal hypodensity	4 (8)	NA
Cortico-subcortical atrophy	8 (16)	NA
Liver enzymes, n = 17		
ALT, U/L	NA	42 (37–58)
AST, U/L	NA	58 (43–76)

*IQR, interquartile range; NA, not applicable; CSF, cerebrospinal fluid; CT, computed tomographic; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
†Observed by parents before admission or measured at admission (≥38°C).
‡Either meningitis signs, stiff neck, Kernig's sign, meningitis rash.

Luc Nam, Luc Ngan, and Son Dong districts, accounting for 56% of all cases. The annual number of cases per commune ranged from 0 to 6. For comparative analysis, we determined the mean proportion of the communes' surface devoted to litchi cultivation over the study period (Figure 3, panel B).

Clinical and Laboratory Results

Detailed clinical and biologic results were available for 88 patients (Table 2). This subgroup of patients did not significantly differ from the whole group for all common measured characteristics. Children were hospitalized within 1 day (median) after symptom onset; most had fever (89%), seizures (82%), and vomiting (64%). At admission, meningeal signs (85%), altered mental status (87%), or coma (10%) were commonly seen. During hospitalization, limb paralysis developed in 13% of children. Twenty-two (25%) of the 88 children died of the disease. Spinal tap was performed in 75 children; CSF was transparent for 73 (97%) children. Leukocyte count in CSF, available for 16 children, was slightly elevated (median 20 cells/mm^3 [reference <5 cells/mm^3]), and with lymphocyte predominance (median 57%). Cranial computed tomography scans were performed in 50 children; no change was detected in 58% of children; edema in 28%; and focal signs in 14%.

Results of Multiple Analyte Profiling are shown for selected analytes in the online Technical Appendix Figure (wwwnc.cdc.gov/EID/pdfs/11-1761-Techapp.pdf).

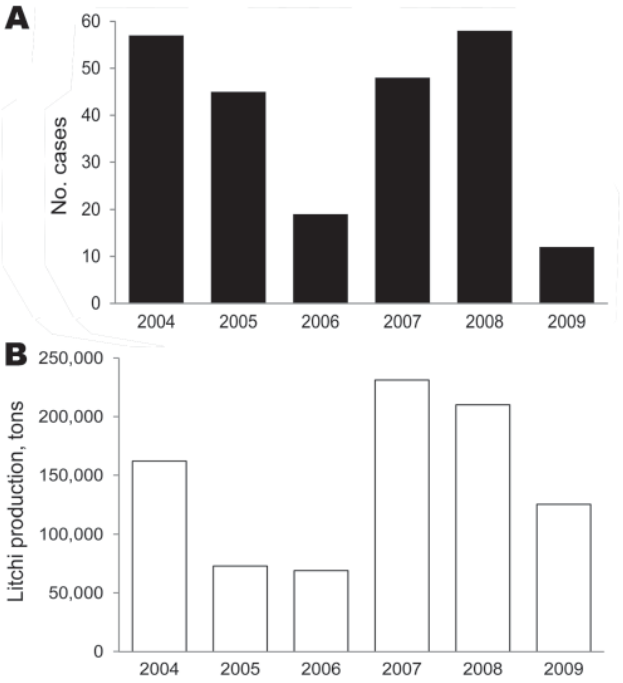

Figure 2. Temporal evolution of Ac Mong encephalitis (AME) and litchi cultivation, Bac Giang Province, Vietnam, 2004–2009. A) Annual number of AME cases; B) annual litchi production.

interferon-α_2 levels (mean ± SEM) were elevated in the serum of patients who survived AME (538.5 ± 196.6) compared with their sibling controls (56.15 ± 55.09) (p = 0.17). Interleukin-8 (IL-8) levels were elevated in the CSF of AME patients of both outcomes but were higher in those who died (930.3 ± 328.7) than in those who survived (237.6 ± 118.4) (p = 0.095). Serum IL-6 also was detected in patients of both outcomes, with higher levels in patients who died (132.2 ± 68.5) than in sibling controls (0.875 ± 0.425) (p = 0.029). IL-6 was elevated in the CSF of patients who died (430.1 ± 148.5) and in those who survived the disease (2151.8 ± 1934.6).

Ecologic Regression Results

The final multivariate model resulted in 3 factors independently associated with AME (Table 3). A positive association between disease incidence and litchi surface proportion was found: the IRRs were 1.52 (95% CI 0.90–2.57), 2.94 (95% CI 1.88–4.60), and 2.76 (95% CI 1.76–4.32) for second, third, and fourth quartiles, respectively, compared with the lowest quartile. A reduced risk was associated with density of poultry: the IRRs were 0.62 (95% CI 0.43–0.91), 0.61 (95% CI 0.42–0.89), and 0.25 (0.15–0.43) for second, third, and fourth quartiles, respectively, compared with the lowest quartile. Relative humidity was negatively associated with disease incidence: the IRRs were 0.84 (95% CI 0.61–1.16) and 0.35 (95% CI 0.23–0.54) for second and third terciles, respectively, compared with the lowest tercile.

Temporal Correlation between the Epidemics and the Litchi Harvests

Figure 4 displays the weekly distributions of AME cases and associated kernel densities for the districts that harvested litchis during May–June (group 1) and during June–July (group 2). The Mann-Whitney U test showed that the distributions of the week numbers differed significantly (p = 0.007) between the 2 groups, with a delay of 1 week for group 2 compared with group 1.

Discussion

We found evidence for a spatiotemporal association between the outbreaks of unknown encephalitis in Bac Giang Province and litchi cultivation. The ecologic regression analysis demonstrated that the annual risk for AME in a commune increased with the proportion of litchi-cultivated surface and that the epidemics occurred earlier in the districts that harvested litchis during May–June than in those that harvested litchis during June–July.

Similar to the clinical features of Chandipura encephalitis (*3*), i.e., acute encephalitis with rapid onset, those reported here suggest a viral etiology. Unfortunately, all viral investigations have thus far been uninformative. New techniques, such as high-throughput sequencing and resequencing micro-arrays, are currently performed on serum, CSF, culture supernatants, and brain homogenates from suckling mice inoculated intracerebrally with patients' CSF. Although electron microscopy of brain tissue is a standard method of identifying new viral pathogens in encephalitis syndromes, we were unable to obtain brain tissue specimens through autopsy because of cultural barriers.

The association between litchis and acute encephalitis remains unclear. As with other emerging viruses, we face a multifactorial problem that seems to have litchi fruit production and harvest as its focal point. One possible scenario is that fruit-bearing litchi trees can attract bats, which might be the reservoir for the putative pathogen. Mosquitoes could feed on the infected bats and transmit the virus to humans who have insufficient protection against mosquito bites. Several species of bats were identified in the province, such as the frugivorous bats *Rousettus leschenaultii*, which can feed on litchi. These bats' highly gregarious, cave dwelling, and migratory characteristics facilitate their role in virus carrying and circulation (*10*). The bat population density is high during April–September (which includes the time of litchi harvest) before migration during October–March.

In addition, several mosquito species were identified in Bac Giang Province: *Anopheles vagus*, *Armigeres*

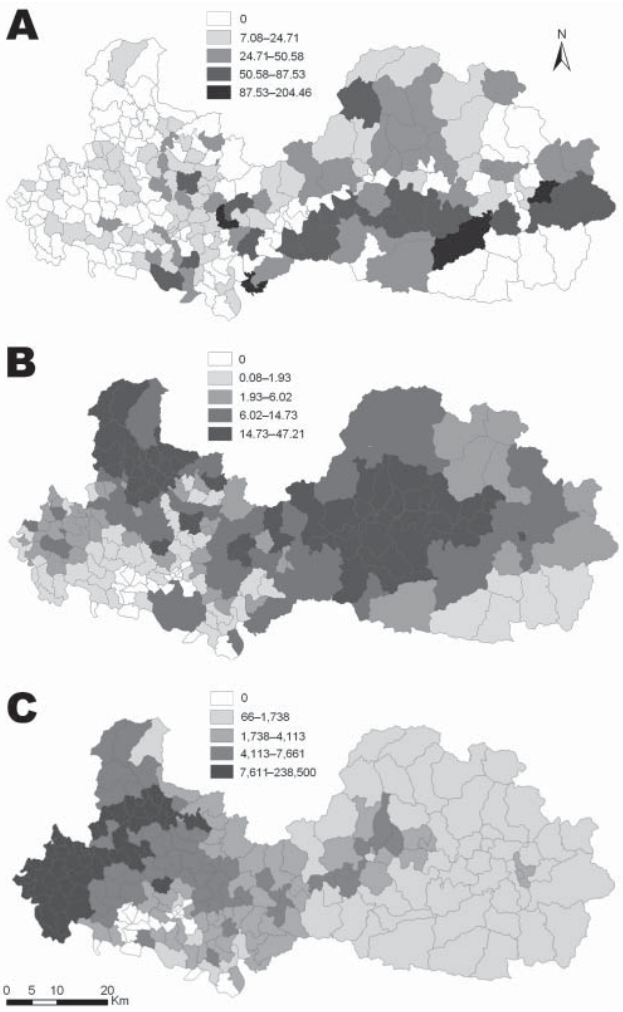

Figure 3. Commune-level maps of Bac Giang Province, Vietnam, 2004–2009. A) Cumulative incidence rate per 100,000 inhabitants of Ac Mong encephalitis. B) Mean percentage of commune surface area devoted to litchi cultivation. C) Mean poultry density (no. per km^2). A color version of this figure is available online (wwwnc.cdc.gov/EID/ article/18/11/11-1761-F3.htm).

subalbatus, *Culex tritaeniorhynchus*, *Cx. vishnui*, *Cx. gelidus*, *Cx. fuscocephalus*, *Cx. quinquefasciatus*, and *Cx. bitaeniorhynchus*. Although the peak incidence of May–July correlates with the rice paddy breeding and development of *Culex* spp. mosquitoes, the paddy fields area was negatively associated with the risk for disease in univariate analysis. No data were collected on vector densities in Bac Giang.

Other modes of transmission, e.g., direct contact with litchis contaminated by bat saliva, urine, or guano (*11*) or with other vectors, such as insects found in litchi trees or phlebotomine sand flies, as in the case of Chandipura virus (*12*), cannot be excluded. Deforestation in Bac Giang to develop the growing of litchi trees because of their high economic value also might have disrupted the ecologic equilibrium of the province, leading to the emergence of a new vector-borne disease (*13*). The putative virus also might be exclusively human; use of human feces as fertilizers to enhance litchi growth in these plantations might have contaminated the soil with enteroviruses, which are known to cause fatal encephalitis in deprived children (*14*).

Last, a toxic origin might be possible; in India, a toxic weed, *Cassia occidentalis*, caused an acute hepatomyoencephalopathy syndrome, which was first assumed to be viral encephalitis (*15*). However, in our study, the presence of fever and meningeal symptoms and the absence of high elevation of liver enzyme favor a viral etiology rather than a toxic origin. Additionally, we demonstrated that children with AME harbor elevated levels of type I interferon in serum and interferon-inducible cytokines, such as IL-6 and IL-8 in CSF, compatible with an infectious process (*16*,*17*); however, these cytokines are expected to be elevated in any inflammatory process.

The protective effects of poultry density and mean relative humidity during January–April (pre–epidemic period) are more difficult to interpret. With respect to the hypothesis of an arbovirus-mediated pathogenesis, a possibility is that poultry is a preferred host for the putative vector (*18*). Regarding negative correlation with humidity, above a certain limit, high relative humidity can be harmful to insects; insects or their eggs can drown or be infected more readily by pathogens (*19*). Litchi growing also depends on climate; ideal conditions include a brief dry, cool, and frost-free winter to lead to flowering, followed by warmer temperatures and moderate rainfall and humidity during fruit development and harvest (*20*). Still, climatic variations alone could not explain the spatial differences in disease risk between communes of the same province. Moreover, even if the association is not proved to be causal, the persistence of both variables (litchi surface and mean

Table 3. Multivariate analysis of Ac Mong encephalitis, Bac Giang Province, Vietnam, 2004–2009

Variable/category	IRR* (95% CI)	p value†
Litchi surface, % commune surface		
<1.49	1	<0.001
1.49–5.84	1.52 (0.90–2.57)	
5.84–12.92	2.94 (1.88–4.60)	
>12.92	2.76 (1.76–4.32)	
Poultry density, no./km^2		
<1,449.79	1	<0.001
1,449.79–3,715.18	0.62 (0.43–0.91)	
3,715.18–7,065.25	0.61 (0.42–0.89)	
>7,065.25	0.25 (0.15–0.43)	
Mean humidity, %, January–April		
<82.07	1	<0.001
82.07–83.55	0.84 (0.61–1.16)	
>83.55	0.35 (0.23–0.54)	

*IRR, incidence rate ratio.
†Indicates global p value.

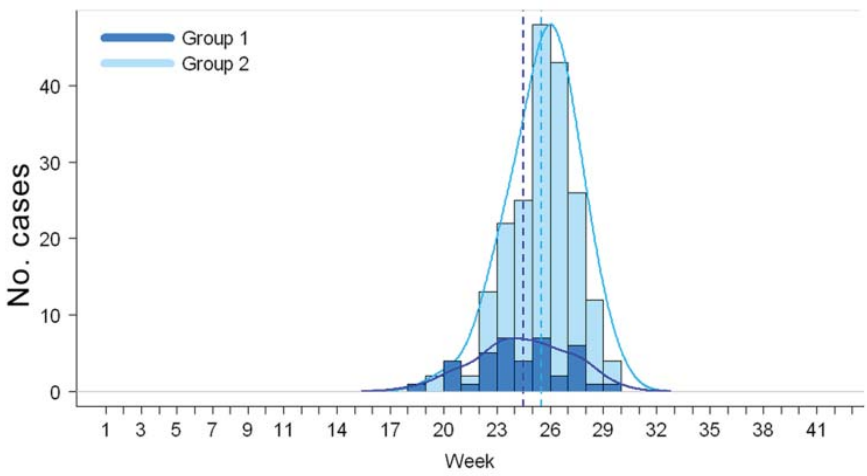

Figure 4. Distribution of weekly numbers of Ac Mong encephalitis cases, Bac Giang Province, Vietnam, 2004–2009, by 2 groups of districts: districts that harvested litchi during May–June (group 1) and districts that harvested litchi during June–July (group 2). Kernel densities are shown in solid lines and medians in dotted lines. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/11-1761-F4.htm).

humidity) in the final model suggests that both factors are independently correlated with the spatiotemporal patterns of the disease. Definitive identification of the infectious agent will help clarify these factors associated with AME incidence.

Because our study concerns an ecologic investigation, the relationship between litchis and AME cannot be inferred at an individual level. Another caveat of the study concerns the nonspecific case definition based on clinical features and negative JEV serologic test results. False-negative results have been observed for JEV serologic tests performed soon after onset of symptoms (*21*); nevertheless, the widespread use of JE vaccination, introduced in 1997, has considerably reduced JE incidence in the region. In addition, after JE vaccine introduction in the WHO Expanded Program on Immunization, AES surveillance might have been intensified, and an apparent increased incidence of AES might be simply an artifact of more active surveillance.

Artifact is not likely to account for the cases described in this manuscript, however, because they were identified clinically among patients seeking care at the hospital with AES rather than through public health surveillance. Conversely, surveillance might have failed to capture some cases because of the rapid deaths of infected children (e.g., those who died before reaching the hospital).

Apart from these cases, given the severity of the disease, we can assume that all parents, including those who lived in remote villages, sought medical attention. Unfortunately, because of logistical difficulties, systematic data and sample collection and analysis could not be conducted for all case-patients in Bac Giang hospital. Nevertheless, we have no reason to believe that cases for which biologic data were available (Table 2) differed from other cases.

Our ecologic analysis of outbreaks of acute encephalitis of unknown origin during the litchi harvest period in Bac Giang Province strengthens the hypothesis that litchis might play a role in these outbreaks by showing that litchi cultivation was spatially and temporally associated with AME. This finding can be useful to guide future prospective studies. The suggested role of litchi trees needs to be more thoroughly investigated to explain disease ecology. Further research should include investigating the specific agricultural practices linked to litchi cultivation, distribution of tasks among adults and children, locations where these activities are conducted, and other activities undertaken around litchi fields before and during the epidemic period; conducting entomologic surveys around litchi plantations; and analyzing potential reservoirs and hosts. Last, research efforts should be continued to identify the causative agent.

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Community Outbreak of Adenovirus, Taiwan, 2011

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In 2011, a large community outbreak of human adenovirus (HAdV) in Taiwan was detected by a nationwide surveillance system. The epidemic lasted from week 11 through week 41 of 2011 (March 14–October 16, 2011). Although HAdV-3 was the predominant strain detected (74%), an abrupt increase in the percentage of infections caused by HAdV-7 occurred, from 0.3% in 2008–2010 to 10% in 2011. Clinical information was collected for 202 inpatients infected with HAdV; 31 (15.2%) had severe infection that required intensive care, and 7 of those patients died. HAdV-7 accounted for 10%, 12%, and 41% of infections among outpatients, inpatients with nonsevere infection, and inpatients with severe infection, respectively ($p<0.01$). The HAdV-7 strain detected in this outbreak is identical to a strain recently reported in the People's Republic of China (HAdV7-HZ/SHX/CHN/2009). Absence of circulating HAdV-7 in previous years and introduction of an emerging strain are 2 factors that caused this outbreak.

Human adenoviruses (HAdVs) are DNA viruses that can cause a variety of human diseases. Since the discovery of these viruses in 1953, more than 50 types have been isolated, some directly linked to specific human diseases, e.g., infantile diarrhea (HAdV 40, 41), epidemic keratoconjunctivitis (HAdV 8, 19, 37, 54), and hemorrhagic cystitis (HAdV 11, 21) (*1*–*3*). Adenoviruses also are common causes of lower respiratory tract infections in children (*4*), but surveillance for these infections is lacking in most countries. Pneumonia caused by adenoviruses cannot be easily differentiated from other types of viral pneumonia, and culturing and typing of adenoviruses is not routinely performed in hospital or public health laboratories. Therefore, community-wide outbreaks of adenovirus are not easily detected; previous reports are limited to those occurring in hospital, school, or military settings (*5*–*7*).

In 1999, after a 1998 enterovirus 71 epidemic, the Taiwan Centers for Disease Control established a nationwide surveillance system using contract virologic laboratories (CVLs) to perform continuous virologic surveillance for respiratory viruses, especially influenza and enteroviruses (*8*). The network consists of 12 CVLs located in the northern, central, southern, and eastern regions of Taiwan (*9*). Early in 2011, the percentage of adenovirus isolated among all respiratory virus isolates evaluated by the CVLs increased from a baseline of 0%–5% to 10% and remained high in the following weeks, indicating a community-wide adenovirus outbreak. The apparent outbreak prompted us to use several existing surveillance systems to describe the characteristics of this outbreak.

Methods

Virologic Surveillance of Outpatients

For respiratory virus surveillance, throat/nasal swabs from outpatients with influenza-like illness (ILI) are collected by sentinel physicians and cultured in the CVL of the corresponding region on a weekly basis. Virus isolates found are then sent to the reference laboratory at the Taiwan Centers for Disease Control for identification, sequencing, and typing. For this study, we reviewed virologic surveillance data from week 1 of 2008 through

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week 43 of 2011 (January 1, 2008–October 30, 2011). The weekly adenovirus-positive rate is defined as the number of adenoviruses isolated from respiratory tract specimens divided by the number of all specimens submitted from patients with ILI for respiratory virus surveillance in the corresponding week. The mean positive rate in 2008–2010 was used as the baseline adenovirus-positive rate, and the epidemic threshold was defined as the baseline adenovirus-positive rate + 2 SD. The start and end of the epidemic were defined accordingly.

Clinical Case Surveillance for Inpatients

We conducted a retrospective study of adenovirus-infected children treated as inpatients in the National Taiwan University Hospital (NTUH), a tertiary hospital in northern Taiwan. We enrolled all patients ≤18 years of age admitted to the NTUH pediatric department from November 1, 2010 (week 44, 2010), through June 30, 2011 (week 26, 2011), who had adenovirus infection identified by virus isolation or PCR from respiratory specimens. All virus isolations and PCRs were performed in the NTUH laboratory, 1 of the 12 CVLs.

All medical records of enrolled inpatients were reviewed. Demographics, medical history, clinical signs and symptoms, diagnoses, and treatments were recorded by using a structured questionnaire. Patients who had been admitted to the intensive care unit were classified as having severe infection; all other patients were classified as having nonsevere infection.

Virus Culture, Identification, and Typing

We typed selected adenovirus isolates collected as part of the virologic surveillance program and all isolates obtained from inpatients from NTUH. We also performed real-time PCR for viral load quantification by using LightCycler (Roche Diagnostics, Mannheim, Germany) for selected patients from the NTUH, according to the method described by Heim et al. (*10*).

Throat/nasal swab specimens from all patients were injected into human embryonic lung fibroblasts, Hep-2, RD, MK-2, and MDCK cells. If a cytopathic effect was observed, the presence of adenovirus was further confirmed by direct immunofluorescence staining with a virus-specific monoclonal antibody. DNA was extracted from the clinical samples by using the QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany); PCR was then conducted targeting a 956-bp region of the hexon gene for typing.

For genetic analyses of HAdV-7 isolates, DNA fragments of the hexon and fiber genes were amplified by PCR. Multiple sequence alignments, protein translation, and phylogenetic analysis were performed on the basis of the nucleotide sequences by using MEGA4 (*11*) and BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html). For phylogenetic analyses, we included the full-length sequences of the hexon and fiber genes (2,805 bp and 978 bp, respectively) from 5 HAdV-7 isolates collected in 2011 in Taiwan and some reference sequences available in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/genbank). A phylogenetic tree was constructed by the neighbor-joining method, and 1,000 bootstrap replications were performed to evaluate the reliabilities of the relationships.

Statistical Analysis

Data from inpatients with versus without severe infection were compared by using the χ^2 or Fisher exact test for categorical variables and the Mann-Whitney U test for continuous variables. A p value <0.05 was considered significant. All statistical operations were 2-tailed and were performed with SPSS version 19.0 software (IBM, Somers, NY, USA).

Results

Virologic Surveillance for Outpatients

From week 1 of 2008 through week 43 of 2011, an average of 276 respiratory tract specimens from ILI outpatients were collected each week by the CVLs (range 27–1,028, SD 130). Weekly adenovirus-positive rates are shown in Figure 1. The baseline adenovirus-positive rate in 2008–2010 was 5.75% (range 0%–13.1%, SD 3.37%); the epidemic started in week 11 and ended in week 41 of 2011. Mean adenovirus-positive rate during the epidemic was 25.9%, with a peak of 37.3% during week 21 of 2011. Ninety-seven percent of all specimens were collected from patients ≤18 years of age.

We typed 883 adenovirus isolates collected during the study period from outpatients ≤18 years of age; temporal distribution is shown in Figure 2. HAdV-3 was the dominant circulating type during 2008–2009 (>50% of all isolates), but in 2010, the proportion of HAdV-3 decreased to ≈30%, similar to that for HAdV-2 and HAdV-1. The proportion of HAdV-5 was consistently ≈5% in 2008–2010, and all other types of adenoviruses were rarely isolated. Among the 844 adenovirus isolates collected during 2008–2010 that were typed, only 3 (0.3%) were HAdV-7; during this outbreak, the proportion of HAdV-7 increased significantly, to 10% (p<0.001). The proportion of HAdV-3 also increased, from 30% to 74%, while the proportions of all other types decreased significantly (p<0.001).

Clinical Case Surveillance for Inpatients

Epidemiology and Typing

We enrolled 202 inpatients at the NTUH pediatric department who tested positive for adenovirus from

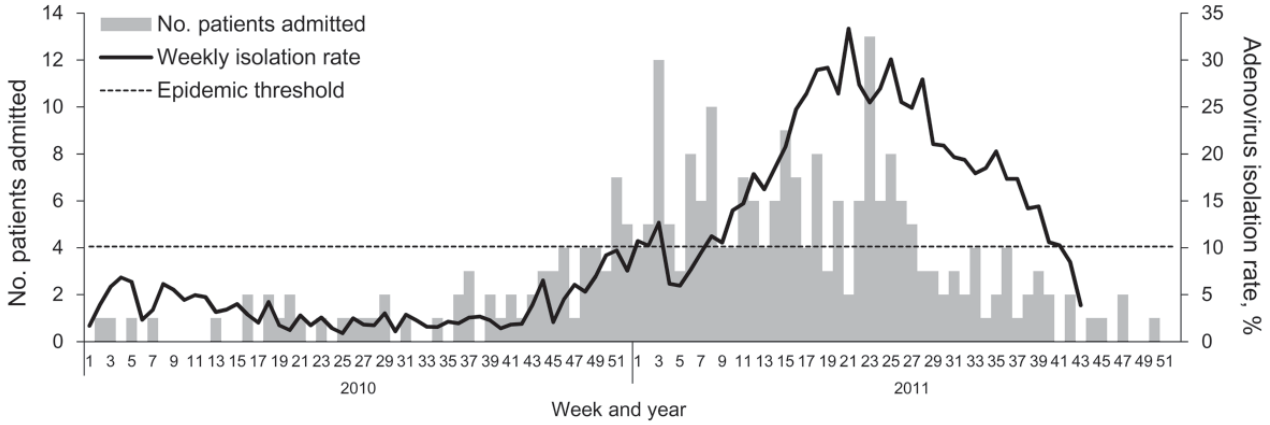

Figure 1. Weekly adenovirus-positive rates for respiratory specimens from patients with influenza-like illness sent to contract virologic laboratories at the Taiwan Centers for Disease Control and weekly number of inpatients infected with adenovirus in the pediatric department of National Taiwan University Hospital, Taipei City, Taiwan, week 1, 2010–week 43, 2011 (January 1, 2010–October 30, 2011). Weekly adenovirus positive rate = no. adenovirus isolates from respiratory tract specimens / no. all specimens submitted to contract virologic laboratories from outpatients with influenza-like illness for respiratory virus surveillance in the corresponding week.

November 1, 2010 (week 44, 2010), through June 30, 2011 (week 26, 2011). All but 3 of these patients had positive test results for adenovirus by culture; the remaining 3 patients had positive test results by PCR only. The number of hospitalized patients increased following week 43 of 2010 and remained high in the following months, a trend that is congruent with virologic surveillance results (Figure 1). Of the 202 patients, 31 had severe infections requiring admission to the intensive care unit and 171 had nonsevere infections.

Hexon gene sequencing showed that HAdV-3 accounted for 64% of all isolates among inpatients; HAdV-7 (19%), HAdV-2 (4%), and HAdV-1 (2%) were the next most prevalent types. HAdV-5, HAdV-6, and HAdV-37 accounted for 2 isolates each (1%). Sixteen isolates (8%) were not typeable because the specimens were inadequate.

The dominant HAdV type differed for patients with versus without severe infection. HAdV-3 infection was more common among patients with nonsevere versus severe infection (68% vs. 42%; p<0.001), whereas HAdV-7 was the dominant type among patients with severe versus nonsevere infection (45% vs. 14%; p<0.001). In the nonsevere infection group, other identified adenovirus types were HAdV-2 (4%), HAdV-1 (2%), HAdV-5 (1%), HAdV-6 (1%), and HAdV-37 (1%). In the severe infection group, only HAdV-2 (10%) and HAdV-6 (3%) were identified in addition to HAdV-7 and HAdV-3.

Demographics and Associated Conditions

Demographic data on inpatients is shown in Table 1. Median age was 40 months (range 1–189 months); the male:female ratio was 1.3:1. Most (59%) patients had a history of contact before illness onset with patients with upper respiratory symptoms in school or in the household. Patients with severe infection had longer hospital stays than did those with nonsevere infection (median 18 vs. 5 days; p<0.001). Thirty-eight percent of all patients had chronic underlying conditions before admission, and more patients with severe infection had underlying diseases, particularly cardiopulmonary and neurologic diseases, than did those with nonsevere infection (77% vs. 30%; p<0.001).

Clinical and Laboratory Characteristics

Clinical and laboratory characteristics of HAdV infections among inpatients are shown in Tables 2 and 3. Fever (98%), cough (85%), and coryza (84%) were the most common symptoms. Compared with patients who had nonsevere infection, patients with severe infection had a longer fever duration (median 11 vs. 5 days; p<0.001) and a higher peak temperature (median 39.8 vs. 39.3°C; p = 0.008). Patients with severe infection also had more signs and symptoms of lower respiratory tract involvement than

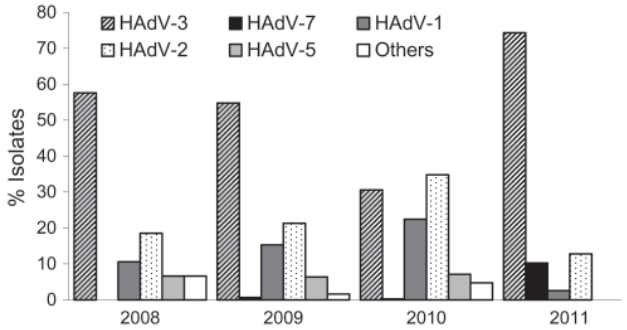

Figure 2. Distribution of adenovirus (HAdV) types in respiratory samples collected from outpatients <18 years of age by contract virologic laboratories in Taiwan, 2008–2011. A color version of this figure is available online (wwnc.cdc.gov/EID/article/18/11/12-0629.htm).

Table 1. Characteristics of 202 children hospitalized for adenovirus infection at National Taiwan University Hospital, by disease severity, Taipei City, Taiwan, November 2010–June 2011*

Characteristic	All	Severe infection, n = 31	Nonsevere infection, n = 171	p value
Median age, mo (range)	40 (1–189)	36 (1–185)	41 (2–189)	0.287
Male sex	115 (57)	21 (68)	94 (55)	0.186
Contact history	119 (59)	15 (48)	104 (61)	0.196
Median hospitalization, d (range)	5 (1–126)	18 (4–126)	5 (1–44)	<0.001
Death rate	7 (4)	7 (23)	0 (0)	<0.001
Any underlying disease	76 (38)	24 (77)	52 (30)	<0.001
Prematurity	36 (18)	8 (26)	28 (16)	0.207
Cardiopulmonary†	31 (15)	12 (39)	19 (11)	<0.001
Neurologic‡	25 (12)	19 (61)	6 (4)	<0.001
Hematologic§	2 (1)	1 (3)	1 (0.6)	0.284
Metabolic¶	7 (4)	4 (13)	3 (2)	0.012
Immunodeficiency#	1 (0.5)	1 (3)	0 (0)	0.153

*Values are no. (%) patients except as indicated. Severe infection indicates patients who were admitted to the intensive care unit.
†Congenital heart diseases (12), asthma (11), chronic lung disease (4), tracheobronchial stenosis (2), and laryngo-tracheo-bronchomalacia (2).
‡Cerebral palsy, epilepsy, and psychomotor retardation.
§Acute myeloid leukemia and Burkitt's leukemia.
¶Pompe disease, metachromatic leukodystrophy, glycogen storage disease, isovaleric acidemia, short-chain acyl-coA dehydrogenase deficiency, renal tubular acidosis, and mitochondrial disease.
#Hypogammaglobulinemia.

did patients with nonsevere infection, e.g., dyspnea (90% vs. 21%; p<0.001), rales (84% vs. 40%; p<0.001), wheezing (61% vs. 18%; p<0.001), and patches or consolidation on a radiograph (71% vs. 19%; p<0.001). In contrast, exudative tonsillitis was more common for patients with nonsevere than with severe infection (41% vs. 13%; p = 0.003). One fourth of all patients had gastrointestinal manifestations, regardless of infection severity.

Compared with nonsevere infection patients, patients with severe infection had lower hemoglobin levels (9.7 vs. 11.8 mg/dL; p<0.001) and more frequently had leukopenia (leukocyte count $<5 \times 10^3$ cells/μL; 58% vs. 6%; p<0.001) and thrombocytopenia (platelet count $<150 \times 10^3$/μL; 77% vs. 11%; p<0.001). Lactate dehydrogenase level was elevated in all patients, but the level was significantly higher among those in the severe infection group (3,281 vs. 937 U/L; p = 0.02). Serum sodium levels tended to be low in all patients, but hyponatremia was significantly more common for patients with severe versus nonsevere infection (81% vs. 41%; p<0.001).

Adenovirus viral load was determined by real-time PCR from throat swabs (n = 26), sputum (n = 5), or pleural effusion (n = 10). Median copy numbers were 2.00×10^7 (range 1.60×10^5–3.00×10^9) for throat swabs, 5.30×10^8 (range 3.10×10^5–9.90×10^{11}) for sputum, and 7.95×10^8 (range 4.80×10^7–2.40×10^{10}) for pleural effusion. Viral load did not differ between patients with severe versus nonsevere infection.

Table 2. Clinical signs and symptoms of 202 children hospitalized for adenovirus infection at National Taiwan University Hospital, by disease severity, Taipei City, Taiwan, November 2010–June 2011*

Signs and symptoms	All	Severe infection, n = 31	Nonsevere infection, n = 171	p value
Fever	198 (98)	31 (100)	167 (98)	1.000
Duration, d (range)	6 (0–28)	11 (1–28)	5 (0–20)	<0.01
Median peak temperature, °C (range)	39.4 (37.0–41.2)	39.8 (38.7–40.7)	39.3 (37.0–41.2)	0.008
Cough	172 (85)	27 (87)	145 (85)	1.000
Coryza	170 (84)	23 (74)	147 (86)	0.111
Dyspnea	64 (32)	28 (90)	36 (21)	<0.01
Abdominal pain	30 (15)	6 (19)	24 (14)	0.419
Vomiting	51 (25)	6 (19)	45 (26)	0.412
Diarrhea	57 (28)	10 (32)	47 (28)	0.587
Sore throat	47 (23)	5 (16)	42 (25)	0.307
Conjunctivitis	35 (17)	2 (7)	33 (19)	0.082
Exudative tonsillitis	74 (37)	4 (13)	70 (41)	0.003
Rash	15 (7)	3 (10)	12 (7)	0.707
Abnormal breath sound				
Rales	95 (47)	26 (84)	68 (40)	<0.01
Wheeze	49 (24)	19 (61)	30 (18)	<0.01
Chest radiograph finding				
Infiltrate	168 (70)	25 (81)	106 (62)	0.045
Patch/consolidation	92 (39)	22 (71)	33 (19)	<0.01
Pleural effusion	6 (3)	3 (10)	3 (2)	0.016

*Values are no. (%) patients except as indicated. Severe infection indicates patients who were admitted to the intensive care unit.

Table 3. Laboratory test results for 202 children hospitalized for adenovirus infection at National Taiwan University Hospital, by disease severity, Taipei City, Taiwan, November 2010–June 2011*

Laboratory results and no. patients tested	All	Severe infection, n = 31	Nonsevere infection, n = 171	p value
Hemoglobin, g/dL (range), n = 201	11.7	9.7 (3.4–13.8)	11.8 (7.0–14.6)	<0.001
Platelets, × 10^3/μL (range), n = 201	237	106 (11–340)	251(14–555)	<0.001
Thrombocytopenia, no. (%) patients†	43 (21)	24 (77)	19 (11)	<0.001
Leukocytes, × 10^3 cells/μL (range), n = 201	10.06	4.4 (1.2–10.3)	11.3 (0.2–35.5)	<0.001
Leukopenia, no. (%) patients‡	28 (14)	18 (58)	10 (6)	<0.001
Sodium, mmol/L (range), n = 138	135	131 (119–137)	135 (127–142)	<0.001
Hyponatremia, no. (%) patients§	68 (49)	22 (81)	46 (41)	<0.001
C-reactive protein, mg/dL (range), n = 200	4.15	6.1 (0.02–30.00)	4.0 (0–47.2)	0.086
LDH, U/L (range), n = 22	2,202	3,281 (1,029–9,782)	937 (493–17,454)	0.023
AST, U/L (range), n = 117	43	135 (38–3,520)	38 (3.5–989)	<0.001
AST >2× upper limit, no. (%) patients	36 (31)	22 (85)	14 (15)	<0.001
ALT, U/L (range), n = 104	26.5	67.5 (12.0–511.0)	24 (8–652)	<0.001
ALT >2× upper limit, no. (%) patients	24 (23)	15 (58)	9 (12)	<0.001
Creatinine, mg/dL (range), n = 155	0.61	0.68 (0.3–1.1)	0.6 (0.2–0.9)	0.088

*Severe infection indicates patients who were admitted to the intensive care unit. LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
†Platelet count <150,000/μL.
‡Leukocyte count <5,000 cells/μL.
§Serum sodium <135 mmol/L.

Diagnosis, Treatment, and Prognosis

The most common clinical diagnosis among patients in this outbreak was pneumonia/bronchopneumonia (31.3%); other notable conditions diagnosed were acute tonsillitis (20.1%), acute sinusitis (9.6%), acute otitis media (9.3%), acute gastroenteritis (5.6%), upper respiratory tract infection (4.4%), pharyngoconjunctival fever (4.0%), acute bronchiolitis/bronchitis (4.0%), encephalitis (1.9%), empyema (0.9%), and croup (0.3%). One child in whom Kawasaki disease was initially diagnosed later was determined to be infected with HAdV-3. HAdV-6 was isolated from the throat of a child in whom acute myocarditis had been diagnosed. Other diagnoses included orbital cellulitis, viral exanthem, acute hepatitis, young infant fever, and febrile convulsions.

Parenteral or oral antimicrobial drugs were given to 73.8% of patients before adenovirus infection was diagnosed. Ten percent of patients required mechanical ventilation during hospitalization; all were in the severe group. Intravenous immunoglobulin (IVIG) was given to 6 patients (3.0%) for diagnoses of acute myocarditis (n = 1), empyema (n = 1), acute respiratory distress syndrome (n = 3), and Kawasaki disease (n = 1). Extracorporeal membrane oxygenation was used for 5 patients with respiratory or cardiovascular failure; 3 survived.

Seven patients died during this outbreak: 4 patients infected with HAdV-7, 2 with HAdV-3, and 1 with HAdV-2. All of these patients were in the severe group and had underlying diseases; 6 were bedridden before hospital admission. During hospitalization, all of these patients had secondary bacterial pneumonia develop; pathogens involved were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Escherichia coli*.

Molecular Studies on Adenovirus Type 7

The hexon gene sequences of HAdV-7 isolates from virologic surveillance and clinical case surveillance, including patients with severe and nonsevere infection, were determined and compared with strains isolated previously in Taiwan and other countries. Hexon nucleotide sequences of 5 HAdV-7 isolates collected in 2011 in Taiwan were identical to the sequence of strain HAdV7-HZ/SHX/CHN/2009 (*6*,*12*), differed by 1 bp of synonymous mutation from genotype 7d of the 383 strain isolated in Japan in 1992, and had 99.7% identity to the sequence of the prototype strain S1058 (Figure 3, panel A). Fiber sequences of these isolates were identical to those of the 383 and Bal strains from Japan, isolated in 1992 and 1995, respectively, and had 99.5% identity to the prototype stain S1058 (Figure 3, panel B). We found no substantial differences between the HAdV-7 isolates collected from outpatients as part of the virologic surveillance program and the isolates from inpatients with severe or nonsevere infection.

Discussion

Adenoviruses circulate year-round in Taiwan, and several community outbreaks have been reported. During 1999–2001, outbreaks were caused, consecutively, by HAdV-7, HAdV-3, and HAdV-4 (*13*); another HAdV-3 outbreak was reported during 2004–2005 (*14*). For these outbreaks, the adenovirus-positive rate increased from 7% during the baseline period to 14%–16% during the outbreak.

The outbreak of co-circulating HAdV-3 and HAdV-7 we report is unique in several ways. The high positive rate during the weeks of the epidemic made this outbreak among the largest reported community-wide adenovirus outbreaks, and the reemergence of HAdV-7 in Taiwan

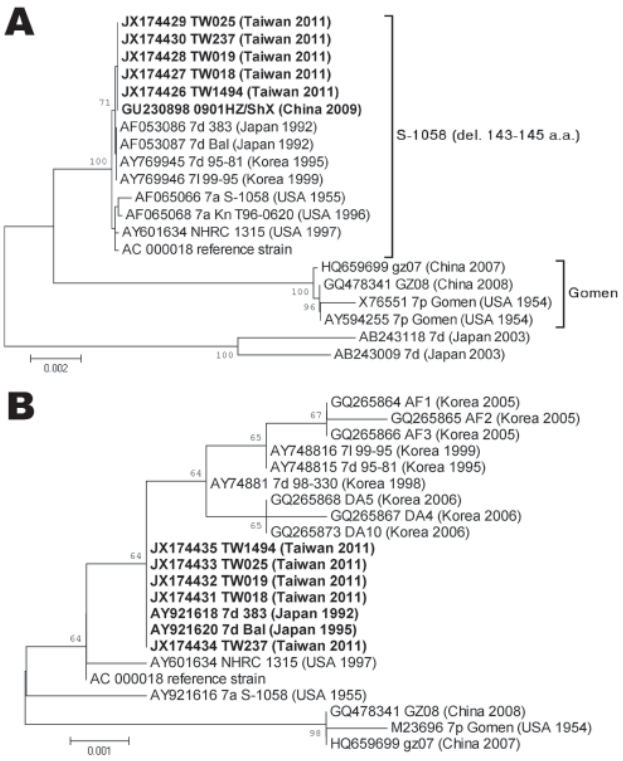

Figure 3. Phylogenetic analysis of hexon (A) and fiber (B) genes of human adenovirus (HAdV) type 7 isolates. Coding sequences of hexon and fiber genes (2,805 and 978 bp) from 5 HAdV isolates from Taiwan in 2011 and reference sequences from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/genbank) were included. Phylogenetic trees were constructed from aligned sequences by using the neighbor-joining method; 1,000 bootstrap replications were performed to evaluate the reliabilities. Bootstrap values are shown at branching points. Taxon names include accession number, available genotype, strain name, isolation country, and year. **Boldface** indicates the isolates collected in Taiwan and reference isolates with identical sequences. TW1494 is from an outpatient; TW018, 019, 025, and 237 are from inpatients. del., deletion. Scale bars indicate nucleotide substitutions per site.

10 years after the last outbreak contributed to severe infection.

HAdV-3 and HAdV-7 are both capable of causing outbreaks but have different circulation patterns. HAdV-3 circulates endemically and causes outbreaks, whereas HAdV-7 is mainly detected during outbreaks (*15*,*16*). DNA restriction analysis has been used to further characterize adenoviruses, and shifts in or replacement of the predominant genome types over time have been reported to be related to outbreaks (*17*,*18*). For a novel genome type of HAdV-7 to spread rapidly and cause a large outbreak, an immunologically naive population and a means of introduction to the susceptible community are needed, and the novel virus must have a greater biologic fitness than that of other circulating strains (*18*). During the 1999 outbreak in Taiwan, 40% of adenovirus isolates were HAdV-7. The proportion of HAdV-7 among all adenoviruses decreased to 20% in 2000, <5% in 2001, and <1% in 2004–2005 and 2008–2010 (*13*,*14*). The near-absence of circulating HAdV-7 in 2001–2010 indicates that a large population was naive to this virus, predisposing that population to the 2011 outbreak.

Genome typing showed that HAdV-7a was the circulating strain in Taiwan in 1983 but was replaced by HAdV-7b by 1999–2001 (*13*). However, phylogenetic analysis of the entire hexon gene showed that the strain from this outbreak has the highest homology with HAdV-7d and HAdV-7d2, which had not reported in Taiwan (*6*). HAdV-7d has been the predominant circulating virus in China since the early 1980s and caused a large outbreak in South Korea during 1995–1997 (*16*). The closely related genotype HAdV-7d2 was reported in Israel in 1992 and has caused outbreaks in chronic care facilities and in communities in several countries (*6*,*19*–*21*). Despite the early introduction of HAdV-7d to neighboring countries, HAdV-7d had not been found in Taiwan until 2011 (*13*).

We sequenced the entire fiber gene, the product of which contributes to virus attachment and infection, to further characterize the circulating strain (*22*). Phylogenetic analysis (Figure 3) showed no substantial genome difference between this HAdV-7d strain and other previously reported HAdV-7d strains. We do not know the means of introduction of this strain into Taiwan, but we believe that an emerging strain and the large, naive population are 2 factors that contributed to this outbreak.

HAdV-7 has been reported to have a strong association with severe illness (*23*). Studies of outbreaks caused by the co-circulation of HAdV-3 and HAdV-7 offer a unique opportunity for us to compare the disease severity resulting from infection with these 2 virus types, but conflicting results have been obtained (*7*,*13*,*24*). During this outbreak, although HAdV-3 was the predominant strain, patients infected with HAdV-7 exhibited more severe disease. HAdV-3 accounted for 74%, 62%, and 41% of all isolates in outpatients, inpatients with nonsevere infection, and inpatients with severe infection, respectively, compared with 10%, 12%, and 41%, respectively, for HAdV-7 ($p<0.01$). Our study offers strong evidence that HAdV-7 is significantly associated with severe infection in a community outbreak setting.

Of the 202 inpatients we analyzed, 31 (15%) had severe infection that required intensive care. As previously reported, children with underlying conditions were more likely to have severe disease develop, especially patients with neurologic, respiratory, and metabolic abnormalities (*23*,*25*–*27*). More than half of inpatients had a history of household or school contact with persons with upper respiratory symptoms before admission, which is an

evidence of the high transmissibility of the virus and the widespread nature of the current outbreak.

Inpatients with severe infection had higher fever, longer fever duration, and more evident lower respiratory tract involvement than those with nonsevere infection. Viremia can be found in up to 72% of immunocompetent children during first infection with HAdV (*28*); our laboratory data revealed that adenovirus-infected patients had more profound systemic involvement than was reflected by clinical signs and symptoms, which may have resulted from viremia. Although renal function remained relatively good, other manifestations (e.g., anemia, leukopenia, thrombocytopenia, elevated serum transaminase and lactate dehydrogenase levels) were indicators of multiorgan involvement. Eighty percent of patients with severe infection had hyponatremia, which suggests a syndrome involving inappropriate antidiuretic hormone (*29*). Viral load in respiratory tract specimens did not differ for patients with severe infection versus nonsevere infection, which suggests host factors may be contributors to severe illness.

Compared with that for other reported outbreaks, the mortality rate we found was relatively low (3.5% for all hospitalized patients) (*5*). Good supportive/intensive care and advanced life support, including the use of extracorporeal membrane oxygenation, are possible explanations. With no proven treatment, various medications, including ribavirin (*30*), cidofovir (*31*) and vidarabine (*32*), and IVIG (*33*), have been used to treat immunocompromised patients who have severe adenovirus infection; results have been inconsistent. Six patients in our study received IVIG and 4 of them died, possibly because of severe underlying conditions. We are not able to draw any conclusions regarding the treatment efficacy of IVIG from this small case series; further study is needed.

In conclusion, by using data from public health and hospital-based surveillance programs, we described a large community outbreak caused by circulating HAdV-3 and emerging HAdV-7. We confirmed the severity of HAdV-7 infection and illustrated the epidemic nature of its circulation. Public health surveillance systems should continue to monitor the molecular epidemiology of adenoviruses to detect outbreaks early.

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Antigenic Diversity of Enteroviruses Associated with Nonpolio Acute Flaccid Paralysis, India, 2007–2009

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Because of the broadened acute flacid paralysis (AFP) definition and enhanced surveillance, many nonpolio AFP (NP-AFP) cases have been reported in India since 2005. To determine the spectrum of nonpolio enterovirus (NPEV) serotypes associated with NP-AFP from polio-endemic and -free regions, we studied antigenic diversity of AFP-associated NPEVs. Of fecal specimens from 2,786 children with NP-AFP in 1 polio-endemic and 2 polio-free states, 823 (29.5%) were positive for NPEVs in RD cells, of which 532 (64.6%) were positive by viral protein 1 reverse transcription PCR. We identified 66 serotypes among 581 isolates, with enterovirus 71 most frequently (8.43%) detected, followed by enterovirus 13 (7.1%) and coxsackievirus B5 (5.0%). Most strains within a serotype represented new genogropups or subgenogroups. Agents for ≈35.0% and 70.0% of culture-positive and -negative cases, respectively, need to be identified. Association of human enterovirus with NP-AFP requires better assessment and understanding of health risks of NPEV infections after polio elimination.

Acute flaccid paralysis (AFP) is defined as sudden onset of weakness and floppiness in any part of the body in a child <15 years of age or paralysis in a person of any age in whom polio is suspected (*1*). It is a complex and broad clinical syndrome associated with a wide range of microbial and nonmicrobial agents and immune processes; clinical presentations and numbers are influenced by environmental and geographic factors. To cast a wider net for poliovirus detection and to maximize sensitivity so that every poliomyelitis case is detected, in 2005, the Global Poliomyelitis Eradication Initiative adapted AFP as a surveillance tool and broadened the case definition of AFP in India. The expanded case definition of AFP encompasses causes of nonpolio AFP (NP-AFP), including Guillian-Barré syndrome, transverse myelitis and traumatic neuritis, and ambiguous cases (*1*).

With the launch of the Global Poliomyelitis Eradication Initiative in 1988 for effective vaccination, surveillance, and monitoring of wild poliovirus transmission toward the target of polio eradication, the number of wild polio AFP cases declined remarkably from ≈300,000 to 974 in 2010 globally (*2*). Introduction of Pulse Polio Immunization, in addition to routine administration of oral polio vaccine, effectively interrupted indigenous wild poliovirus transmission and led to a remarkable decline in the number of poliomyelitis cases from ≈35,000 cases annually during 1994–1995 to 66 in 2005 in India. However, during 2006–2009, the number of polio cases hovered at ≈559–874 each year (*2–5*), with most cases reported primarily from the 2 northern states of Uttar Pradesh and Bihar, in which wild poliovirus remained endemic. The last case of type 2 wild poliovirus globally occurred in 1999 in India. Introduction of bivalent oral polio vaccine types 1 and 3 resulted in a dramatic decline in wild poliovirus cases to 42 in 2010 and only 1 case reported in January 2011 (*2–5*). India is now considered a polio-free nation by the Government of India, National Polio Surveillance Project (NPSP) and by the World Health Organization (WHO)/South-East Asia Regional Office and WHO.

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However, analysis of WHO-monitored polio surveillance data on the number of AFP, polio AFP, and NP-AFP cases available at the public domains (www.polioeradication.org/; www.searo.who.int/vaccine; www.npspindia.org/) (*2*–*5*) from 1998 through June 2, 2012, in India shows that concomitant with the phenomenal elimination of wild poliovirus transmission in India was an annual increase in the number of reported AFP cases from 2005 to date throughout the country (*2*–*4*). Although 8,103–9,705 were reported during 1998–2003, a total of 55,782 and 60,883 cases were reported during 2010 and 2011, respectively. Through June 2, a total of 20,677 AFP cases were reported in India during 2012, compared with 18,625 during the corresponding period in 2011 (*4*). This large increase in NP-AFP cases, which represent AFP cases caused by agents other than poliovirus, probably reflects the excellent implementation of the expanded definition of AFP and highly sensitive surveillance and detection methods used by NPSP in India from 2005 onwards, in contrast to the other polio-endemic countries, i.e., Pakistan, Nigeria, and Afghanistan, where the expanded AFP surveillance is not in place (*1*–*5*). The large increase in the NP-AFP rate from 1.45 and 1.97 per 100,000 children during 1998–2003 to 16.20 in 2011 (*3*–*5*) further reflects the excellent operational performance of the expanded AFP surveillance in India.

The genus *Enterovirus* within the family *Picornaviridae* comprises a diverse group of 10 species, of which 7 are associated with a wide spectrum of acute and chronic human diseases (*6*,*7*). Human enteroviruses (HEVs) are ubiquitous, infecting ≈1 billion persons worldwide. Although the actual incidence of enteroviral diseases is not known, most infections are thought to be asymptomatic, with ≈1% resulting in severe illness with high rates of death, especially in infants and young children (*6*). The >100 HEV serotypes comprising echoviruses (E), coxsackieviruses A (CAV) and B (CBV), polioviruses, and newer enteroviruses (EV) have been grouped into 4 species—HEV-A, HEV-B, HEV-C, and HEV-D—with poliovirus being part of HEV-C. Recently, rhinoviruses also have been included in the genus *Enterovirus* (*8*).

Molecular typing methods based on reverse transcription PCR (RT-PCR) amplification, nucleotide sequencing of the complete or the 3′ portion of the viral protein (VP) 1 gene, and comparison of the derived sequences with those of prototype and variant HEVs in the databases are widely used to identify EV types in clinical samples (*9*,*10*). In the current most commonly used molecular typing scheme, homotypic viruses generally share at least 75% nt identity and 85%–88% aa identity in VP1 (*9*,*10*).

Although nonpolio enteroviruses (NPEVs) are a major cause of AFP (*6*,*7*,*11*) and NP-AFP cases are being detected in large numbers, detailed knowledge is lacking about the serotypes associated with NP-AFP or other enteroviral diseases in India. We aimed to determine the spectrum of NPEV serotypes associated with NP-AFP from polio-endemic and polio-free regions of India with a view to develop strategies against the so-far unrecognized viral infections.

Materials and Methods

AFP Samples and Processing Laboratory

The National Institute of Virology Bangalore Unit in Victoria Hospital (Bangalore, India) is a WHO-accredited National Polio Laboratory for receiving, processing, and analyzing AFP specimens for polio and NPEVs (*5*,*12*). At least 2 specimens, obtained ≈24 hr apart, from each person with AFP were collected during 2007–2009. The National Institute of Virology–National Polio Laboratory receives fecal specimens of persons with AFP in accordance with WHO NPSP guidelines from the southern states of Karnataka and Kerala, 5 districts of the polio-endemic Uttar Pradesh State (Pilibhit, Badaun, Bareilly, Rampur, and Shahjahanpur), and other districts as and when required by the NPSP.

The chloroform-treated supernatants from 2,786 first-collection fecal samples from NP-AFP patients and second specimens from 310 of the patients were examined for EVs by observing cytopathic effects in L20B and RD cells. Poliovirus-positive isolates were further identified by neutralization tests, RT-PCR and ELISA following WHO-prescribed protocols (*12*) and sequence analysis of VP1 gene for determining vaccine-derived polioviruses. RT-PCRs have replaced all the earlier methods for identifying wild and vaccine polioviruses. All fecal specimens are stored for 1 year at −20°C and discarded by autoclaving. The virus-positive cell culture supernatants were stored at –20°C until completion of reporting, and only NPEVs were retained for further studies. RNA was extracted from 1,129 samples from children with NP- AFP, which include 823 NPEV-positive individual cases and 138 NPEV-positive second specimens. A total of 174 NPEV-negative fecal samples, including 16 second specimens, were used as controls to determine whether any of the samples negative for EV in cell culture became positive in RT-PCR.

Healthy Children

Fecal specimens from 780 apparently healthy children 3 months–3 years of age who did not receive vaccine and did not have diarrhea or any other illnesses during the 2 weeks before sample collection from different localities in the Bangalore community were used as healthy controls to determine the frequency of EV detection in healthy children. We obtained the necessary approvals from

Institutional Biosafety and Ethical committees for carrying out the work.

Laboratory Analyses

Total RNA was extracted from 200 μL of NPEV–positive RD cell culture supernatants or clarified chloroform extracted fecal samples by using RNeasy mini kit (QIAGEN, Hilden, Germany). RNA was eluted in 100 μL of RNase-free water and stored at −80°C. EV species–specific degenerate primers were constituted into 4 sets (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1457-Techapp.pdf). Set 1 contained EV-B and EV-C–specific primers; set 2 contained primers specific for EV-A, EV71, and EV-D and sets 3 and 4 consisted of primers specific for Aichi virus/kobuvirus and klasseviruses and for cardioviruses and human cosaviruses, respectively. VP1 region was amplified by 1-step RT-PCR (QIAGEN) with reverse transcription at 40–42°C for 45 min depending on the primer, followed by heating at 94°C for 15 min. The first 2 PCR cycles were performed with melting, annealing, and extension at 94°C (40 s), 45–50°C (30 s), and 68°C (2 min), respectively. PCR was continued for 40 cycles with annealing and extension at 55°C (30 s) and 72°C (2 min), respectively. VP1 PCR fragments were sequenced (Macrogen Inc., Seoul, South Korea; SciGenom, Cochin, India) either directly by using primers corresponding to arbitrary sequences present at the 5′ end of the PCR primers or by using M13 forward and reverse primers after cloning the inserts between *Eco*RI or *Bam*HI and *Hin*dIII sites in pBluescript KS$^+$ (Agilent Technologies, Santa Clara, CA, USA).

Phylogenetic Analyses

EV serotypes were identified by comparing VP1 nucleotide and deduced amino acid sequences from NP-AFP isolates among themselves and with those of other prototype or variant strains belonging to all known serotypes available in GenBank. Phylogenetic analyses were conducted by using MEGA5 (*13*) as described in the legends to the figures. GenBank accession numbers of reference strains used for sequence comparisons are available at www.pcornastudygroup.com, some of which are indicated in the trees. The VP1 gene GenBank accession numbers of 618 India NP-AFP isolates are HQ454497–454499 and JN203499–JN204113.

Results

Frequency of NPEV Detection in Children with NP-AFP and in Healthy Children

A total of 2,786 fecal specimens from individual NP-AFP patients and second-collection specimens from 310 of the patients were examined for EV. Samples from 823 (29.5%) NP-AFP patients were positive for virus growth in RD cells. The percentage of virus positivity varied from 24.0% to 33.0% in different years from the 3 states. The remaining ≈70% samples were negative for EV in RD cells (Table). Of the 823 NPEV-positive cases, 532 (64.6%) yielded VP1-specific PCR fragments corresponding to EV-A, EV-B, or EV-C species. Sequencing of VP1 from 154 second specimens confirmed the serotype specificity of the isolate determined from the first sample (Tables 1 and 2 in online Technical Appendix). Whereas the species-specific primers yielded a PCR product of ≈1,200 bp, the EV71–specific primers F1 and R1 yielded an 850-bp fragment. VP1 region from 291 (35.4%) samples could not be amplified by using primers designed for EV-A, EV-B, EV-C, and EV-D, bovine enterovirus, porcine enterovirus, klassevirus, kobuvirus, human cosavirus, or parechovirus under various PCR conditions (*8*,*14*–*20*). Four (2.3%) of 174 NPEV-negative samples showed positivity in RT-PCR (Table; Table 1 in online Technical Appendix).

Of the 780 fecal specimens from apparently healthy children, ≈20 (2.6%) fecal samples were positive for NPEV in cell culture, and ≈23 (3.0%) were RT-PCR-positive with the same primers used for AFP samples (Table). Although only 4 (0.9%) of 450 samples collected during winter months (November–March) were positive for NPEV,

Table. Analysis of fecal samples in 2,786 children with AFP and in 780 healthy children for virus growth in RD cells and for viral protein 1 gene RT-PCR, India, 2007–2009*

State/RD cell status	Total no. AFP samples (second samples)	No. samples used for RNA extraction			Total no. (%) RD-positive patients	No. (%) RT-PCR-positive patients/RD-positive or -negative patients
		2007	2008	2009		
Karnataka	929 (253)					
Positive	NA	51	75	87	213/676 (31.51)	141/213 (66.2)
Negative	NA	48	60	32	0	4/158 (2.5)†
Healthy children	NA	240	250	290	20/780 (2.56)†	18/20 (90.0)†
Uttar Pradesh: positive	1,833 (39)	129	165	232	526/1,794 (29.32)	331/526 (62.93)
Kerala	334 (18)					
Positive	NA	22	45	17	84/316 (26.6)	60/84 (71.7)
Negative	NA			18		
Total	3,096	490	595	676	823/2,786 (29.54)	532/823 (64.64)

*AFP, acute flacid paralysis; RT-PCR, reverse transcription PCR; NA, not applicable.
†Not included in RD cell–positive or RT-PCR–positive samples from children with AFP.

most virus-positive samples (16 [4.8%] of 330) were those collected in other months, suggesting seasonal incidence of NPEV infections in Bangalore.

Extreme Antigenic Diversity of EVs Associated with NP-AFP

Comparison of the complete or partial VP1 gene sequences of the clinical isolates with those of prototype and other EVs available in GenBank and phylogenetic analyses (*13*) showed 66 serotypes among isolates associated with NP-AFP (Figures 1, 2). Among the 66 serotypes, EV71 was more frequently detected than others, representing ≈8.4% of the characterized isolates, followed by E13 (7.1%) and CBV5 (5.0%). Although strains belonging to 6 serotypes (E6, E7, E11, E14, E19, and E33) each accounted for 3.3%–4.5% of the characterized strains, those belonging to 13 serotypes (CVA4, CBV1, CBV2, CBV4, CBV6, E1, E20, E24, E25, E29, E30, EV69, and EV75) each represented 1.7%–2.9%. The frequency of detection of each of the other serotypes ranged from 0.2% to 1.6% (Figure 1; Tables 1 and 2 in online Technical Appendix). Only 15 serotypes (CAV4, CBV2, CBV4, E6, E7, E13, E14, E17, E19, E25, E30, E33, EV71, EV75, EV80) were detected in all 3 states. Forty-eight serotypes, including CBV5, were not detected in Kerala, probably because of the relatively small number of samples available from that state or noncirculation of these serotypes. Strains representing 16 serotypes were each detected only 1× or 2× during the period and represented <0.2%–0.3% of the characterized isolates (Figure 1). Strains belonging to EV-C species, to which poliovirus belongs, were rarely observed, with 1 CAV17 strain in Karnataka and 1 CAV21 strain in Uttar Pradesh being detected in samples collected during 2008. Of the 4 NPEV-negative samples that showed positivity in RT-PCR, 3 belonged to CAV4 and the other to CAV8.

Of the 532 NPEV-positive cases, 41 (7.7%) were identified as exhibiting mixed infection by >1 strain, as indicated by RT-PCR and sequence analysis of the cloned fragments. Although 37 cases of mixed infections involved 2 different serotypes, infections involving 3 different serotypes were detected in 4 cases. Although 32 serotypes were detected in mixed infections, CBV4, CBV5, E6, EV69, and EV71 accounted for 12.2%, 12.2%, 14.6%, 17.1%, and 31.7% of these infections, respectively. Mixed infections involving EV69 and CBV4 accounted for 58.3% and 41.7%, respectively, of their total detections (Table 3 in online Technical Appendix).

A notable observation was detection of EV71 as the single most prevalent serotype; it accounted for ≈8.5% of the characterized isolates. Although EV71 was undetectable in 2007 and only 1 isolate was detected during 2008 in Karnataka, it was the most frequently detected serotype in Kerala during these 2 years. EV71 appears to have spread from Kerala to the neighboring Karnataka, resulting in its frequent detection in 2009. EV71 was frequently detected in Uttar Pradesh only during 2008 and 2009. Although 26.5% of total EV71 detections involved mixed infection, it alone accounted for 31.7% of mixed infections (Table 3 in online Technical Appendix).

Genetic Relatedness of EV71, E13, and CBV Strains in India with Strains from Other Countries

To understand the genetic relatedness of isolates in India that belonged to an EV type to those from other countries, we performed initially phylogenetic analyses of VP1 sequences of isolates from India that belonged to the prevalent EV71, E13, and CBV types with a large number of VP1 sequences representing different genogroups or subgenogroups within the types available in GenBank. Final phylogenetic trees, generated by using a few representative strains belonging to different genogroups and subgenogroups of EV71, E13, and CBV serotypes, are shown in Figure 3, panels A and B, and Figure 4. These studies showed that most of the India isolates segregated into either distinct genogroups or subgenogroups within known genogroups. Among the India EV71 isolates, 3 distinct new genogroups tentatively assigned as D (represented by N975c), E (N493), and F (N390), were identified (Figure 3, panel A). Isolate N975c showed ≈76%-nt sequence identity with a few EV71 strains but exhibited <70% aa identity, which suggests that it represents a new EV71 lineage. Other EV71 India strains segregated into different subgenogroups within C (C6 and C7) and B (B6) genogroups (*21*–*23*). Most India E13 strains formed 2 distinct subgenogroups within 2 E13 genogroups (Figure 3, panel B) (*24*–*26*). Most India CBV isolates, although grouped according to serotypes CBV1 to

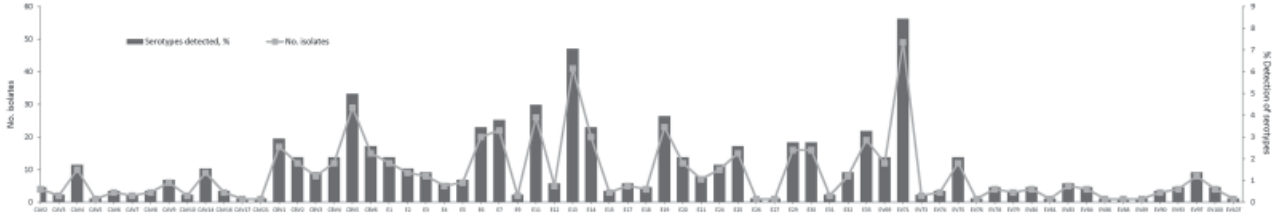

Figure 1. Strains belonging to each serotype detected in children with acute febrile paralysis, India, 2007–2009. CA, coxsackievirus; E, echovirus; EV, enterovirus.

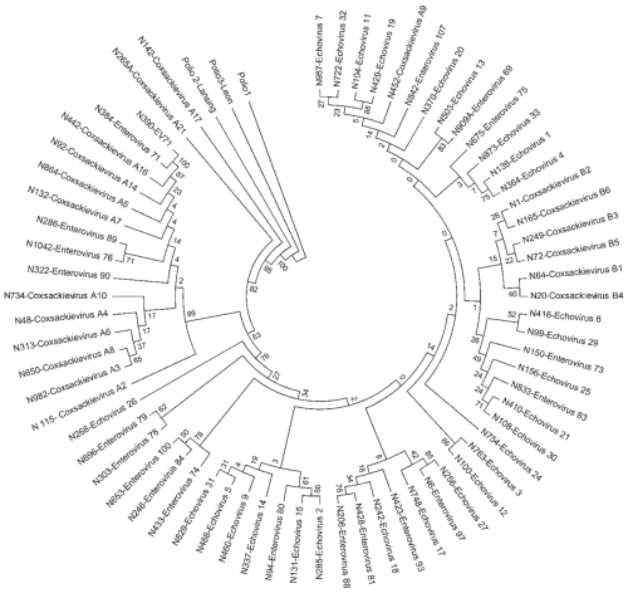

Figure 2. Phylogenetic tree based on viral protein 1 gene nucleotide sequences of strains representing each of the 66 enterovirus serotypes detected in children with acute febrile paralysis, India, 2007–2009. The phylogenetic tree was constructed by using the maximum-parsimony method and with search level 1 close-neighbor-interchange algorithm. The percentage of replicate trees in which the associated types clustered together in the bootstrap test (500 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA5 (*13*). The viral protein 1 gene sequences of the 3 poliovirus prototypes were used as reference. The strain numbers with their serotype association are indicated. The GenBank accession numbers of VP1 gene for 618 acute febrile paralysis isolates are HQ454497–454499 and JN203499–JN204113.

CBV6, represented a different subgenogroup within each type (Figure 4) (*27*,*28*). Preliminary phylogenetic analyses of VP1 sequences of strains belonging to a few other types also showed similar results (data not shown). These data suggest that most India EVs represent evolutionary lineages, within a given serotype, that differ from those prevalent in other countries.

Discussion

This study, which represents a comprehensive molecular epidemiologic investigation of EVs associated with NP-AFP in India, was feasible because of the detection of a large number of AFP cases during the last several years from all regions of the country. The few previous studies from India and Pakistan identified 6–15 serotypes (*11*,*29*) in AFP cases by using methods that are limited in scope in identifying the >100 known HEV serotypes. Although studies in other countries (*14*,*15*,*30*,*31*) identified 6–12 serotypes, those in Romania and the People's Republic of China observed 20 and 40, respectively (*32*,*33*). The serotypes that were prevalent varied in each of these studies. In contrast, we identified 66 NPEV serotypes in samples collected during 2007–2009 from children with NP-AFP in India. Detailed phylogenetic analysis of strains belonging to a few prevalent serotypes showed that NP-AFP is associated with circulation of strains belonging to different genogroups and subgenogroups of a serotype in widely separated geographic regions of the country (Figure 3, panels A and B; Figure 4).

Our results raise several issues that require immediate attention for the future direction of clinical and basic research and for better understanding of the health risks

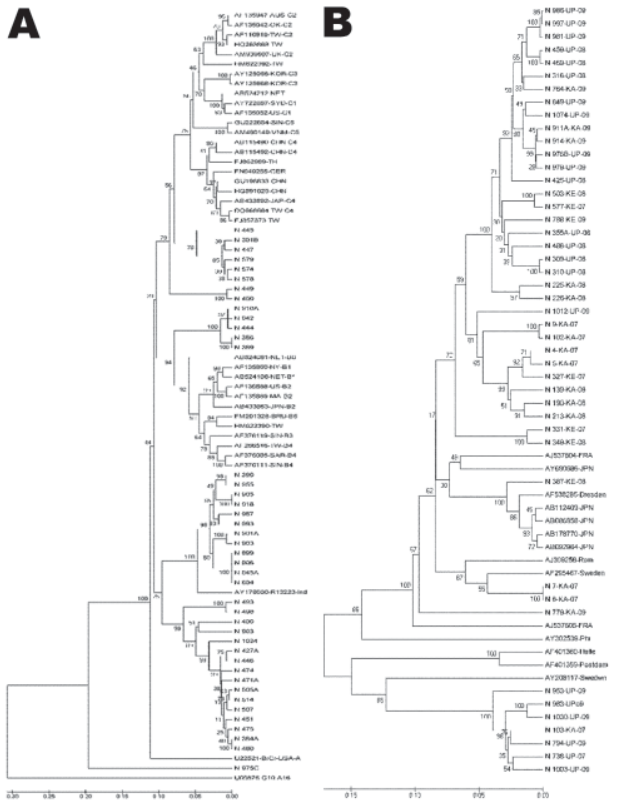

Figure 3. Phylogenetic analyses of viral protein 1 sequences of enterovirus 71 and echovirus 13 strains with those of reference strains representing different genogroups and subgenogroups within a serotype, India, 2007–2009. Multiple sequence alignments were performed by using ClustalW program (www.genome.jp/tools/clustalw/) and phylogenetic analysis by MEGA5 program (*12*) with pairwise comparison and maximum composite likelihood nucleotide substitution model. Phylogenetic trees were constructed by UPGMA (unweighted pair group method using arithmetic averages) with statistical significance of the phylogenetic analyses estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. A and B and represent phylogenetic trees of viral protein 1 sequences of enterovirus 71 and echovirus 13 isolates, respectively. The serotype, state and year of isolation of each strain and GenBank accession numbers of reference strains used are indicated. 1000B is an echovirus 1 strain. Scale bars indicate nucleotide substitutions per site.

posed by NPEV infections. First, no virus was isolated in ≈70% of the AFP cases that remain to be identified. Second, the genetic nature of the viral agent(s) in ≈35% cases that were positive for virus in RD cells remains to be determined. Third, the association of a wide spectrum of serotypes with NP-AFP poses a challenging problem toward development of effective anti-enteroviral strategies. However, the need for effective antiviral strategies, including vaccines, is a major leap from the findings reported here and requires an in-depth analysis of the true effect of disease.

Detection of EV71 as the single most prevalent EV type associated with NP-AFP is of clinical significance because it is regarded as the most virulent neurotropic EV, next to poliovirus, associated with poliomyelitis-like paralytic disease, meningitis, meningoencephalomyelitis, Guillain-Barré syndrome, transeverse myelitis, cerebellar ataxia, opsoclonus-myoclonus syndrome, benign intracranial hypertension, brainstem encephalitis (*34*–*36*), and frequent epidemics of hand, foot, and mouth disease with substantial illness and death worldwide affecting >500,000 children in the Asia-Pacific region and causing >200 deaths in China during the past decade (*37*–*39*). The observation that >30% of EV71 detections involved mixed infections could be of clinical significance in terms of severity and spectrum of the diseases associated with these infections. Detection of 3 new genogroups of EV71 in widely separated geographic regions in India suggests uniform spread of EVs of different lineages of EV71 and other types in India. Long-term assessment of the clinical outcome of the EV71 infections would be of interest to better understand the severity of the disease in children with AFP.

Our study provides a wealth of information about NP-AFP in India. It suggests the necessity for the WHO-NPSP programs, which are primarily directed for a 60-day follow-up of poliomyelitis cases, to design and implement a long-term strategic plan for understanding and addressing the short-term and long term health risks in children arising from NPEV infections after poliomyelitis elimination in India.

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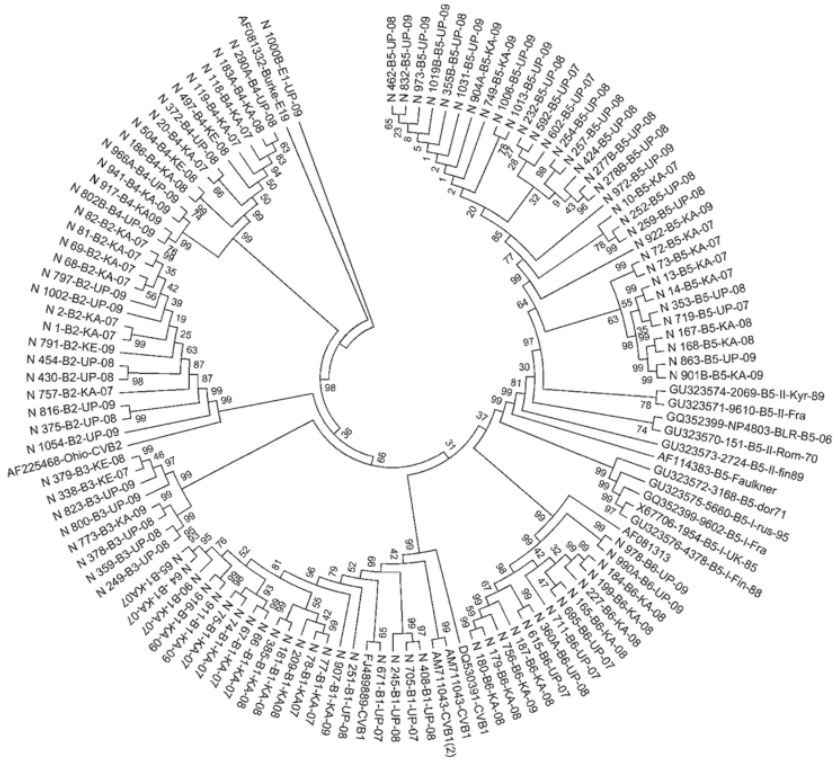

Figure 4. Phylogenetic tree of viral protein 1 sequences of coxsackievirus B1-B6 isolates generated in comparison with those of strains belonging to different genotypes of B1, B2, B3, B4, B5, and B6 serotypes, India, 2007–2009. Multiple sequence alignments were performed by using ClustalW program (www.genome.jp/tools/clustalw/) and phylogenetic analysis by MEGA5 program (*13*) with pairwise comparison and maximum composite likelihood nucleotide substitution model. Phylogenetic trees were constructed by UPGMA (unweighted pair group method using arithmetic averages) with statistical significance of the phylogenetic analyses estimated by bootstrap analysis with 1,000 pseudoreplicate datasets.

Dr Rao is a professor at the Indian Institute of Science, Bangalore, India. His research interests include molecular epidemiology, molecular biology and mechanism of pathogenesis of rotavirus and EVs, and posttranscriptional regulation of gene expression.

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Livestock Density as Risk Factor for Livestock-associated Methicillin-Resistant *Staphylococcus aureus*, the Netherlands

Beth J. Feingold, Ellen K. Silbergeld, Frank C. Curriero, Brigite A.G.L. van Cleef, Max E.O.C. Heck, and Jan A.J.W. Kluytmans

To determine whether persons living in areas of high animal density are at increased risk for carrying livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), we used an existing dataset of persons in the Netherlands with LA-MRSA carriage and controls who carried other types of MRSA. Results of running univariate and multivariate logistic regression models indicated that living in livestock-dense areas increases the odds of nasal carriage of LA-MRSA. We found that doubling pig, cattle, and veal calf densities per municipality increased the odds of LA-MRSA carriage over carriage of other types of MRSA by 24.7% (95% CI 0.9%–54.2%), 76.9% (95% CI 11.3%–81.3%), and 24.1% (95% CI 5.5%–45.9%), respectively, after adjusting for direct animal contact, living in a rural area, and the probable source of MRSA carriage. Controlling the spread of LA-MRSA thus requires giving attention to community members in animal-dense regions who are unaffiliated with livestock farming.

Staphylococcus aureus is a zoonotic and human pathogen that can cause a range of health outcomes in humans from minor to life-threatening infections of the skin, bloodstream, respiratory system, urinary tract, and surgical sites (*1*). An increasing proportion of *S. aureus* infections involve drug-resistant strains, including methicillin-resistant *S. aureus* (MRSA) (*2*). In 2007, 171,200 MRSA infections occurred in European Union member states plus Iceland and Norway, resulting in 1,050,000 extra days spent in the hospital (*3*) This translates into excessive hospital inpatient and outpatient costs because of the need to isolate patients and because patients require longer stays and more extensive treatments (*3*). In 2005 in the United States, an estimated 94,000 MRSA infections resulted in >18,000 deaths (*4*).

MRSA has, in the past, been largely associated with hospitals and other healthcare facilities, but since 2000, the majority of MRSA infections in most countries are acquired in the community outside of healthcare settings (*5*,*6*). These strains of community-acquired MRSA are vital public health concerns, but less is known of their origins and routes of transmission. Among these strains of community-acquired MRSA, livestock-associated (LA) strains have been detected in several regions of the world (*7*).

Originally, the LA-MRSA strain studied here was denoted as nontypeable MRSA because of the inability to type it by using standard methods of pulsed-field gel electrophoresis (*8*). It was first detected in the Netherlands in 2003 (*9*) and, as of 2010, accounts for >40% of the MRSA cases in that country (*10*). LA-MRSA has now been identified largely as a single clonal complex on the basis of multilocus sequence typing (ST398), and this clonal complex has a demonstrated association with pigs, cows, and other animals (*11*,*12*), although other clonal complexes have also been shown to be associated with livestock as well. In several European countries, increased risks of carriage have been reported in persons in contact with pigs and veal calves, including farmers, veterinarians, and slaughterhouse workers (*13*,*14*). In the Netherlands, among these occupational groups, the prevalence of ST398 carriage is roughly 42% (*15*), whereas the prevalence of any strain of MRSA in the general population is <1% (*16*).

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The emergence and transmission of LA-MRSA among humans and animals (such as poultry, horses, companion animals, pigs, and cattle) have recently been reviewed (*17*). Most epidemiologic studies have focuses on identifying individual and farm level characteristics associated with LA-MRSA carriage and on studying those in direct contact with livestock. In 1 study among pig farmers and their household members, 30% were carriers of MRSA ST398, and the risk for carriage was related to direct exposure to pigs (*15*). A study among veterinarian field workers found that after short-term occupational exposure to pigs, 17% of them carried MRSA. However, >90% of those lost this carriage the next day (*18*). Another study found a clear association between carriage and the duration of contact with veal calves and that carriage was strongly reduced after a period of absence from animal contact (*11*,*19*). These studies, which indicate a high risk for carriage from livestock contact but little persistence of carriage after interruption of animal contact, bring into question the ability of LA-MRSA to spread into the population. Only 1 study examined the role of living in a livestock-dense region as a risk factor as well but did not find it to be a risk factor (*20*). This study used a random mailing in the 3 most pig-dense municipalities in the Netherlands. Of the 534 adult respondents without livestock contact, 1 person was positive for MRSA (0.2%), compared with 13 of 49 persons who worked or lived on a livestock farm (26.5%).

In 2007, risk factors for LA-MRSA carriage in the Netherlands were investigated by van Loo and colleagues (*8*). They found that risk factors for increased odds of LA-MRSA carriage included contact with livestock, acquiring MRSA through known risk factors such as travel to a foreign country or recent interaction with the healthcare environment, and living in a rural area. Our study, conducted during 2008–2011, built upon this work to test the hypothesis that persons living in areas of high pig density may be at increased risk for carrying LA-MRSA. We did this by combining information about where persons lived and what the livestock density was in these areas for which existing information on risk factors had been determined in the 2007 study.

Methods

Data

We used data from van Loo et al. (*8*), which consisted of records of all index case-patients with nontypeable MRSA carriage (now referred to as LA-MRSA) from the Netherlands from its emergence in 2003 through September 2005, before the country adopted active surveillance of high risk populations. Information on case-patients and controls was obtained from a national MRSA surveillance program through the country's Institute for Public Health and the Environment (www.rivm.nl/mrsa). Case-patients were LA-MRSA index patients, that is, the first in a cluster of persons who tested positive for LA-MRSA from a given reference laboratory. Each LA-MRSA case-patient was matched with 2 controls from the same laboratory. The controls were also index patients, but had tested positive for a typeable strain of MRSA (T-MRSA) instead of to LA-MRSA. Further details on subject selection can be found in the original article (*8*).

The same variables used in the 2007 study were used in this current study: contact with pigs, contact with cows, the probable source of MRSA, and whether one lived in a rural area. Probable source of MRSA was placed by the original authors into the following categories: healthcare setting, foreign source (such as travel to another country), other source, or an unknown source (*8*). Our study added municipality level variables of livestock and human population densities and location of residence of study participants. (Municipalities are administrative boundaries in the Netherlands that comprise provinces. In 2005, there were 498 municipalities.) To accomplish this, our inclusion criteria were residence in the Netherlands, availability of information on individual contact with livestock, and geographic information sufficient to support mapping each person to the 6 digit postal code of his or her residence.

We then assigned spatial coordinates to LA-MRSA case-patients and T-MRSA controls on the basis of their 6-digit postal code using the geographic information system software, ArcGIS version 9.3 (*21*). When this method was not sufficient, we used Google Earth (*22*).

We downloaded municipality level statistics of population; land area; and numbers of swine, veal calves, and cows in 2005 from CBS StatLine (*23*). Livestock densities and population density were calculated as the number of animals (pigs, cows, and veal calves) per hectare of land in a municipality. In ArcGIS, we determined in which municipality participants lived, and assigned to them municipality-level characteristics of animal and population densities. We determined counts of case-patients and controls for each of the municipalities using ArcGIS. This study was reviewed and approved by the Institutional Review Board at Johns Hopkins Bloomberg School of Public Health.

Statistical Analysis

Summary statistics for all relevant variables were reported by using STATA version 10 (*24*). We explored the spatial variation in risk for LA-MRSA—or the concept that the risk or odds of acquiring LA-MRSA varies geographically—using spatial methods available in the R statistical package (*25*). We estimated spatial intensity of case-patients and controls for locations across the study area, defined respectively as the expected numbers of case-patients and controls per km^2. Spatially varying intensity

provides an estimate of regions of high and low densities of case-patients and controls. Spatial intensity is often measured as weighted counts as described (*26*). We used the quartic kernel as our weighting scheme in this study. Using the intensity estimates, we calculated the spatial odds of LA-MRSA to compare the geographic variation of case-patients and controls across the study area. The spatial odds of LA-MRSA per km^2 compared with those for T-MRSA per km^2 were calculated as the ratio of estimated case-to-control intensities (*26*).

Contact with pigs, contact with cows, and rural (versus urban) residence were modeled as binary variables. Probable MRSA source was a categorical variable. We compared probable acquisition of MRSA from a foreign country, acquisition from another source, or acquisition from an unknown source with the referent group of healthcare-related acquisition.

We determined goodness of fit of the models using Akaike information criteria and the Hosmer-Lemeshow goodness-of-fit test. Likelihood ratio tests were used to compare multivariate nested models. The densities of livestock were right skewed; thus, we log-transformed the variables to create a more linear relationship between animal density and log odds of LA-MRSA. For ease of interpretation, instead of the 1 log increase in livestock densities, we used a doubling of livestock densities, which is calculated by raising 2 to the power of the β of the density coefficient in the logit model (*27*). Variograms were used to diagnose possible spatial variation in regression residuals, with inference on regression parameters adjusted accordingly.

In a separate but related analysis, we identified specific clusters of LA-MRSA. We used SaTScan version 9.0 (*28*) to conduct this cluster detection analysis with a Poisson model of counts of case-patients per municipality after adjusting for population size as described (*28*). SaTScan is a software package that is used to analyze spatial and temporal patterns in data. It uses a moving window method (in this application, over a set of contiguous municipalities) and determines the presence of a cluster on the basis of whether the estimated risk within a window is significantly greater than the estimated risk outside of the window. Statistical significance is based on the null hypothesis of Poisson constant risk (*28*). We created maps showing the identified clusters after adjustment of population density per municipality in ArcGIS. We made similar maps after further adjusting for pig, cow, and veal calf densities per municipality.

Table 1. Characteristics of population in study of LA-MRSA carriage, the Netherlands, 2003–2005*

Characteristic	MRSA status, no. (%) persons		Total no. (%) persons	p value†
	LA-MRSA	T-MRSA		
Total, N = 87	27	60	87	
Probable source				
Health care setting	3 (11.11)	30 (50.00)	33 (37.93)	
Foreign	2 (7.41)	3 (5.00)	5 (5.75)	
Unknown	13 (48.15)	20 (33.33)	33 (37.93)	
Other	9 (33.33)	7 (11.67)	16 (18.39)	0.001
Contact with livestock and farms				
With pigs	10 (37.04)	3 (5.00)	13 (14.94)	0.000
With cows	7 (25.93)	1 (1.67)	8 (9.20)	0.001
Residential location				
Rural	12 (44.44)	4 (6.67)	16 (18.39)	0.000
Livestock density in municipality, animals/ hectare/ municipality				
Pigs				
Quartile 1, 0.000–0.004	0	16 (26.67)	16 (18.39)	
Quartile 2, 0.005–0.651	5 (18.52)	15 (25.00)	20 (22.99)	
Quartile 3, 0.652–3.268	6 (22.22)	12 (20.00)	18 (20.69)	
Quartile 4, 3.269–45.477	16 (59.26)	17 (28.33)	33 (37.93)	0.003
Cows				
Quartile 1, 0.00–0.340	3 (11.11)	30 (50.00)	33 (37.93)	
Quartile 2, 0.341–0.848	8 (19.63)	8 (13.33)	16 (18.39)	
Quartile 3, 0.849–1.496	3 (11.11)	14 (23.33)	17 (19.54)	
Quartile 4, 1.497–5.920	13 (48.15)	8 (13.33)	21 (24.14)	0.000
Veal calves				
Quartile 1, 0.000–0.000	0	20 (33.33)	20 (22.99)	
Quartile 2, 0.001–0.013	4 (14.81)	13 (21.67)	17 (19.54)	
Quartile 3, 0.014–0.178	10 (37.04)	13 (21.67)	23 (26.44)	
Quartile 4, 0.179–4.818	13 (48.15)	14 (23.33)	27 (31.03)	0.000
Population density				
Quartile 1, 0.250–2.027	11 (40.74)	6 (10.00)	17 (19.54)	
Quartile 2, 2.028–3.649	11 (40.74)	11 (18.33)	22 (25.29)	
Quartile 3, 3.650–9.175	2 (7.41)	18 (30.00)	20 (22.99)	
Quartile 4, 9.176–57.11	3 (11.11)	25 (41.67)	28 (32.18)	0.000

*MRSA, methicillin-resistant *Staphylococcus aureus*; LA-MRSA, livestock associated MRSA; T-MRSA, typeable MRSA.
†Fisher exact test for differences in MRSA status by covariate categories.

Results

Study Population Characteristics

Descriptive statistics of the study population are shown in Table 1. From the total population used in the Van Loo analysis of 111 persons (35 case-patients, 76 controls) (*8*), 87 persons (27 case-patients, 60 controls) were included in our study after we excluded persons who lived outside of the Netherlands (n = 4), persons for whom spatial information was insufficient (n = 3), and persons for whom information about individual contact with livestock was lacking (n = 17).

Of the 87 subjects with complete case information, most of those who had contact with pigs (10/13, 76.9%) and cows (7/8, r 87.5%) were LA-MRSA case-patients. Three subjects had contact with both pigs and cows, 2 of whom were case-patients. Twelve of 27 persons without any direct contact with livestock were LA-MRSA positive (44.4%).

Specific locations of case-patients and controls are plotted against municipality level population (Figure 1, panel A), cow density (Figure 1, panel B), pig density (Figure 1, panel C) and veal calf density (Figure 1, panel D). Case-patients and controls had significant differences in human and livestock densities per municipality (Table 1).

Spatial Odds

Relatively high concentrations of controls are seen in general areas of high population density while higher spatial concentrations of case-patients are seen in the more agricultural areas of the country (Figure 2, panels A, B). We estimated spatial odds to give a visual assessment of the spatial variation in risk across the Netherlands (Figure 2, panel C). It is evident that the greatest differences in odds between case-patients and controls are in general areas of high pig density, as was originally reported by van Loo and colleagues (*8*). The elevated spatial odds in the northern part of the country are a spurious result because of small

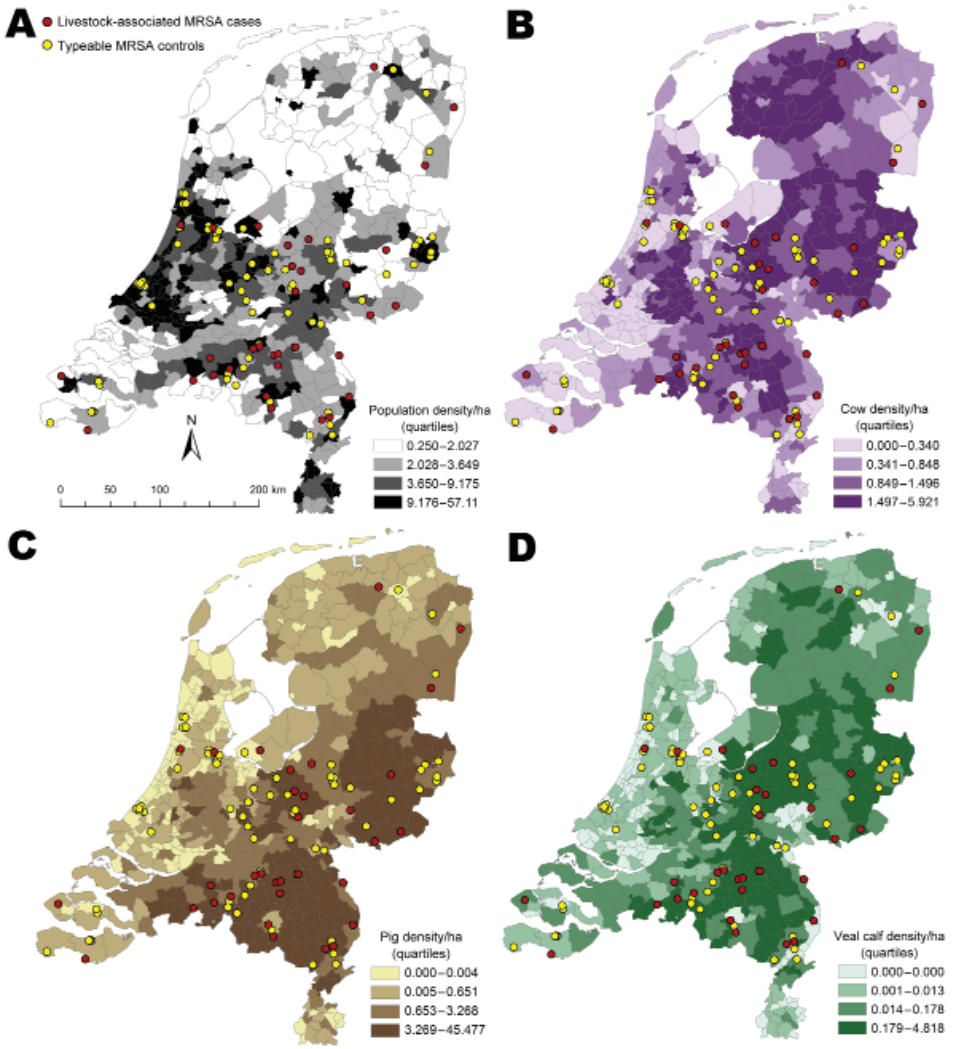

Figure 1. A) Case-patients with livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) and controls with typeable MRSA, according to population density, the Netherlands, 2003–2005. B) Density of cattle per municipality. C) Density of pigs per municipality. D) Density of veal calves by municipality.

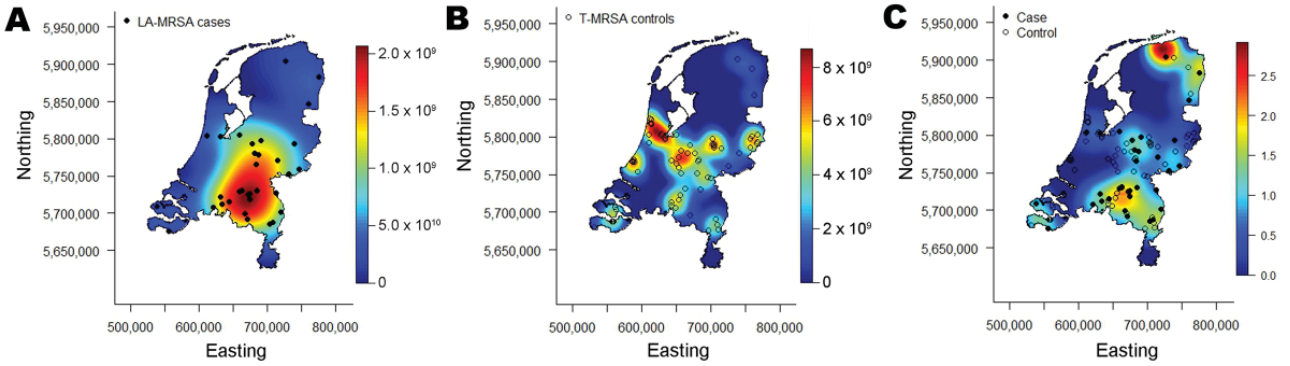

Figure 2. A) Spatial intensity of case-patients with livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA); B) spatial Intensity of controls with typeable MRSA (T-MRSA); and C) calculated spatial odds for LA-MRSA compared with those for T-MRSA, the Netherlands, 2003–2005. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/11-1850-F2.htm).

numbers of case-patients and controls and not something that we put forth as a valid result.

Univariate Logistic Regression

Univariate models results are reported in Table 2. Persons who have contact with pigs have 11.18 times the odds of carrying LA-MRSA (compared with odds of carrying T-MRSA) than persons without pig contact (95% CI 2.76–45.30; p<0.001). A similar relationship is seen when persons with and without contact with cows are compared (odds ratio [OR] 20.65; 95% CI 2.39–178.31; p <0.006). Living in a rural area rather than living in an urban area is associated with 11.2 times the odds for LA-MRSA compared with T-MRSA (95% CI 3.15–39.76; p<0.000). Carriage of MRSA from an unknown or "other" source, as compared to healthcare settings (a priori known to be associated with typeable MRSA), was significantly (p<0.05) associated with the odds of LA-MRSA carriage as compared to T-MRSA.

We found that when pig density per hectare is doubled within a municipality, the odds of acquiring LA-MRSA in a univariate model are increased by 29.5% over the odds of acquiring T-MRSA (p<0.003). Similarly, doubling cow and veal calf densities increases the odds of acquiring LA-MRSA compared with those for acquiring T-MRSA by 75.4% (p<0.001) and 19.8% (p<0.001), respectively.

When the inclusion criteria required by our study were used, 16% of original data were lost. We conducted sensitivity analyses by coding all persons with missing livestock contact information as either all having contact or as all not having contact to produce what might be bounds for high and low extremes. In all cases, livestock densities remained significant independent risk factors at the 0.05% level (Table 3).

Multivariate Models

Multivariate model results are reported in Table 4. Model 1 is based only on the original individual level variables from van Loo's study (*8*): contact with pigs and cows, rural versus urban residence, and information on patient's probable source of MRSA. In model 1, both contact with pigs and rural residence remain significant predictors as they were in the univariate models, even when adjusting for contact with cows and probable MRSA source. Odds for LA-MRSA compared with those for T-MRSA were 11.6 times higher for a foreign source of MRSA than they were

Table 2. Risk factors for LA-MRSA in comparison with those for T-MRSA, the Netherlands, 2003–2005*

Variable	Univariate models		AIC
	OR (95% CI)	p value	
Contact with pigs	11.176 (2.76–45.29)	0.001	97.81
Contact with cows	20.65 (2.39–178.31)	0.006	99.42
Rural	11.20 (3.15–39.76)	0.000	95.21
Probable source of MRSA		0.083	101.02
Foreign vs. health care setting	6.67 (0.78–57.06)		
Unknown vs. health care setting	6.50 (1.64 25.76)	0.008	
Other vs. health care setting	12.86 (2.75–60.22)	0.001	
Livestock density/municipality			
Log (pig)	1.45 (1.13–1.86)	0.003	95.58
Log (cow)	2.25 (1.40–3.60)	0.001	96.32
Log (veal calf)	1.30 (1.11–1.52)	0.001	98.70
Log (population)	0.36 (0.20–0.64)	0.001	94.78

*MRSA, methicillin-resistant *Staphylococcus aureus*; LA-MRSA, livestock-associated MRSA; T-MRSA, typeable MRSA; AIC, Akaike information criteria.

Table 3. Results of univariate logistic regression including missing data on contact with livestock, the Netherlands, 2003–2005*

Contact with livestock	All missing having livestock contact		All missing having no livestock contact	
	OR (95%CI)	p value	OR (95% CI)	p value
Pigs	3.83 (1.56–9.41)	0.003	9.86 (2.49–38.94)	<0.001
Cows	3.20 (1.26–8.13)	0.015	18.85 (2.21–160.68)	<0.007

*OR, odds ratio.

with a healthcare source (95%CI 1.04–129.63; p<0.046). Similarly, the odds were 9.56 when persons with an unknown source were compared with those with a healthcare source (95% CI 1.76–51.93; p<0.009). Acquiring MRSA from another (other) source compared with healthcare acquisition also had increased odds, but this result was not significant (OR 4.3, 95% CI 0.55–33.56; p<0.164).

Models 2–4 build on model 1 (the base model) by adding in the logs of pig, cattle, and veal calf densities per municipality, respectively, with the same individual level variables used in model 1 (Table 4). Model 2 builds on model 1 by adding a term for the log of pig density. The odds ratio comparing LA-MRSA to T-MRSA for a 1 log increase in pig density per hectare after adjusting for the individual risk factors (the variables in model 1) for LA-MRSA was 1.37 (95% CI 1.01–1.87, p<0.041). A doubling of the pig density per municipality increases the odds of LA-MRSA carriage compared with T-MRSA carriage by 24.7% after adjustment for individual level risk factors. Model 3 builds on model 1 by incorporating the log of the cow density per municipality. Adjusting for the individual level predictors, a 1 log increase in cow density yields a 2.28 increase in odds for LA-MRSA compared with T-MRSA (95% CI 1.17–4.45, p<0.016). Here, a doubling of cow density in a municipality increased the odds of LA-MRSA compared with T-MRSA by 76.9%. The odds ratio of carrying LA-MRSA compared with those of carrying T-MRSA in model 4 for a 1 log increase in veal calf density after adjustment for individual variables was 1.37 (95% CI 1.08–1.72, p<0.009). Thus, a doubling of the veal calf density per municipality yields a 24.09% increase in the odds of carrying LA-MRSA compared with carrying T-MRSA.

The Hosmer-Lemeshaw goodness-of-fit tests indicate that all models fit the data sufficiently well. The Akaike information criteria and likelihood ratio values for models 2–4 indicate that adding area-level animal density variables improves the original model (model 1). Variograms of re-

Table 4. Results of multivariate logistic regression for LA-MRSA carriage compared with those for T-MRSA carriage, the Netherlands, 2003–2005*

	Model 1: individual level		Model 2: model 1 + pig density		Model 3: model 1 + cow density		Model 4: model 1 + veal calf density	
Variable	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
Individual level								
Contact with pigs	8.63 (1.23–60.40)	0.030	6.41 (0.77–53.35)	0.086	6.84 (0.86–54.49)	0.069	9.41 (1.24–71.26)	0.030
Contact with cows	7.37 (0.57–94.68)	0.125	8.39 (0.55–129.18)	0.127	5.10 (0.39–65.87)	0.212	6.18 (0.53–71.83)	0.146
Rural	5.63 (1.02–31.17)	0.048	4.14 (0.64–26.65)	0.135	5.55 (0.89–34.56)	0.066	4.94 (0.802–30.41)	0.085
Probable source of MRSA								
Foreign vs. health care	11.61 (1.04–129.63)	0.046	8.53 (0.72–100.45)	0.088	8.71 (0.74–102.73)	0.086	14.36 (1.06–193.53)	0.045
Unknown vs. health care	9.56 (1.76–51.93)	0.009	11.47 (2.01–65.64)	0.006	14.03 (2.25–87.47)	0.005	13.31 (2.02–87.75)	0.007
Other vs. health care	4.30 (0.55–33.56)	0.164	4.12 (0.54–31.32)	0.032	2.91 (0.36–23.77)	0.319	4.11 (0.51–33.00)	0.184
Municipality level								
Log (pig density)			1.37 (1.01–1.87)	<0.041				
Log (cow density)					2.28 (1.17–4.45)	0.016		
Log (veal calf density)							1.37 (1.08–1.72)	0.009
Regression diagnostics								
AIC	84.76		79.98		79.14		77.90	
Hosmer-Lemeshow†	2.52	0.6407	5.48	0.7050	7.52	<0.4817	6.09	<0.6374
Likelihood ratio test	NA			0.0092		<0.0058		<0.0029

*MRSA, methicillin-resistant *Staphylococcus aureus*; LA-MRSA, livestock-associated MRSA; T-MRSA, typeable MRSA; OR, odds ratio; AIC, Akaike information criteria; NA, not applicable.
†Hosmer-Lemeshow, Hosmer-Lemeshow goodness-of-fit test.

siduals from the 4 models did not reveal any substantial spatial variation.

Cluster Detection

Cluster detection analysis results indicate that after adjusting for the size of the population in a given municipality, 1 significant cluster of LA-MRSA cases (relative risk 5.2, p<0.014) was found when a maximum of 20% of the population at risk was designated as the maximum spatial cluster size. Figure 3 (panels A–C) shows the cluster detection results mapped on top of veal calf density, all cattle density, and pig density for visual identification of associations.

To test whether accounting for livestock density at the municipality level would eliminate the existence of this hot spot of LA-MRSA, we ran additional analyses in SaTScan with adjustment for the density of each animal population separately (*28*). These results indicated that adjusting for animal densities eliminated the presence of the cluster, further supporting the hypothesis that livestock densities per municipality are key risk factors for LA-MRSA carriage.

Discussion

Our findings indicate that regional density of livestock is a notable risk factor for nasal carriage of LA-MRSA for persons with and without direct contact with livestock. This finding has been emphasized in recent research that found LA-MRSA carriage in persons without connections to the farm environment (*29*). A recent study indicated that proximity to farms is a potential risk factor, even in absence of direct contact between humans and animals (*30*). In addition, MRSA has been found in meat; diet may therefore provide another route of exposure for the general population (*31*,*32*).

We observed in the multivariate analysis that living in a region with high cattle density conferred higher odds of LA-MRSA carriage than did living in a region of high pig or veal calf density. We are not certain what may explain this association, but it does warrant further investigation. We found in our multivariate models that some of the risk factors previously identified by univariate analysis by van Loo and colleagues (*8*) dropped out as being less significant when regional livestock density was included in a multivariate model, such as direct contact with pigs and cows and living in a rural location. Intriguingly, acquiring MRSA from an "unknown" source remained highly significant in all of the multivariate models. These results highlight the value of considering these individual-level variables, together with regional level data, as an update of the univariate analysis conducted by van Loo and colleagues in 2007.

This analysis is limited by the small size of the dataset. However, even with such a small dataset, and after adjusting for known and supposed LA-MRSA risk factors, the densities of livestock per municipality remain strong and independent risk factors for LA-MRSA carriage.

A second limitation of the study is that the case-patients were initially restricted to index case-patients, which inherently selected against detecting secondary transmission. Conducting a future study that includes non–index case-patients would produce a more accurate picture.

A final limitation is the possibility of recall bias in the participants' reports of exposures to livestock, leading to a misclassification of exposure. Such a nondifferential information bias may have biased our results toward the null hypothesis.

This work has potential policy implications for MRSA surveillance in countries where a substantial percentage of total MRSA cases are LA-MRSA, such as the Netherlands. Starting in 2006, health policy in the Netherlands has required testing for MRSA carriage on admission to the hospital for persons living or working on pig farms. This study

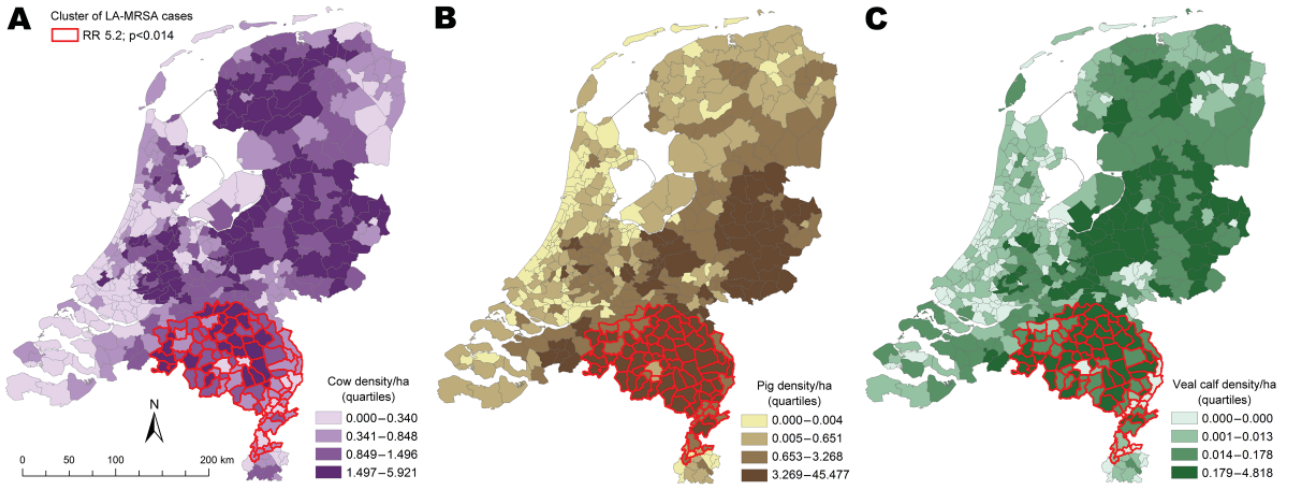

Figure 3. Clusters of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in the Netherlands, 2003–2005, taking into account 20% population at risk with overlays showing veal calf density (A), cow density (B), and pig density (C). RR, relative risk.

suggests that this screening program may need to be expanded to include other persons from municipalities where livestock densities are high.

Although research has indicated that LA-MRSA is not readily transmitted from person to person (*20*,*33*), cases continue to be reported with no identified livestock-associated risk factors. Some possible modes of exposure could involve contact with other domesticated animals, person-to-person contact, and contact with contaminated meat or, in some cases, environmental pathways such as air or waste releases from farms to the surrounding community. Future research should assess these factors in terms of their relationship to living in livestock-dense areas and the likelihood of exposure to MRSA with a larger sample sizes. Information from the statistically significant cluster in the cluster detection analysis can be used to target interventions in the Netherlands. Future work should investigate more recent cases, specifically those without direct links to livestock farming.

We confirm what has been suggested in other studies that veal calf farming (not just pig farming) is a risk factor for LA-MRSA. We also demonstrate a relationship between nontypeable MRSA and all cattle, not just veal calves. The hypothesis that a relationship exists between other types of cattle farming and LA-MRSA carriage should also be explored in further research.

These findings also have the potential to affect countries beyond the Netherlands. Although pig farming is an important industry in the Netherlands, its scale there is greatly overshadowed by the density of pig-farming operations in the United States. In the United States, in 2007, there were 75,442 pig farms, 8,206 of which have >2,000 pigs on them (10.9%) (*34*). For comparison, in the Netherlands in 2000, of the 14,524 pig farms, only 983 housed >2,000 swine (6.8%). Future work could investigate the relationship between these more intensive livestock operations and drug-resistant microorganisms, especially LA-MRSA, which at present has not been widely detected in the United States. These research findings will be useful for generating hypotheses regarding the epidemiology of LA-MRSA in the Netherlands and can provide a warning that where one lives may play a critical role in one's risk of disease.

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Sources of Dengue Viruses Imported into Queensland, Australia, 2002–2010

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To assess risk for importation of dengue virus (DENV) into Queensland, Australia, and sources of imported viruses, we sequenced the envelope region of DENV isolates from symptomatic patients with a history of travel during 2002–2010. The number of imported dengue cases greatly increased over the surveillance period, some of which were associated with domestic outbreaks. Patients reported traveling to (in order) Asia, Papua New Guinea, Pacific Island countries, and non–Asia-Pacific countries. By using phylogenetic methods, we assigned DENV isolates from returning residents and overseas visitors with viremia to a specific genotypic group. Genotypes circulating in Asia were extremely diverse. Genotyping and molecular clock analysis supported Asian origination of a strain that caused an outbreak of DENV-4 in Pacific Island countries during 2007–2009, and subsequently, in Innisfail, Australia, in 2009. Our findings indicate that Asia is a major source of DENVs that are imported into Australia, causing a risk for epidemics.

Queensland, a state located in the tropical and subtropical northeastern area of Australia, has a long history of dengue virus (DENV) activity. Dengue was present in the late 19th century (*1*) and, following a lull for most of the 20th century, dengue importation and epidemic transmission have been increasingly reported in the past 20 years (*2*,*3*). Epidemics of the disease have occurred historically in other states of Australia, but only in Queensland have epidemics been reported in recent times. These epidemics were caused by the distribution of the vector, *Aedes aegypti* mosquitoes. The species was once found in other Australian states, but its area of distribution has now contracted so that it lies almost exclusively within Queensland's borders (*4*,*5*).

Despite repeated transmission events, dengue is not endemic to Queensland, and transmission requires a viremic traveler to import the virus to initiate epidemic spread (*6*). Rapid identification of cases and disease tracking, incorporating targeted vector surveillance, and control measures adopted rigorously to limit epidemic potential have been major factors in preventing local transmission and in reducing the cost of managing mosquito-borne disease (*7*).

With the apparent increasing frequency of dengue epidemics and imported cases, the disease has become a major public health issue. Exposure to multiple serotypes of DENV, of which there are 4 in total, may result in a higher probability of potentially life-threatening conditions such as dengue hemorrhagic fever and dengue shock syndrome, a potentially life-threatening condition (*8*). Perhaps not coincidentally, 2 fatal cases of dengue hemorrhagic fever were reported in 2004 in Queensland, and the serologic profile of the case-patients indicated secondary infection consistent with dengue shock syndrome (*9*). Of additional concern is the possibility that the virus may become endemic if case numbers were to rise to a point at which vector control measures became ineffectual at controlling virus spread.

Recent DENV infection is diagnosed by serologic testing, through virus isolation or by nucleic acid amplification by reverse transcription PCR (RT-PCR). The advantage of the latter is that sequencing of reaction products enables a definitive diagnosis of acute infection, identification of the virus serotype, and genotyping. As an adjunct to isolation techniques, sequencing and genotyping can provide valuable evidence of importation or can confirm local transmission and enable differentiation between multiple circulating strains and serotypes.

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Analysis of DENV sequence data facilitates rapid disease tracking and vector control. However, not all specimens are suitable for RT-PCR because infected persons usually exhibit a relatively short-lived viremia early in the febrile period (*10*). In addition, clinicians may find it difficult to obtain acute-phase samples, particularly if patients delay their initial consultation or are still in transit during their viremic phase.

As part of control measures by Queensland Public Health, we sequenced the envelope region of DENV isolates from symptomatic patients with a history of travel during 2002–2010. The proportion of the 4 DENV serotypes that were imported was determined, as well as the geographic origin of each serotype. Phylogenetic trees containing imported DENV viruses and others strains circulating throughout the world (from GenBank) were constructed by using a maximum likelihood model. From this analysis, we ascertained the likely geographic origin of imported viruses. This enabled us to assess the risk for importation of DENV from various sources by travelers entering Australia.

Materials and Methods

Virus Samples

Serum samples from patients with suspected DENV infection were referred to the Public Health Virology Laboratory, Queensland Health Forensic and Scientific Services, following the directive of Queensland Public Health medical officers, or were obtained through the public or private laboratory network. Acute-phase specimens underwent RT-PCR and serologic testing, and those that successfully yielded an RT-PCR product (after specific DENV serotype amplication) were sequenced and genotyped by phylogenetic analyses to assist public health investigations. This work was approved by the Ethics Committee of Queensland Health Forensic and Scientific Services.

Viral RNA Extraction and Nucleotide Sequencing

RNA was extracted from 200 μL of serum, either manually (QIAamp viral RNA extraction kit; QIAGEN, Hildren, Germany) or by using the EZ1 Virus Mini Kit and (QIAGEN) according to the manufacturer's instructions. Amplification was performed for each DENV serotype by using the Superscript III/Platinum Taq High Fidelity One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with specific RT-PCR primers (Table 1, Appendix, wwwnc.cdc.gov/EID/article/18/11/12-0014-T1.htm). Nucleotide sequencing of the complete envelope gene region (DENV-1, DENV-2, and DENV-4: 1,485 bp; DENV-3: 1,479 bp) was performed by using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence data obtained were deposited in GenBank (Table 2, Appendix, wwwnc.cdc.gov/EID/article/18/11/12-0014-T2.htm).

Phylogenetic Analysis of Envelope Protein Sequence

Full-envelope protein sequences for each serotype were aligned by using the multiple alignment tool of MEGA5 (www.megasoftware.net). Unrooted trees were then constructed by using a maximum likelihood estimation with a Jukes-Cantor model and γ-distributed rates, and by constructing 1,000 replicates to generate bootstrap support values. Divergence time from a common ancestor was estimated by using the molecular clock calculator.

Results

Increasing Incidence of Dengue Outbreaks and Serotype Diversity

Previous reports (*3*,*11*) and anecdotal evidence indicated that there has been an increase in the number of dengue outbreaks occurring in Queensland. To investigate this apparent trend, we combined recent and historical outbreak data (*3*) over a 20-year period. A 5-year moving average does indeed show trends of increasing dengue outbreak incidence and increasing diversity of DENV serotypes that cause such outbreaks (Figure 1, panels A, B). A line of best fit revealed a significant increase with time ($r^2 = 0.48$; $p<0.05$ by Student *t* test, 2-tailed). All 4 DENV serotypes caused outbreaks; DENV-2 was the most common cause (50.0%), followed by DENV1 and DENV-3 (19.4% each) and DENV-4 (11.1%) (Figure 1, panel C). The increase in outbreak incidence reflects changes in international travel over this period, which has increased 3.5-fold since the early 1990s (*12*). This increase is consistent with increased importations of virus carried by viremic travelers and the recognized increase in DENV infections throughout the world (*13*). Also of note is the dramatic increase in infections caused by imported viruses in 2010 (Figure 1, panel D).

Geographic Origins and Diversity of Imported DENVs, 2002–2010

We ascertained the number and diversity of imported DENV serotypes from infected travelers during 2002–2010 (Table 3). This period was chosen because the most comprehensive patient sequence data were available. Information was analyzed from viremic travelers, for whom an RT-PCR amplification product and serotype designation could be obtained. The possible strain origins, which were determined after phylogenetic analyses, were compared with available travel histories to ascertain likely geographic origins of the infecting virus. The data were categorized into 4 separate regions: Asia, Papua New

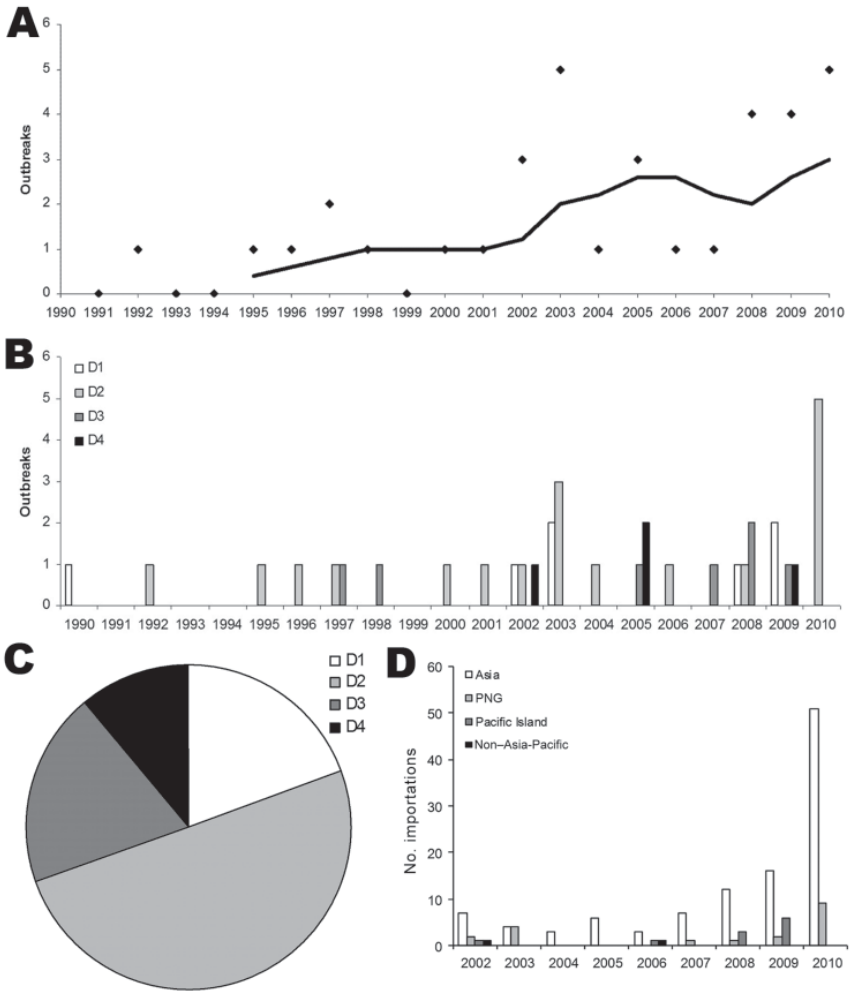

Figure 1. Number and diversity of dengue outbreaks in northern Queensland, Australia. A) Outbreaks of dengue causing epidemic spread in Queensland 1990–2010 showing 5-year moving average. B) Outbreaks shown as individual serotypes. C) Proportion of dengue virus serotypes responsible for the outbreaks shown in A and B. D) Geographic origins of dengue viruses imported into Queensland by viremic travelers. D1–D4, DENV-1–DENV-4; PNG, Papua New Guinea.

Guinea (PNG), the Pacific Islands, and countries outside of the Asia-Pacific region (non–Asia-Pacific).

Most infected travelers (77.9%) reported spending time abroad in Asia. In particular, 26.4% of all virus importations could be traced to Indonesia alone. All 4 DENV serotypes were detected in the specimens sequenced from persons with a travel history to that country. Notably, patients reported traveling to other Asian countries, including Thailand and the Philippines, where all 4 DENV serotypes have been found. Most other countries to which travel was reported had at least 3 DENV serotypes (Timor-Leste, PNG, and Vietnam), 2 serotypes (Cambodia, India, Fiji, Malaysia, and Laos), or 1 serotype (Brazil, Guyana, Samoa, Singapore, Solomon Islands, Sri Lanka, Tonga, and Vanuatu). A greater degree of diversity cannot be excluded in many of these countries because sampling numbers for individual countries were often low.

We calculated the proportion of the 4 DENV serotypes for infected travelers (Figure 2, panel A), which was slightly different from the proportion associated with outbreaks within Queensland (Figure 1, panel C). The serotype most commonly imported by travelers was DENV-1 (39.3%), followed by DENV-2 (25.7%), DENV-3 (21.4%), and DENV-4 (13.6%). Strains of all 4 DENV serotypes originated mainly in Asia (Figure 2, panel B). DENV-1 had the most diverse origins, with patients reporting travel mainly to Asia, but also to PNG, the Pacific Islands, and non–Asia-Pacific regions. In addition to Asia, DENV-2 was found to originate in PNG; DENV-3 originated in PNG and a non–Asia-Pacific area (Brazil); and DENV-4 originated in the Pacific Islands.

Origins of DENVs Imported by Returning Residents

Infected travelers were either returning Queensland residents or international visitors. Returning residents were the largest proportion of patients (96.4%) who sought treatment from the health system with dengue viremia. Further analysis was conducted on the subset of returning residents (135 of 140 patients with imported cases). Similarly to the analysis of all travelers above, infected

Table 3. Number and diversity of imported dengue serotypes, Queensland, Australia, 2002–2010*

Region and country	No. (%) cases	Dengue serotypes (genotypes)
Asia		
Indonesia	37 (26.4)	1 (I, IV), 2 (Cosmopolitan), 3 (I), 4 (II)
Thailand	15 (10.7)	1 (I), 2 (Asian genotype I), 3 (II), 4 (I)
Philippines	10 (7.1)	1 (IV), 2 (Cosmopolitan), 3 (I), 4 (I)
India	9 (6.4)	1 (V), 2 (Cosmopolitan)
Timor-Leste	9 (6.4)	1 (IV), 2 (Cosmopolitan), 4 (II)
Vietnam	7 (5.0)	1 (I), 2 (Asian genotype I), 3 (II)
Malaysia	5 (3.6)	1 (I, IV), 4 (II)
Laos	2 (1.4)	1 (I), 2 (Asian genotype I)
Cambodia	2 (1.4)	1 (I), 3 (II)
Singapore	1 (0.7)	1 (I)
Sri Lanka	1 (0.7)	1 (V)
Asia, not specified	11 (7.9)	–
Papua New Guinea	19 (13.6)	1 (I, IV), 2 (Cosmopolitan), 3 (I)
Pacific Islands		
Fiji	4 (2.9)	1 (IV), 4 (II)
Samoa	2 (1.4)	4 (II)
Solomon Islands	1 (0.7)	1 (ND)
Tonga	3 (2.1)	4 (ND)
Vanuatu	1 (0.7)	4 (ND)
Non–Asia-Pacific		
Brazil	1 (0.7)	3 (ND)
Guyana	1 (0.7)	1 (V)
Total	140	

*ND, not determined

returning residents (Table 4) reported that they had most frequently returned from Asia (77.0%), followed by PNG (13.3%), then the Pacific Islands (8.1%), and least often from non–Asia-Pacific areas (1.5%). The overall ratio was significantly different from that expected on the bases of the proportion of all Queensland residents who reported returning from dengue-endemic countries of those 4 regions, as calculated by using data from the Australian Bureau of Statistics for 2002–2010 (χ^2 analysis, p = 0.0004). The proportion of patients reporting travel to Asia, and to PNG in particular, was higher than expected. The overrepresentation of cases from PNG is best explained by

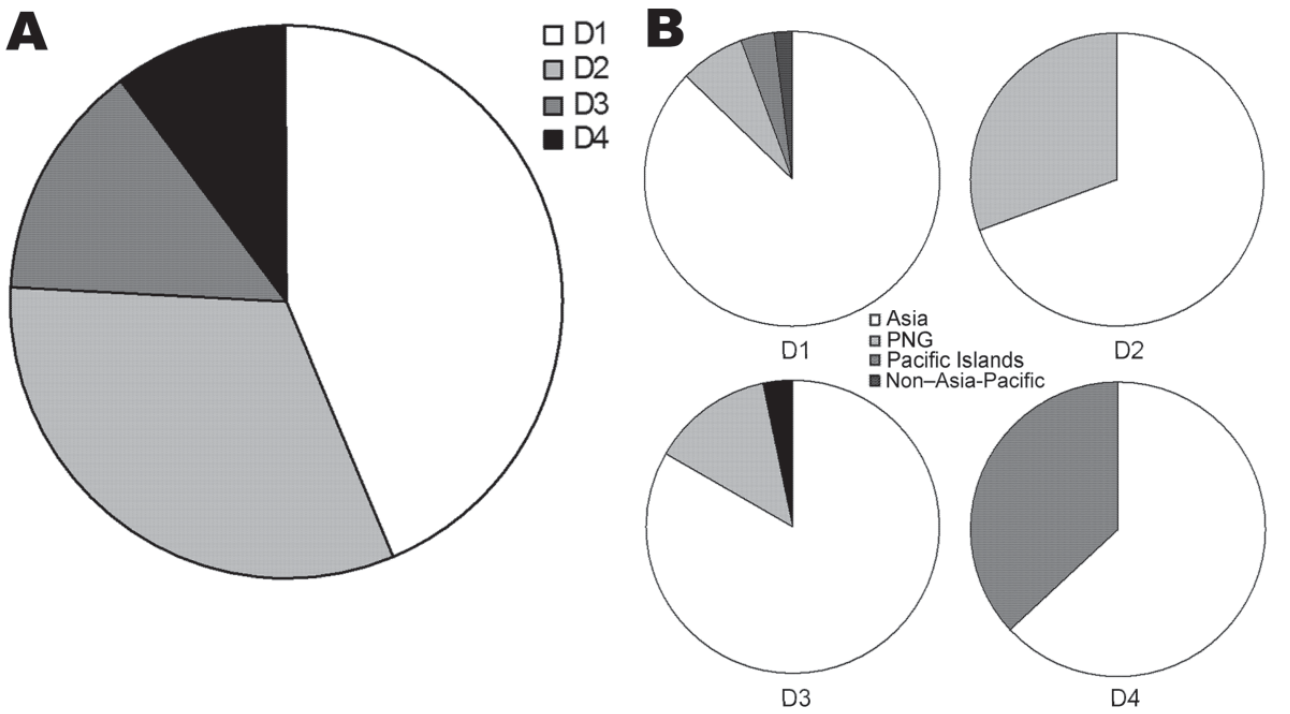

Figure 2. Importation of dengue viruses (DENVs) into Queensland, Australia, 2002–2010. A) Proportion of imported DENV serotypes. B) Geographic origins of the 4 imported DENV serotypes. D1–D4, DENV-1–DENV-4; PNG, Papua New Guinea.

a recent increase in DENV activity in that country, which is consistent with a large number of importations from PNG (47.4%) during 2010 (Figure 1, panel D) and, in addition, other recent reports (*14*). In comparison, the proportion of patients who reported travel to the Pacific Islands was lower than expected. Because only 2 cases originated in the non–Asia-Pacific region, it was subsequently difficult to draw conclusions about this region for statistical purposes.

Genotype Assignment of Imported DENVs

The genotypic mix for the various regions from which dengue was imported is shown in Table 3 and Figures 3–6. Across all regions, viruses could be classified into 1 of 2 genotypic groups within each serotype; the exception was DENV-1, which had 3 groups. In countries which were a source of imported viruses, generally 1 genotypic group for each serotype predominated.

DENV genotypic groups generally circulate in particular regions (*15*). The viruses imported into Queensland were consistent with DENV genotypes which had previously been reported to circulate in those countries to which patients had reported travel (*16*–*19*). For example, DENV-4 genotypic group II has been reported in Indonesia, Tahiti, the Caribbean Islands, and Central and South America (*17*). In 2007–9, DENV-4 was introduced into the Pacific Islands, displacing DENV-1 in the process (*20*,*21*). Our genotypic analysis confirms classification of the Pacific Island DENV-4 in genoptypic group II, as recently reported (*21*). This was the first time this genotypic group had been reported in the Pacific region, and suggested that the origin of this strain of DENV-4 may have been Southeast Asia.

In support of this suggestion, a closely related DENV-4 strain from the Torres Strait (Figure 6, JN575595) with 99.1% envelope nucleotide identity to a DENV-4 strain from Samoa (Figure 6, JN575592), was detected before the Pacific outbreak in 2005. A maximum likelihood test of the phylogenetic tree determined that a molecular clock was applicable (H_o not rejected; $p = 0.06$). Using a previously published substitution rate for dengue 4 of 1×10^{-3} substitutions/site/year (*22*), we calculated that divergence from a common ancestor occurred in ≈2002 with an error of ± 2 years. Thus, the Pacific Island outbreak strain (Figure 6, Pacific Island clade) is geographically and temporally closely related to the Torres Strait 2005 virus. Both of these virus strains are mostly closely related to DENV-4 strains which originated in Indonesia (Figure 6, JN575583). These data support suggestions the Pacific Island outbreak strain originated in Indonesia and made its way to the Torres

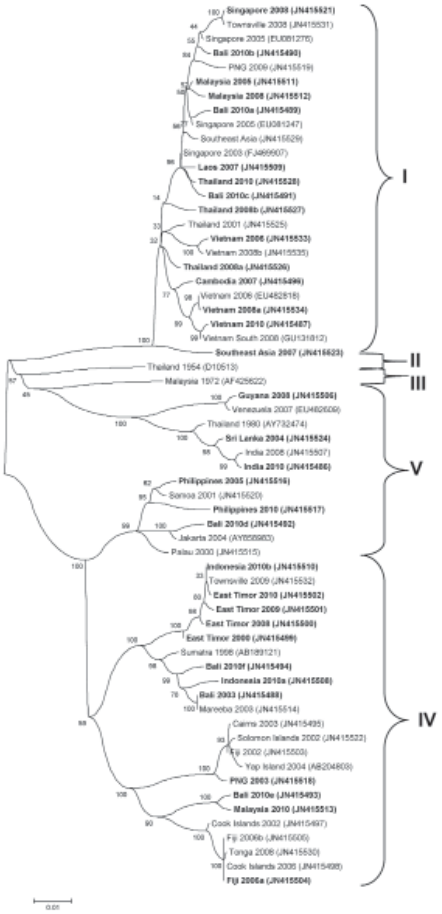

Figure 3. Phylogenetic tree showing the relationship of dengue viruses, serotype 1, imported into Queensland, Australia, 2001–2010, based on sequencing of the envelope gene. Viruses are designated according to reported origin and GenBank accession number, and imported cases are shown in **boldface**. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

Table 4. Observed and expected numbers of imported DENVs by region, Queensland, Australia, 2002–2010*

Imported DENVs	No. (%) from Asia†	No. (%) from Papua New Guinea	No. (%) from Pacific Islands	No. (%) from non–Asia-Pacific region
Observed	104 (77.0)	18 (13.3)	11 (8.1)	2 (1.5)
Expected‡	92 (68.2)	11 (7.9)	28 (20.8)	4 (3.2)

*χ^2 is 18 ($p<0.0004$; 2-tailed test). DENVs, dengue viruses.
†Asia includes travelers to Indonesia, Timor-Leste, Thailand, India, Malaysia, Philippines, Vietnam, Singapore, Cambodia, Sri Lanka and Laos; Papua New Guinea; Pacific Islands includes travelers to Fiji, Samoa, Solomon Islands, Tonga, and Vanuatu; non–Asia-Pacific region was defined as all cases outside the Asia-Pacific region which included Brazil and Guyana.
‡Based on travel data from the Australian Bureau of Statistics (www.abs.gov.au/) for departing Queensland residents who named the country where they planned to spend the most time, selected for those countries designated as having an ongoing dengue transmission risk, according to the Centers for Disease Control and Prevention Dengue Map (www.healthmap.org/dengue).

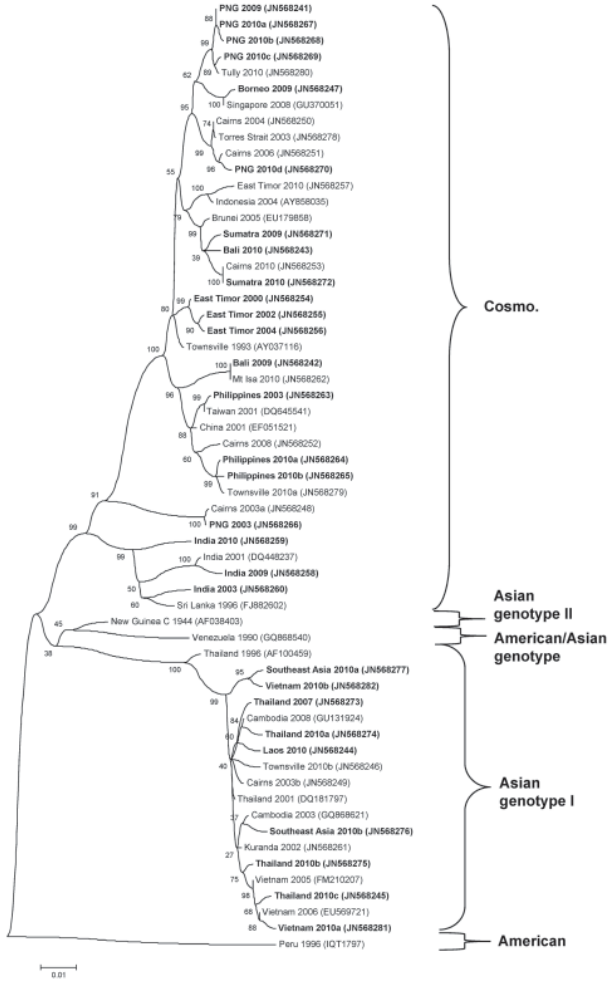

Figure 4. Phylogenetic tree showing the relationship of dengue viruses, serotype 2, that were imported into Queensland, Australia, 2002–2010, based on sequencing of the envelope gene. Viruses are designated according to reported origin and GenBank accession number, and imported cases are shown in **boldface**. Genotypes are indicated on the right. Cosmo., Cosmopolitan. Scale bar indicates nucleotide substitutions per site.

Strait (Australia) in 2005, probably through PNG, and into the Pacific in 2007 where it is currently circulating. A virus most closely related to the Pacific Island strain was then imported into Innisfail in northern Queensland in 2009, where it caused an outbreak (Figure 6). This incident highlights the epidemic potential of DENV strains that are imported into Queensland (*3*,*23*,*24*).

Discussion

In this study we have analyzed the importation of DENVs into northern Queensland, the only area within Australia where domestic epidemic spread is a risk. Two issues are apparent from these analyses. First, DENV infections, in terms of the number of importations and outbreaks, have increased in recent years. This issue is most apparent when it is considered that 42.9% of all instances of virus importation identified in this study occurred in 2010. The greatest risk was from residents returning from travel overseas, rather than overseas visitors. However, cases in the latter may be somewhat underreported because they may be more reluctant to seek medical assistance in a foreign country.

The second issue is the large degree of risk that Asia represents as a primary source of DENVs that can cause epidemics in Australia. Not only does Asia represent the biggest source of imported viruses in terms of number and serotype diversity, but it is also a source of viruses that can be imported into the Pacific region and, subsequently, a secondary source of importation into Australia as can be seen from the outbreak of the Pacific Island DENV-4 genotypic group II in Innisfail in 2009. If suggestions that the Pacific Island states are unable to sustain long-term DENV circulation are correct (*20*), then Asia may also be an important source of new outbreaks in the Pacific by

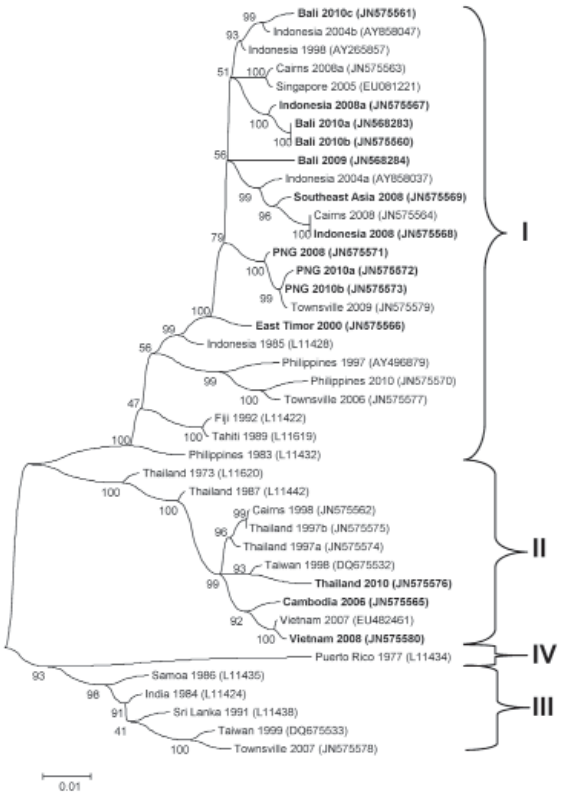

Figure 5. Phylogenetic tree showing the relationship of dengue viruses, serotype 3, imported into Queensland, Australia, 2002–2010, based on sequencing of the envelope gene. Viruses are designated according to reported origin and GenBank accession number, and imported cases are shown in **boldface**. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

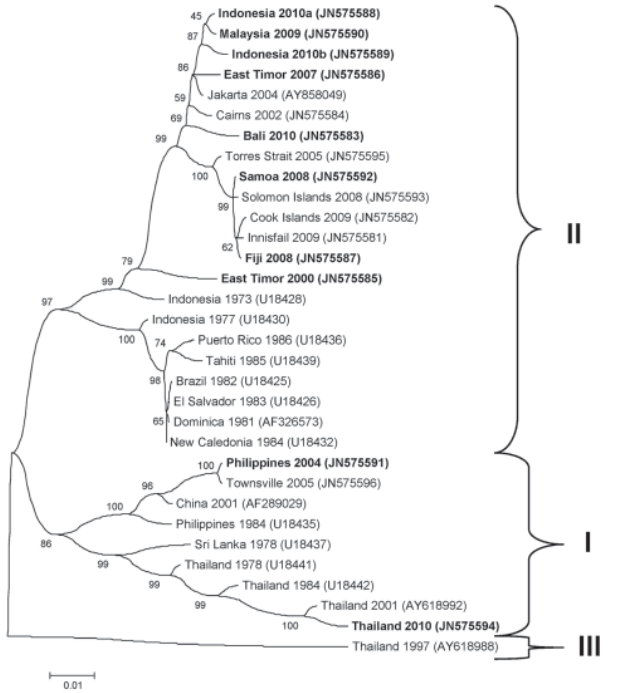

Figure 6. Phylogenetic tree showing the relationship of dengue viruses, serotype 4, imported into Queensland, Australia, 2002–2010, based on sequencing of the envelope gene. Viruses are designated according to reported origin and GenBank accession number, and imported cases are shown in **boldface**. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

incursions perhaps from either PNG or the islands of the Torres Strait.

To determine whether travelers returning from the 4 regions were either underrepresented or overrepresented in the dataset, we compared travelers departing Australia (using information obtained from outgoing passenger cards). A subset of Queensland residents was used as this information was available from the Australian Bureau of Statistics only for outgoing residents. The overrepresentation of infections imported from Asia and PNG relative to the Pacific Island countries may be due to higher levels of DENV activity in those countries. In the case of PNG, this result was mostly due to a large increase in imported DENVs from that country in 2010 (47.4% of all imported DENVs from PNG). Data from 2011 continue this trend to higher levels of DENVs imported from that country (data not shown). A previous study noted a decline in imported DENVS from PNG from 51% over the period 1999–2003 to 12% from 2004 to 2008 (*25*). The findings from this study may indicate a return to the historically higher proportion of imported DENVs from that region with the likelihood that recent dengue activity in PNG has intensified.

Little is known about overt disease in adults who acquire DENV-2 and DENV-4 infections. Disease may only be seen in those persons with previous antibody responses to another dengue serotype (*26*). This circumstance has implications for vaccine development because those persons with DENV antibodies may experience disease when exposed to vaccine formulations that contain apparently attenuated DENV-2 and DENV-4 (*26*). Susceptible adults who contract dengue while traveling represent an opportunity to study the factors associated with overt disease. To explore the pathogenicity of DENV-2 and DENV-4, serologic responses should be correlated, in the context of patient age, with molecular diagnostics in future studies of dengue surveillance.

This work clearly shows the increasing risk that viremic travelers pose to Australia, and to Queensland in particular, as a means for importing DENVs that could have substantial outbreak potential. Molecular epidemiologic studies have identified Asia as the greatest source of DENV infections that have been imported into Queensland recently. The increase in imported DENV strains and the number of outbreaks is of major public health importance and has been largely exacerbated by the heightened frequency and affordability of modern air travel. As this trend continues, the chance of the virus becoming endemic and the likelihood of the recurrence of disease also increase. Although additional studies are required to investigate the clinical implications of the imported viruses and specific patient anomalies, the sequence information presented here could assist future understanding of viral markers in relation to symptomatic disease and their association with pathogenesis.

Acknowledgments

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Dr Warrilow is currently the research and development coordinator at Public Health Virology, Queensland Health Forensic and Scientific Services. He has investigated viruses of human health importance, including arboviruses, Australian bat lyssavirus, and HIV, and his current research interests focus on RNA virus replication, diagnostics, and virus discovery.

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Phylogeography of Dengue Virus Serotype 4, Brazil, 2010–2011

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Dengue virus serotype 4 (DENV-4) reemerged in Roraima State, Brazil, 28 years after it was last detected in the country in 1982. To study the origin and evolution of this reemergence, full-length sequences were obtained for 16 DENV-4 isolates from northern (Roraima, Amazonas, Pará States) and northeastern (Bahia State) Brazil during the 2010 and 2011 dengue virus seasons and for an isolate from the 1982 epidemic in Roraima. Spatiotemporal dynamics of DENV-4 introductions in Brazil were applied to envelope genes and full genomes by using Bayesian phylogeographic analyses. An introduction of genotype I into Brazil from Southeast Asia was confirmed, and full genome phylogeographic analyses revealed multiple introductions of DENV-4 genotype II in Brazil, providing evidence for >3 introductions of this genotype within the last decade: 2 from Venezuela to Roraima and 1 from Colombia to Amazonas. The phylogeographic analysis of full genome data has demonstrated the origins of DENV-4 throughout Brazil.

Dengue virus (DENV), a widespread arthropod-borne virus that commonly affects humans, belongs to the family *Flaviviridae*, genus *Flavivirus*, and is classified into 4 distinct serotypes (DENV 1–4). DENV is most prevalent in tropical and subtropical areas, where eco-epidemiologic conditions appear to sustain the virus. In particular, these regions harbor 2 competent DENV vectors (*Aedes aegypti* and *A. albopictus* mosquitoes) and have environments favorable for DENV (*1*). In past decades, the number of countries reporting DENV cases and those with endemic DENV has increased dramatically. These increases reflect the expanding habitat of the *Aedes* spp. mosquito vectors, the poorly planned urbanization of many cities in developing countries, an increased number of susceptible human hosts, and the rapid spread of DENV serotypes through global human travel networks (*2*–*4*). According to the World Health Organization, ≈3 billion persons living in >100 countries are at risk of being infected at least once annually by 1 of the 4 DENV serotypes (*4*).

In Brazil, DENV-1–3 were responsible for ≈5 million cases of DENV infection during 1990–2009, resulting in >15,000 reported cases of dengue hemorrhagic fever and ≈1,000 DENV-related deaths (*5*–*7*). DENV-4 reemerged in Brazil in 2010, 28 years after it was last detected in the country; the site of the reemergence was Roraima State, northern Brazil (*8*), the same state in which DENV-4 had last been detected in 1982 (*9*). Brazilian Ministry of Health data for 2010 and 2011 show there were 1,666,208 cases of DENV infection, including 26,659 severe cases and 1,097 associated deaths (*10*).

We describe the genetic characterization and spatiotemporal patterns of spread for DENV-4 strains isolated from 4 Brazilian states: Pará, Amazonas, and Roraima in northern Brazil and Bahia in northeastern Brazil. To characterize the origins of DENV-4 reemergence, we performed discrete Bayesian phylogeographic analysis on

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98 full-length DENV-4 genomes and compared the results with those of a similar analysis on 314 envelope gene sequences.

Material and Methods

Viral Strains

We included 16 DENV-4 isolates in this study (Table). The viruses corresponded to low-passage virus strains (passage no. 1) from C6/36 cells obtained from the Department of Arbovirology and Hemorrhagic Fevers, Instituto Evandro Chagas, Brazilian Ministry of Health (Ananindeua, Brazil).

Sequencing, Assembly, and Accession Numbers

Nearly complete genome sequences were obtained by using high-throughput sequencing on a GS FLX+ System (454 Life Sciences, Branford, CT, USA) as described (*11*). The sequences of the 5′ and 3′ termini were obtained by using 5′ and 3′ rapid amplification of cDNA ends systems (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, with the following specific sets of primers: DENV-4 5′SP1 (5′-AKCCCTGTCTTGGGTCCAGC-3′), DENV-4 5′SP2 (5′- TTGAACGCCTCTTGAAGGTC-3′), DENV-4 5¢SP3 (CTCTTGAAGGTCCAGGTCTA), DENV-4 3′SP1 (5′-ATATCTGAATGGCAGCCATC-3′), and DENV-4 3′SP2 (5′-TCACTGGCTGTTTCTTCTGCT-3′). All 5′ and 3′ rapid amplification of cDNA ends amplicons were cloned into a plasmid bacterial system by using the TOPO TA Cloning Kit (Invitrogen) and directly sequenced (in both directions) by using the ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit on an ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA) with the M13F/M13R set of primers (Invitrogen).

Data generated by the GS FLX+ System and ABI 3130 platforms were assembled by using the mapping reference method as implemented by the GS Reference Mapper Program (available in Newbler v.2.6 software, http://454.com/products/analysis-software/index.asp). The mapper program was used to rearrange the reads against a given reference sequence by using the following default parameters: input = 20 bp, all contig threshold = 100, large contig threshold = 200, minimum overlap length = 40, minimum overlap identity = 70%, *k*-mer = 12 (seed step), and *k*-mer = 16 (seed length). A total of 16 new full-length DENV-4 sequences were obtained and deposited in GenBank (accession nos. JQ513330–JQ513345).

Nucleotide Data Compilation and Alignment

We complemented our data with available full genome and envelope protein sequences for a total of 98 full genome (10,624 nt) and 314 full-length DENV-4 envelope protein (1,485 nt) sequences. These included 18 sequences from Brazil (3 from Belém, Pará State; 1 from Santarém, Pará State; 5 from Manaus, Amazonas State; 8 from Boa Vista, Roraima State; and 1 from Salvador, Bahia State); 26 full genome and 75 envelope protein sequences from the Caribbean region (Puerto Rico, Dominica, Dominican Republic, Jamaica, Puerto Rico, Trinidad and Tobago, Barbados, Montserrat, Martinique, and Bahamas); 8 full genome and 10 envelope protein sequences from Colombia; 34 full genome and 48 envelope protein sequences from Venezuela; 9 full genome and 88 envelope protein sequences from Mainland Southeast Asia (Thailand-Bangkok, Vietnam, Cambodia, Myanmar, and Peninsular Malaysia); and 3 full genome and 22 envelope protein sequences from Maritime Southeast Asia (Philippines, Indonesia, Singapore, and East Malaysia).

We aligned the sequences by using MAFFT (*12*). After we manually edited the resulting alignment by using Se-Al (*13*), the total lengths of the full genome and envelope protein alignments consisted of 10,624 bp and 1,485 bp, respectively. All sequences were screened for recombination by using the Phi-test (*14*), which is available in the SplitsTree4 program (*15*).

Table. Dengue virus type 4 strains isolated in Brazil used for genetic characterization and phylogeographic analyses

Identification	Strain	Year of isolation	Municipality, state, of isolation	GenBank accession no.
ROR 7542	Be H 774846	2010	Boa Vista, Roraima	JQ513333
ROR 7591	Be H 780090	2010	Boa Vista, Roraima	JQ513340
ROR 7620	Be H 780120	2010	Boa Vista, Roraima	JQ513341
ROR 7357	Be H 772846	2010	Boa Vista, Roraima	JQ513330
ROR 7363	Be H 772852	2010	Boa Vista, Roraima	JQ513331
ROR 7365	Be H 772854	2010	Boa Vista, Roraima	JN559741
STM 31	Be H 775222	2010	Santarém, Pará	JQ513334
BEL 83791	Be H 778494	2011	Belém, Pará	JQ513335
BEL 83846	Be H 778887	2011	Belém, Pará	JQ513337
BEL 83804	Be H 778504	2011	Belém, Pará	JQ513336
AM 5079	Be H 779652	2011	Manaus, Amazonas	JQ513339
AM 5105	Be H 780571	2011	Manaus, Amazonas	JQ513344
AM 4963	Be H 779228	2011	Manaus, Amazonas	JQ513338
AM 5090	Be H 780556	2011	Manaus, Amazonas	JQ513342
AM 5097	Be H 780563	2011	Manaus, Amazonas	JQ513343
BHI 3681	Be H 781363	2011	Salvador, Bahia	JQ513345

Evolutionary Reconstruction of DENV-4 Dispersal

The parameters of a full probabilistic model of evolutionary history, including timed sequence evolution and spatial dispersal, were estimated by using a discrete Bayesian asymmetric diffusion approach (*16*) implemented in the BEAST software package (*13*,*17*). We used the Bayesian skyride model (*18*) as a flexible tree prior and a general time-reversible model with a discretized gamma distribution (general time-reversible + 4Γ) to account for among-site rate variation. To calibrate the time scale of the trees, we obtained isolation dates (in years) from the GenBank annotations, and to accommodate rate variation among lineages, we used a lognormal relaxed molecular clock approach (*19*).

This approach enabled us to estimate ancestral spatial locations throughout the phylogenetic history while accounting for uncertainty in the phylogenetic and diffusion process (*17*). For the full genome and envelope protein datasets, 3 Markov-chain Monte Carlo analyses were run for 50 million states and sampled once every 10,000 states. We used the BEAGLE library (*13*) together with BEAST (*13*) to augment the computational speed. After we removed 10% of the burn-in, we combined the runs by using LogCombiner (www.molecularevolution.org/software/phylogenetics/beast). Maximum clade credibility (MCC) trees were summarized by using TreeAnnotator and visualized by using FigTree (*12*). We use the SPREAD application (*20*) to visualize and convert the estimated divergence times and spatial estimates annotated in the MCC trees to a keyhole markup language file (the files are available from authors upon request). All evolutionary parameters are reported as posterior means along with their 95% Bayesian credibility intervals.

To obtain the expectations for the location state transitions, we estimated Markov jump counts (*18*,*21*) along the branches of the posterior tree distribution (*22*). We applied kernel density estimation in R to summarize Markov jump densities through time from particular locations.

Results

Asian DENV-4 Genotype I in Northern Brazil

The analysis (Phi-test implemented in SplitsTree) of the 98 DENV-4 full genomes did not provide support for recombination. Therefore, all 98 complete nucleotide (10,624 nt) and 314 (1,485 nt) envelope protein sequences were included in the Bayesian phylogeographic analyses by using a discrete asymmetric diffusion model and a flexible demographic prior (online Technical Appendix Table, wwwnc.cdc.gov/EID/pdfs/12-0217-Techapp.pdf).

The complete genome and envelope protein MCC genealogies confirmed that 3 major phylogenetic groups were compatible with the genotypes currently established for DENV-4: genotypes I–III (Figure 1). In particular, genotype I (n = 5 full genome, n = 85 envelope protein sequences) included strains that were isolated in Southeast Asia and a unique strain (BHI 3581 Be H 781363) that was isolated from an autochthonous febrile patient in 2011 in Salvador, Bahia State. The dense sampling in the envelope protein dataset made it possible for us to establish a relatively recent

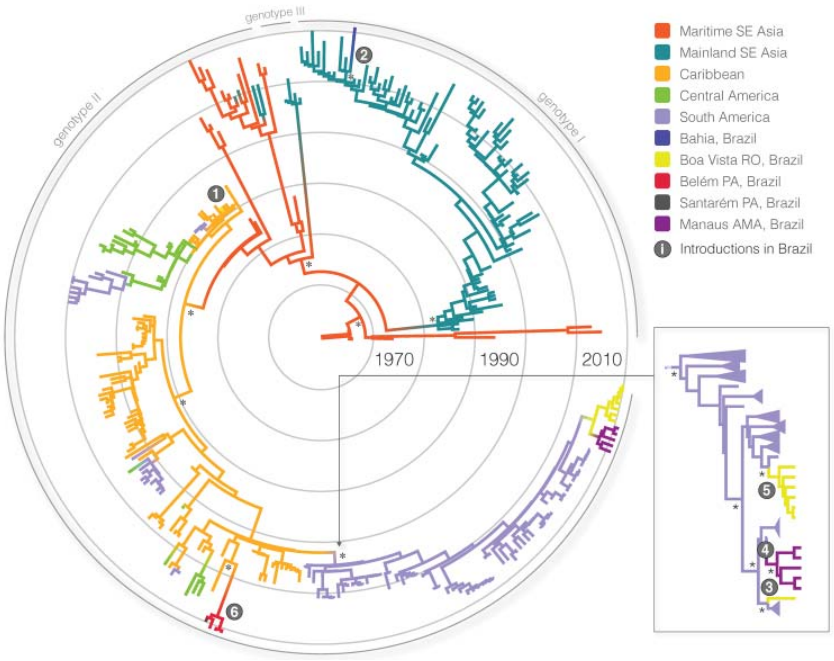

Figure 1. Maximum clade credibility tree demonstrating the phylogenetic relationships of the 314 dengue virus type 4 (DENV-4) envelope genes (1,485 nt). The major groups (genotypes I–III) are indicated. Branch lengths are scaled in time units; scale bars, representing the chronology of DENV-4 emergence, are color-coded according to the most probable geographic location of the descendent node. Introductions of DENV-4 in Brazil are indicated by numbered circles; numbering follows the temporal order of the mean estimate of divergence time for that particular sequence or clade. In selected nodes, * indicates posterior probability support of 1.00. Panel on right shows a detail of the maximum clade credibility tree (built by using an alignment of 98 DENV-4 full genomes [10,624 nt]) that reveals 3 distinct well-supported introductions of genotype II cluster from neighboring South American countries into Brazil. SE, Southeast; RO, Roraima State; PA, Pará State; AMA, Amazonas State. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/12-0217-F1.htm).

common ancestor with an Asian genotype I dating back to 2004 (95% Bayesian credible interval [BCI] 2002–2007). In particular, results from the genomic and envelope protein phylogeographic analyses confirmed with high posterior probability (>0.99) that the Brazilian strain from Bahia was introduced from Mainland Southeast Asia.

Multiple Introductions of DENV-4 Genotype II in Brazil

Genotype II (n = 86 full genome, n = 223 envelope protein sequences) included the other 17 strains sampled in Brazil (Municipalities of Manaus, Belém, Santarém, and Boa Vista) and in Venezuela, Colombia, Central America, the Caribbean, and Southeast Asia. Genotype III (n = 2 full genome, 4 envelope protein sequences) included strains that were exclusively isolated from Southeast Asia (Thailand). On the basis of the envelope gene analysis, we reconstructed 3 introductions of genotype II DENV-4 in Brazil (Figure 1, left panel). First, the envelope protein MCC tree indicated a Brazilian cluster of strains identified in Boa Vista and Manaus as part of the major South American genotype II cluster. Second, the full genome MCC tree broke the cluster in Boa Vista and Manaus into 3 different lineages in the same genotype II cluster. Third, the large South American genotype II cluster, as part of the full genome MCC genealogy (Figure 1, right panel), indicates additional introductions of genotype II into Brazil. Thus, we identified a total of 6 introductions of DENV-4 in Brazil.

The most recent common ancestor of genotypes I–III is believed to have a Southeast Asian origin (7). In line with recent findings (*23*), we estimate that genotype II, the predominant genotype, was likely first introduced into the Americas (Caribbean region) around 1978 (95% BCI 1976–1979) (Figure 1; online Technical Appendix Figure). Following a period of circulation in the Caribbean region, this genotype independently entered South American countries >4 times, as inferred respectively by the full genome and envelope protein analyses. Most of these introductions into South America seem to have led to relatively shallow phylogenetic clusters that probably reflect short-term survival of the virus in South America.

Nevertheless, our data provide support for a major genotype II cluster that reflects the successful establishment of this genotype in South American countries neighboring Brazil in or around 1994 (95% BCI 1992–1995). From Venezuela and Colombia, the genotype spread to Brazil >3 times in the last decade, as inferred by the full genome phylogeographic analyses (Figure 1, right panel). In contrast, the DENV-4 outbreak in Pará State (Santarém and Belém) was identified around 2008 (95% BCI 2006–2009) and is directly linked to strains from the Caribbean region. Overall, we found >3 distinct DENV-4 introductions into Roraima State: 1 from the Caribbean region ≈3 decades ago (indicated as 1 in Figure 1, left panel), and 2 from the strain that has become endemic in Venezuela within the last decade (indicated as 3 and 5 in Figure 1, right panel). One of these strains resulted in a well-supported cluster that dated back to about 2006 (95% BCI 2005–2007) and comprised 6 strains.

Global Patterns of DENV-4 Dissemination

To identify the major export and import locations for DENV-4 at a global scale, we summarized the number of introductions from and to each of the locations that were estimated by using Markov jump count approaches as part of the full genome analysis (Figure 2, panel A). Our results pinpoint Maritime Southeast Asia, the Caribbean region, and Venezuela as the 3 most prominent sources of DENV-4 export. In addition, to investigate the temporal dynamics of viral dispersal from the 3 locations in the evolutionary history of DENV-4, we estimated the Markov jump density from these locations through time (Figure 2, panel B). We distinguished different peaks, which appear to reflect 3 distinct waves of DENV-4 migration; the most recent wave corresponded to exportations from South American countries bordering Brazil, particularly Venezuela, where DENV-4 has been established longer.

After an initial establishment in the Caribbean region, genotype II most likely first dispersed to Brazil (Roraima State), Venezuela, Colombia, and, more recently, to Brazil (Belém, Pará State) again (Figure 3). Locations such as Santarém, Pará State, and Manaus, Amazonas State, do not have a direct relation to the Caribbean locations; instead, the strain found in Santarém is linked to a cluster of sequences from other locations in Pará State. This finding further indicates that there is ongoing circulation of DENV-4 within Brazil. Nevertheless, most recent introductions of genotype II in Brazil in the last decade seem to have been fueled by strains originating from bordering countries.

Discussion

DENV-4 was not detected in Brazil for 28 years after the first clinical and laboratory reports of dengue fever cases in Roraima State during 1981–1982 (*9*). Since then, no additional cases were reported in the country until the reemergence of DENV-4 in Boa Vista, Roraima State, in 2010 (*8*). Following those first recent detections of DENV-4 in Brazil, the virus was identified in 2011 in the northern Brazilian states of Amazonas, Amapá, and Pará. Furthermore, DENV-4 was serologically detected in persons in several other states, demonstrating the potential for this virus to spread quickly to several regions (*8*).

Since the first detection of DENV-4 in Brazil in 1982 (*8*,*9*), partial genomic studies have confirmed that the original virus was directly associated with the Caribbean strains, which are known as genotype II. Genotype II has

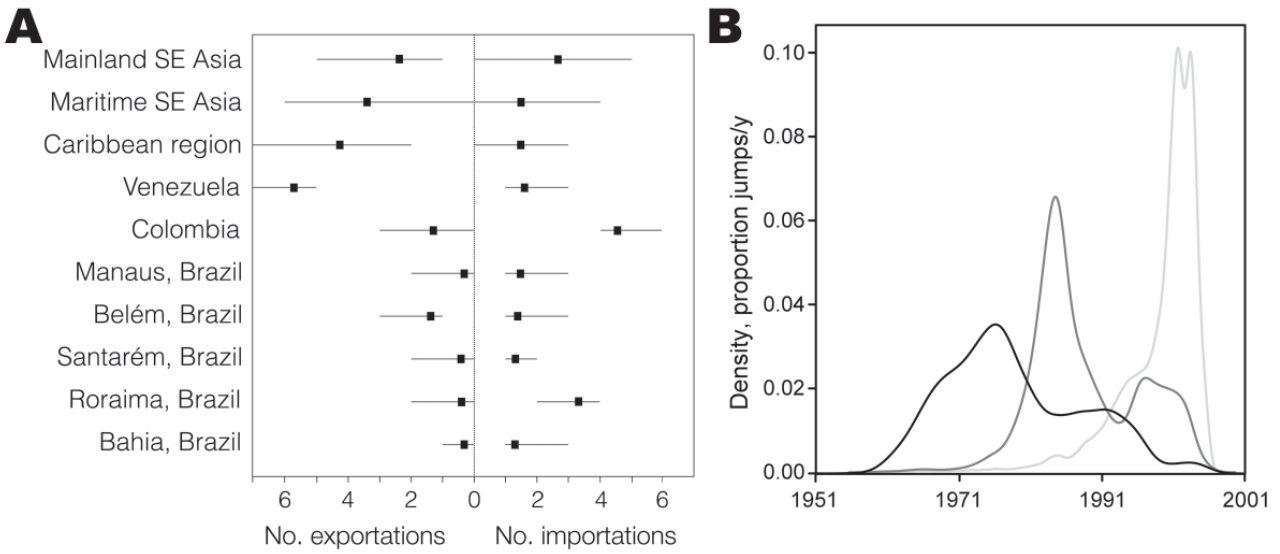

Figure 2. A) Estimated number of dengue virus type 4 exportations and importations (mean and 95% Bayesian credible intervals) (A). The full genome dataset was used and available data were discretized for countries neighboring Brazil. SE, Southeast. B) Markov jump density of viral exportations over time for the 3 major exporters of dengue virus type 4 (black line, Maritime Southeast Asia; dark gray line, Caribbean region; and light gray line, Venezuela).

been responsible for several outbreaks in many countries in the Caribbean and South America (*8*,*24*–*28*). Phylogenetic analyses of different strains demonstrated the presence of 2 distinct genotypes (genotypes I and II) of DENV-4 in Brazil. The introduction of a new serotype and a distinct DENV-4 genotype (Asian genotype I) into Brazil after nearly 3 decades of no circulation highlights the potential for future outbreaks of genotype I in this country. The presence of a serotype against which the population is not immunized indicates that the country is at risk for a sharp increase in the number of dengue virus infections, including severe cases (*8*).

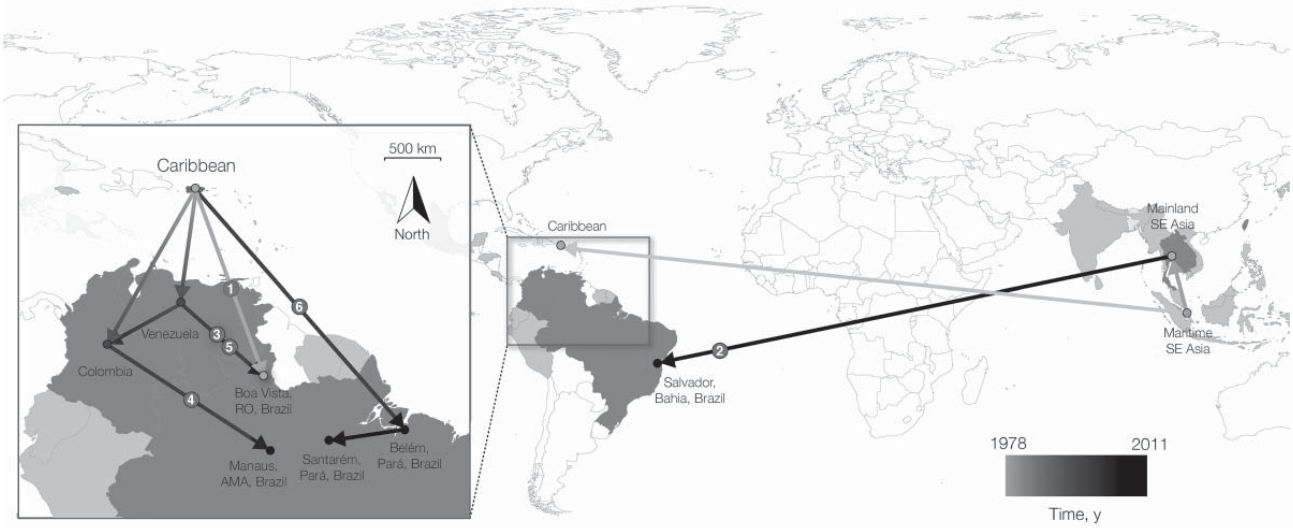

Figure 3. Overview of spatiotemporal dispersal of dengue virus type 4 (DENV-4) from Southeast (SE) Asia to the Caribbean region and then to South America. Links between geographic locations represent phylogeny branches of the full genome maximum clade credibility tree, as projected by using SPREAD software (*20*). The time gradient is coded to the arrows and depicts the relative time elapsed since the earliest inferred viral migration out of Southeast Asia (i.e., 1978, 95% Bayesian credible interval 1977–1980). Introductions are numbered as in Figure 1. Medium gray indicates the presence of DENV-4 where genomic data were available; dark gray indicates sampled countries where complete genomic sequence data were available; white indicates no genomic data available; and circles indicate sampled locations and are colored according to the earliest migration that was detected from the sink location. RO, Roraima State; AMA, Amazonas State. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/12-0217-F2.htm).

The phylogeographic analyses of full genomes and envelope genes confirmed the co-circulation of 2 distinct DENV-4 genotypes (I and II) in Brazil. Genotype II is most common in South America and the Caribbean region, and genotype I was represented by 1 strain isolated from Bahia State in northeastern Brazil. The analyses also confirmed the introduction of DENV-4 into Pará State from the Caribbean region and suggest that this introduction is very recent (online Technical Appendix Figure).

Previous phylogenetic analyses have been performed on partial and a few complete DENV-4 genomes (*8*,*29*–*31*). The results we obtained by using the new set of complete genomes suggest that DENV-4 genotype II emerged and reemerged in Brazil from >3 distinct origins (Southeast Asia, the Caribbean region, and Venezuela), which demonstrates a dispersal pattern in Brazil that is far more complex than expected from standard epidemiologic data.

We demonstrate that full genome analysis complements the analysis of more widely available envelope protein sequences. Although a dense sampling may have its own particular benefits for divergence time and spatial diffusion estimation, full genomes can provide more phylogenetic resolution to unravel individual introductions from populations of closely related strains.

Our results indicate that DENV-4 was exported multiple times from the Caribbean region to northern South American locations before the virus became established in South America. Overall, the spatiotemporal patterns of DENV-4 revealed in South America over a long time scale are reminiscent of a source-sink model of virus dispersal (*32*), in which the location of primary source virus populations shifted from Maritime Southeast Asia to the Caribbean region and, most recently, to northern South America, albeit with co-circulation in each location. This dispersal pattern differs from the source-sink dynamics of seasonal influenza, in which each epidemic wave seems to be caused by emergence from the same source (*33*). Active molecular epidemiologic surveillance will be essential for better characterizing local source populations of DENV-4.

In conclusion, we found that 2 distinct genotypes (I and II) of DENV-4 are circulating in Brazil, and we provide insight into the origin and dispersal of the DENV throughout northern Brazil and areas of several South American countries. Further studies are needed to analyze complete genomes from other countries to which DENV-4 is endemic; such studies will more fully elucidate the geographic dispersal dynamics of DENV-4 in regions of the Americas where it is endemic.

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Coxsackievirus B3, Shandong Province, China, 1990–2010

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To determine the cause of a 2008 outbreak of aseptic meningitis in Shandong Province, China, we analyzed samples from outbreak patients and coxsackievirus B3 samples collected during 1990–2010 surveillance. The cause of the outbreak was coxsackievirus B3, genogroup D. Frequent travel might increase importation of other coxsackievirus B3 genogroups.

Coxsackievirus B3 (CVB3) (family *Picornaviridae*, genus *Enterovirus*) is a major human pathogen (*1*–*3*), and CVB3-associated aseptic meningitis is an emerging concern (*4*). In the summer of 2008, an aseptic meningitis outbreak occurred in southern Shandong Province, People's Republic of China. Shandong is a coastal province with a population of 94.7 million. The huge number of hospitalized children caught the attention of public health officials and media and triggered an extensive study on the causative agent. To help determine the cause of the outbreak, we analyzed samples from outbreak case-patients and conducted a molecular epidemiology study of CVB3 isolates collected in Shandong during 1990–2010.

The Study

The 2008 outbreak occurred during June–September and peaked in early July. A total of 887 patients, 596 male and 291 female, were hospitalized. Patient ages ranged from 2 months to 64 years; most (98%) were <15 years of age. Epidemiologic investigation showed that 617 (69.6%) case-patients had a history of contact with aseptic meningitis case-patients, and 159 (17.9%) had drunk untreated well water. The most common clinical manifestations were fever (89.6%), vomiting (57.2%), headache (48.3%), lethargy (14.5%), and rash (1.5%). No sequelae or deaths were reported.

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To investigate the causative agent, we collected 120 cerebrospinal fluid samples and 22 fecal samples from 142 case-patients. Using the RD and HEp-2 cell lines, we isolated 82 enteroviruses (67 from cerebrospinal fluid and 15 from feces); serotypes were CVB3 (n = 81) and echovirus 30 (n = 1). We sequenced and analyzed the virus capsid protein 1 (VP1) regions of 34 randomly selected CVB3 strains as described (*5*–*7*). Only strain TC177 had 8.1%–9.7% genetic divergence with the other 33 strains, which shared 98.6%–100.0% identity among themselves.

We also analyzed details of clinical and environmental CVB3 strains collected in Shandong since 1990 (Table) by using data from the acute flaccid paralysis (AFP) surveillance system. Because China has no specialized enterovirus surveillance system, no comprehensive molecular epidemiologic data are available for CVB3 or other nonpolio enteroviruses that cause aseptic meningitis. However, the AFP surveillance system, developed for the polio eradication program and conducted in Shandong since 1990, can provide baseline data of local nonpolio enterovirus circulation (*8*).

According to the AFP surveillance system, during 1990–2010, a total of 768 nonpolio enteroviruses were isolated, and 59 (7.7%) strains were typed as CVB3 by use of a molecular method (*6*). Frequency of CVB3 isolation fluctuated from year to year and peaked during 1996, 2000–2002, 2005, and 2008 (Figure 1). The low level of CVB3 before 1996 might be a surveillance artifact because AFP surveillance in China increased substantially throughout the 1990s. CVB3 accounted for ≈21% of all nonpolio enterovirus isolates from AFP case-patients in 2008. Another outbreak of aseptic meningitis outbreak occurred in 2005, during which 57 CVB5 strains were recovered (*9*) and 2 CVB3 strains were identified. From environmental surveillance, CVB3 was isolated in 2008 (n = 7), 2009 (n = 1), and 2010 (n = 1). In addition, CVB3 was isolated from patients with hand-foot-and-mouth disease (n = 2) and sporadic aseptic meningitis and encephalitis syndrome (n = 4).

To study the molecular epidemiology of CVB3 in Shandong, we used MEGA version 4.0 (*10*) to phylogenetically analyze the complete VP1 sequences of global CVB3 strains, including 72 Shandong isolates from AFP surveillance (n = 59), aseptic meningitis case-patients (n = 7), hand-foot-and-mouth disease case-patients (n = 2), and environmental surveillance (n = 4) (Figure 2). Global CVB3 strains were segregated into 4 genogroups, A–D, with at least 15% complete VP1 nucleotide diversity between clusters. All strains from Shandong, together with 5 strains from the Chinese provinces of Guangdong, Jilin, Anhui, Yunnan, and Beijing, fell into genogroup D, which was further divided into 3 subgenogroups, D1–D3, repre-

[1]These authors contributed equally to this article.

Table. Sources of 157 coxsackievirus B3 in Shandong Province, People's Republic of China, 1990–2010*

Source	Specimen	No. isolates	Years of isolation
AFP surveillance	Feces	59	1990–2010
Environmental surveillance	Sewage	9	2008–2010
Patients with aseptic meningitis and encephalitis syndrome in Jinan	CSF	4	2006–2008
Patients with aseptic meningitis during outbreak in Tancheng	CSF and feces	81	2008
Patients with aseptic meningitis during outbreak in Yanzhou	CSF	2	2005
Patient with hand-foot-and-mouth disease	Throat swab	2	2008

*AFP, acute flaccid paralysis; CSF, cerebrospinal fluid.

senting isolates from 1990–2003, 2002–2010, and 2004–2009, respectively. Homologous analysis indicated that nucleotide identities among subgenogroups D1–D3 were 90.9%–100%, 89.6%–100%, and 94.4%–99.6%, respectively. The representative strain from the aseptic meningitis outbreak (TC012) belonged to subgenogroup D2, and TC177 belonged to subgenogroup D3.

When partial VP1 sequences (393 nt) were aligned, the relationship between Shandong strains in 2008 and Hong Kong aseptic meningitis strain 26362/08 was close: strains 08153 and 08281 had the highest similarities (98.7%) with 26362/08. A close relationship (97.7%–98.5% similarity) was also observed between 26362/08 and the other aseptic meningitis strains from Shandong (TC047, CVB3 strain 08AM.199, hand-foot-and-mouth disease strain Y019, environmental stains in 2008, and the strains from Beijing, Anhui, and Yunnan from 2008).

The VP1 sequences were deposited into GenBank. Accession numbers are GQ246518, GQ329744–GQ329767, FJ919564, FJ919566–FJ919598, GU272011–GU272013, and JQ364844–JQ364885.

Conclusions

Our results indicate that CVB3 was the cause of the outbreak and that most CVB3 isolates were closely related. The AFP surveillance in Shandong, although insufficient for monitoring enterovirus infections of humans, revealed a similar fluctuating epidemic mode for CVB3 with a temporal peak in 1–3 years and quiescence for 2–3 years. The temporal dynamics of echovirus 30, causing nationwide epidemics of 2–4 years separated by periods of quiescence, have been reported (*11*). The CVB3 peak detected by AFP and environmental surveillance in 2008 correlated well with the aseptic meningitis outbreak, demonstrating high CVB3 activity at that time. Of note, aseptic meningitis surveillance in Hong Kong detected increased CVB3 activity during 2001, 2005, and 2008 (*4*), consistent with the temporal peak of CVB3 in Shandong. The close genetic relationship between the strains from mainland China and the Hong Kong strains in 2008 suggests that the same strain was circulating in both regions.

Most global CVB3 strains belong to genogroups B and D. During 1970–2006, genogroup B came from the United States, Australia and Europe. However, this genogroup has not yet been found in mainland China. Although genogroup D was composed entirely of strains from China, our data are insufficient for us to propose that genogroup D is confined to China only. Nevertheless, our study demonstrates that genogroup D has been predominantly circulating in mainland China for the past 20 years and is responsible for all documented outbreaks and sporadic cases of CVB3-associated aseptic meningitis. Because of the more frequent population exchange between China and the rest of the world, the chance for importation of other CVB3 genogroups to mainland China is greatly increased.

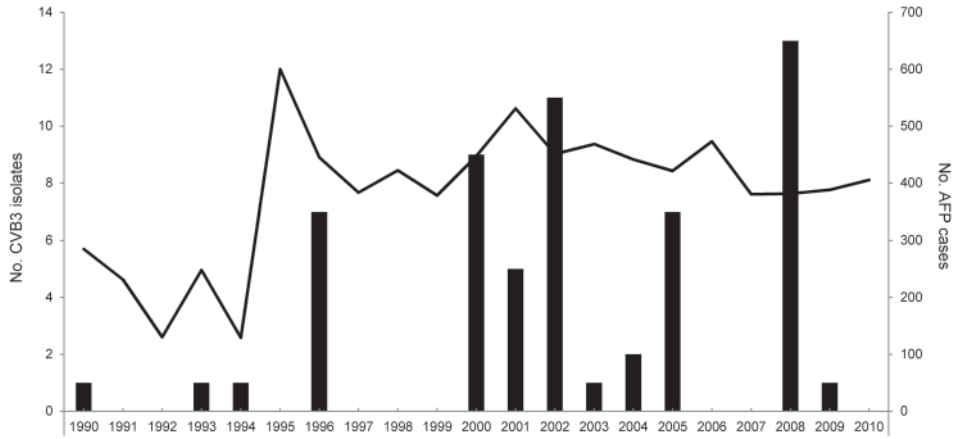

Figure 1. Frequency of isolation of coxsackievirus B3 (CVB3) in patients with acute flaccid paralysis, Shandong Province, People's Republic of China, 1990–2010. Bars indicate number of CVB3 isolates from acute flaccid paralysis (AFP) surveillance; line indicates number of cases of AFP.

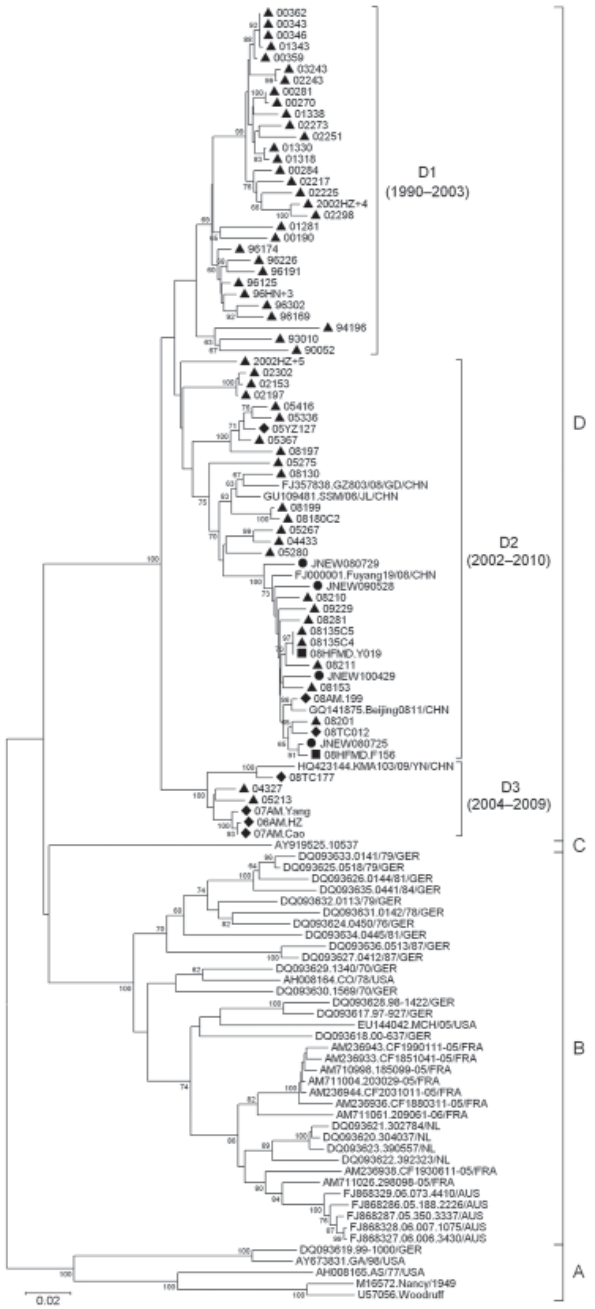

Figure 2. Phylogenetic tree based on the alignment of the entire virus capsid protein 1 coding regions of coxsackievirus B3 isolates from Shandong, People's Republic of China, and around the world. Triangles indicate isolates from patients with acute flaccid paralysis; diamonds indicate isolates from patients with aseptic meningitis; circles indicate isolates from the Shandong environment; and squares indicate isolates from patients with hand-foot-and-mouth disease; the arrow indicates the representative strain 2008TC012 from the aseptic meningitis outbreak in 2008. Strain 26362/08 from patients with aseptic meningitis in Hong Kong is not shown in the tree because only part of the virus capsid protein 1 sequence was available. AUS, Australia; CHN, China; FRA, France; GER, Germany; NL, the Netherlands; USA, United States. Scale bar indicates nucleotide substitutions per site.

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Vibrio fluvialis in Patients with Diarrhea, Kolkata, India

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We identified 131 strains of *Vibrio fluvialis* among 400 nonagglutinating *Vibrio* spp. isolated from patients with diarrhea in Kolkata, India. For 43 patients, *V. fluvialis* was the sole pathogen identified. Most strains harbored genes encoding hemolysin and metalloprotease; this finding may contribute to understanding of the pathogenicity of *V. fluvialis*.

Many members of the family *Vibrionaceae* cause diarrheal disease; among these, *Vibrio cholerae* O1/O139 and *V. parahaemolyticus* are responsible for several epidemics and pandemics (*1*,*2*). In Indonesia, >20% of diarrheal infections are caused by pathogenic *Vibrio* spp. (*3*). Some of these *Vibrio* spp. can grow in thiosulfate–citrate–bile salts–sucrose agar as yellow colonies and do not agglutinate with *V. cholerae* O1 antiserum. These species are broadly defined as nonagglutinating (NAG) vibrios.

The emerging etiologic agent *V. fluvialis* has caused sporadic cases and outbreaks of diarrhea in several countries (*4*–*6*). Species-specific minimal biochemical tests, e.g., lysine decarboxylase, ornithine decarboxylase, arginine didydrolase, and L-arabinose, are used to identify *V. fluvialis*; without these tests, it may be confused with NAG vibrios, *V. cholerae*, and even *Aeromonas* spp. In most resource-poor countries, these tests are not performed, which may lead to labeling of *V. fluvialis* as a NAG vibrio.

Although *V. fluvialis* is known to cause diarrhea, the mechanisms involved in its pathogenicity are not well established. To evaluate the prevalence of *V. fluvialis* in India and possible mischaracterization as an NAG vibrio, we examined cases in which isolates from hospitalized patients with diarrhea were identified as NAG vibrios and characterized the strains using phenotypic and genetic methods.

The Study

We examined 400 isolates identified as NAG vibrios that were collected during 2002–2009 from 11,904 stool specimens from patients with diarrhea admitted to the Infectious Diseases and Beliaghata General Hospital, Kolkata, India. Specimens were screened for common enteric pathogens, according to standard protocols (*7*). Oxidase, string test, and arginine dihydrolase–positive strains that did not agglutinate with *V. cholerae* O1 polyvalent or O139 monovalent antiserum were further confirmed as *V. fluvialis* by using a multiplex PCR targeting the *toxR* gene of *V. fluvialis* and the *ompW* gene of *V. cholerae* (*8*,*9*). Isolates were also subjected to PCRs targeting different virulence-associated genes encoding the repeat in toxin (*rtxA, rtxC*), heat-stable enterotoxin (*stn*), type 3 secretion system (*vcsC2, vcsV2, vcsN2* and *vspD*), cholera toxin (*ctxA*), toxin co-regulated pilus (*tcpA*), thermostable direct-hemolysin (*tdh*), TDH-related hemolysin (*trh*), *V. fluvialis* hemolysin (VFH), and metalloproteases, according to published methods (*10*–*12*).

Expression of VFH was determined in vitro by using erythrocytes from rabbit and sheep. Cytotoxin assay was performed with HeLa and Chinese hamster ovary cell lines by using sterile culture filters of the *V. fluvialis* strains that were isolated as a sole pathogen. Antimicrobial drug susceptibility testing was performed by using the disk diffusion method with commercially available disks (Becton Dickinson, Sparks Glencoe, MD, USA), according to Clinical and Laboratory Standards Institute criteria (*13*). Because these guidelines do not include interpretive criteria for *V. fluvialis*, breakpoints for *Enterobacteriaceae* were adopted. *Escherichia coli* ATCC 25922 was used as a quality control strain.

Pulsed-field gel electrophoresis was performed according to the PulseNet standardized protocol for *V. cholerae* (*14*). Gel Compare II software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used for electrophoresis pattern comparison that runs on Dice similarity index and unweighted pairgroup with arithmetic mean method.

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Table 1. Prevalence of *Vibrio fluvialis* among patients with diarrhea, Kolkata, India, 2002–2009

Year	No. samples	No. (%) *V. fluvialis* isolates	No. (%) patients	
			Sole infection	Mixed infection
2002	2,285	16 (0.7)	5 (0.2)	11 (0.5)
2003	1,673	8 (0.5)	1 (0.1)	7 (0.4)
2004	2,430	19 (0.8)	6 (0.2)	13 (0.5)
2005	1,472	17 (1.1)	7 (0.5)	10 (0.7)
2006	930	12 (1.3)	4 (0.4)	8 (0.9)
2007	842	9 (1.1)	2 (0.2)	7 (0.8)
2008	1,124	24 (2.1)	8 (0.7)	16 (1.4)
2009	1,153	26 (2.2)	10 (0.9)	16 (1.4)
Total	11,909	131 (1.1)	43 (0.4)	88 (0.7)

Among the 400 isolates presumptively identified NAG vibrios, multiplex PCR confirmed 131 and 269 strains (each strain representing a case) as *V. fluvialis* and *V. cholerae*, respectively. The overall prevalence rate of *V. fluvialis* among 11,904 hospitalized patients with diarrhea was 1.1%. Abrupt appearance of *V. fluvialis* was identified in 2002, although the surveillance of diarrheal infection was initiated at the Infectious Diseases and Beliaghata General Hospital in 1996 (www.niced.org.in/annual_reports.htm). The isolation rate of *V. fluvialis* gradually increased from 0.7% in 2002 to 2.2% in 2009 (Table 1). Of the 131 strains of *V. fluvialis*, 43 (33%) were identified as the sole pathogen; the remaining 88 (67%) were isolated as a co-pathogen with either *V. cholerae*, *V. parahaemolyticus*, *E. coli*, *Shigella* spp., parasites, or enteric viruses (data not shown). Among the mixed infections, *V. fluvialis* with *V. cholerae* was isolated most often (17%), followed by *V. fluvialis* and *V. parahaemolyticus* (8%). The presence of *Vibrio* spp. as mixed pathogens indicates that these patients likely acquired the infection from contaminated water or food. We analyzed the date of admission and place from where the patients resided and found no evidence for clusters of infection or small outbreaks caused by *V. fluvialis*.

V. fluvialis infection was much more often detected in adults (73%) than in children <5 years of age (27%). Clinical symptoms of sole infection caused by *V. fluvialis* were similar to that of cholera: watery diarrhea (86%), severe dehydration status (28%), and abdominal pain (12%) (Table 2). Several previous investigations have identified cholera-like diarrheal outbreaks caused by *V. fluvialis* (*4*,*5*).

All the *V. fluvialis* strains were negative for the virulence genes commonly reported in *V. cholerae* and *V. parahaemolyticus*, but >90% were positive for genes encoding VFH and metalloproteases. More than 80% of the strains expressed hemolysin against rabbit and sheep red blood cells. Hemolysin is a widely distributed virulence factor in most pathogenic *Vibrio* spp. Metalloprotease produced by *V. fluvialis* is related to hemagglutination proteases of *V. vulnificus*, which enhances permeability and hemorrhagic activities (*12*). These factors may increase the virulence of *V. fluvialis* and contribute to diarrhea.

When the culture filtrates were tested, cytotoxic effect was readily noticed in the Chinese hamster ovary and HeLa cell lines, i.e., cytoplasmic vacuolation, cell rounding, and destruction of the monolayer. In most strains isolated as a sole pathogen, the cytotoxic endpoint titer was 2–256 (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/12-0520-Techapp.pdf). The cell vacuolation phenomenon has been reported as a virulence factor in several enteric pathogens (online Technical Appendix References).

In this study, *V. fluvialis* strains were highly resistant to ampicillin (92%), streptomycin (85%), furazolidone (85%), and sulfamethoxazole/trimethoprim (70%) (online Technical Appendix Table 2). About half the number of strains were resistant to ciprofloxacin and 45% to nalidixic acid; the lower resistance rate for nalidixic acid compared with fluoroquinolones is unexpected and warrants further investigation to confirm the additional mechanisms. In a previous study, we found that some *V. fluvialis* strains carried the plasmid-mediated quinolone resistance gene allele *qnrA1* and a gene encoding the aminoglycoside acetyltransferase (*aac(6′)-Ib-cr*), which reduces ciprofloxacin activity (*15*). Fluroquinolone resistance and intermediate susceptibility to erythromycin (92%) are the unique features of the *V. fluvialis* isolated in this study;

Table 2. Clinical features of *Vibrio fluvialis*–infected patients with diarrhea, Kolkata, India, 2002–2009

Clinical feature	No. (%) patients	
	Sole infection	Mixed infection
Type of diarrhea		
Watery	36 (86)	72 (81)
Bloody mucus, loose	7 (16)	16 (19)
Dehydration status		
Severe	12 (28)	14 (16)
Some or rare	31 (72)	74 (84)
Fever		
Yes	4 (9)	9 (10)
No	39 (91)	79 (90)
Abdominal pain		
Yes	5 (12)	11 (12)
No	38 (88)	77 (88)
Age		
>5 y	30 (70)	66 (75)
≤5 y	13 (30)	22 (25)
Sex		
M	23 (53)	58 (66)
F	20 (47)	30 (34)

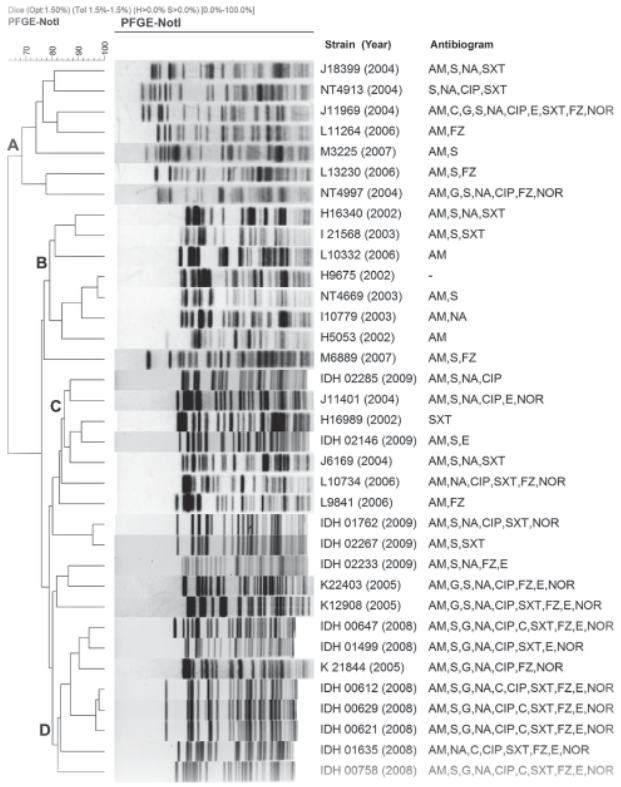

Figure. Dendrogram of *Not*I-digested pulsed-field gel electrophoresis (PFGE) profiles with representative *Vibrio fluvialis* isolates. Clustering identified 4 clades (A–D). AM, ampicillin; S, streptomycin; G, gentamicin; NA, nalidixic acid; CIP, ciprofloxacin; C, chloramphenicol; E, erythromycin; SXT, sulfamethoxazole-trimethoprim; FZ, furazolidone; NOR, norfloxacin. Scale bar indicates degree of similarity.

this trend was not recorded in other *Vibrio* spp., e.g., *V. cholerae* and *V. parahaemolyticus*.

Although the *V. fluvialis* strains exhibited distinct *Not*I restriction profiles in the denrogroam analysis, at least 4 major clades were identified (Figure). Clades A and B, with strains isolated during 2002–2007, exhibited less antimicrobial drug resistance than did clade C and D strains identified during 2008–2009; multidrug-resistant strains, especially those resistant to fluroquinolones, were identified in higher numbers in clades C and D (Figure).

Conclusions

Our results demonstrate an emerging trend of prevalence of *V. fluvialis* among patients with acute diarrhea patients in Kolkata. The expression of cytotoxic activity and hemolysin may contribute to understanding the pathogenicity of *V. fluvialis*. Further epidemiologic studies are necessary to elucidate the public health importance of *V. fluvialis*–mediated diarrhea.

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etymologia

Coxsackievirus

[kok-sak′e-vi′rəs]

Named for Coxsackie, the small town on the Hudson River where they were first isolated, human coxsackieviruses are nonenveloped, positive-sense, single-stranded RNA viruses in the family *Picornaviridae*, genus *Enterovirus*. They were first described by Gilbert Dalldorf, who was investigating suspected poliomyelitis outbreaks in upstate New York in the summer of 1947. Coxsackieviruses are divided into 2 groups, A and B. In suckling mice, group A viruses cause generalized myositis and flaccid paralysis, and group B viruses cause focal myositis and spastic paralysis. With the discovery of coxsackieviruses, Dalldorf also helped popularize the suckling mouse as an inexpensive laboratory animal model.

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Legionella pneumophila Serotype 1 Pneumonia in Patient Receiving Adalimumab

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We describe a case of severe pneumonia caused by *Legionella pneumophila* serotype 1 in a woman receiving the tumor necrosis factor–α antagonist to treat rheumatoid arthritis. As use of tumor necrosis factor–α inhibitors increase, clinicians should consider their possible association with legionellosis.

Legionella pneumophila, a gram-negative rod normally inhabiting aquatic environments, causes severe infections, including pneumonia (legionellosis) that can be acquired in the community or in hospitals. Traditional risk factors for legionellosis include smoking, corticosteroid use, and chronic lung disease (*1*). Receipt of a tumor necrosis factor–α antagonist (TNF-α antagonists: infliximab, adalimumab, etanercept) generally has not been considered a risk factor for legionellosis.

In the past 2 decades, use of TNF-α antagonists have revolutionized the treatment of rheumatoid arthritis, inflammatory bowel disease, psoriasis, and other inflammatory conditions. After introduction of these agents, a growing body of postmarketing literature has shown an increased risk for disease from *Mycobacterium tuberculosis*, endemic mycoses, and intracellular bacterial pathogens, including *Legionella pneumophila* (*2*). We report a severe case of right upper lobe pneumonia caused by *L. pneumophila* serotype 1, mimicking *M. tuberculosis* reactivation, in a patient receiving the TNF-α antagonist adalimumab for rheumatoid arthritis.

Case Report

In June 2010, a 67-year-old woman sought care at an emergency department in Winnipeg, Manitoba, Canada, with a 7-day history of fevers, rigors, and progressive breathlessness. She reported diarrhea, nausea, and vomiting during the preceding 4 days. A chronic nonproductive cough was unchanged. Her medical history included rheumatoid arthritis, hypothyroidism, hypertension, and dyslipidemia. Her immunosuppressive regimen consisted of azathioprine, 150 mg per day (unchanged over the previous 2 years) and adalimumab, 40 mg per month, initiated 10 weeks earlier. She reported no history of travel or contact with persons who had tuberculosis. She denied hot tub use or other exposures to aerosolized droplets. The result of a tuberculin skin test, performed at initiation of TNF-α inhibitor, was nonreactive.

On examination, the patient appeared acutely ill. Her respiratory rate was 30 breaths per minute. Her peripheral saturation of oxygen was 96% while receiving 5 L/min oxygen by face mask. Blood pressure and heart rate were 90/60 mm Hg and 140 beats per minute, respectively; oral temperature was 38.3°C. Breath sounds were rapid with crackles noted bilaterally to the lung fields and occasional wheezes. Abdominal examination disclosed some tenderness in the right lower quadrant.

Laboratory investigations showed a leukocyte count of 5.9 cells/L (reference 4.5–11.0 × 10^9 cells/L) (90% neutrophils), with toxic granulation, left shift, and Dohle bodies on the peripheral blood smear. Renal function was acutely impaired (creatinine 286 mmol/L [reference 35–97 μmol/L]); liver enzyme levels were moderately elevated (aspartate aminotransferase 150 U/L [reference 10–32 U/L], alanine aminotransferase 440 U/L [reference <25 U/L], alkaline phosphatase 75 U/L [reference 30–120 U/L]), but liver synthetic function was normal (total bilirubin 15 μmol/L [reference 3–19 mmol/L], albumin 19 g/L [reference 33–45 g/L], international normalized ratio 1.0 [reference 0.9–1.1]). An arterial blood gas suggested acidemia resulting from metabolic and respiratory acidosis with pH 7.23, pCO_2 41 mm Hg, HCO_3 17 mmol/L, and an anion gap of 23 (reference 10–12). The initial chest radiograph demonstrated right upper lobar consolidation (Figure 1).

The patient was intubated, placed on mechanical ventilation, and transferred to the intensive care unit. Her immunosuppressive agents were held, and antimicrobial drug treatment was initiated with vancomycin (because of concern about methicillin-resistant *Staphylococcus aureus* pneumonia), ceftriaxone, and azithromycin. Bronchoscopy demonstrated frank pus in the right upper lobe bronchi, cultures of which ultimately grew 2+ *L. pneumophila* serotype 1 and 2+ yeast and 1+ *Stenotrophomonas maltophilia*. Mycobacterial cultures were negative. Initial *Legionella* direct fluorescent antibody staining from the bronchoalveolar lavage was negative, as were blood cultures. A *Legionella* spp. urinary antigen test result was positive.

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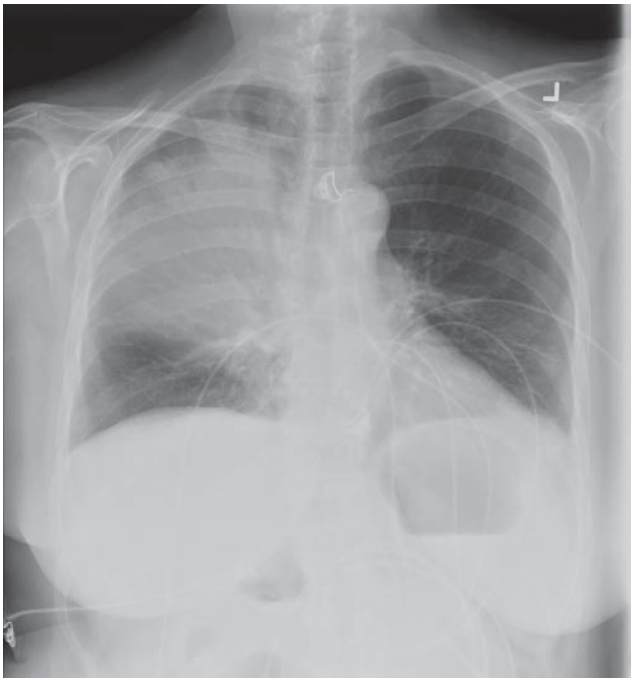

Figure 1. Chest radiograph demonstrating right-upper lobe consolidation in a 67-year-old woman with *Legionella pneumophila* serotype 1 pneumonia.

Antimicrobial drugs were switched on day 5 of hospitalization to levofloxacin (500 mg intravenous daily) and rifampin for *Legionella* spp. and trimethoprim–sulfamethoxizole to treat *S. maltophilia*. Over 24 hours, her clinical condition improved dramatically, leading to extubation and transfer to the general medicine ward. A computed tomographic scan of the chest, obtained 2 weeks after admission because of worsening hypoxemia, showed areas of necrosis, development of cavitary lesions within the right upper lobe, and a segmental pulmonary embolism. Anticoagulation was initiated. The antimicrobial drugs were continued for 3 weeks, then substituted for azithromycin, for a total duration of 6 weeks, which was considered an adequate duration of treatment given the cavitation. Mobilization and weaning from oxygenation were slow because of marked deconditioning and malnutrition.

With the support of physiotherapists and other members of the integrated health care team, the patient eventually recovered and was discharged home 5 weeks after admission. Repeat computed tomographic scan 2 months after her initial illness showed evolution of the pulmonary cavity and reduced consolidation (Figure 2).

Conclusions

Thirty-three cases of legionellosis have been described in patients receiving infliximab, adalimumab, or etanercept for rheumatoid arthritis, inflammatory bowel disease, psoriasis, or other inflammatory conditions (*3*–*5*). Similar to the case with other intracellular bacterial pathogens, such as *Listeria monocytogenes* (*2*), receipt of a TNF-α antagonist is emerging as a risk factor for pneumonia from *Legionella* spp. Lung cavitation or necrosis, which occurred in this case, is an uncommon manifestation of legionellosis and has been reported more commonly in immunocompromised hosts (*6*); we are aware of only 1 other case of lung cavitation caused by *Legionella* spp. in a patient receiving a TNF-α antagonist (*3*).

Registry data of all patients on a TNF-α antagonist in France suggested the relative risk for *L. pneumophila* infection was 16.5–21, compared with the general population (*7*). Although confounding factors, such as the effect of concomitant immunosuppressive medications or disease, are possible contributors, TNF-α antagonists themselves are likely to contribute substantially to the high risk for legionellosis in these patients. To our knowledge, azathioprine has not been associated with increased risk for *Legionella* infection.

Including the current report, 11 cases of *Legionella* spp. infection have been documented in patients receiving adalimumab. The researchers from France presented data indicating a higher risk for legionellosis in patients receiving infliximab or adalimumab, compared with etanercept (*8*).

The cytokine TNF-α appears to play a direct role in the immunologic response to *Legionella* spp. infection. TNF-α promotes macrophage recruitment and factors in

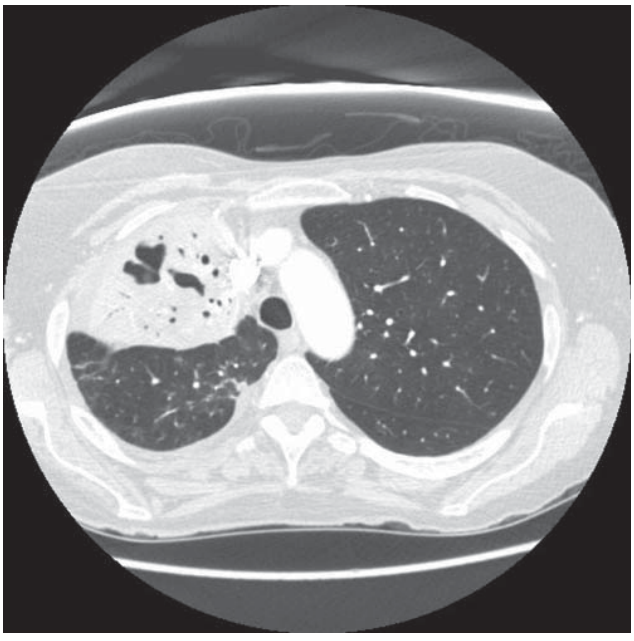

Figure 2. Results of repeat computed tomographic scan in a 67-year-old woman with *Legionella pneumophila* serotype 1 pneumonia 2 months after hospital admission. The scan shows reduction in the amount of consolidation and evolution of the lung cavity.

host response to infection with intracellular pathogens (*2*). Induction of increased levels of TNF-α to macrophage cultures resulted in resistance to subsequent infection with *L. pneumophila* serotype 1; however, susceptibility was restored with addition of TNF-α antibodies to the culture (*9*). Furthermore, a mouse knockout model demonstrated 90% mortality and decreased clearance of *L. pneumophila*–infected, TNF receptor–deficient mice, in comparison with wild-type mice (*10*). *L. pneumophila* grew in TNF receptor-1 deficient macrophage culture but not when this receptor was present (*10*). The exact mechanism by which cytokine TNF-α contributes to protection from infection by *Legionella* spp. has not yet been elucidated.

Guidelines for preventing *Legionella* infection in patients receiving a TNF-α antagonist are not available; however, minimizing aerosolized exposure to untreated water sources (such as decorative fountains) is reasonable (*1*). Current data do not support avoiding the consumption of tap water.

As use of TNF-α inhibitors increase, we urge clinicians to consider this association with legionellosis. Empiric therapy for pneumonia should include an agent with activity against Legionella, such as a fluoroquinolone or macrolide.

Dr Wuerz is a fellow in his final year of clinical infectious disease training at the University of Manitoba. His research interests include the epidemiology and role of infectious diseases in northern Canada.

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Susceptibility of Children to Sapovirus Infections Nicaragua, 2005–2006

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We describe the genetic diversity of sapovirus (SaV) in children in Nicaragua and investigate the role of host genetic factors and susceptibility to SaV infections. Our results indicate that neither ABO blood group, Lewis phenotype, nor secretor status affects susceptibility to SaV infection in Nicaragua.

Human sapovirus (SaV), family *Caliciviridae*, is a causative agent of gastroenteritis in children and adults. Symptoms of SaV infection seem to be milder than symptoms of rotavirus and norovirus (NoV) infections; thus, SaV infection prevalence is higher in nonhospitalized children than in hospitalized children.

Some calciviruses bind to histo-blood group antigens (HBGA) expressed on cells in the gastrointestinal tract (*1*). Human NoV strains demonstrate strain-dependent binding patterns to HBGAs (*1*,*2*). ABO blood groups and Lewis phenotype also play a role in NoV infections, either as ligands or as restriction factors (*2*). Moreover, a non-sense mutation in the fucosyltransferase gene 2 (*FUT2*), which gives rise to the nonsecretor phenotype, has been found to provide almost complete protection from experimental and natural NoV infection (*2*,*3*).

Although human NoV susceptibility is highly associated with secretor status, and thus with mutations in *FUT2* (*4*), no information is yet available on whether host genetic factors determine susceptibility to SaV. We describe here the genetic diversity of SaV in a Central American population of hospitalized and nonhospitalized children and investigate the role of host genetic factors and susceptibility to SaV infections.

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The Study

A total of 292 children ≤5 years of age (205 symptomatic and 87 asymptomatic) were randomly selected among 694 symptomatic and 158 asymptomatic children who participated in a community- and hospital-based study of sporadic acute diarrhea in León, Nicaragua, during 2005–2006. In brief, clinical cases were evaluated according to the World Health Organization strategy for diarrhea management (*5*), and fecal, blood, and saliva samples were collected from each child.

Viral RNA was extracted from stool suspensions by use of a QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions. RNA underwent reverse transcription (RT) to produce cDNA by using 50 pmol of random hexadeoxynucleotides (pd[N]6) (Amersham Biosciences, Chalfont St. Giles, United Kingdom) and RT-PCR beads (Amersham Biosciences), according to manufacturer´s instructions.

The RT products were amplified by SaV PCR using forward JV33 and reverse SR80 primers (*6*,*7*). This amplification was followed by an outer PCR with a primer pool of 2 forward (SV-F13 and SV-F14) and 2 reverse (SV-R13 and SV-R14) primers (*8*). Nested PCR was performed under identical conditions to the outer PCR with a primer pool consisting of universal forward primers (SV-F13 and SV-F14) and genogroup-specific reverse primers (SV-G1-R, SV-G2-R, SV-G4-R, and SV-G5-R). Primers for genogroup 3 were not available.

The N terminal and shell region of the SaV genome were then sequenced by using DYEnamic dye terminator kit (GE Healthcare, Little Chalfont, United Kingdom) (*9*). All samples included in the study were also screened for the presence of co-infection with the most common enteric pathogens (*5*). Any samples showing co-infection were then removed from further analysis.

Hemagglutination tests were performed to define blood types. The ABO blood group and Lewis phenotype in saliva were determined for persons found to be secretors as described (*10*).

A single nucleotide polymorphism at position 428 in the *FUT2* gene was investigated by pyrosequencing (*3*,*11*). Each person was classified as a homozygous secretor (SeSe), a heterozygous secretor ($Sese^{428}$), or a nonsecretor ($se^{428}se^{428}$).

SaV-positive specimens were compared with specimens from a control group of children who tested negative for SaV, which was matched in terms of sex, age group, clinical status, and HBGAs using 2-tailed significance with Fisher exact tests. GraphPad (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.

Of 292 samples analyzed, 33 (25 from symptomatic and 8 from asymptomatically infected children) (11%)

[1]These authors contributed equally to this article.

Table 1. Epidemiologic profile of SaV infections, León, Nicaragua, 2005–2006*

Characteristic	Symptomatic, n = 205			Asymptomatic, n = 87		
	Total	No. (%) SaV strains	OR	Total	No. (%) SaV strains	OR
Setting						
Community	160	20 (12)	0.9	87	8 (9)	
Hospital	45	5 (11)		0		
Both	205	25 (12)		87	8 (9)	
Patient sex						
M	125	14 (11)	0.8	43	6 (14)	3.405†
F	80	11 (14)		44	2 (5)	
Patient age, mo						
≤6	36	5 (14)	1.2	11	2 (18)	2.6‡
7–12	57	12 (21)	2.8§	15	2 (13)	1.7
13–24	67	7 (10)	0.8	48	4 (8)	0.8
25– 60	45	1 (2)	0.1	13	0 (0)	NA

*SaV, sapovirus; OR, odds ratio; NA, not applicable.
†Fisher test, 95%, CI, p = 0.15.
‡Fisher test, 95%, CI, p = 0.26.
§Fisher test, 95%, CI, p = 0.02.

were SaV positive. This study found that diarrhea is as common in SaV-infected children as it is in NoV-infected children in Nicaragua (*5*), and 11% of SaV-infected children were hospitalized (Table 1). Why the prevalence of SaV infections in Nicaraguan children is comparable to the high rate of NoV infections in these children is unknown and can only be speculated upon. Two likely key factors are poor sanitary conditions, which enables the virus to spread easily between susceptible persons, and malnourishment, which makes these children more susceptible to infection. Because we used conventional PCR with widely used JV33 and SR80 primers (*6*), the method of choice should not have created a bias.

Results of a previous seroepidemiologic study of SaV infections suggested that SaV infections are frequent in children during the first 2 years of life because >90% of these children had antibodies against SaV (*12*). Our study supports this proposal because most SaV infections, either symptomatic or asymptomatic, occurred in children <2 years of age (Table 1).

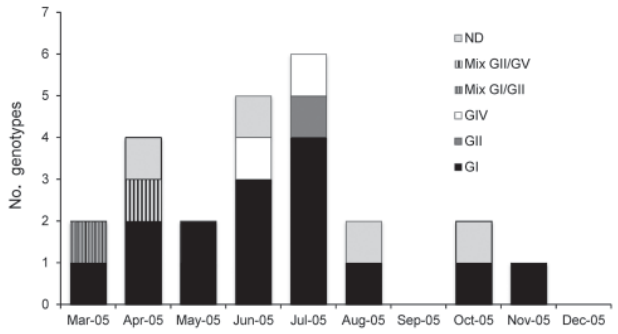

Figure 1. Distribution of sapovirus (SaV) genotypes in children ≤5 years of age from Nicaragua, March–December 2005. A total of 16 (64%) children were infected; 4 children (16%) were infected with genogroup II (GII), and 3 (12%) were infected with GIV. SaV infections were most frequently diagnosed during June–July 2005, in the rainy period. ND, not determined

SaV infections were most frequently detected during June–July 2005, in the rainy period (Figure 1), and were only found during March–November. The most common symptoms in SaV-positive children with diarrhea were loss of appetite (52%), vomiting (36%), fever (32%), and abdominal distension (32%).

To investigate whether certain SaV genogroups (GI, GII, GIV, or GV) were associated with clinical severity, we examined 25 SaV-positive symptomatic and asymptomatic children. All human SaV genogroups were found in study participants as observed (*13*). Notably, SaV GI was the dominant (64%) genogroup in Nicaragua, and infection with this genogroup could lead to asymptomatic disease as well as severe disease requiring hospitalization. This finding suggests that factors contributing to severity of SaV disease in Nicaragua are not only related to viral properties, but also to host factors and the surrounding environment. Co-infections with GI:GII and GI:GV were found in 2 symptomatic children, respectively. No association between genogroup and clinical status was observed; GI, GII, and GIV viruses infected symptomatic and asymptomatic children at similar rates (data not shown). Nucleotide sequencing of 12 SaV-positive samples revealed 8 additional strains related to the SaV Manchester strain, isolated in the United Kingdom in 1993, than to the SaV strain isolated in Japan in 1982, both representing genotype 1 within SaV GI (GI/1) (Figure 2).

We have previously investigated whether HBGA were susceptibility markers for NoV infections in children in Nicaragua and observed that secretors of blood type O were highly susceptible to infections with different NoV genotypes (*14*). No nonsecretors who were carrying the G428A *FUT2* mutation were infected with NoV (*9*,*14*). To investigate whether HBGAs were associated with SaV susceptibility similar to NoV susceptibility (*2*,*3*,*11*), we examined 22 of 33 SaV-infected children who were either symptomatic (n = 18) or asymptomatic (n = 4) in relation to ABO

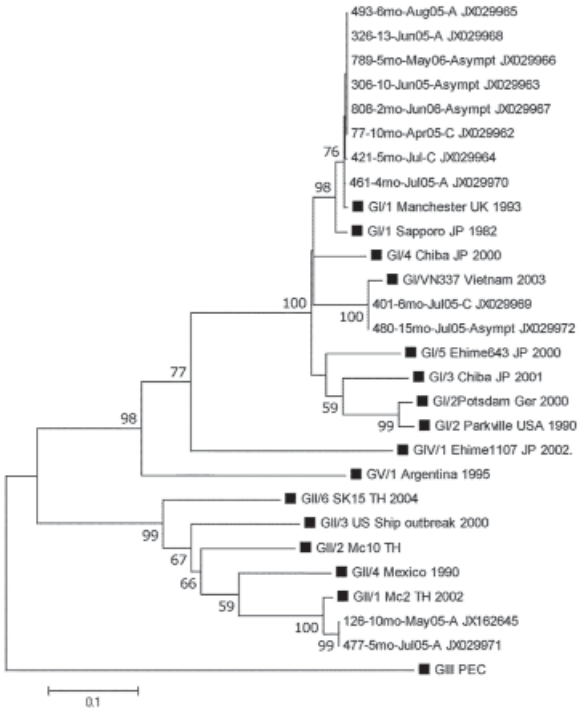

Figure 2. Phylogenetic analysis of the partial N terminal capsid gene (339 bp) of SaV strains identified in a pediatric population in Leon, Nicaragua, March 2005–September 2006. The tree was constructed on the basis of the Kimura 2-parameter and neighbor-joining methods with MEGA5 software (www.megasoftware.net). Bootstrap values are shown at the branch nodes (values <50% are not shown). The black squares represent SaV reference strains GI–GV. For Nicaraguan strains, the number of the strain is given, followed by age in months, month and year of sample collection, and clinical status. A, mild; C, severe; Asympt, asymptomatic. Scale bar indicates nucleotide substitutions per site.

blood types, Lewis phenotypes, and secretor genotype. Notably, we did not find any significant association between ABO blood groups, Lewis phenotypes, or secretor genotype and susceptibility to SaV infection (Table 2). Children who were secretors (SeSe and $Sese^{428}$) and nonsecretors ($se^{428}se^{428}$ and Le^{a+b-}) were susceptible to SaV infections. Our data suggest that SaV can infect secretors and nonsecretors, those who are Lewis phenotype positive as well as Lewis phenotype negative, and persons of all ABO blood groups. However, it must be noted that these data represent susceptibility to symptomatic GI SaV infection. To our knowledge, only 1 report in the literature has explored binding properties of recombinant SaV GI/1 (Mc114) and GV/1 (NK24) to HBGAs and revealed that the recombinant SaV strains investigated showed no specific binding activity to HBGAs from or to synthetic carbohydrates (*15*).

Conclusions

Our results demonstrate that SaV, similar to NoV, frequently causes acute gastroenteritis in children in Nicaragua (*5*). However, in contrast to the case with NoV, neither the G428A nonsecretor mutation, nor any of the ABO blood groups or Lewis phenotypes protected children against SaV infection.

Acknowledgments

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Table 2. Distribution of ABO blood groups, Lewis antigens, and *FUT2* 428 genotypes among SaV- infected children and healthy controls, León, Nicaragua, 2005–2006*

Variable	No. (%) SaV infected	No. (%) controls†	OR (95% CI)	p value‡
Blood group				
O	16 (73)	87 (66)	1.3 (0.49–3.7)	0.63
A	2 (9)	26 (20)	0.4 (0.09–1.8)	0.37
B	3 (13.5)	13 (10)	1.4 (0.37–5.5)	0.80
AB	1 (4.5)	5 (4)	1.2 (0.13–11)	1.00
Lewis phenotype				
Le^{a-b+}	17 (77.5)	93 (71)	1.3 (0.46–3.91)	1.00
Le^{a+b-}	2 (9)	5 (4)	2.5 (0.45–13.7)	0.26
Le^{a-b-}	3 (13.5)	32 (25)	0.48 (0.13–1.7)	0.41
FUT2 SNP (428G→A)				
SeSe	13 (59)	69 (53)	1.3 (0.52–3.2)	0.64
$Sese^{428}$	7 (32)	58 (44)	0.59 (0.22–1.5)	0.35
$se^{428}se^{428}$	2 (9)	4 (3)	3.2 (0.55–18.00)	0.2
Secretor status				
Secretor	20 (91)	123 (94)	0.65 (0.13–3.3)	0.63
Nonsecretor	2 (9)	8 (6)	1.5 (0.3–7.8)	0.63

*SaV, sapovirus; OR, odds ratio; *FUT2*, fucosyltransferase gene 2; SNP, single nucleotide polymorphism; SeSe, homozygous wild type at *FUT2* 428; $Sese^{428}$, heterozygous carrier of *FUT2* 428 non-sense mutation; $se^{428}se^{428}$, homozygous carrier of the non-sense mutation at nt 428 in the *FUT2* gene
†From Bucardo et al. (*14*).
‡Fischer exact test.

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Effect of Latitude on Seasonality of Tuberculosis, Australia, 2002–2011

Jennifer H. MacLachlan, Caroline J. Lavender, and Benjamin C. Cowie

Seasonal variation in tuberculosis diagnoses recently has been reported in various populations. In Australia, seasonality of tuberculosis diagnoses was more pronounced in areas where UV exposure is reduced and vitamin D deficiency is more prevalent. Our findings suggest vitamin D deficiency as a factor in disease activation.

Tuberculosis profoundly affects human health, with 5.7 million new or recurrent cases reported and >1 million deaths attributed to the infection by the World Health Organization in 2010 (*1*). Tuberculosis has afflicted humans for millennia, and the potential of sunlight in prevention and treatment has been recognized for more than a century. Before the introduction of antimycobacterial therapy, several therapeutic approaches for tuberculosis were attempted. One example was creation of sanatoria, a positive aspect of which was thought to have been exposure to sunlight:

> The ultraviolet rays are absorbed and are beneficial to the general health, even counterbalancing a deficiency in vitamins, to some extent. They have been… used as a treatment for tuberculosis of bones and joints, in which they seem to have a direct effect on the bacteria. (*2*)

The nexus between exposure to sunlight and risk for active tuberculosis has been increasingly recognized, with a putative mechanism being vitamin D deficiency that reduces the ability of macrophages to kill intracellular *Mycobacterium tuberculosis* (*3*). Vitamin D deficiency has been associated with latent (*4*) and active (*5*) tuberculosis, and seasonality in the number of tuberculosis diagnoses has recently been reported in several regions (*3*,*6*).

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Australia extends from latitudes 10° to 44° south, from equatorial to temperate climatic zones (Figure 1); as a consequence, incident sunlight intensity and vitamin D synthesis vary widely (*7*). We hypothesized that active tuberculosis cases diagnosed in Australia would display seasonality in regions further from the equator but less so in the tropics and that this seasonality would manifest as increased tuberculosis activity several months after nadir sunlight levels.

Tuberculosis is a nationally notifiable disease in Australia, with medical practitioners and testing laboratories legally required to report all tuberculosis cases in all Australian states and territories. Each jurisdiction reports all confirmed tuberculosis cases to the National Notifiable Diseases Surveillance System, managed by the Australian Government Department of Health and Ageing (*8*). Confirmed tuberculosis cases are classified as either new or relapsed at the time of infection, consistent with World Health Organization case notification definitions (*1*,*8*).

The Study

We obtained all notifications of tuberculosis to the National Notifiable Diseases Surveillance System during January 2002–December 2011. States and territories were divided on the basis of latitude into north, central, and south regions (Figure 1). Australia data for seasonal UV exposure (*9*,*10*) and vitamin D levels (*7*,*10*) in these regions were obtained and compared with tuberculosis notifications to identify potential patterns in incidence according to these

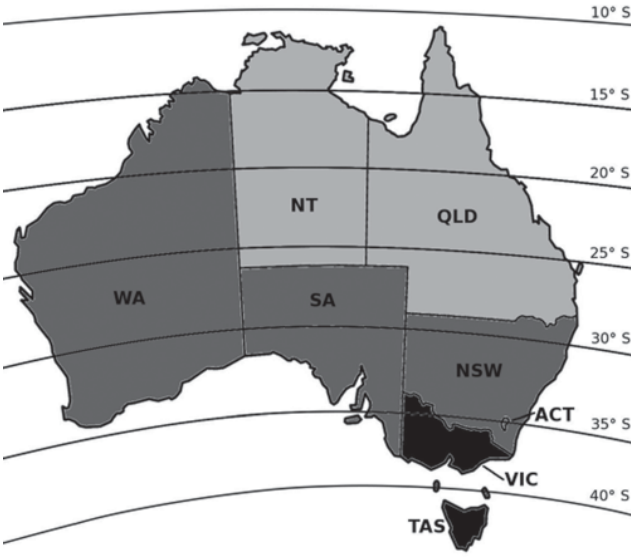

Figure 1. Australia with latitude lines, divided into north, central, and south regions according to latitude and ultraviolet (UV) exposure. Although Western Australia extends to the tropics, >90% of the state's population lives below latitude 30°S (*7*). ACT, Australian Capital Territory; NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia. Black, south region; dark gray, central region; light gray, north region.

changing environmental factors. We derived the number of tuberculosis notifications per 100,000 population using midpoint population denominator estimates for each state taken from the 2006 census (*11*).

Seasonality in tuberculosis notifications was assessed for the whole of Australia and individually for each region by using Edward's test of seasonality, and the difference in magnitude of seasonality between regions was compared by using the amplitude of cyclic variation. The Wilcoxon rank-sum test was used to test the hypothesis that the mean tuberculosis notifications per 100,000 persons in the south did not differ from those in central states, separately for January–June and July–December.

During 2002–2011, a total of 11,576 tuberculosis cases were reported in Australia (Table), with an apparent increase in mean number of cases reported during September–December. This effect was more pronounced in temperate than in tropical areas (Figure 2). Notifications of tuberculosis began to rise in temperate areas in July and peaked in October in the central region and in December in the south. Furthermore, incidence of tuberculosis diagnosis per 100,000 population was similar in the central and south regions in the first half of the calendar year ($p = 0.20$) but diverged significantly for July–December ($p = 0.006$).

When sunlight exposure was taken into account, the fall in UV index <3 in the south and central regions was followed 2–3 months later by notable increases in tuberculosis diagnoses. In northern Australia, where the average UV index does not drop below 4 even during winter, the seasonal trend was less marked (Figure 2).

Formal tests of seasonality indicated that the number of tuberculosis notifications per 100,000 varied significantly by month for the whole of Australia and for each region ($p<0.001$). The effect was more pronounced in the south than in the central and north regions (amplitudes of cyclic variation 0.207, 0.094, and 0.101, respectively).

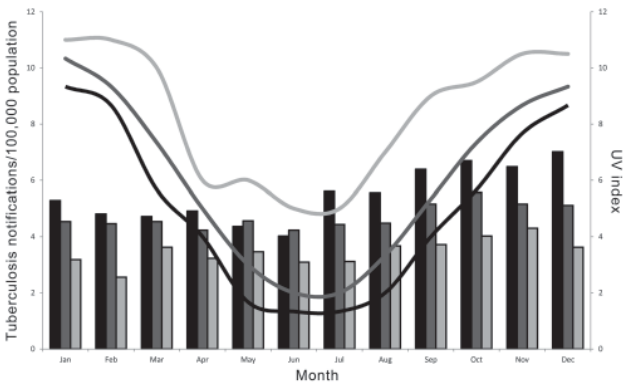

Figure 2. Tuberculosis notifications per 100,000 population (bars) and ultraviolet (UV) index (lines), Australia 2002–2011. Black, south region; dark gray, central region; light gray, north region.

Conclusions

The cyclical patterns in tuberculosis activity in this study have been observed in other settings (*3*,*12*), and a difference according to latitude has been found in India, with more seasonality in northern regions (*13*). No effect of latitude was found in a recent study of tuberculosis seasonality in the United States (*6*). This difference may be due to the relative latitudes of these locations; all latitudes of the continental United States are further from the equator (30°N–55°N) than are latitudes in India (8°N–37°N) and Australia (10°S–44°S).

The seasonal pattern according to latitude corresponds with findings about vitamin D deficiency by region in Australia in a national study of 11,247 adults (*7*), which found that the odds of vitamin D deficiency were 3–4 times higher in winter/spring than in summer and were more than double for persons residing in latitudes >35°S (Figure 1). Although ecologic studies, such as that presented here, cannot address questions of causation, the temporal lag between low UV exposure, vitamin D deficiency, and increased tuberculosis diagnoses supports the argument that vitamin D deficiency leads to tuberculosis, and not the converse.

Persons born overseas accounted for 86.4% of tuberculosis cases in Australia in 2007, despite constituting ≈30% of the population. Aboriginal and Torres Strait Is-

Table. Number and rate* of tuberculosis notifications, Australia, 2002–2011

Month	South		Central		North		Total	
	Mean cases (range)	Rate	Mean cases (range)	Rate	Mean cases (range)	Rate	Mean cases (range)	Rate
Jan	30.2 (25–37)	5.3	48.4 (35–62)	4.5	13.7 (8–20)	3.2	92.3 (73–103)	4.5
Feb	27.6 (21–37)	4.8	47.5 (38–58)	4.5	11 (5–14)	2.6	86.1 (74–105)	4.2
Mar	27.8 (17–40)	4.7	47.6 (36–56)	4.5	15.6 (8–24)	3.6	91.0 (66–109)	4.4
Apr	29.4 (18–41)	4.9	43.8 (21–65)	4.2	13.9 (6–20)	3.2	87.1 (54–114)	4.2
May	25.3 (10–35)	4.4	48.4 (40–58)	4.6	14.9 (10–29)	3.5	88.6 (74–104)	4.3
Jun	24 (16–39)	4.0	44.2 (35–56)	4.2	13.3 (8–26)	3.1	81.5 (62–98)	3.9
Jul	32.9 (26–49)	5.6	46.4 (36–65)	4.4	13.4 (7–24)	3.1	92.7 (73–110)	4.5
Aug	31.9 (18–42)	5.6	47.6 (34–57)	4.5	15.8 (10–26)	3.7	95.3 (72–121)	4.6
Sep	37.2 (27–44)	6.4	54.3 (45–70)	5.2	16 (8–26)	3.7	107.5 (85–135)	5.2
Oct	39.7 24–61)	6.7	57.9 (42–89)	5.6	17.3 (13–30)	4.0	114.9 (81–166)	5.5
Nov	38.1 (27–50)	6.5	53.9 (44–78)	5.2	18.5 (13–22)	4.3	110.5 (94–122)	5.3
Dec	41.6 (25–58)	7.0	52.9 (33–67)	5.1	15.6 (9–31)	3.6	110.1 (81–139)	5.3

*Per 100,000 population.

lander peoples represented 23% of cases in Australia-born persons, despite constituting 3.7% of the Australian-born population (*11*). The predominant countries of birth of Australians born overseas and in whom tuberculosis was diagnosed during that year were (in descending order) India, Vietnam, the Philippines, People's Republic of China, Indonesia, Papua New Guinea, Sudan, Myanmar, Nepal, Bangladesh, and Pakistan (*8*). In addition to the increased risk for exposure to tuberculosis early in life in persons born overseas, increased susceptibility to vitamin D deficiency also might increase the risk for reactivation of tuberculosis in these groups, with vitamin D deficiency more common among indigenous Australians (*14*) and migrants from high tuberculosis prevalence countries (*7*,*15*).

These findings should guide clinical and public health practice in Australia and similar countries that have high migration from tuberculosis-endemic populations and substantial risk for vitamin D deficiency. Clinicians should provide individualized advice to persons at higher risk for tuberculosis, especially those who have migrated from tuberculosis-endemic areas and those in whom latent tuberculosis already has been diagnosed, about vitamin D levels and consideration of supplementation as appropriate.

Ms MacLachlan is an epidemiologist at the Victorian Infectious Diseases Reference Laboratory, Melbourne. Her research interests include viral hepatitis and tuberculosis, particularly the use of infectious diseases surveillance data to inform public health initiatives.

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Pandemic Influenza Virus Surveillance, Izu-Oshima Island, Japan

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A population-based influenza surveillance study (using PCR virus subtyping) on Izu-Oshima Island, Japan, found that the cumulative incidence of influenza A(H1N1) pdm09 virus infections 2 seasons after the pandemic was highest for those 10–14 years of age (43.1%). No postpandemic A(H1N1)pdm09 case-patients had been infected with A(H1N1)pdm09 virus during the pandemic season.

The dynamics of an influenza epidemic are difficult to determine because they vary for each circulating influenza subtype. We evaluated the epidemiology of influenza A subtypes at Oshima Medical Center and Maeda Internal Medicine Clinic on Izu-Oshima Island, Japan. The island is a semiclosed community, which facilitates population-based surveillance. Access to the island is limited; ≈18,000 persons travel to and from the island each month, primarily to and from Tokyo (*1*). Izu-Oshima Island is 120 km southwest of Tokyo; population was 8,856 in January 2009.

The Study

Clinical information was collected retrospectively for January 1 through July 31, 2009, and prospectively from August 1, 2009 through April 30, 2011. Retrospectively, we identified patients who had been tested for influenza by use of a rapid test kit and extracted personal and clinical information from medical records. Prospectively, we collected the same information from patients tested for influenza by rapid test kit and used nasopharyngeal swab extracts from rapid test kits for virus typing. Typing was performed by reverse transcription nested-PCR (RT-nPCR): multiplex for seasonal influenza virus (subtypes A/H1, A/H3, B-, and A/H5) and simplex for influenza A(H1N1)pdm09 virus (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/11-1681-Techapp.pdf). By amplifying product exclusively for influenza A(H1N1)pdm09 and for traditional influenza (H1N1) viruses and by producing a different length product for each subtype, this method enabled us to easily define the subtypes visually. We started using RT-nPCR in week 33 of 2009. Protocols were approved by Keio University School of Medicine Ethical Committee.

For the retrospective period, we identified 1,066 suspected cases of influenza; 20 patients were nonresidents of Izu-Oshima Island; the address for 1 patient was unknown. For the prospective period, we identified 2,348 patients with suspected influenza; 3 were excluded because they did not consent to study participation. Patients from the prospective period were tested with a rapid test. In total, 97.8% (2,293/2,345) of the samples were also tested by RT-nPCR. The total number of patients with suspected influenza in the prospective study was 2,219 (a patient with multiple visits within 7 days was counted as 1 patient), of which 78 were not residents of Izu-Oshima Island (online Technical Appendix Table 2).

The sensitivity of the rapid test kit compared with RT-nPCR was ≈90% for type A but lower (≈80%) for type B (online Technical Appendix Table 3). Among patients with positive rapid test results, >90% received anti-influenza agents (Table 1). No cases were severe or fatal.

The same influenza subtypes circulated in Izu-Oshima as in other areas (*2*,*3*). We assessed the period from when a novel virus was introduced through the postpandemic season. The introduction of A(H1N1)pdm09 virus in Izu-Oshima occurred 11 weeks after confirmation of the first case in Japan (*4*) and 10 weeks after confirmation in Tokyo (*5*). After the first case of A(H1N1)pdm09 infection was identified in Japan on May 16 (week 20) (*4*), no other influenza A cases were diagnosed by a rapid test in Izu-Oshima until August 1 (week 31), when a patient with influenza A (unspecified) was determined to have had contact with a person with confirmed A(H1N1)pdm09 infection on mainland Japan.

The first outbreaks of influenza A in Izu-Oshima ceased within 5 weeks (Figure 1). After 3 weeks with no cases, starting at the end of September (week 39), clusters of influenza cases were observed in schools and families. Although immediate school or class closures were implemented, the pandemic began in the middle of November and peaked during week 50, which was 6 weeks later than the Tokyo peak (*5*).

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Table 1. Rapid diagnostic test results and prescription of anti-influenza agents in Izu-Oshima

Influenza season and influenza virus type*	Prescriptions written at clinics, no. (%)					Total
	Oseltamivir†	Zanamivir‡	Peramivir§	Laninamivir¶	None	
2008–09						
A	193 (53.5)#	45 (12.5)#	0	0	124 (34.3)	361
B	39 (32.5)	29 (24.2)	0	0	52 (43.3)	120
A + B	2 (3.3)	0	0	0	4 (66.7)	6
Negative	32 (5.5)	10 (1.7)	0	0	537 (92.8)	579
2009–10						
A	219 (48.5)	209 (46.2)	0	0	24 (5.3)	452
Negative	42 (4.7)	33 (3.7)	0	0	828 (91.7)	903
2010–11						
A	147 (66.2)	52 (23.4)	3 (1.4)	0	20 (9.0)	222
B	60 (38.0)	82 (51.9)	1 (0.6)	1 (0.6)	14 (8.9)	158
Negative	44 (7.2)	18 (3.0)	0	0	548 (89.8)	610

*Influenza virus type determined by rapid diagnostic test.
†Tamiflu, Chugai Pharmaceutical Co., Tokyo, Japan.
‡Relenza, GlaxoSmithKline, Research Triangle Park, NC, USA.
§Rapiacta, BioCryst Pharmaceuticals Inc., Durham, NC, USA; marketed since January 27, 2010.
¶Inavir, Daiichi Sankyo Co., Ltd., Tokyo, Japan; marketed since October 19, 2010.
#Includes 1 case for which Tamiflu and Relenza were prescribed.

On the island, the incidence of A(H1N1)pdm09 infection during the pandemic season was higher than the incidence caused by other subtypes during the 2009–2011 seasons (Table 2). However, the overall incidence on the island (5.0%) during the pandemic season was one third of the estimated incidence for all of Japan (16.2%) (*3*).

The introduction and dissemination of A(H1N1) pdm09 virus varied by age (Figure 2). On the island, as on the mainland, introduction of the emerging virus preceded the outbreak among high school–age children (*4*,*6*). However, at the end of the season, the incidence among persons 5–14 years of age exceeded that among persons 15–19 years of age (Table 2).

During the postpandemic season, in addition to A(H1N1) pdm09 virus, epidemics of influenza A/H3 and B viruses occurred. No patient with confirmed A(H1N1)pdm09 infection during the postpandemic season had a history of influenza in the previous season; but 28% (50/180) of patients with influenza B and 3% (2/58) with influenza A/H3 virus did. The cumulative incidence of A(H1N1)pdm09 infection 2 seasons after the pandemic was estimated at 7.3% and was highest (43.1%) among persons 10–14 years of age.

Conclusions

This population-based surveillance study determined the incidence of the influenza virus subtypes circulating in

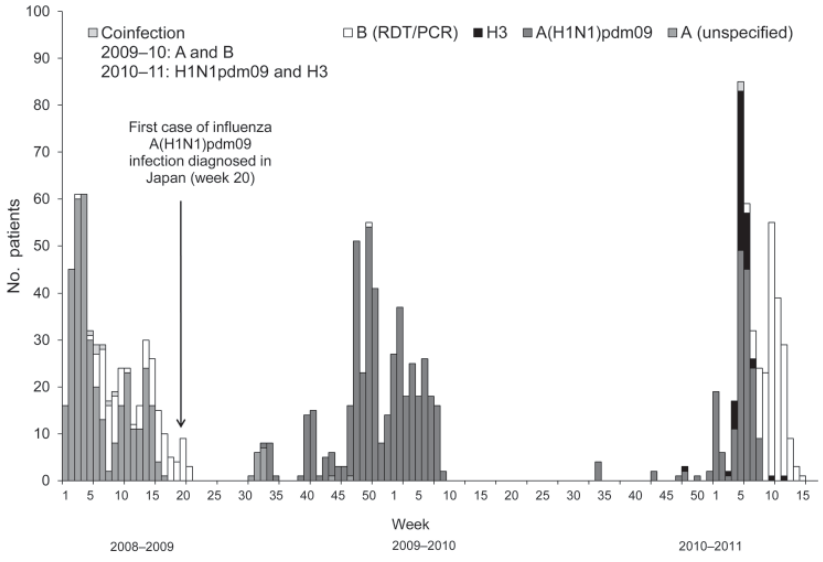

Figure 1. Cases of influenza and influenza-like illnesses on Izu-Oshima Island, Japan, from week 1 of 2009 through week 17 of 2011. The number of influenza cases and influenza-like illnesses are plotted weekly from the disease onset. Influenza cases were defined as illnesses diagnosed by a rapid test combined with a reverse transcription nested PCR (RT-nPCR) or by a rapid diagnostic test (RDT) alone, during the retrospective period (unspecified). Influenza-like illnesses were defined as cases for which influenza was ruled out by negative RT-nPCR or cases for which influenza was ruled out by RDT results and further tests were not performed. Multiple visits within 7 days were counted as a single case. Disease onset was defined by the date when the patient first reported fever or upper respiratory symptoms. The disease onset for the case that had no date in the clinical records was defined as the day before the first clinical visit according to the median day of visit from the available study data. A and B, co-infection, cases diagnosed by RDT. B (RDT/PCR), cases diagnosed by a RDT or RT-nPCR. A(H1N1) pdm09 and H3, co-infection cases with 2 virus subtypes confirmed by RT-nPCR. Influenza seasons were defined as follows: week 1–30 of 2009 was the 2008–09 prepandemic season, week 31 of 2009–week 33 of 2010 was the 2009–10 pandemic season, and week 34 of 2010–week 17 of 2011 was the 2010–11 postpandemic season. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/11-1681-F1.htm).

Table 2. Incidence of major influenza virus subtypes endemic to Izu-Oshima Island, Japan, by influenza season

	Incidence of influenza cases among Izu-Oshima residents, no. (%)									
	2008–09			2009–10		2010–11				Cumulative incidence of
		Influenza virus type			Influenza virus		Influenza virus type			
Age, y*	No.†	A‡	B	No.†	type A/H1§	No.†	A/H1§	A/H3	B	A/H1¶
0–4	326	65 (19.9)	11 (3.4)	323	36 (11.1)	316	20 (6.3)	21 (6.6)	16 (5.1)	41 (13.0)
5–9	366	98 (26.8)	51 (13.9)	367	105 (28.6)	349	39 (11.2)	6 (1.7)	91 (26.1)	137 (39.3)
10–14	316	53 (16.8)	44 (13.9)	316	97 (30.7)	332	26 (7.8)	3 (0.9)	48 (14.5)	143 (43.1)
15–19	475	10 (2.1)	4 (0.8)	480	91 (19.0)	475	24 (5.1)	0	6 (1.3)	129 (27.2)
20–29	541	19 (3.5)	3 (0.6)	492	25 (5.1)	486	12 (2.5)	2 (0.4)	2 (0.4)	39 (8.0)
30–39	987	56 (5.7)	1 (0.1)	991	39 (3.9)	951	22 (2.3)	9 (0.9)	5 (0.5)	62 (6.5)
40–49	977	27 (2.8)	2 (0.2)	971	21 (2.2)	996	18 (1.8)	7 (0.7)	5 (0.5)	42 (4.2)
50–59	1,396	15 (1.1)	1 (0.1)	1,283	14 (1.1)	1,179	10 (1.8)	5 (4.2)	4 (0.3)	24 (2.0)
≥60	3,472	12 (0.3)	3 (0.1)	3,540	8 (0.2)	3,568	2 (0.1)	5 (0.1)	2 (0.1)	11 (0.3)
Total	8,856	355 (4.0)	120 (1.4)	8,763	436 (5.0)	8,652	173 (2.0)	58 (0.7)	179 (2.1)	628 (7.3)

*Age during January 2009 (for 2008–09), 2010 (for 2009–10), and 2011 (for 2010–11).
†Population recorded in the Resident Registry in January 2009 (for 2008–09), 2010 (for 2009–10), and 2011 (for 2010–11).
‡Influenza A (unspecified). The number of second diagnoses in the season was excluded.
§Influenza A (H1N1) pdm09.
¶The sum of influenza A(H1N1)pdm09 cases among residents in Izu-Oshima during the study divided by the population during January 2011.

pandemic and postpandemic seasons. The estimated incidence of symptomatic cases was accurate because of the easy access to health care on Izu-Oshima Island. Care was sought for almost all (97%) children with upper respiratory symptoms and fever, although the proportion of adults who sought clinical care was not high (*7*). Most (83.6%; 1,774/2,122) patients for whom disease onset was identified had visited a medical institute within 2 days of disease onset despite their symptoms being only mild to moderate. Furthermore, all suspected cases of influenza, except 2, were confirmed by a rapid test; most were tested further and isolates were subtyped by RT-nPCR.

The cumulative incidence 2 seasons after the pandemic indicates that early introduction of A(H1N1)pdm09 virus to those 15–19 years of age was not caused by differential

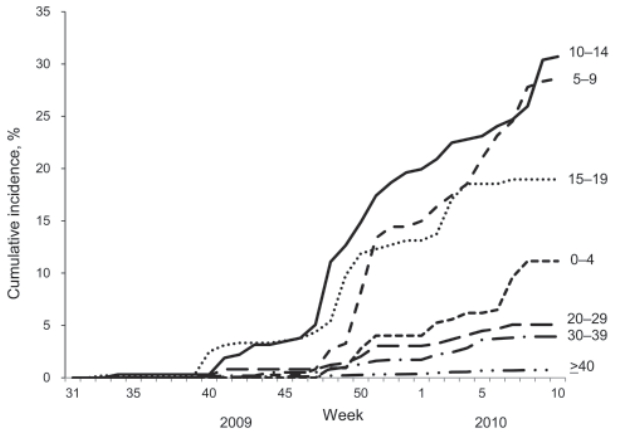

Figure 2. Cumulative incidence of influenza A(H1N1)pdm09 infections by age group during the 2009–10 season. The cumulative incidence of A(H1N1)pdm09 infections for 2009–10 was calculated for the sum of A(H1N1)pdm09 virus cases among residents on Izu-Oshima Island, Japan, divided by the population at the end of December 2009 and plotted by week in the 2009–10 season. The numbers adjacent to the lines indicate the age groups, in years. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/11-1681-F2.htm).

sensitivity to the virus. Rather, it was probably caused by more frequent exposure to the emerging virus, possibly because of higher mobility of persons in this age group. Considering the conservative antigenic property of A(H1N1)pdm09 virus in the postpandemic season (*8*), the absence of A(H1N1)pdm09 infection in this season among those who had experienced it in the pandemic season suggests that immune memory persisted in the postpandemic season. The cumulative incidence suggests that nearly half of the school-age children had immunity to A(H1N1)pdm09 virus by infection after 2 seasons. The remaining virus-naive elderly population should be considered for future preventive intervention, although they might have some immunity against A(H1N1)pdm09 virus (*9–12*).

The delayed introduction of A(H1N1)pdm09 virus might primarily be explained by the isolated environment of the island; introduction would be mediated solely by visitors carrying the virus. The delayed start and peak of the epidemic and the low incidence could be attributed early case identification plus early and extensive therapy (including prompt initiation of antiviral medication according to results of proactively performed rapid tests); easy access to health care; and public health interventions (such as school closures).

In addition, unique social features might also have contributed to the delayed pandemic and low disease incidence. The proportion of children <15 years of age (≈12%) is the same on Izu-Oshima Island as in Tokyo; whereas, the proportion of those ≥65 years of age is 31% on the island and only 20% in Tokyo. Assuming that persons ≥65 years of age had preexisting immunity against A(H1N1)pdm09 virus, as suggested by other studies (*9–12*), the community possibly had a larger number of nonsusceptible persons. Limited public transport and low population density (96/km^2) might have reduced disease spread. School closures might have more effectively reduced the chance of transmission in such settings than in other areas. This study

provides a sound basis for modeling studies that consider social structures to help explain the effects of public health interventions for influenza spread in a community.

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Fatal Respiratory Infections Associated with Rhinovirus Outbreak, Vietnam

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During an outbreak of severe acute respiratory infections in 2 orphanages, Vietnam, 7/12 hospitalized children died. All hospitalized children and 26/43 children from outbreak orphanages tested positive for rhinovirus versus 9/40 control children (p = 0.0005). Outbreak rhinoviruses formed a distinct genetic cluster. Human rhinovirus is an underappreciated cause of severe pneumonia in vulnerable groups.

The World Health Organization estimates that ≈2 million children die each year from acute respiratory tract infection (ARI), and most live in developing countries (*1*). Human rhinovirus (HRV), a common cause of mild upper respiratory tract infections, may also cause severe ARI in children. We report on an outbreak of severe ARI caused by HRV in children living in orphanages in Vietnam.

The Study

During December 2007–February 2008, twelve infants <6 months of age with severe ARI infection were admitted to 2 hospitals in Hanoi. Because all 12 infants lived in 2 orphanages in Hanoi, the National Hospital of Pediatrics (NHP) initiated an outbreak investigation. Data on demographic characteristics, clinical features, and outcomes were collected for the 12 infants. Researchers visited 1 of the outbreak orphanages and 1 control orphanage (40 km apart, no severe ARI observed). Patient histories were obtained from orphanage staff; all infants were examined, and nasal and pharyngeal swab specimens were collected. Respiratory specimens were tested by bacterial culture and multiplex reverse transcription PCR (RT-PCR, Seeplex RV12 Kit; Seegene, Seoul, South Korea) for the following respiratory viruses: influenza A/B viruses, respiratory syncytial virus (RSV), rhinovirus, coronavirus (OC43/HKU1 and 229E/NL63), adenovirus, parainfluenzavirus, and human metapneumovirus (*2*). This outbreak investigation was approved by the Scientific Committee of NHP.

Twelve patients with severe ARI were admitted to the NHP intensive care unit over 38 days during the cool months of December 2007–February 2008 (temperature 5°C–14°C). The 12 hospitalized infants (11 female) were 2- to 4-months-old. Most (10/12) were from 1 orphanage, which was selected for investigation. Two hospitalized children had a known underlying condition: congenital hypothyroidism (n = 1) and HIV infection (n = 1). Seven infants were underweight (sex-specific weight-for-age; *z* score <2 SDs). All exhibited cough, coryza, wheezing, and dyspnea, and 6 (50%) had a documented fever.

Acute respiratory distress syndrome developed in all 12 a mean of 8.3 (95% CI ± 5 days) days after onset. Mean pressure of arterial oxygen/fractional inspired oxygen ratio was 66.0 (95% CI ± 30.6, range 25.1–131.0). Chest radiographs showed extensive bilateral infiltrates. Blood cultures for bacteria were negative. Despite mechanical ventilation and administration of intravenous broad-spectrum antimicrobial drugs, 7 patients died and 3 patients recovered (2 were lost to follow up). HRV was detected by RT-PCR in all infants; 2 patients were co-infected with RSV and adenovirus (Table). In addition, 1 bronchoalveolar lavage specimen from 1 patient was HRV positive.

The outbreak orphanage was visited within 1 week of outbreak detection. The visit revealed that several other infants had been hospitalized elsewhere, but we could not obtain detailed data about them. We tested nasal–pharyngeal swab specimens (pooled) from all 43 infants (100% <12 months of age) living in the outbreak orphanage and nasal swab specimens from the 40 youngest children (97.5% <12 months of age) at the control orphanage within 2 weeks of the outbreak. In both orphanages, 5–9 children lived in 1 room and shared the same bed, wiping cloths, basic utensils such as cups, and clothing.

Most children in the outbreak orphanage were female (31/43 [72%]), compared with 50% (20/40) in the control orphanage. Of children whose specimens were tested by RT-PCR, 98% (42/43) of the infants from the outbreak orphanage had at least 1 symptom of respiratory tract infection compared with 14 (35%) of 40 infants at the control orphanage. Among children from the outbreak orphanage (both hospitalized and nonhospitalized [n = 55]), a single pathogen was identified in 31 (56.4%) infants and ≥2 pathogens were found in 9 (16.4%) children (Table). The most frequently detected pathogen was HRV (n = 38). In the control orphanage, 9 (22.5%) children tested positive

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Table. Detected viruses in respiratory specimens from hospitalized children, children at the outbreak orphanage, and children from the control orphanage, Hanoi, Vietnam, December 2007–February 2008*

Viral diagnosis†	Outbreak orphanage infants		Control orphanage infants
	No. (%) hospitalized, n = 12	No. (%) not hospitalized, n = 43	No. (%) not hospitalized, n = 40
Single infection			
Rhinovirus	10 (83.3)	19 (44.2)	4 (10.0)
Adenovirus	0	0	6 (15.0)
RSV	0	2 (4.7)	0
Co-infection, 2 pathogens			
Rhinovirus and adenovirus	0	2 (4.7)	4 (10.0)
Rhinovirus and RSV	0	4 (9.3)	1 (2.5)
Rhinovirus and parainfluenzavirus	0	1 (2.3)	0
Co-infection, 3 pathogens			
Rhinovirus, adenovirus, and RSV	2	0	0
Any rhinovirus	12 (100)	26 (60.5)	9 (22.5)‡

*RSV, respiratory syncytial virus.
†By multiplex reverse transcription PCR (Seeplex RV12; Seegene, Seoul, South Korea).
‡χ^2 test p = 0.0005

for HRV, and 5 children were infected with 2 pathogens. Only nonpathogenic bacteria were cultured from the respiratory specimens (data not shown).

Because HRV was the predominant pathogen detected, we genotyped all HRV isolates directly from the specimens using a molecular typing assay based on phylogenetic comparisons of a 260-bp variable sequence (P1–P2) in the 5′-noncoding region with homologous sequences of the 101 known serotypes (*3*,*4*). We were able to sequence the P1–P2 fragment of HRV genome from 23 specimens positive for HRV: 2 were from hospitalized patients (08040R and 08043R) (Figure), 19 were from the outbreak orphanage (identification numbers starting with DA), and 5 were from the control orphanage (identification numbers starting with BaVi; Figure). The P1–P2 phylogenetic tree showed that sequences obtained from isolates from the 2 hospitalized infants were closely related to a subclade of cluster A sequences from nonhospitalized infants at the outbreak orphanage (Figure). HRVs from the control orphanage are also part of the A cluster but formed a distinct subcluster. Several children (not hospitalized) from the outbreak orphanage were also infected with HRVs from the C cluster. No children had B cluster strains.

Conclusions

We found that HRV was the main pathogen detected in an outbreak of severe ARI in children living in an orphanage in Vietnam. Our findings support recent studies showing that HRV may be associated with severe respiratory tract infection in infants and children (*4*–*6*). A study in central Vietnam also showed that HRV is a notable cause of ARI in children in Vietnam (*7*). Because we detected rhinovirus in all hospitalized infants, we believe that HRV was the main causal agent in this outbreak, although 2 hospitalized infants were co-infected with RSV, an unambiguous respiratory pathogen. A deep lung specimen from 1 hospitalized infant was also positive for HRV, which supports a causal relationship. Furthermore, the outbreak orphanage had significantly more HRV-positive patients than did the control orphanage, and sequence analysis showed that the outbreak isolates formed a distinct cluster, which also supports a causal role for HRV.

In addition, we found that several infants from the outbreak orphanages were infected with HRV strains belonging to the C cluster, according to P1-P2 sequence analysis. HRV-C has been associated with more severe infections and circulates worldwide (*3*,*8*–*10*). We may have missed the HRV-C strains in the clinical case-patients, because we were only able to sequence the virus from samples from 2 patients. We had to sequence directly from the specimens because we had no viral culture facility at the time of the outbreak. Phylogenetic analysis of P1–P2 sequences can distinguish HRV-B rhinoviruses, but it is limited in distinguishing HRV-A and HRV-C. Full HRV sequence analysis would be able to provide this detail, but it was not feasible for this investigation.

This outbreak investigation has some limitations. Only a small number of controls were selected from a single orphanage, and the controls were sampled later than the hospitalized patients and children from the outbreak orphanage. These limitations may have led to underdiagnosis of HRV in controls. Bacterial co-infection cannot be ruled out as a cause of more severe infection in the hospitalized infants because most were receiving antimicrobial drugs at the time respiratory specimens were collected.

Viral respiratory infections can be more severe in malnourished infants, which was likely the case for the hospitalized infants in this outbreak. In this study, co-infections (16.4%) with other respiratory viruses were detected at a similar rate as in another study in central Vietnam (12.5%) (*7*). This finding is also consistent with previous work indicating that HRV infections can occur with other respiratory viruses and lead to more severe disease (*2*,*7*). This outbreak illustrates that HRVs can cause severe pneumonia, leading

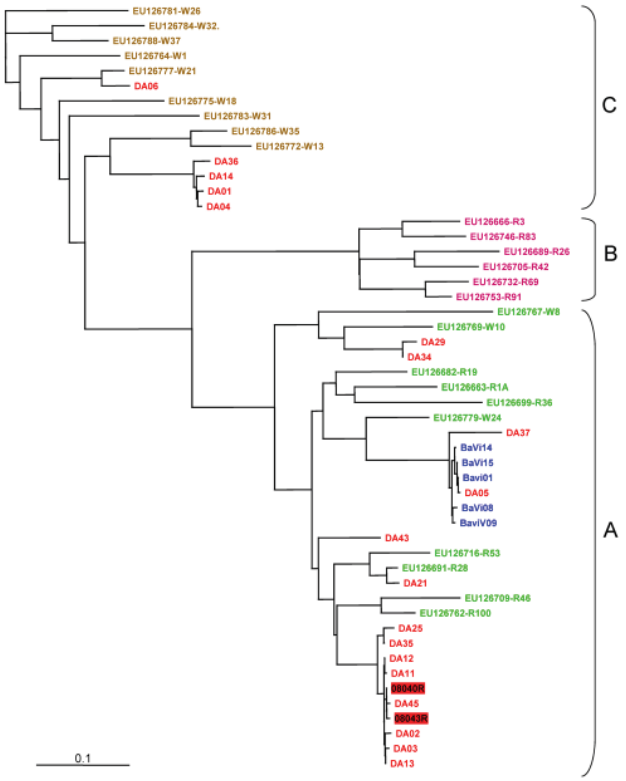

Figure. Genotyping of human rhinovirus (HRV) from outbreak and control specimens by P1–P2 sequence analysis. We compared 19 HRVs from the outbreak orphanage (black), 5 HRVs from control orphanage (italics), and the following P1-P2 sequences in GenBank, according to Lee et al. (*3*). HRV-A group (dark gray): EU126769, EU126779, EU126767, EU126699, EU126682, EU126663, EU126716, EU126691, EU126709, EU126762; HRVB group (black): EU126732, EU126689, EU126666, EU126753, EU126705, EU126746, HRV-C group (light gray): EU126786, EU126772, EU126775, EU126777, EU126783, EU126788, EU126784, EU126781, EU126764. Two HRV sequences from hospitalized patients were included (HRV number in box). Multiple sequence alignment was performed by using the BioEdit program package (Ibis Biosciences, Carlsbad, CA, USA). Nucleotide distances were analyzed with DNAdist, the neighbor-joining tree of BioEdit package. The consensus tree was visualized by TREEVIEW v1.6.6 (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK). Scale bar indicates nucleotide substitutions per site. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/11/12-0607-F.htm).

to acute respiratory distress syndrome in young, vulnerable infants. HRV remains an underappreciated cause of severe pneumonia in vulnerable groups.

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Streptococcus pneumoniae Serotype 15A in Psychiatric Unit, Rhode Island, USA, 2010–2011

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During a pneumococcal disease outbreak in a pediatric psychiatric unit in a hospital in Rhode Island, USA, 6 (30%) of 20 patients and staff were colonized with *Streptococcus pneumoniae* serotype 15A, which is not included in pneumococcal vaccines. The outbreak subsided after implementation of antimicrobial drug prophylaxis and enhanced infection control measures.

Streptococcus pneumoniae, or pneumococcus, causes an estimated 4 million illnesses in the United States annually (*1*). Pneumococcal disease outbreaks often occur in closed settings such as childcare facilities and hospitals. Control measures include vaccination, antimicrobial drug prophylaxis, and infection control (*2*). On January 26, 2011, the Rhode Island Department of Health was notified of 2 cases of invasive pneumococcal disease (IPD) and 2 cases of pneumonia that were associated with a unit (Unit 1) in a pediatric psychiatric hospital that had unusual infection control challenges. We investigated the outbreak to confirm the etiologic agent and prevent disease transmission.

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The Study

Children who have developmental disabilities and aggressive or self-injurious behavior are treated in Unit 1. Inpatient capacity is 17, and <5 outpatients are sometimes present during the day for group and individual therapy. The unit employs >100 staff members. Patients require constant supervision and intensive help with activities of daily living.

At the beginning of this investigation, we established outbreak case definitions. We defined confirmed pneumococcal disease as IPD (isolation of *S. pneumoniae* from a normally sterile site such as blood) or noninvasive (laboratory confirmation of *S. pneumoniae* from a nonsterile site in the setting of a compatible clinical illness, such as isolation from ear drainage samples from a patient with otitis media). We defined confirmed pneumonia as pneumonia diagnosed by a clinician, using chest radiographs confirmed as showing pneumonia by a radiologist. We defined suspected pneumonia as pneumonia diagnosed by a clinician with no radiologic studies obtained. Cases occurred in Unit 1 staff, patients, and visitors during the study period. We maintained active surveillance through May 1, but no cases occurred after February 23.

To identify risk factors for pneumococcal disease (*3*,*4*), we abstracted medical records of all Unit 1 patients (n = 30) during November 1, 2010–January 30, 2011. Unit 1 staff members completed questionnaires about respiratory illnesses experienced during December–February. The hospital informed families of then-current Unit 1 patients of the outbreak. Family members or visitors who reported illness were interviewed. All pertinent medical records were reviewed.

We collected available clinical specimens and conducted a survey to identify respiratory pathogens carried by patients and staff (Figure 1). We collected nasopharyngeal and oropharyngeal calcium alginate swab specimens from then-current Unit 1 patients (n = 16) and staff with ongoing respiratory symptoms (n = 4) during January 29–February 2. For pneumococcal carriage, swab specimens were processed as described (*5*,*6*). Three pneumococcal isolates were recovered (2 from blood, 1 from ear drainage samples). Swab specimens and isolates underwent specific real-time PCR that targeted the *lytA* gene (*6*), PCR-based serotyping (*7*,*8*), and multilocus sequence type determination (*9*). Antibacterial drug susceptibility testing was performed by using broth microdilution (*10*). Additional swabs stored in viral transport media were tested by solid-phase real-time PCR on TaqMan Array Cards (Life Technologies, Carlsbad, CA, USA), for 20 additional respiratory pathogen targets (*11*).

Twenty patients resided on Unit 1 during December 25, 2010–January 31, 2011 (Table 1). Among Unit 1 patients, staff, and visitors, the following cases were iden-

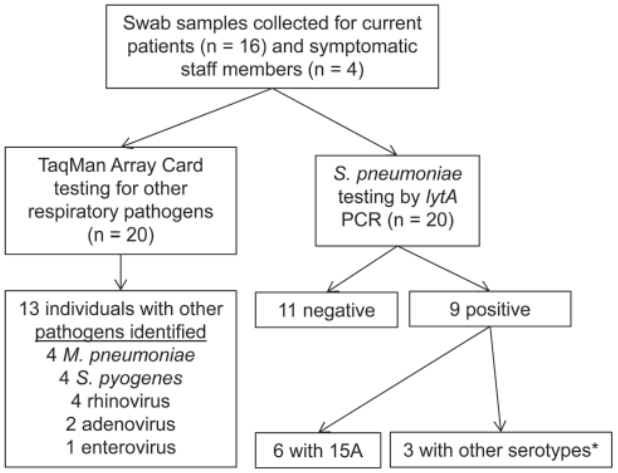

Figure 1. Respiratory pathogen carriage survey related to *Streptococcus pneumoniae* serotype 15A outbreak in a pediatric psychiatric hospital, Rhode Island, USA, December 25, 2010–January 31, 2011, performed on Unit 1 patients (n = 16) and symptomatic staff (n = 4) during January 29–February 2. No visitors were screened for respiratory pathogen carriage. Nasopharyngeal and oropharyngeal swab specimens were taken from each participant. TaqMan Array Card used to test for influenza A (H1 and H3) and B, respiratory syncytial virus, human parainfluenza viruses 1–3, human metapneumovirus, rhinovirus, enterovirus, parechovirus, adenovirus, *Legionella* species, *Haemophilus influenzae, Streptococcus pyogenes, Mycoplasma pneumoniae, Chlamydophila pneumoniae,* and *Bordetella pertussis.* Asterisk indicates other pneumococcal serotypes identified in 3 persons, including 11A/D, 20, 34, and serotypes that were nontypeable by real-time PCR: 1, 2, 3, 4, 5, 6A/B/C, 6C, 7F/7A, 9V/9A, 11A/11D, 12F/(12A/44/46), 14, 15A/15F, 16F, 18/(18A/18B/18C/18F), 19A, 19F, 22F/22A, 23A, 23F, 33F/33A/37.

tified: 3 confirmed pneumococcal disease, 6 confirmed pneumonia, and 2 suspected pneumonia (Figure 2). Three case-patients were hospitalized (2 with IPD, 1 with confirmed pneumonia). Among the 20 patients, the cases of 5 (attack rate 25%) met an outbreak case definition (Table 2). In addition, 1 staff member had IPD, 1 had confirmed pneumonia, and 1 had suspected pneumonia (Table 2, attack rate <3%). These staff members provided direct care to all Unit 1 patients. Three visitors had confirmed pneumonia. Adults (3 staff and 3 visitors) who became ill during the outbreak and for whom ages were available (5 of 6) ranged in age from 27 to 56 years.

All 3 clinical isolates were identified as *S. pneumoniae* serotype 15A, sequence type 63, (Table 2) with matching antimicrobial drug susceptibility patterns. Nine (45%) of 20 persons tested carried pneumococcus; of those, 6 (30%) carried serotype 15A. Rhinovirus, *Streptococcus pyogenes*, and *Mycoplasma pneumoniae* were additional pathogens most frequently identified by using TaqMan Array Cards (Figure 1, Table 2).

We assessed Unit 1 infection control practices; in particular, for staff compliance with hand and respiratory hygiene. The hand hygiene audit revealed that staff members had performed hand hygiene in 4 (24%) of 17 instances before patient contact and 11 (79%) of 14 times after patient contact, for an overall compliance of 15 (48%) of 31 opportunities. In addition,, supplies (e.g., gloves) were kept in locked cabinets because of safety concerns. Staff reported that patients, because of their developmental delays, were often unable to appropriately manage their respiratory secretions.

The presence of 6 carriers of serotype 15A on Unit 1 during the carriage survey indicated the potential for con-

Table 1. Characteristics of 20 psychiatric unit patients during *Streptococcus pneumoniae* serotype 15A outbreak, Rhode Island, USA, December 25, 2010–January 31, 2011

Characteristic	No. (%) patients
Male sex	14 (70.0)
Median age, y (range)	13 (4–24)
Race	
White	16 (80.0)
Black	4 (20.0)
Other/unknown	0
Underlying medical conditions	
Asthma*	6 (30.0)
Chronic heart disease	2 (10.0)
Other risk factors for pneumococcal infection†	0
Median length of stay, days on Unit 1 as of January 30, 2011 (range)	71 (1–706)
Receiving systemic antimicrobial drugs during January for illness unrelated to outbreak	1 (5.0)
No. patients on Unit 1 as of January 30, 2011	
Current in-patient	15 (75.0)
Day program patient‡	1 (20.0)
Discharged (since December 25, 2010)	4 (5.0)

*None were treated with systemic steroids (i.e., none had an indication for vaccine) (*3,4*).
†Includes chronic lung disease (other than asthma), diabetes mellitus, cerebrospinal fluid leaks, cochlear implant, systemic steroid use, sickle cell disease, congenital or acquired asplenia, HIV infection, chronic renal failure, nephritic syndrome or kidney disease with dialysis, bone-marrow or organ transplant, diseases associated with treatment with immunosuppressive drugs or radiation therapy, including malignant neoplasms, leukemia, lymphoma, and Hodgkin disease (*3,4*).
‡Day program patients were treated in Unit 1 during the day and returned home to stay overnight with their families.

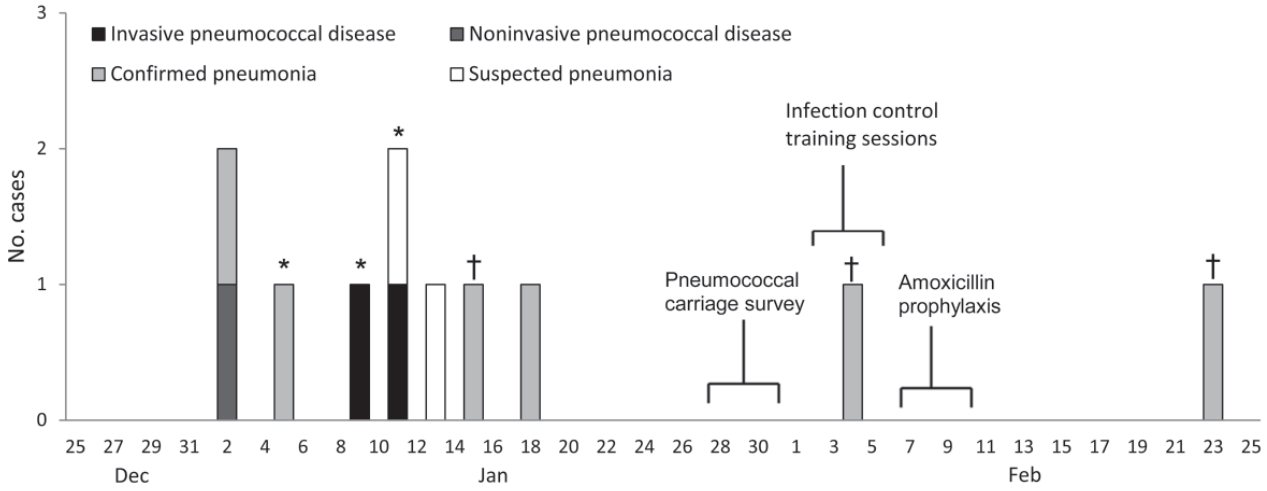

Figure 2. Epidemic curve of *Streptococcus pneumoniae* outbreak in a pediatric psychiatric hospital, Rhode Island, USA, December 25, 2010–January 31, 2011 for invasive pneumococcal disease (IPD), noninvasive pneumococcal disease, confirmed pneumonia, and suspected pneumonia. *Staff members; †family members.

tinued transmission and disease. The hospital mandated hand and respiratory hygiene training for all Unit 1 staff, administered during February 4–8. We recommended high-dose amoxicillin prophylaxis (90 mg/kg/day divided into 2 doses, maximum of 1,000 mg, for 5 days) for all Unit 1 patients, which began on February 7. After control measures were fully implemented, 1 case of pneumonia was confirmed in a patient's parent on February 23, but the etiology was not identified. No additional cases occurred among Unit 1 patients and staff members during the subsequent 3 months.

Conclusions

Laboratory and epidemiologic evidence indicates that an outbreak of *S. pneumoniae* serotype 15A infection occurred on Unit 1. Increased transmission opportunities likely resulted from infection control challenges in the unit. Because serotype 15A is not included in any current pneumococcal vaccine, we used antimicrobial drug prophylaxis as an immediate intervention to reduce transmission. However, infection control was the critical long-term control measure. We modified acute-care hospital infection control practices for the unique environment of Unit 1, such as scheduling hand hygiene sessions every 2–3 hours for all patients, rather than expecting patients to understand when to perform hand hygiene. Furthermore, other respiratory pathogens were present on Unit 1, which could have contributed to disease and facilitated pneumococcal transmission by increasing nasopharyngeal colonization (*12*). The same infection control lapses that led to the *S. pneumoniae* serotype 15A outbreak likely led to transmission of these respiratory pathogens.

Since the introduction of the 7-valent pneumococcal conjugate vaccine, nasopharyngeal carriage of serotype 15A has increased (*13*). Among Massachusetts children 3 months–7 years of age, serotype 15A carriage prevalence was 4% in 2007 (*14*), compared with 30% in this outbreak. Since the introduction of the 7-valent pneumococcal conjugate vaccine, serotype15A sequence type 63 has also caused an increasing proportion of penicillin-nonsusceptible IPD in the United States (*15*). The sequence type and antimicrobial drug susceptibilities of the serotype 15A strain from this outbreak are characteristic of a single, internationally disseminated serotype 15A strain (*15*). No other increases in IPD or serotype 15A were detected by the Rhode Island Department of Health during this period, indicating that the outbreak was restricted to Unit 1.

This investigation had several limitations. First, diagnostic tests were performed at the discretion of treating clinicians, and specific diagnostic testing was not performed for most pneumonia case-patients. In addition, we used TaqMan Array Cards <3 weeks after symptom onset, which decreased our ability to detect all bacterial and viral co-factors that possibly contributed to this outbreak. Because of the small size of the patient cohort, statistical tests would not have power to assess risk factors for pneumococcal disease or carriage. Finally, we did not assess the knowledge of hand hygiene among staff after the training to evaluate its effectiveness.

In conclusion, this outbreak was associated with *S. pneumoniae* serotype 15A, a serotype not included in available pneumococcal vaccines. The outbreak subsided after patients received antimicrobial drug prophylaxis. The hospital instituted a hand hygiene monitoring program in

response to our recommendations and enhanced infection control practices were implemented, especially careful adherence to hand and respiratory hygiene. Hand and respiratory hygiene training and monitoring are critical for infection control in units that serve patients with special cognitive needs.

Acknowledgments

We gratefully acknowledge the patients, staff members, and family members of Unit 1 and the staff at the hospital who assisted with the investigation.

Dr Fleming-Dutra is an Epidemic Intelligence Service officer with the Respiratory Diseases Branch, Division of Bacte-

Table 2. Cases, clinical isolates, and results of pneumococcal carriage and respiratory pathogen survey associated with *Streptococcus pneumoniae* serotype 15A outbreak, Rhode Island, USA, December 25, 2010–January 31, 2011*

Case status	Case no.	Onset date	Culture date, specimen and result	Chest imaging results	Treatment	Outcome	Carriage	Sero	Other pathogens by TAC†
IPD	S1	Jan 9	Jan 14, blood, *S. pneumoniae* 15A	Right upper lobe pneumonia	Moxifloxacin	Rec	Neg		Neg
IPD	P1	Jan 11	Jan 25, blood, *S. pneumoniae* 15A	Negative	Ceftriaxone	Rec	Pos	15A	Rhinovirus, adenovirus
Noninvasive pneumococcal disease	P2	Jan 2	Jan 28, otorrhea, *S. pneumoniae* 15A		Cefuroxime, topical ofloxacin	Rec	Pos	15A	*Mycoplasma pneumoniae*
Confirmed pneumonia	P3	Jan 2		Left lower lobe pneumonia	Amoxicillin/ clavulanate, azithromycin	Rec	Pos	NT‡	Rhinovirus
Confirmed pneumonia	S2	Jan 5		Left upper lobe pneumonia	Amoxicillin/ clavuanate, ciprofloxacin	Rec	NS		NS
Confirmed pneumonia	V1	Jan 15		Right basilar pneumonia	Moxifloxacin	Rec	NS		NS
Confirmed pneumonia	P4	Jan 18		Right lower lobe pneumonia	Ceftriaxone, ampicillin, amoxicillin	Rec	Neg		*M. pneumoniae*
Confirmed pneumonia	V2	Feb 4		Left lower lobe pneumonia	Doxycycline, levofloxacin	Rec	NS		NS
Confirmed pneumonia§	V3	Feb 23		Left lower lobe pneumonia	Unk	Unk	NS		NS
Suspected pneumonia	S3	Jan 11			Azithromycin	Rec	NS		NS
Suspected pneumonia	P5	Jan 13			Clindamycin	Rec	Neg		Neg
Non-case	P6						Neg		*S. pyogenes*
Non-case	P7						Neg		Adenovirus
Non-case	P8						Neg		Neg
Non-case	P9						Pos	34	*S. pyogenes*
Non-case	P10						Neg		Neg
Non-case	P11						Pos	15A	Enterovirus
Non-case	P12						Pos	15A	Neg
Non-case	P13						Pos	11 A/D, 20	*S. pyogenes*
Non-case	P14						Neg		Neg
Non-case	P15						Pos	15A	Rhinovirus, *M. pneumoniae*
Non-case	P16						Pos	15A	*M. pneumoniae*
Non-case	S4						Neg		Rhinovirus
Non-case	S5						Neg		Neg
Non-case	S6						Neg		*S. pyogenes*

*Sero, serotype; TAC, TaqMan Array Card; IPD, invasive pneumococcal disease; S, staff; rec, recovered; neg, negative; P, patient; pos, positive; NS, no carriage screen; V, visitor; NT, nontypeable; Unk, unknown.
†TAC used to test for influenza viruses A (H1 and H3) and B, respiratory syncytial virus, human parainfluenza viruses 1–3, human metapneumovirus, rhinovirus, enterovirus, parechovirus, adenovirus, *Legionella* species, *Haemophilus influenzae*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Bordetella pertussis*.
‡NT by real-time PCR (for serotypes: 1, 2, 3, 4, 5, 6A/B/C, 6C, 7F/7A, 9V/9A, 11A/11D, 12F/(12A/44/46), 14, 15A/15F, 16F, 18/(18A/18B/18C/18F), 19A, 19F, 22F/22A, 23A, 23F, 33F/33A/37).
§Refused further involvement with investigation.

rial Diseases, National Center for Immunizations and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta. Her research interests include outbreaks of bacterial respiratory diseases and pneumococcal epidemiology.

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Seroprevalence of Influenza A(H1N1) pdm09 Virus Antibody, England, 2010 and 2011

Katja Hoschler, Catherine Thompson, Nick Andrews, Monica Galiano, Richard Pebody, Joanna Ellis, Elaine Stanford, Marc Baguelin, Elizabeth Miller, and Maria Zambon

The intense influenza activity in England during the 2010–11 winter resulted from a combination of factors. Population-based seroepidemiology confirms that the third wave of influenza A(H1N1)pdm09 virus circulation was associated with a shift in age groups affected, with the highest rate of infection in young adults.

Seroepidemiologic data collected in England during the first 2 influenza pandemic waves suggested that another wave of infection with influenza A(H1N1)pdm09 virus was unlikely during 2010–11 (*1*). However, a substantial third wave occurred that affected persons in older age groups (*2*). Severity indicators suggested a higher level of illness and death, with increased cases in critical care and deaths. We conducted further seroepidemiologic study in England during 2010–11 to identify possible reasons for these observations.

The Study

This observational study used anonymized, residual serum samples from routine microbiological testing. Patient age and sex, date of sample collection, and source laboratory information were available (*3*).

Samples were from patients 0–99 years of age, of whom 53% were female. Samples were grouped according to collection date: pre–first wave (before April 2009 [1,403 samples]) and post–first wave (August–October 2009 [3,091 samples]); post–second wave (January–April 2010 [2,225 samples]); and pre–third wave (June–October 2010 [1,782 samples]) and post–third wave (February–April 2011 [1,257 samples]) (Figure 1). Availability of samples by region and patient age was not consistent. With the objective of measuring age-dependent incidence, we prioritized serum samples by patient age. Samples were spread across 7 age groups (<5, 5–14, 15–24, 25–44, 45–64, 65–74, and ≥75 years) and came from the 9 regions of England (East, East Midlands, London, North East, North West, South East, South West, West Midlands, and Yorkshire and Humber).

Viruses were characterized and sequenced as described (*4*). All samples were tested by hemagglutination-inhibition (HI) assay; samples with sufficient material also were tested by microneutralization assay according to standard methods (*1*). Samples with titers ≥32 or ≥40 by HI or microneutralization assay, respectively, were considered seropositive.

We determined antibody persistence by comparing antibody levels in the post–second wave panel with those of the pre–third wave panel on a subset of samples from 3 regions (North East, North West, and South West) where samples were available for both time points. Results were assessed with 95% confidence intervals. The full analysis of the seroprevalence preceding the 2010–11 season is detailed elsewhere (*1*).

In samples from all persons except those in the youngest age group (<5 years), antibody declined from the end of the 2009–10 winter season (post–second wave) to before

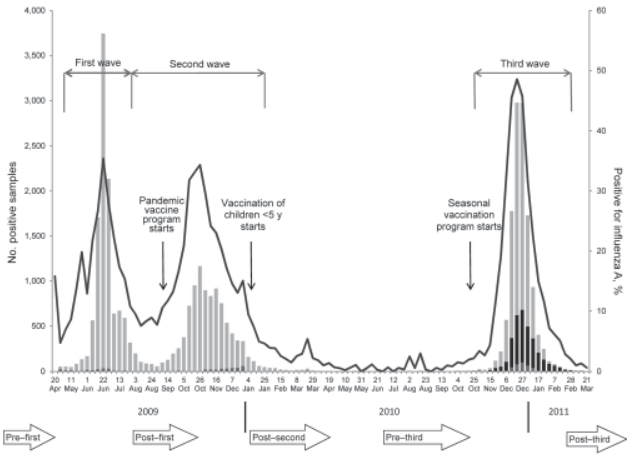

Figure 1. Number of influenza A(H1N1)pdm09 virus detections (and percentage positive) detected through a network of Health Protection Agency laboratories (the Respiratory DataMart system) from the start of the pandemic in week 17 (week of April 27) 2009 until the end of the 2010–11 winter season. It demonstrates the 3 waves of pandemic influenza activity in summer 2009, autumn 2009, and winter 2010–11 and the key events in relation to the timing of the national influenza vaccination program. The timing of the serum collections are illustrated at the bottom of the figure. Samples were grouped into panels according to their collection. Light gray, A(H1N1)pdm09 virus; medium gray, untyped influenza A virus; black, influenza B virus; line, overall percentage positive. Study periods were defined as follows: pre–first wave, before April 2009; post–first wave, August–October 2009; post–second wave, January–April 2010; pre–third wave, June–October 2010; and post–third wave, February–April 2011.

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Table 1. Seroprevalence of influenza A(H1N1)pdm09 virus antibody, England, 2010 and 2011*

	No. seropositive samples/no. total samples† (% seropositive samples, 95% CI)					
	Post–second wave‡		Pre–third		Post–third wave	
Characteristic	HI	MN	HI	MN	HI	MN
Age group, y						
<5	36/98 (0.37, 0.27–0.47)	31/77 (0.4, 0.29–0.52)	94/182 (0.52, 0.44–0.59)	88/174 (0.51, 0.43–0.58)	99/160 (0.62, 0.54–0.69)	93/150 (0.62, 0.54–0.7)
5–14	132/213 (0.62, 0.55–0.69)	69/107 (0.64, 0.55–0.73)	142/244 (0.58, 0.52–0.64)	152/237 (0.64, 0.58–0.7)	155/200 (0.78, 0.71–0.83)	146/199 (0.73, 0.67–0.79)
15–24	68/154 (0.44, 0.36–0.52)	44/101 (0.44, 0.34–0.54)	152/405 (0.38, 0.33–0.42)	156/400 (0.39, 0.34–0.44)	216/320 (0.68, 0.62–0.73)	188/311 (0.6, 0.55–0.66)
25–44	66/200 (0.33, 0.27–0.4)	31/83 (0.37, 0.27–0.49)	106/370 (0.29, 0.24–0.34)	114/370 (0.31, 0.26–0.36)	187/294 (0.64, 0.58–0.69)	155/283 (0.55, 0.49–0.61)
45–64	59/220 0.27, 0.21–0.33)	42/110 (0.38, 0.29–0.48)	69/320 (0.22, 0.17–0.26)	93/318 (0.29, 0.24–0.35)	62/138 (0.45, 0.36–0.54)	52/135 (0.39, 0.3–0.47)
65–74	36/145 (0.25, 0.18–0.33)	27/87 (0.31, 0.22–0.42)	35/168 (0.21, 0.15–0.28)	42/167 (0.25, 0.19–0.32)	38/74 (0.51, 0.39–0.63)	38/74 (0.51, 0.39–0.63)
≥75	55/172 (0.32, 0.25–0.4)	77/163 (0.47, 0.39–0.55)	16/93 (0.17, 0.1–0.26)	25/92 (0.27, 0.18–0.37)	46/71 (0.65, 0.53–0.76)	39/71 (0.55, 0.43–0.67)
Region§						
Total	1,202		1,782		1,257	
North West	561		624		337	
South West	404		232		265	
North East	237		526		179	
East	0		292		122	
Yorkshire and Humber	0		108		354	

*HI, hemagglutination inhibition assay; MN, microneutralization assay.
†Samples with titers ≥32 by HI or ≥40 by MN,
‡For data consistency, samples from only North West, South West, and North East regions were included in this analysis because these regions also were among the 5 regions in pre– and post–third wave samples.
§Numbers by region describe total available number of samples (all analyzed at least by HI assay).

the onset of the 2010–11 season (pre–third wave). This decline was limited (<10% reduction by HI and microneutralization assays in persons 5–74 years of age), with the largest reduction in the ≥75-year group (–15% and –20% by HI and microneutralization assays, respectively). In children <5 years, antibody levels increased (15% and 10% by HI and nicroneutralization assays, respectively) during the same time period (Tables 1,2; Figure 2).

We assessed changes in antibody levels during the 2010–11 season using data from all 5 available regions (East, North East, North West, South West, and Yorkshire and Humber) (Tables 1, 2; online Technical Appendix Table, wwwnc.cdc.gov/EID/pdfs/12-0720-Techapp.pdf). For all age groups, HI and microneutralization assays demonstrated similar trends, although the increase by microneutralization assay in elderly persons was lower than by HI assay (48% vs. 28% increase). We found no evidence for association of titer with sex or region.

Children in the 2 youngest groups (≤14 years) had the highest titers overall and highest percentage of seropositive samples (Tables 1, 2; Figure 2; online Technical Appendix Table). The highest increases in seroprevalence during the third wave were observed in the oldest age group (≥75 years, from 17% to 65% seropositive by HI assay), followed by young adults (15–44 years, from 33% to 66% seropositive by HI assay) (online Technical Appendix Figure).

Conclusions

Clinical surveillance data obtained during the course of acute illness (*2*) and seroepidemiology through population sampling are consistent and together point toward a

Table 2. Seroincidence estimates of influenza A(H1N1)pdm09 virus antibody, England, 2010 and 2011

	Change in antibody level, % (95% CI)			
	Post–second wave to pre–third wave*		Pre–third wave to post–third wave†	
Age group, y	Hemagglutination inhibition	Microneutralization	Hemagglutination inhibition	Microneutralization
<5	15 (3–27)	10 (–3 to 24)	10 (0–21)	11 (1–22)
5–14	–4 (–13 to 5)	0 (–11 to 11)	19 (11–28)	9 (1–18)
15–24	–7 (–16 to 3)	–5 (–15 to 6)	30 (23–37)	21 (14–29)
25–44	–4 (–12 to 4)	–7 (–18 to 5)	35 (28–42)	24 (16–31)
45–64	–5 (–13 to 2)	–9 (–19 to 1)	23 (15–33)	9 (0–19)
65–74	–4 (–13 to 5)	–6 (–18 to 6)	31 (18–43)	26 (13–39)
≥75	–15 (–25 to –4)	–20 (–32 to –8)	48 (34–61)	28 (13–42)

*Antibody levels at the end of the 2009–10 winter season compared with those before the 2010–11 season.
†Antibody levels before and after the 2010–11 season.

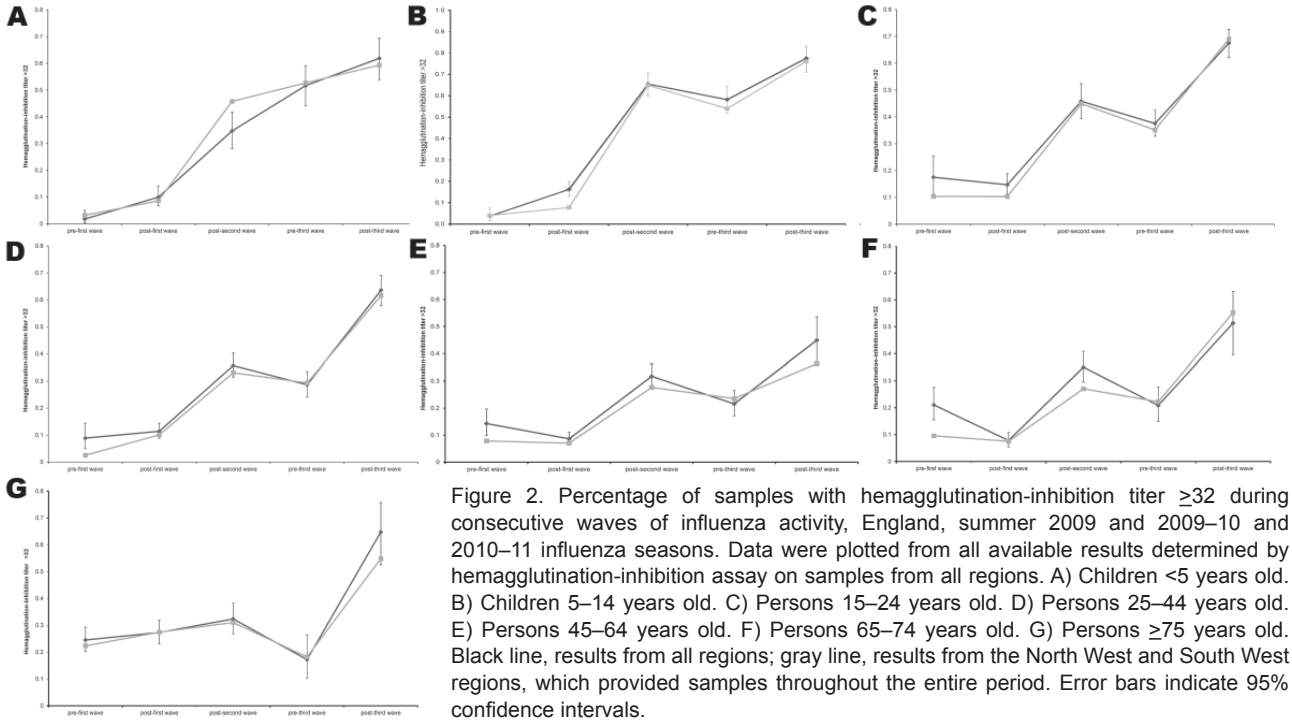

Figure 2. Percentage of samples with hemagglutination-inhibition titer ≥32 during consecutive waves of influenza activity, England, summer 2009 and 2009–10 and 2010–11 influenza seasons. Data were plotted from all available results determined by hemagglutination-inhibition assay on samples from all regions. A) Children <5 years old. B) Children 5–14 years old. C) Persons 15–24 years old. D) Persons 25–44 years old. E) Persons 45–64 years old. F) Persons 65–74 years old. G) Persons ≥75 years old. Black line, results from all regions; gray line, results from the North West and South West regions, which provided samples throughout the entire period. Error bars indicate 95% confidence intervals.

shift in the age range for infection with A(H1N1)pdm09 in the first season after the 2009 pandemic. This finding is similar to those in earlier pandemics (*5*) and other countries (*6*). Historical data, including from 1918, suggest that the initial impact in children is followed by a dramatic shift in age distribution of infected persons, with the probability of infection in adults exceeding those of children until the age distribution returns to the normal seasonal pattern (*5*,*7*). This adaptation process may take 3–10 years (*7*).

The rates of decline in antibody to A(H1N1)pdm09 from the 2009–10 to the 2010–11 winters are similar to historic data (*8*) and A(H1N1)pdm09 vaccine trials (*9*,*10*). The implications of such reduction are uncertain. The seroprevalence data suggested susceptibility in young adults pre–third wave, but not in children who were targeted by an extended vaccination program in the United Kingdom from January 2010. Up to 30% of children <5 years were vaccinated (*11*).

During the 2010–11 season, antibody was acquired primarily by young and old adults. The largest increase in antibody levels after the 2010–11 winter occurred in persons >75 years of age. Clinical surveillance data suggests that elderly persons (>65 years of age) were relatively spared from infection with A(H1N1)pdm09 virus (*12*). We propose that the increase resulted primarily from seasonal influenza vaccination in 2010–11 with vaccine uptake of 72.8% (*13*). In young adults (15–44 years), we believe that acquisition of antibody occurred as susceptible persons became infected during the winter. Children were relatively spared from infection with A(H1N1)pdm09 during winter 2010–11; their high rate of infection in the 2 previous pandemic waves, together with vaccination, left a limited number of susceptible persons (Figure 2) .

Our study design—a retrospective, periodic, cross-sectional collection—has certain limitations. We analyzed similar but not identical groups and persons at different time points. For each sample, only limited information was available. Without information about vaccination status or influenza exposure history during the season, our interpretation of antibody levels and their changes has to be taken with caution. However, in this descriptive analysis we also used supportive evidence from UK influenza surveillance programs and take into account the date of vaccination timing and uptake, which strengthens our interpretation of the serologic data.

The collections for each sample set were distributed over time periods of up to 21 weeks, during which antibody levels would have changed, depending on the combined effects of seroconversion, antibody waning and availability of vaccination. A novel likelihood-based approach, described previously has therefore been developed to overcome some of the limitations of the conventional statistical method (*1*).

We found no evidence of substantial antigenic drift in circulating viruses that could affect seroepidemiology results (online Technical Appendix Table). We conclude that the intense A(H1N1)pdm09 virus activity in the England during the 2010–11 winter must have resulted from a combination of factors.

The change in age distribution of infection is likely to have caused increased severity, resulting from a larger number of patients with underlying concurrent conditions (*12*) or from age-dependent changes in pathology. Defining antibody correlates of protection becomes more complex with rising patient age as other immune mechanisms increasingly contribute to protection, e.g., CD4+ T cells, as demonstrated in human challenge experiments (*14*). Moreover, a murine model identified the role of age in susceptibility to pathogenesis and transmission of influenza virus infection (*15*). These observations might help to provide some mechanistic insights for the shift in age distribution of infection and severity in the season after the 2009 pandemic. Genetic drift in circulating virus over time affecting human airway adaptation and varying climatic conditions during different pandemic waves also should be investigated.

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Call to Action on World Pneumonia Day

Rana Hajjeh and Cynthia G. Whitney

This month, on November 12, the world will recognize the fourth annual World Pneumonia Day. First launched in 2009 by a coalition of global health leaders (*1*), World Pneumonia Day aims to raise awareness about pneumonia's toll on the world's children and to promote interventions to protect against, treat, and prevent the disease. Pneumonia continues to be the leading killer of young children around the world, causing ≈14% of all deaths in children 1 month to 5 years of age (*2*). It is a critical disease for countries to conquer in order to reach Millennium Development Goal 4: reducing the child mortality rate by two thirds from 1990 to 2015 (*3*). Most children who die from pneumonia live in developing countries, where such factors as malnutrition, crowding, and lack of access to quality health care increase the risk for death. Pneumonia kills few children in industrialized countries, although it remains among the top 10 causes of deaths in the United States, for example, because of deaths in older adults (*4*).

Fortunately, many interventions are now available to reduce deaths due to pneumonia among children throughout the world. On the first World Pneumonia Day in 2009, the World Health Organization and the United Nations Children's Fund, together with many global experts and partners, launched the Global Action Plan for Prevention and Control of Pneumonia (GAPP) (*5*). GAPP recommends a strategy of prevention, protection, and treatment that is designed to implement readily available interventions that can reduce pneumonia deaths in children. GAPP focuses on improving nutrition (through measures such as exclusive breastfeeding), increasing access to vaccines that protect from agents that cause pneumonia (such as *Haemphilus influenzae* type b and pneumococcal vaccines), reducing exposure to indoor air pollution, and increasing access to antimicrobial drugs that can treat pneumonia.

In 2010, the World Health Assembly passed a resolution recognizing the role of pneumonia as the leading cause of deaths in children, setting out the goal of reducing pneumonia deaths as a global health priority (*6*), and the World Health Organization began tracking implementation of GAPP. One notable area of success has been the introduction of new vaccines to prevent pneumonia. During the last few years, because of funding and technical support from the Global Alliance for Vaccines and Immunizations and various partners, the introduction of new vaccines in developing countries has had unprecedented momentum. *Haemophilus influenzae* type b vaccines have been introduced or are ready to be introduced in all countries eligible for Global Alliance for Vaccines and Immunizations funding by 2013, and pneumococcal conjugate vaccines are expected to be introduced in 54 countries by 2015 (*7*).

Despite recent progress in the effort to decrease the number of pneumonia cases, pneumonia is still an urgent problem. This month's issue of Emerging Infectious Diseases presents results of recent research on pneumonia conducted around the world. The work, mostly from high-income settings, highlights some of the remaining difficulties involving pneumonia prevention, treatment, and control. For example, although van Deursen et al. show the remarkable benefits of the pneumococcal conjugate vaccination program in the Netherlands (*8*), Fleming-Dutra et al. report how pneumococcal pneumonia outbreaks can occur even in a highly vaccinated population if crowding and poor health are common, because currently available vaccines do not cover all pneumococcal serotypes (*9*). By describing cases of pneumonia that occurred after the megaquake in Japan in 2011, Takahashi et al. show how natural disasters might lead to increases in pneumonia risk or create large shifts in needed health care (*10*).

Many challenging research questions remain. A recent priority-setting exercise outlined the most urgent studies needed to reduce pneumonia deaths in low-income countries (*11*). Priority items ranged from assessments of vaccine effects on disease in low-income settings to evaluation of measures to improve community management of pneumonia. In addition, a large, multicenter study to identify the etiologic agents of pneumonia in developing countries, supported by the Bill and Melinda Gates Foundation, was launched in 2011 (*12*). This study is expected to generate

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data that will better guide prevention and treatment strategies, particularly in countries that are already using new vaccines.

This November 12, World Pneumonia Day, we urge the global community to consider the massive problems of pneumonia. Better yet, take a moment to consider what you can do to solve this problem. Health care providers, researchers, policy makers, and the greater public health community all need to contribute if we are to make rapid, substantial progress toward reducing disease and deaths due to pneumonia. Progress is being made, but much more can be done.

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Pandemic Influenza Outbreak on a Troop Ship—Diary of a Soldier in 1918

Jennifer A. Summers

A newly identified diary from a soldier in 1918 describes aspects of a troop ship outbreak of pandemic influenza. This diary is the only known document that describes this outbreak and provides information not officially documented concerning possible risk factors such as overcrowding and the suboptimal outbreak response by military leaders. It also presents an independent personal perspective of this overwhelming experience.

A previous study described the epidemiology and risk factors for death from pandemic influenza in 1918 aboard a World War I (WWI) New Zealand troop ship (*1*). This outbreak aboard His Majesty's New Zealand Transport (HMNZT) Tahiti represents a worst-case scenario for a novel infectious disease outbreak, occurring in a crowded setting with limited medical resources. Although this ship outbreak of pandemic influenza was not the most severe reported during 1918 (*2*,*3*), it was a notable outbreak that had a cumulative mortality rate of 68.9 cases/1,000 population and estimated cumulative morbidity rate of 90%.

A newly identified diary from a soldier on this ship has recently been transcribed by the author's grandson for July 10, 1918, through January 31, 1919 (Figure) (J Hansen, unpub. data). The diary accounts were not part of the official inquiry made into this outbreak or subsequent report (*4*,*5*). Therefore, this diary, the only known document of its nature, is an independent and unofficial account of this outbreak. The author of the diary, now deceased, was a rifleman in the 40th Reinforcements of the New Zealand Expeditionary Force aboard HMNZT Tahiti. Before the war, he was a telegraphist, and later served in the New Zealand Air Force during World War II (*6*).

HMNZT Tahiti left New Zealand on July 10, 1918, and, after stops in Australia and South Africa, joined a military convoy in Freetown, Sierra Leone, in western Africa. This voyage was among the last to supply new New Zealand reinforcements to Europe during WWI.

10 July (Diary entry, day of embarkment, Wellington, New Zealand):

> We left the wharf at 1.55 pm and finally set sail at 5 pm. Started off in fine weather and all well. There were 4 companies of infantry, A B C & E, and one coy of artillery and our own coy, the 40th Specs on board in this trip & no doubt the total number will be about 1200, truly a large number to be packed on this vessel, which I hear is about 7000 tonnes, and where I am domiciled, we are packed in like sardines in the bows of the ship 'tween decks. Our bunks consist of hammocks strung to the ceiling and we are packed right across the ship longways & sideways & every inch of space is utilised, for underneath are our mess tables which are also our writing tables etc, & we have to live down here in rough weather. The food is very good and plenty of it. We have a band aboard & I joined same. Played some tunes before we finally sailed.

The language in the diary suggests that the respondent was a fairly thoughtful observer and not prone to exaggeration, because his diary entries accurately describe, when compared with the official report, ship hospital admission numbers and details aboard during the outbreak. For example, the first cases of influenza (n = 35) were reported in the diary on August 26: "… the hospital is over full and also a dozen patients on deck." This is consistent with the official report, which documents that when "… the number of cases exceeded the capacity of the Hospital (36) more and more deck space was utilised for Hospital purposes."

The soldier himself contracted influenza during the outbreak, but his illness was not incapacitating, and he maintained daily entries into his diary throughout the outbreak.

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27 August (diary entry; 1 day after setting sail from Freetown, Sierra Leone):

> Fairly warm and showers of rain occasionally. An epidemic of influenza seems to have broken out on board. Sixty odd on this morning's sick parade and 24 admissions to the hospital. Have caught it myself of course but so far am able to get about & think I will hang out all right but feel very crook. Saw a few porpoises quite near the boat today. We are still travelling in the same formation but the distances from each boat varies. No band this morning, as a lot of the members are not well. This afternoon we pasted some music on to cards instead of practicing. This morning we had a lecture for half an hour on transport etc in England and was a most idiotic lecture too. Had no object in it that I could see.

2 September (diary entry; part of military convoy off the coast of Western Africa):

> Fine day and sea like glass. Quite a gloom was cast round the cabin as soon as we were up when three deaths were reported on board. The burials took place at 11am and there were 4 burials. The Colonel read the service and it was quite a touching scene. This afternoon two more burials took place and there is another death. The other ships are also busy with burials. One of the deaths is our Clarionette player in the band and a beautiful player he was too. The strange thing about this sickness is that the big strong men seem to get it the worst and are the ones that die. One of the deaths is namesake of myself I hear. Today was mess orderly again. Was also able to eat a bit today and feel much better.

10 September (diary entry; arriving in England):

> Fine but very cold & sea fair. Land was in sight this morning and all very excited in the thought of getting on land again for good.… The country here looks really beautiful green fields and ploughed land with forests here and there. … One man died as we were coming into port.

Despite the obvious ongoing outbreak, officials continued with war preparation, regardless of the potential transmission risk. The diary states that after the outbreak and situated in England, the troops were required to parade, although they were weak: "most of us are not too strong yet and got a bit fagged." However, later treatment of the troops does imply that military officials were not lacking empathy, because a brigadier general promised the troops "that when all danger of infection was over we would be granted a week's leave and free rail pass anywhere, so we are on a win if all goes well." There is no reference made in the inquiry regarding this leave; however, the soldier documents on October 10: "Tomorrow we are to get our weeks leave which we have been looking forward to for so long." Also, the following week's diary entries describe travel to Edinburgh and Glasgow: "Quite a treat to get in a decent bed again and makes one feel almost homesick. Hurray, won't need to be up with the blasted bugle in the morning."

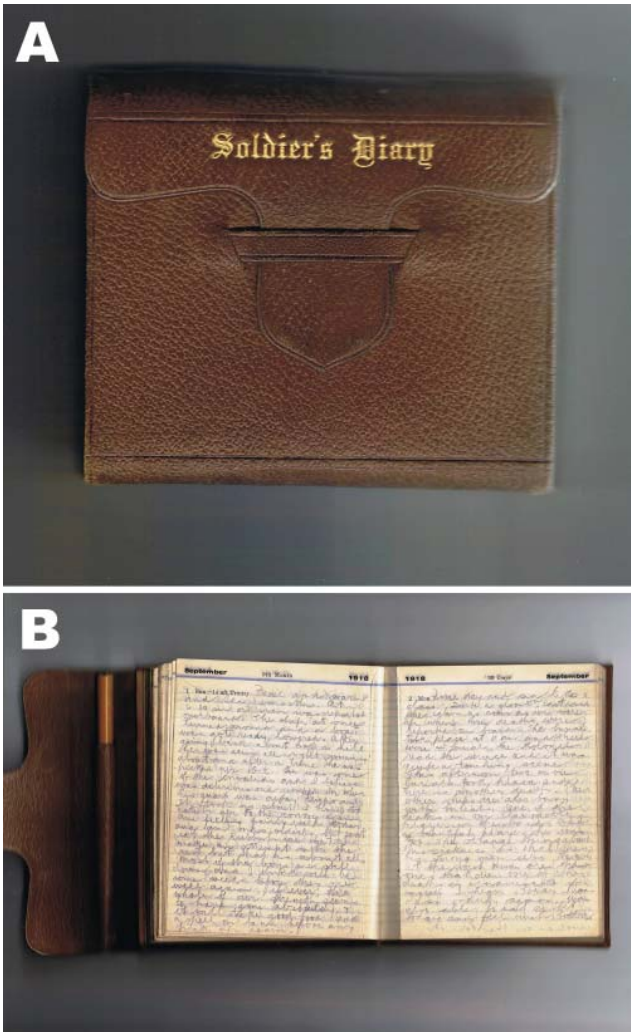

Figure. A) Front cover and B) entry of a diary from a soldier on board His Majesty's New Zealand Transport Tahiti during a period of pandemic influenza, July 10, 1918, through January 31, 1919.

A crowded environment is an acknowledged risk factor for influenza transmission and probably played a role in this particular outbreak (*1*). Furthermore, the official inquiry concluded that the cause of the high death rate on the

ship was caused by "the virulent nature of the infection, which affected a large number of men massed together on a ship where the ventilation was defective owing to the enforced closing of the ports and the absence of any form of recognized artificial ventilation."(*4*) The diary makes several references to the disagreeable conditions aboard. On July 10, (pre-outbreak), the soldier writes, "we are packed in like sardines," and "we are packed right across the ship longways & sideways," on August 29 (mid-outbreak), "men are packed in such a small space." On September 5 (mid-outbreak), he elaborates, "more deaths and burials total now 42. A crying shame but it is only to be expected when human beings are herded together the way they have been on this boat." The inquiry concluded that the crowding aboard ship was justified given the necessities of war and that it was no more excessive than on other troop ships during this period.

It was clear at the time of the inquiry that medical provision on board the ship was inadequate for coping with an outbreak of this scale. The inquiry and report documented that the nursing staff were "overwhelmed with work," criticized the work of the medical orderlies, and indicated that there was a lack of medical supplies (*4*,*5*). The August 31 entry in the diary supported this: "the ship has also run short of medicine now, this sickness must have been something they did not bargain for." However, as the previous study concluded (*1*), medication for influenza during 1918 was unlikely to have had an effect on the risk for death. Referring to the overwhelmed medical staff, the diary corroborates the findings of the inquiry: "two or three hundred more on sick parade today and the nurses and doctors have their hands full all right now."

Food supplies on board were discussed in the diary and the inquiry, with some conflicting entries. A pre-outbreak diary entry stated: "The food we are having is very good & plenty of it so far." However, even after the ship was stocked up with provisions in Freetown, Sierra Leone, a later entry (mid-outbreak) noted: "There are tons of meat etc wasted just for the want of cooking into a decent dish… but the muck we get to eat does not tempt any one to eat." This statement suggests that the overall food quality during the outbreak may have been poor. This account is partially consistent with the inquiry findings, which acknowledged some problems with food quality. However, the inquiry does state: "it is apparent from the evidence that there was an abundance of other foods to supplement the bread and meat on the occasions on which they were not satisfactory" (*4*). However, it is not clear from either source whether food provision was the problem or whether there was a failure to organize able military staff to cook food (assuming the ship's original cooks were unwell).

Regarding unsatisfactory food, a diary entry made at mid-outbreak noted: "Heard today that the Sergeant's mess have received from the skipper a certificate asking them to sign to effect that the food and general comfort on this ship was all that could be desired and not one would sign it." This entry suggests that military leaders aboard the Tahiti were aware that the overcrowding situation existed and that the outbreak response was lacking (as was to some extent confirmed by the subsequent inquiry).

The last influenza-related death among the 40th Reinforcements occurred in England on October 3, 1918, at the beginning stages of the second wave of pandemic influenza (*7*). The October 24 diary entry states: "Very foggy and bitterly cold first thing in the morning. Band parades as usual. Learnt how to counter march this morning.… We were all inoculated this morning for the purpose of counteracting the influenza plague. I believe there is an epidemic of it in camp at present."

This diary provides an insightful and relevant snapshot of one soldier's experience during an influenza outbreak. The chronological account in this soldier's diary of this troop ship outbreak of pandemic influenza also supports issues raised in other historical documents: the potential for crowded quarters to amplify influenza transmission and the inadequate provision of medical resources (both supplies and staff), which were also identified in the official inquiry. However, new issues are raised by the information noted in this diary: the likelihood for poor organization of the provision of food, interference in official accounts of conditions aboard the ship, and military rigidity in the wake of an overwhelming influenza outbreak. Release of this account enforces the idea that historical data sources should be routinely sought by researchers examining the complex issues related to the epidemiology and control of past pandemics.

None of the 40th Reinforcements soldiers experienced combat as part of WWI; the war ended on November 11, 1918. However, of the 1,117 members of the 40th Reinforcements on board, >90% were sickened by the pandemic influenza strain, and 77 died. This caused one of the highest mortality rates from any cause among New Zealand military units. The diarist refers to the outbreak as "quite a catastrophe," and it was an experience not likely to have been forgotten by those aboard this ill-fated voyage of HMNZT Tahiti.

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I thank the diarist's grandson, John Hansen, who kindly transcribed the diary and provided it to me.

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Extensively Drug-Resistant Tuberculosis, Central China, 2007–2009

To the Editor: Multidrug-resistant (MDR) tuberculosis (TB), defined as TB caused by *Mycobacterium tuberculosis* resistant to isoniazid and rifampin, is threatening global control of TB. The emergence of extensively drug-resistant (XDR) TB, defined as MDR TB resistant to at least 1 quinolone and 1 of 3 injectable second-line drugs (kanamycin, amikacin, or capreomyin), further jeopardizes TB control and prevention.

In the People's Republic of China, a country in which the economic cost of TB is high, incidence of MDR TB is higher (10%) (*1*) than the average global incidence (4.8%) (*2*). Published reports of XDR TB prevalence indicate that XDR TB is probably an underestimated problem in mainland China (*3*–*7*). China is a geographically large country, and the characteristics of drug resistance of TB might vary among provinces (*2*). Five regional surveys reported drug-resistance patterns of XDR TB in mainland China, and 3 were conducted in eastern China (*3*–*7*). To determine prevalence of XDT TB in central China, we characterized the resistance of MDR *M. tuberculosis* to second-line drugs, specifically identifying XDR strains, in Henan, a major province of central China. Henan Province has the country's third largest provincial population (94 million) and high rates of drug resistance to any agent (35.5%) (*2*).

The bacterial population retrospectively analyzed in this study has been described (*8*). In brief, from 2007 through 2009, clinical isolates were collected consecutively by the Henan Center for Disease Control and Prevention TB surveillance system and screened for resistance to 4 first-line drugs. Proportion method–based drug susceptibility testing was conducted for the following critical concentrations: isoniazid 0.2 μg/mL, rifampin 40.0 μg/mL, ethambutol 2.0 μg/mL, and streptomycin 4.0 μg/mL. As a result of that study, 150 MDR TB isolates from TB patients were obtained. The genotyping of all MDR isolates was identified by variable number tandem repeat of mycobacterial interspersed repetitive units based on 16 loci with high discriminatory power.

For the study reported here, we performed additional drug susceptibility testing of 4 second-line drugs at the Henan Center for Disease Control and Prevention TB reference laboratory. We used the Löwenstein-Jensen proportion method, recommended by the World Health Organization, according to the following critical drug concentrations: ofloxacin 3.0 μg/mL, kanamycin 30.0 μg/mL, amikacin 30.0 μg/mL, and capreomycin 40.0 μg/mL (*9*).

Susceptibility results for all second-line drugs tested were reported for 143 (95.3%) of 150 MDR *M. tuberculosis* isolates. Among these 143 isolates, 49 (34.3%) were resistant to ofloxacin, 23 (16.1%) to kanamycin, 17 (11.9%) to amikacin, and 25 (17.5%) to capreomycin (Table). All 17 amikacin-resistant isolates were also resistant to kanamycin, and 16 were also resistant to capreomycin. Also among the 143 MDR isolates, 18 (12.6%) showed resistance to ofloxacin and at least 1 second-line injectable drug and were defined as XDR strains. All 18 XDR *M. tuberculosis* isolates were resistant to isoniazid, rifampin, streptomycin, and ofloxacin; 14 (77.8%), 16 (89.9%), 12 (66.7%), and 17 (94.4%) were resistant to ethambutol, kanamycin, amikacin, and caperomycin respectively. Twelve amikacin-resistant XDR isolates were also resistant to kanamycin and capreomycin (Table).

Genotyping results demonstrated that XDR strains were distributed diversely in the phylogenetic tree, suggesting that these strains evolved independently. Our results indicated that 12.6% of MDR TB isolates from Henan Province meet the definition of XDR TB, which is less than that found by hospital-based studies performed in Shandong (18.7%), Shanghai (30.0%), and Beijing (14.9%) (*3*,*5*,*6*) but higher than that found by 2 other studies conducted in Beijing and Shanghai (6.3% each) (*4*,*7*). The discrepancy between the percentages of XDR TB and MDR TB strains found in these studies might be explained by the probable inclusion of patients who had been previously treated and patients with chronic TB.

Previous studies found high cross-resistance among all 3 second-line injectable drugs in MDR and XDR TB strains (*5*,*10*). Our results support these observations; capreomycin resistance of MDR and XDR strains (17.5%

Table. Second-line drug resistance patterns for 143 strains of multidrug-resistant tuberculosis, Henan Province, China, 2007–2009*

Drugs	No. (%) strains
INH + RIF	84 (58.7)
INH + RIF + KAN	1 (0.7)
INH + RIF+ CAP	3 (2.1)
INH + RIF + KAN + AMI	1 (0.7)
INH + RIF+ KAN + CAP	1 (0.7)
INH + RIF+ KAN + AMI + CAP	4 (2.8)
INH + RIF+ OFX	31 (21.7)
INH + RIF + OFX + KAN	1 (0.7)
INH + RIF+ OFX + CAP	2 (1.4)
INH + RIF+ OFX + KAN + CAP	3 (2.1)
INH + RIF+ OFX + KAN + AMI + CAP	12 (8.4)
Total	143 (100%)

*INH, isoniazid; RIF, rifampin; KAN, kanamycin; CAP, capreomycin; AMI, amikacin; OFX, ofloxacin.

and 94.4%, respectively) in Henan Province were higher than the average levels (10.2%, 62.5%, respectively) reported by a worldwide study (*10*). Pyrazinamide is an essential drug recommended by World Health Organization guidelines for treatment of MDR TB. Among the population with MDR TB that we studied, 10 (76.9%) of 13 XDR isolates were sensitive to pyrazinamide (data not shown), suggesting that pyrazinamide is still an effective first-line anti-TB drug for most XDR TB patients in Henan Province.

We restricted our investigation to 1 province. However, given the average national prevalence of XDR TB (8% of MDR TB) (*1*) and the magnitude of the population of Henan Province, our findings indicate that the prevalence of XDR TB might be higher in central China than previously documented.

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Seroprevalence of Pandemic Influenza Viruses, New York, New York, USA, 2004

To the Editor: Exposures to influenza viruses can lead to immune responses that substantially affect susceptibility to infection with related viruses. Characterization of preexisting immunity within a population can inform public health, as highlighted during the influenza A(H1N1)pdm09 virus pandemic, when surveillance data demonstrated that older persons (≥65 years old) were less likely than younger persons to have influenza (*1*). Seroprevalence studies of prepandemic samples show that older persons had preexisting antibody responses to A(H1N1) pdm09 virus, presumably because of prior exposure to related strains (*2*). The A(H1N1)pdm09 virus possesses hemagglutinin and neuraminidase genes derived from classical swine influenza virus (*3*).

Epidemiologic and molecular data indicate that prior exposure to early twentieth century H1N1 viruses conferred immunity to A(H1N1) pdm09 virus. Human antibodies that neutralize A(H1N1)pdm09 virus and H1N1 subtype viruses from earlier in the twentieth century have been characterized, and animal studies have demonstrated that antibodies to the earlier H1N1 subtype viruses cross-neutralize A(H1N1)pdm09 virus and protect from virus challenge (*2*,*4*–*6*). Prior exposure to antigenically related viruses can explain the relationship between age and susceptibility to infection.

To determine the seroprevalence of preexisting hemagglutinin inhibition (HAI) antibody titers to influenza strains with pandemic potential, we tested serum samples for antibodies to A(H1N1)pdm09 virus and the 1918, 1957, and 1968 pandemic viruses.

The samples had been collected in 2004 from a representative sample of adults in New York City (NYC), USA, as part of the NYC Health and Nutrition Examination Survey (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0156-Techapp.pdf). For the 1918 and A(H1N1)pdm09 viruses, the highest prevalence of HAI titers >40 was among persons born before 1940 (>65 years old in 2004), although younger adults also had antibodies. Antibody prevalence to the 1957 H2N2 subtype virus was highest among persons born during 1942–1961, and >70% in persons born before 1971 had antibody to the 1968 H3N2 subtype virus (Figure). For all pandemic viruses, there was no significant difference in seroprevalence by sex or by US birth and only minor differences by race/ethnicity (online Technical Appendix Table 1).

We examined A(H1N1)pdm09 virus seroprevalence by the age of persons tested and by antibody titer. The mean age for persons with no serologic evidence of prior exposure (titer <20) was 50 years, compared with 72 years for those with titers of 20–40 and 80 years for those with titers >40 (online Technical Appendix Table 2). In a multivariate logistic regression model, presence of antibody to the 1918 H1N1 subtype virus was strongly associated with antibody to A(H1N1)pdm09 virus (online Technical Appendix Table 3). No demographic factor was independently associated with positivity to A(H1N1)pdm09 virus. By using a nonlinear regression model for the probability of A(H1N1)pdm09 antibody prevalence compared with birth year, we found the model that best fit the age-stratified seroprevalence data inflected near 1927 (online Technical Appendix Figure), indicating that persons born before 1927 were most reliably protected.

Our findings show that the prevalence of pandemic influenza virus antibody in a representative population-based 2004 sample of NYC residents correlated with birth year and year(s) of circulating virus. These data reveal the immunologic background during the emergence of A(H1N1)pdm09 virus in NYC beginning in late April 2009 (*7*) and help explain why fewer cases of A(H1N1)pdm09 infection were detected among older persons than younger persons, supporting the conclusion that the difference was a result of, at least in part, antibodies elicited by prior H1N1 subtype infection in older persons.

Viruses antigenically resembling the 1918 pandemic strain circulated among humans earlier in the twentieth century; cross-reactivity with antibodies to those viruses likely provided protection against the 1918 virus. Most (*2*,*4*), but not all (*8*), previous A(H1N1)pdm09 virus seroprevalence studies demonstrated an increase in immunity with age. In our study, more persons born before than after 1927 (i.e., persons >82 vs. those 65–82 years of age in 2009) had HAI assay results positive for A(H1N1)pdm09 virus. Protection among persons 65–82 years old during the 2009 pandemic may be explained by the presence of preexisting immunity not measured by standard HAI tests (e.g., antibodies that target the hemagglutinin stalk) or by T-cell responses (*9*). More positive test results were recorded with the 1918 than the A(H1N1)pdm09 virus; this finding is consistent with the model in which preexisting immunity to A(H1N1)pdm09 virus was derived from exposure to the 1918 pandemic strain or to antigenically related strains that evolved since then (*10*). The 1918 and 2009 strains used in testing may have exhibited different sensitivities in HAI assays. Immunity in older populations is not surprising and was seen in the 1918, 1957, and 1968 pandemics, during which newly introduced pandemic viruses were more likely to cause clinical illness in younger persons, presumably because prior exposure to similar viruses resulted in cross-reactive antibodies (*11*).

Study limitations include a relatively small sample size and a lack of history regarding influenza virus infection or vaccination. Nevertheless, the ability to evaluate seroreactivity in a representative sample of adults helps validate and reinforce previously published findings on H1N1 subtype viruses and clarifies levels of immunity to H2N2 and H3N2 subtype viruses.

Figure. Seroprevalence of cross-reactive antibodies to the 1918, 1957, 1968, and 2009 pandemic influenza viruses among persons >23 years of age, New York, New York, 2004. LOESS (locally weighted scatterplot smoothing) curves represent the estimated prevalence of hemagglutination-inhibition antibody titers of ≥40 (positive titers) by year of birth.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Pulmonary *Streptomyces* Infection in Patient with Sarcoidosis, France, 2012

To the Editor: *Streptomyces* spp. are aerobic, gram-positive bacteria of the order Actinomycetales, known for their ability to produce antimicrobial molecules such as streptomycin. *Streptomyces* spp., usually saprophytic to humans, can cause local cutaneous fistulized nodules known as actinomycetoma or mycetoma. Severe invasive infections have seldom been reported, but most cases reported have occurred in immunocompromised patients (*1–5*). We report a case of invasive pulmonary infection caused by a *Streptomyces* sp. in a splenectomized patient with sarcoidosis.

In 2003, multiorgan sarcoidosis was diagnosed in a man, 57 years of age; the disease involved lungs, skin, joints, and lymph nodes. Corticosteroids were initially given but quickly discontinued because of a severe psychiatric reaction. In 2007, a splenectomy was performed on this patient to remove an intestinal obstruction caused by a severely enlarged spleen, identified as a specific localization of sarcoidosis.

In April 2008, the patient was admitted to the internal medicine unit of Saint-André Hospital in Bordeaux, France with fever (38.9°C/102°F), progressive asthenia, anorexia, weight loss, productive cough, and New York Heart Association grade III dyspnea. Bilateral basal crackles could be heard in the lungs; physical examination findings were otherwise within normal limits. Biological tests showed inflammatory syndrome with elevated C-reactive protein (74 mg/L, reference value <5 mg/L) without any other consequential abnormality. Gamma globulin levels were normal. A chest radiograph showed bilateral interstitial infiltrate. A computed tomogra-

phy scan of the chest confirmed an interstitial micronodular infiltrate with thickening of the peribronchovascular interstitium, associated with paratracheal and left anterior mediastinal supracentimetric lymph nodes.

To determine whether this infiltrate was linked to sarcoidosis, tuberculosis, or another opportunistic infection, bronchoscopy and bronchoalveolar lavage (BAL) were performed and showed multiple submucous nodules of the left superior bronchus. Biopsy samples contained epithelioid granulomas and a nonspecific, amorphous eosinophilic material without focal necrosis, but no bacteria, by using periodic acid–Schiff, Ziehl–Neelsen, and auramine-rhodamine stains. BAL culture isolated a *Streptomyces* sp. (2×10^5 CFU/mL) but no other pathogens.

Treatment with intravenous imipenem (2 g/day for 14 days) and amikacin (1 g/day for 3 days) was initiated. After antimicrobial susceptibility tests, the treatment was changed to oral rifampin (1.2 g/day) and ciprofloxacin (1.5 g/day) for 6 months. After 3 days of treatment, clinical signs and symptoms resolved; a thoracic computed tomography scan performed 6 months later showed complete regression of pulmonary infiltrates. Bronchoscopy at that time showed no nodules, and BAL culture showed no pathogens.

Streptomyces spp. are widespread environmental bacteria that rarely cause severe invasive infections. During our literature search, we found 21 cases of invasive *Streptomyces* infections, including 8 pulmonary infections. A contributing factor was found for all cases: immunosuppression linked to HIV infection (*1*), antineoplastic chemotherapy (*2*), Crohn disease (*3*), use of oral (*4*) or inhaled corticosteroids (*5*), and presence of foreign material such as a central venous catheter (*6*) or a prosthetic aortic valve (*7*).

Specific features of pulmonary *Streptomyces* infection are summarized in the online Technical Appendix Table (wwwnc.cdc.gov/EID/pdfs/12-0797-Techapp.pdf). Death related to such an infection is mostly dependent on the underlying disease associated with *Streptomyces* infection. Deaths have not been linked to *Streptomyces* infections described in the literature when *Streptomyces* sensibility testing was performed and treatment length recommendations were followed.

To understand how the patient was infected with a *Streptomyces* sp., we explored 2 possibilities. First, sarcoidosis induces immune deficiency (*8*). This phenomenon is clinically well known as anergy to tuberculin or other immunogenic haptens after subcutaneous injections. Expansion of regulatory T lymphocytes (*8*) and attenuated myeloid dendritic cell functions (*9*) decrease cellular immunity efficiency and increase infectious episodes in affected patients. Second, splenectomy can increase susceptibility to infection, such as bloodstream infections with encapsulated bacteria or opportunistic infections with *Campylobacter jejuni*, *Pneumocystis jiroveci*, or *Babesia* spp. The lung infection with *Streptomyces* in the patient described was not acquired through the bloodstream, but through direct airway contact. However, we could not exclude other immune mechanisms not related to blood, such as dysregulation or lack of some lymphocyte populations.

A pathologic feature of pulmonary infections with *Streptomyces* spp. is the presence of granulomas sometimes associated with focal necrosis. This feature makes differentiating infection with these species from that of tuberculosis difficult. Bacterial culture is often used to confirm the diagnosis. Histologic differences between the 2 entities are not well defined because of the rarity of invasive *Streptomyces* infections. In our observation of this patient, histologic examination revealed granulomas potentially linked to sarcoidosis and a nonspecific, amorphous eosinophilic material that was not caseous necrosis. Both lesions could have also resulted from the *Streptomyces* infection. For further identification, Dunne et al. added the presence of sulfur granules to the specific histological description of *Streptomyces* infection (*1*).

An overall literature review for results of in vitro testing for *Streptomyces* spp. identified a common susceptibility to aminoglycosides, macrolides, imipenem, or trimethoprim/sulfamethoxazole. This finding suggests that the first-line treatment against invasive *Streptomyces* infections should begin with imipenem and aminoglycosides for at least 6 weeks (online Technical Appendix Table). Quinolones have an immunomodulatory effect that might be therapeutic in patients with disease-induced immunosuppression such as sarcoidosis or after splenectomy (*10*). In conclusion, invasive *Streptomyces* infection of the lungs should be included in differential diagnoses of interstitial pneumonia in immunocompromised patients.

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Pneumonia after Earthquake, Japan, 2011

To the Editor: The earthquake that occurred in Japan on March 11, 2011, triggered an extremely destructive tsunami (*1*), which destroyed cities along the Pacific coastline in the Tohoku area and resulted in the loss of >19,000 human lives. Water from the tsunami inundated ≈33.7% of Tagajo City (population ≈61,000) and caused 188 deaths. Many local residents were left without lifeline utilities, including electricity, gas, water, or any means of transportation and thus were forced to live in crowded shelters or limited small spaces (e.g., the upper floor of their home); ≈11,000 persons were displaced from their damaged or destroyed homes to crowded school gymnasiums or community centers. In March, the mean daily maximum air temperature in Tagajo City was cold (8°C/46.4°F). After the earthquake, cases of pneumonia increased rapidly.

Saka General Hospital is located in this region near the coast. The destruction around the hospital was so severe that persons were without electricity, water, gas, and fuel for several weeks. Fortunately, the hospital laboratory was almost completely functional and could perform bacterial and other tests at a near-normal level, despite the earthquake. However, several other hospitals in the area were severely damaged and thus had difficulty treating patients with severe pneumonia.

To determine the characteristics of pneumonia after the earthquake, we conducted a retrospective study of patients who had pneumonia during the 6 weeks before the earthquake and the first 9 weeks after the earthquake. To identify patients with pneumonia, we checked all chest radiographs and computed tomography scans of adult patients (>16 years of age) who had visited the hospital. We examined clinical and bacteriologic data for these patients. We excluded from the study patients without sputum culture and patients with other conditions, such as lung cancer, pulmonary infarction, or cardiac failure.

During the 6 weeks before the earthquake, pneumonia had been diagnosed for 49 adults (controls), and within the 9 weeks after the earthquake, community-acquired or health care–associated pneumonia was newly diagnosed for 172 adults. Patient data from 2 pre-earthquake periods and 3 postearthquake periods are shown in the Table. Although the number of patients with pneumonia in the first 3 weeks after the earthquake increased sharply, no substantial differences were noted in mean age, death rates, or underlying concurrent conditions among these patients. The interval between the onset of respiratory signs and symptoms and a diagnosis of pneumonia did not increase after the earthquake. The proportion of patients who received antimicrobial drugs before the diagnosis of pneumonia (premedication) in the early postearthquake period did not differ significantly. The number of patients with pneumonia peaked in the first 3 weeks after the earthquake, followed by a gradual decrease starting from 4 weeks after the earthquake.

Chest radiographs were taken and hematologic examinations were performed for all patients; computed tomography of the chest and rapid diagnostic tests for influenza were performed for 42.2% and 54.2% of 83 patients, respectively, who had pneumonia in the early postearthquake period. During the first 3 weeks after the earthquake, *Haemophilus influenzae* and *Moraxella catarrhalis* were more predominant than *Streptococcus pneumoniae*; most strains were isolated from purulent sputum specimens. In contrast, pneumonia caused by enterobacteria, staphylococci, or atypical pathogens did not increase after earthquake.

Table. Characteristics of patients with pneumonia before and after the earthquake and tsunami, Japan, 2011*

Characteristic	Weeks before disaster		Weeks after disaster		
	4–6	1–3	1–3	4–6	7–9
Patients with pneumonia, no.	26	23	83	51	38
CAP	20	19	57	39	24
HCAP	6	4	26	12	14
Isolates from sputum culture, no. (%)					
Streptococcus pneumoniae	8 (30.8)	2 (8.7)	19 (22.9)	10 (19.6)	4 (10.5)
Haemophilus influenzae	4 (15.4)	4 (17.4)	27 (32.5)	8 (15.7)	4 (10.5)
Moraxella catarrhalis	1 (3.8)	0	26 (31.3)	9 (17.7)	3 (7.9)
Purulent sputum, (Geckler 4 or 5), %	14 (53.8)	10 (43.5)	51 (61.4)	24 (47.1)	16 (42.1)
Mean age, y	73.7	76.0	75.5	76.0	74.9
Location of patient at illness onset, no.					
Shelter	NA	NA	36	13	6
Own or friend's home	NA	NA	37	29	23
Nursing home / home visit by doctor	NA	NA	10	9	9
Patient's hospital status, no.					
New patient	7	3	32	19	20
Routinely examined at hospital	19	20	51	32	18
Rate of hospital admission, %	76.9	73.9	77.1	64.7	78.9
Deaths, no. (%)	3 (11.5)	1 (4.3)	6 (7.2)	2 (3.9)	3 (7.9)
Underlying disease, %					
Respiratory disease	8 (30.8)	12 (52.2)	29 (34.9)	19 (37.3)	13 (34.2)
Other	18 (69.2)	11 (47.8)	54 (65.0)	31 (60.8)	23 (60.5)
Healthy	1 (3.8)	0	8 (9.6)	5 (9.8)	2 (5.3)
Interval from onset to diagnosis, mean no. days	3.96	2.43	2.51	3.22	2.89
Antimicrobial premedication, no., %	2 (7.7)	0	4 (4.8)	11 (21.6)	3 (7.9)

*CAP, community-acquired pneumonia; HCAP; health care–acquired pneumonia; NA, not applicable.

Detection rates of *H. influenzae* remained constant at 15.4% (4/26 patients); before the earthquake the rate was 17.4% (4/23), and soon after the earthquake it increased to 32.5% (27/83). Detection rates of *M. catarrhalis* increased from 0–3.8% before the earthquake to 31.3% (26/83) after the earthquake ($p<0.01$). These bacterial strains were isolated widely from refugees at shelters and from persons living at home without running water and/or electricity. Soon after the earthquake, it was thought that infections with these strains were not part of a localized outbreak but were widespread in the region. Most patients from whom *M. catarrhalis* was isolated were located throughout the area flooded by the tsunami. In contrast, many patients with *H. influenzae* were mainly located outside the flooded area. There was no regional imbalance in isolation of *S. pneumoniae*.

It was reported that living in a multiple-bedroom residence and the winter season were risk factors for outbreaks of *M. catarrhalis* (*2–4*). Similar outbreaks of *H. influenza* infections were reported (*4*,*5*). Cold shock at a physiologically relevant temperature of 26°C promotes *M. catarrhalis* adherence to upper respiratory tract cells and can contribute to virulence (*6*).

The possibility of a pseudo-epidemic must also be considered. The substantial increase in the number of new patients at Saka General Hospital, as a result of the severe damage to other hospitals in this area and the changed patient profiles (community-acquired pneumonia, hypothermia, trauma), might have largely affected the etiology of pneumonia. We found no increase in cases of severe pneumonia caused by resistant bacteria or aspiration pneumonia in elderly patients. We conclude that multiple localized small community outbreaks might have occurred widely in this area after earthquake.

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Severe Pneumonia Caused by *Legionella pneumophila* Serogroup 11, Italy

To the Editor: *Legionella pneumophila* serogroups (SGs) 1–16 cause pneumonia in humans. Although SG 1 is the serogroup most commonly associated with disease (*1*), we report a case of community-acquired legionellosis caused by SG 11.

In November 2010, a 42-year-old man was admitted to Modena University Hospital, Modena, Italy, with a 4-day history of fever, dyspnea, and cough. His vital signs were as follows: temperature 40.0°C, pulse 135 beats/min, blood pressure 110/60 mm Hg, respiratory rate 30 breaths/min, and oxygen saturation 85% in room air. Inspiratory crackles were heard in the left lower lung lobe. Chest radiographs and successive high-resolution computerized tomography revealed left lobar infiltrates (Figure, panels A and B). Blood count documented severe pancytopenia together with high levels of inflammation markers: fibrinogen (1,031 mg/dL), C-reactive protein (33 mg/dL), and procalcitonin (28.5 ng/mL). The patient's medical history was unremarkable; however, results of tests conducted at the time of hospital admission led to the diagnosis of acute leukemia.

Empirically prescribed antimicrobial treatment for neutropenic patients was initiated and consisted of meropenem (3 g/day) and levofloxacin (500 mg/day), combined first with vancomycin (2 g/day) and later with linezolid (1,200 mg/day). A few days later, antifungal therapy was empirically added to the treatment regimen (liposomal amphotericin B at 3 mg/kg/day). The patient received continuous positive airway pressure, which resulted in progressive improvement of blood gas exchange, until normalization was achieved.

Serologic and molecular examination and culture of bronchoalveolar lavage fluid, blood, urine, and feces produced negative results for fungal, viral, and bacterial pathogens. Test results for *L. pneumophila* urinary antigen (Biotest AG, Dreieich, Germany) and IgM and IgG against *L. pneumophila* (Serion-Immundiagnostica GmbH, Würzburg, Germany) were negative. Culture of sputum collected at the time of

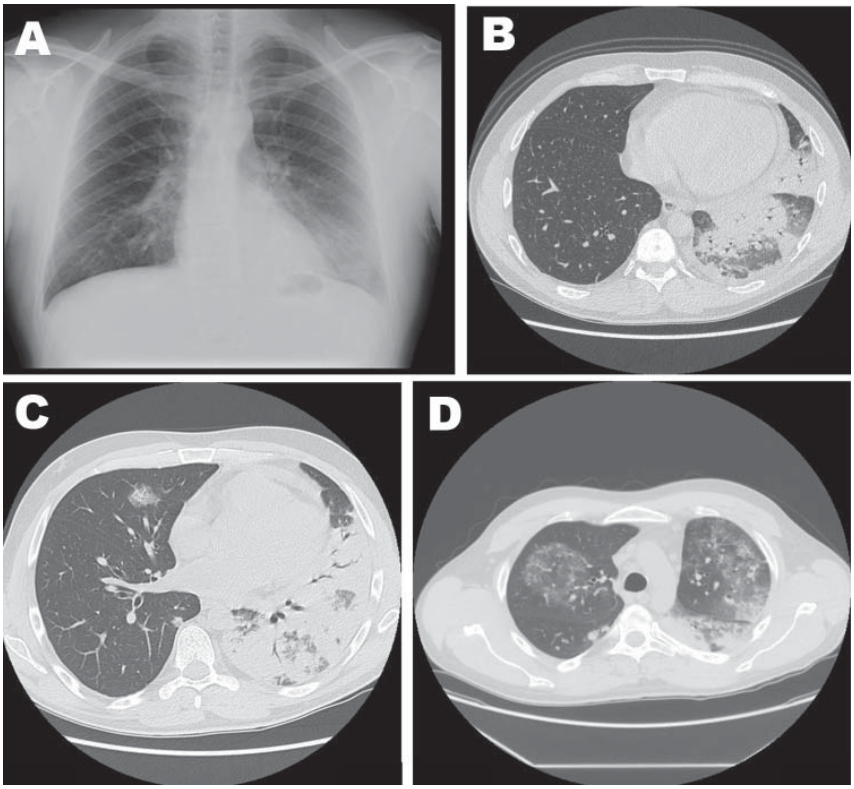

Figure. Imaging studies of 42-year-old man with severe pneumonia caused by *Legionella pneumophila* serogroup 11, showing lobar consolidation of the left lower lung lobe, with an air-bronchogram within the homogeneous airspace consolidation. Consensual mild pleural effusion was documented by a chest radiograph (A) and high-resolution computed tomography (B). A week after hospital admission, repeat high-resolution computerized tomography of the chest showed extensive and homogeneous consolidation of left upper and lower lobes, accompanied by bilateral ground-glass opacities (C and D).

hospital admission showed growth of legionella-like colonies on buffered charcoal yeast extract, with and without the addition of antimicrobial drugs (Oxoid, Basingstoke, UK). The colonies were identified as *L. pneumophila* SGs 2–14 by the *Legionella* latex test (Oxoid). The strain was further characterized as *L. pneumophila* SG 11, according to a polyclonal latex reagent set (Biolife, Milan, Italy). Environmental investigations were conducted in the patient's house and workplace, but *L. pneumophila* SG 11 was not detected in any of the locations tested.

A week after hospital admission, the patient was persistently febrile and experienced pain in the left thorax. High-resolution computerized tomography of the chest was repeated and showed increased pulmonary infiltrate (Figure, panels C and D) that was consistent with *L. pneumophila* pneumonia (*2*). Highly potent antimicrobial therapy against *L. pneumophila* was administered, consisting of high-dosage levofloxacin (1 g/day) combined with azithromycin (500 mg/day), while the other antimicrobial agents were progressively reduced (*3*). The fever subsided 14 days after the onset of targeted antimicrobial drug treatment; at that time, the sputum culture and test results for urinary *L. pneumophila* antigen were negative, but serologic assay results were positive for IgG and negative for IgM against *L. pneumophila.* Subsequent computerized tomographic scans of the chest documented progressive improvement of lung infiltrates, and nearly complete resolution was obtained 3 months after hospital admission.

L. pneumophila SG 11 infection has, thus far, been reported only rarely in humans. The first SG 11 strain was isolated in the United States in 1982 from a patient with multiple myeloma (*4*). Since then, few other cases of SG 11 strains have been reported in Europe (*5*,*6*); it is conceivable that this strain is not as widely distributed and is less pathogenic than other SGs, especially SG 1. It can be argued that infections caused by SG 11 have been underdiagnosed. *L. pneumophila* SG 11 cannot be detected by *Legionella* urinary antigen or serologic tests, the assays most frequently used to diagnose legionellosis (*7*–*9*). The negative urinary antigen test result for this patient is consistent with a non–SG 1 infection. The single positive serologic result for IgG was probably caused by cross-reactivity because the commercial assay kit was designed to recognize only *L. pneumophila* SGs 1–7. Culture is the only useful diagnostic tool for identifying SGs. However, this tool is not always feasible because it requires specialized media and skills to identify the organism. In addition, sensitivity is low, depending on the severity of the disease and the availability of adequate respiratory specimens (*9*). Despite these limitations, culture is needed to detect all SGs of *L. pneumophila*, especially in immunocompromised patients, who are more susceptible to infections caused by strains of non–SG 1 *L. pneumophila* (*10*).

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Novel Human Enterovirus C Infection in Child with Community-acquired Pneumonia

To the Editor: Human enteroviruses (HEVs) are small, nonenveloped viruses with a positive-stranded RNA genome that includes regions P1, P2, and P3 (*1*). Most experts believe that HEV strains with >75% nt and >85% aa identity in complete or partial viral protein 1 (VP1) sequences should be considered the same type (*2*). However, more stringent criteria of 75% nt and 88% aa identity have been suggested for routine typing (*3*). We report the isolation and characterization of a novel HEV type within the species HEV-C (designated EV-C117 by the *Picornaviridae* Study Group, www.picornastudygroup.com/).

A 45-month-old boy was admitted to a hospital in Vilnius, Lithuania, in December 2010 after 1 day of high fever (temperature 40°C) with cough and a moderately increased respiratory rate (36 breaths/min). Decreased breath sounds were heard at the base of the left lung during auscultation, and a chest radiograph showed alveolar pneumonia with partial consolidation of the lower lobe of the left lung. The patient had a leukocyte count of 23,900 cells/mm^3 and C-reactive protein level of 9.6 mg/dL. Blood culture results were negative for bacteria and fungi. The patient was treated with cefuroxime (500 mg every 8 h) for 7 days. Oxygen administration was not required because the saturation level of peripheral oxygen was always >97%. The patient was discharged in good clinical condition after 7 days and did not experience clinical problems in the following 4 weeks.

For research purposes, a nasopharyngeal sample was collected from the boy at hospital admission by using a flexible pernasal flocked swab; written informed consent was obtained from the parents. The swab was immediately placed in a minitube containing 1 mL of universal transport medium (UTM-RT Kit; Copan Italia, Brescia, Italy). The sample was stored at 4°C in the hospital laboratory before being sent to the central laboratory at the University of Milan, Italy in a refrigerated package. We extracted viral nucleic acids from the swab sample by using an automated extraction system (NucliSens easyMAG; Biomeriéux, Craponne, France), and we tested the extract for respiratory viruses by using the Respiratory Virus Panel (Fast assay (Luminex Molecular Diagnostics Inc., Toronto, Ontario, Canada) in accordance with the manufacturer's instructions (*4*).

The assay result was positive for bocavirus and enterovirus/rhinovirus, so we retested the sample to identify the rhinovirus. We performed real-time reverse transcription PCR by using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) and primers and probe sequences reported by Lu et al. (*5*). Phylogenetic analysis of the VP4/VP2 region showed that some nucleotide sequences belonged to enterovirus species. We obtained a partial VP1 sequence by using the primers described by Nix et al. (*6*), and we obtained the remaining sequence of the VP1 capsid region by using in-house amplification and sequencing protocols (available upon request). The complete P1 sequence was submitted to the *Picornaviridae* Study Group, compared with other enterovirus sequences, and designated as a proposed new type of enterovirus, EV-C117 (GenBank accession no. JQ446368).

To obtain additional viral sequences, we analyzed and sequenced the complete P1 capsid region. This region was compared with the matching region of all of the complete enterovirus genomes available in the GenBank database (as of January 20, 2012). On the basis of the nucleotide sequences, we reconstructed a phylogenetic tree by using maximum likelihood methods with the Tamura 3-parameter model as the evolutionary model; rates among sites were heterogeneous, and gamma distribution was used for the relative rate (*7*). The closest genotypes were EV-C104 (GenBank accession no. EU840733) and EV-C109 (GenBank accession no. NC014336) (Figure). The VP4 genomic region had the greatest identity with other HEV strains; the VP1 coding region had the lowest identity.

We report the identification of a novel enterovirus (designated EV-C117) in a child hospitalized with community-acquired pneumonia in Vilnius, Lithuania. EV-C117 was detected in the child in association with bocavirus. Although it is not possible to say whether this new enterovirus was the etiologic cause of the disease, a close relationship has been found (mainly in children) between the development of severe lower respiratory tract infections requiring hospitalization and infections caused by EV-68 (*8*) and EV-C104 and EV-C109 (*9*), which are molecularly similar to EV-C117.

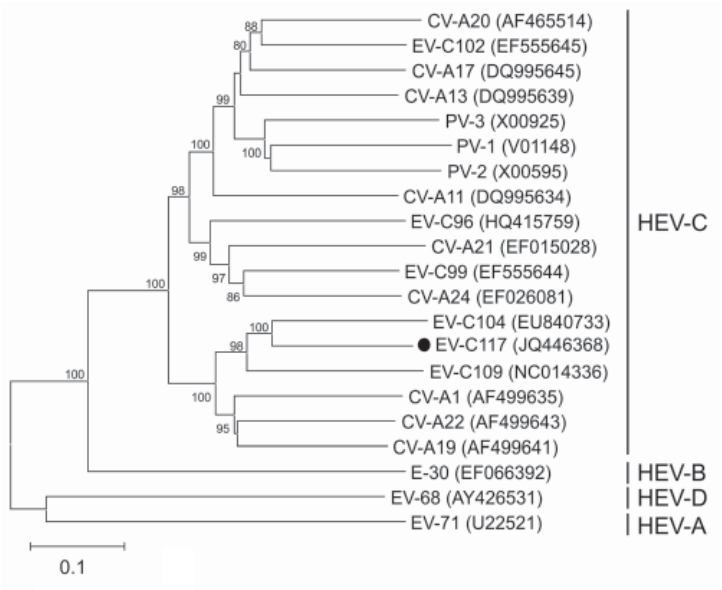

Figure. Phylogenetic relationships of human enterovirus C (HEV-C) and the new strain EV-C117 (dot), as determined on the basis of the complete capsid protein coding region sequences. The phylogeny of the nucleotide sequences was reconstructed by using maximum likelihood methods with the Tamura 3-parameter model as the evolutionary model rates among sites were heterogeneous, and gamma distribution was used for the relative rate (*7*). Branch support was assessed by means of bootstrap analyses of 1,000 replicates; a bootstrap value of 70% was used as the cutoff point for cluster analysis. Enterovirus strains EV-68 and EV-70 were used as the outgroup. Scale bar indicates nucleotide substitutions per site.

In addition, bocavirus is a frequently reported co-pathogen in children with community-acquired pneumonia. (*10*). No bacteria or fungi were observed in the blood culture. It is therefore reasonable to think that this new virus may have played a major role in the development of community-acquired pneumonia.

Our findings serve as a reminder that all HEV infections should be closely monitored; knowing the molecular characteristics of virus strains involved in lower respiratory tract infections will help determine appropriate prophylactic and therapeutic measures. However, further studies are needed to determine the tissue tropism and possible pathogenesis of EV-C117 in vivo, and epidemiologic studies are needed to clarify the circulation of this virus strain in countries other than Lithuania.

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Genome Sequencing of Pathogenic *Rhodococcus* spp.

To the Editor: Infections caused by non-equi *Rhodococcus* spp. are uncommon but can cause severe pneumonia and bloodstream infections with sepsis (*1*–*4*). Increasing prevalence of immune-compromising illnesses and use of immunosuppressive agents might contribute to reemergence of these pathogens (*5*,*6*). Rhodococci infections may go undiagnosed or misclassified because of difficulties in laboratory identification, nomenclatural instability, and similarity of signs and symptoms to *Mycobacterium tuberculosis* infection (e.g., increasing cough, dyspnea, hemoptysis, and weight loss; failure to respond to broad-spectrum antimicrobial drugs; progressive cavitary lesion on repeated chest imaging).

A 73-year-old immunocompetent man with chronic obstructive pulmonary disease who lived on a ranch in the southwestern United States was evaluated for possible malignancy of the left lung. Computed tomography showed expansion of an upper lobe cavitary lesion, and he underwent a partial lobectomy. *Rhodococcus* spp. infection was suspected on the basis of the finding of the progressive lesion, salmon-pink colony growth on chocolate agar, and gram-positive coccobacilli on Gram stain. The isolate, R1101, could not be further identified by using commercial automated systems in the laboratory and so was subjected to genome study.

Alignment of 16S rRNA gene sequences showed that R1101 was most closely related to *R. rhodochrous*, with identities of 98.78%, 99.32%, and 99.86% to the 16S rRNA gene for type strains *R. rhodochrous* DSM43271, *R. pyridinivorans* PDB9, and *R. gordonae* W4937 (*7*), respectively. In contrast, sequence identity with *R. equi* type strain DSM20307 was 95.57%. Phylogenetic analysis with *Rhodococcus* 16S rRNA genes suggested that R1101 was a member of *R. rhodochrous* and evolutionarily separate from *R. equi* (Figure, panel A). A query against the National Center for Biotechnology Information nonredundant DNA database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) found 60 rhodococcal 16S rRNA sequences that are 99%–100% identical to R1101, which demonstrates worldwide distribution of these closely related strains in diverse environments.

Optical genome mapping (*8*) of R1101 generated a consensus full-length map showing a circular chromosome map of »4.32 Mb (± 5%) in length. The whole-genome restriction map for R1101 was compared with in silico *Nco*I restriction maps of 33 complete genome sequences from the family *Nocardiaceae*, retrieved from GenBank; these sequences included *R. equi*. No substantial similarity (alignment scores >15%) was detected between R1101 and these genomes. Thus, we determined that R1101 was not an *R. equi* isolate.

Pyrosequencing of the R1101 genome yielded an average coverage depth of 14-fold. The total length of 1,020 assembled de novo contigs was 4.65 Mb, with a G + C content of 68%. A total of 3,969 putative protein-encoding genes were identified. Whole-genome phylogeny was used to show evolutionary distances between R1101 and the sequenced *Rhodococcus* species (Figure, panel B). We sampled ≈50.4 kb of sequences, encoding 67 putative proteins shared among all selected rhodococci, to generate the

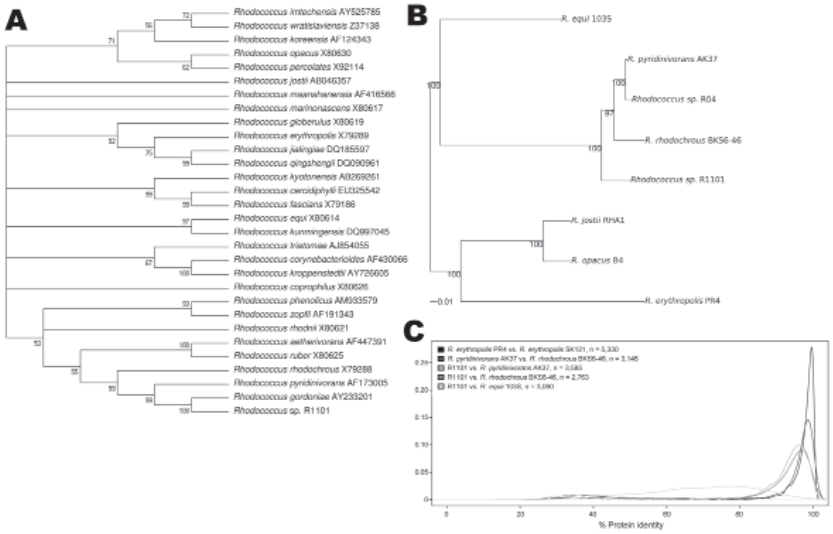

Figure. Phylogenetic and proteomic comparison of isolate R1101 and *Rhodococcus* spp. strains. A) 16S rRNA–based phylogenetic analysis. Complete or partial 16S rRNA gene sequences for 29 *Rhodococcus* spp. type strains were aligned with complete sequence of 16S rRNA gene for isolate R1101. GenBank accession numbers are provided. B) Genome-wide phylogenetic analysis. A sample of 67 protein-coding genes shared among genomes of isolate R1101 and 6 *Rhodococcus* spp. strains were analyzed. Scale bar indicates 0.01 nt substitutions per site. An expanded version of this figure including panel C is available online (wwwnc.cdc.gov/EID/article/18/11/12-0818-F1.htm).

phylogenetic tree. Results confirmed that R1101 is a member of *R. rhodochrous* and is phylogenetically distant from *R. equi*.

We used a BLASTP search to determine the similarity of predicted R1101 proteins to homologous proteins from *R. equi* 103S, *R. erythropolis* (PR4 and SK121), and *R. pyridinivorans* (AK37 and BKS6-46) (Figure, panel C, Appendix, wwwnc.cdc.gov/EID/article/18/11/12-0818-F1.htm). These data indicate that R1101 is highly similar to but distinct from *R. pyridinivorans* and *R. rhodochrous* at the protein sequence level and is more distantly related to *R. equi*.

Each R1101 protein ≥1 database blast hit with an E-value $\leq 1 \times 10^{-10}$ was assigned a best match to another species on the basis of its bit score. Consistent with the 16S rRNA–based phylogenetic analysis, >92% of the R1101 proteins showed greatest similarity to a protein from *R. pyridinivorans*, *R. rhodochrous*, or *Rhodococcus* sp. R04. However, 120 proteins (3%) have highest homology to their counterparts in the pathogen *R. equi*; 10 of these are unique to R1101 and *R. equi*. Furthermore, genes with the greatest similarity to *R. equi* are not randomly distributed throughout the R1101 genome.

We calculated the probability of observing groups of adjacent R1101 genes that are most similar to *R. equi* and observed 1 cluster of 5 adjacent genes ($p = 2.33 \times 10^{-8}$), 2 groups of 6 adjacent genes ($p = 6.75 \times 10^{-10}$), and 2 blocks of 7 contiguous genes ($p = 1.94 \times 10^{-11}$). Nucleotide BLAST analysis showed that R1101 sequence contigs 604, 456, 139, and 610 align to nt 4454241–4469589 of the *R. equi* 103S chromosome, with >97% identity at the DNA level. The 12 proteins wholly or partially encoded within this 15.3-kb region show >99% identity at the amino acid level with their R1101 orthologs. Among them, contig 139 encodes a cluster of 7 consecutive proteins with high homology to *R. equi*; this portion of the R1101 genome is likely to have been acquired from *R. equi* through horizontal gene transfer. R1101 may have acquired clusters of virulence-related genes, such as those crucial iron-uptake proteins (*9*), from the phylogenetically distant species *R. equi*.

In conclusion, infections caused by non-equi *Rhodococcus* spp. are rare, especially in immunocompetent patients, but may represent an emerging threat. Specialized diagnostics such as genome sequencing may be needed to accurately identify these pathogens.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Atypical Pestivirus and Severe Respiratory Disease in Calves, Europe

To the Editor: The article by Decaro et al. (*1*) described an outbreak of severe respiratory disease in calves in Italy caused by an atypical bovine pestivirus. This report confirms our concern that this group of viruses is probably widespread (*2*) and present on >3 continents. Moreover, it demonstrates that atypical bovine pestiviruses are capable of causing disease in calves in the field and a clinical picture consistent with most bovine viral diarrhea virus (BVDV) infections that occur naturally or experimentally (*3*,*4*).

Some key issues remain unknown. The origin of the bovine pestivirus and the route of introduction into the herd are unclear, although phylogenies demonstrated a close relationship between this virus strain from Italy and atypical bovine pestiviruses from Brazil. Batches of fetal bovine serum from Brazil have repeatedly been found to be contaminated with atypical bovine pestiviruses (*2*,*3*), and there is a risk for contamination of vaccines with these viruses. Animal trade and vaccines should be considered when conducting further investigations into this outbreak.

The evolutionary relationship between the atypical and the recognized pestivirus species (*1*) needs to be clarified. Maximum-likelihood and Bayesian analyses of a concatenated dataset positioned atypical pestiviruses consistently in a clade sister to BVDV-1 and BVDV-2, and the larger clade was sister to pestivirus of giraffes (*5*). The same pattern was observed in complete genome phylogeny (*1*).

The term atypical is not informative, and a new name has been proposed for these bovine pestiviruses (*5*). Outcomes of infections with these viruses are typical, but the viral antigens and phylogenies are unique.

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In Response: We read with interest the comment by Liu et al. (*1*) to our article reporting overt disease in calves caused by infection with atypical or 'Hobi'-like pestivirus (*2*). Although we can rule out that vaccination was the source of infection because only killed vaccines were administered on the farm, the most recent findings about this emerging group of viruses deserves further discussion regarding their possible taxonomical, diagnostic, and prophylactic implications.

The association of 'Hobi'-like pestivirus with clinical disease in cattle was confirmed by reports of experimental and natural infections (*3*,*4*). A cytopathogenic/noncytopathogenic 'Hobi'-like virus pair was isolated from a diseased heifer, demonstrating that the new group of viruses includes 2 biotypes already known for bovine viral diarrheal viruses 1 (BVDV-1) and 2 (BVDV-2) (*5*).

Although the clinical presentation might mimic that of extant BVDV-1/BVDV-2, distinctive features were also evident in the reported cases (*1*,*4*). At the antigenic and phylogenetic levels, 'Hobi'-like strains showed distinct differences from the other bovine pestiviruses: they were at least as different from BVDV-1/2 as are border disease virus and classical swine fever virus (*6*; N. Decaro et al., unpub. data). The antigenic and genetic uniqueness of 'Hobi'-like viruses will likely lead to the definition of a new species within genus *Pestivirus*, but how this species should be designated remains unclear.

Some scientists have tentatively proposed to name the virus bovine viral diarrhea 3, but others believe this nomenclature would be problematic from regulatory and scientific standpoints (J. Ridpath, pers. comm.). Molecular assays standardized for BVDV-1/2 might not be able to detect 'Hobi'-like strains because of the presence of mismatches in the oligonucleotide binding regions (*7*). Prophylactic measures should take into account the circulation of 'Hobi'-like pestiviruses in cattle herds. Whether commercial BVDV vaccines are effective against the emerging pestivirus is unknown, and requires future in vivo cross-protection studies.

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Bartonella spp. Bacteremia and Rheumatic Symptoms in Patients from Lyme Disease–endemic Region

To the Editor: We believe the recent article by Maggi et al. (*1*) contains serious flaws in content and underlying message, including a poorly defined study population, lack of appropriate controls, improper use of the term bacteremia, and incongruent laboratory findings. Selection criteria were vague: the authors state only that participants were a "biased" collection of "patients selected by a rheumatologist," with no control population included for comparison. The diagnosis of Lyme disease and other previously diagnosed conditions was solely by self-report. Although blood samples were collected from every participant, the authors apparently neglected to perform standardized testing for *Borrelia burgdorferi* or other conditions.

The term "bacteremia" signifies presence of viable bacteria in the bloodstream, which is not substantiated solely by a positive PCR result. True bacteremia was documented in only 1.7% of participants from whom a viable *Bartonella* species isolate was cultured, rather than the purported 41.1% of participants.

Surprisingly, many participants whose PCR results were positive for *Bartonella* spp. had no serologic evidence of infection (e.g., 82.5% of samples that had positive PCR results for *Bartonella henselae* were not seroreactive). Although anergy has been reported, samples from most immunocompetent and immunocompromised patients infected with *Bartonella* spp. are seroreactive (*2–4*), calling into question the authors' findings. Furthermore, 24% of samples that were positive by PCR revealed no identifiable *Bartonella* spp. by DNA sequencing; these participants should have been excluded from analysis.

Maggi et al. hypothesize that *Bartonella* spp. infection is causally related to a variety of chronic ailments. In fact, there was no association within the study population between positive *Bartonella* spp. PCR results and chronic illness, self-reported Lyme disease, or even a prior diagnosis of bartonellosis.

Efforts to define the clinical and public health importance of *Bartonella* spp. require scientific rigor. The above issues challenge the validity of the study, and results should be interpreted with caution.

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To the Editor: Some chronic diseases, including multiple sclerosis, chronic arthritis (*1*), cognitive disorders, and chronic fatigue remain unexplained, yet patients and patient advocacy groups are anxious to find an explanation and a cure. For 50 years, zoonotic agents have been wrongly considered as the cause of many of these diseases because diagnoses were based on results of serologic tests with low specificity. In France, at the beginning of my career, serologic testing for rickettsia was used as a diagnostic tool for many of these diseases and prompted inappropriate antimicrobial drug use because micro-agglutination on a slide is a nonspecific serologic technique (*2*). I had a hard time reversing this practice.

Results of serologic testing for nanobacteria were also unconfirmed because they were based on nonspecific antibodies (*3*). Results of Lyme disease serologic tests lacking specificity were also associated with these chronic diseases and led to the same results and conflicts between the Infectious Diseases Society of America and alternative users of *Borrelia burgdorferi* diagnostic tests (*4*). Currently, Google search pages display more results for alternative interpretations than for scientific information. Again, I have tried to limit the damages in France without success (*5*).

Now the *Bartonella* spp. appear as the new candidates to explain chronic illness (*1*). Once more, I am confronted with the problem in France. Some patients whose test results are negative in my laboratory were tested at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA (*3*) and received positive results (using a technique that I have not been able to reproduce). Now one of my patients is arguing and menacing because I do not confirm his infection by *Bartonella* spp.

We need to follow rigorous standards of causal influence before claiming that a bacterium is causing an unexplained chronic disease, to avoid facing the same problem that we had with Lyme disease: a mess with open conflicts between most scientists and some atypical investigators and patient advocacy groups.

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In Response: We offer the following comments to Beard et al. (*1*) and Raoult (*2*) regarding their respective responses to our recent article (*3*). Before 1990, *Bartonella* species were not known to infect animals or humans in North America. If not for the AIDS epidemic, the expansion of literature about *Bartonella* spp. might not have occurred (Figure). In 2010, in collaboration with Raoult (*4*), we posed a question in Emerging Infectious Diseases, "Could ticks transmit *Bartonella* spp.?" That article elicited an editorial response emphasizing the

lack of evidence supporting tick transmission of *Bartonella* spp. (*5*). Subsequently, *Bartonella birtlesii* transmission by *Ixodes ricinus* ticks was proven experimentally (*6*).

We now hope that this article will stimulate others to investigate a potential role for *Bartonella* spp. in rheumatologic diseases. Whether caused by politics or priorities, over the past 22 years, National Institutes of Health funding for *Bartonella* spp. research has been minimal and the US Centers for Disease Control and Prevention (CDC) has not critically investigated the medical impact of this genus of bacteria in US citizens. On 2 occasions, researchers at CDC declined to examine serum from these patients for antibodies against *Borrelia burgdorferi*. Because our research was not funded by any governmental agency, testing beyond our focus was not financially feasible.

We do not agree with the assertion that our study "contains serious flaws in content and underlying message, including a poorly defined study population, lack of appropriate controls, improper use of the term bacteremia, and incongruent laboratory findings." As indicated in the Materials and Methods section of our article, a physician, B. Robert Mozayeni, recipient of a Yale residency and rheumatology fellowship and predoctoral and postdoctoral molecular immunology fellowships at the National Institutes of Health, selected all study participants. In this exploratory cross-sectional study, entry criteria were not rigid and controls were not selected at patient recruitment but were defined later from the study population. Strikingly, serologic and molecular prevalence was higher among selected patients than among occupationally high-risk veterinary professionals (*7*) tested in the same laboratory by using the same diagnostic techniques. In our article, associations were reported, causation was not argued, and caution in results interpretation was addressed in the discussion.

Bacteremia is defined as the presence of bacteria in the blood. To suggest that agar plate isolation is the only way to document bacteremia is inappropriate. *B. burgdoferi* does not grow on an agar plate, and its isolation was challenging before development of insect-based liquid growth media. PCR testing is routinely used in human and veterinary medicine to diagnose bacteremic infections by *Anaplasma, Ehrlichia,* hemotrophic *Mycoplasma*, and *Rickettsia* spp. For example, *Ehrlichia ewingii*, a recognized pathogen of canids and humans, has never been successfully isolated, whereas bacteremia is routinely diagnosed by using PCR.

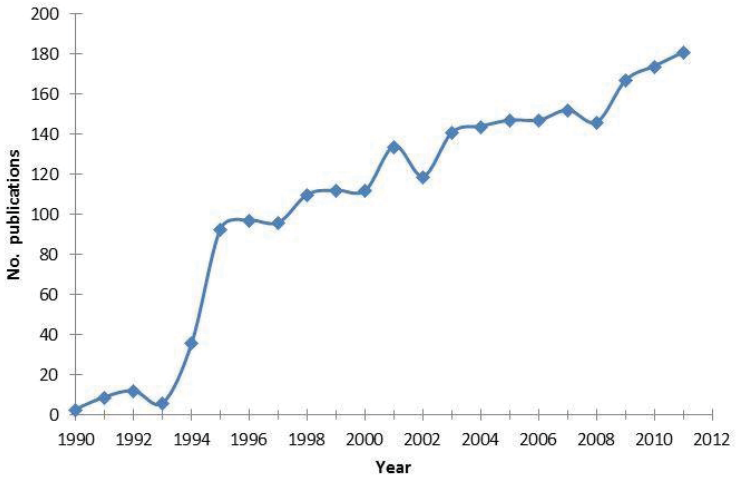

Figure. Annual worldwide number of published articles about *Bartonella* spp., 1990–2011. Data source: www.pubmed.gov.

In the spirit of collaboration, we have distributed *Bartonella* α Proteobacteria growth medium, an insect cell culture-based growth medium developed at and patented by North Carolina State University, to researchers around the world. Recipients included Michael Kosoy at CDC, who subsequently used this medium to isolate *Candidatus* Bartonella tamiae from febrile patients in Thailand (*8*). Subsequent studies have validated insect cell culture-based media for growth of *Bartonella* spp. For reasons that remain less than clear, there is incongruence between results of serologic testing and results of enrichment blood culture and PCR, which was addressed in our discussion and previous publications (*7*). In contrast to reports of the lack of antibodies in some bacteremic patients, we have reported specific serologic responses to infecting *Bartonella* spp. (*9*,*10*). The dated references provided by the correspondents relative to serologic testing do not address our bacteremic study population or their diseases.

We agree with Raoult that sensitive and specific diagnostic tests are critically needed to define the pathophysiology of bartonellosis. We also agree that bartonellosis is not borreliosis, and the 2 diseases should not be confused by patients, advocacy groups, Lyme disease researchers, or governmental agencies.

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Paul Signac (1863–1935) *La salle à manger*, *Breakfast* (1886–1887) Oil on canvas (89.5 cm × 116.5 cm) Kröller-Müller Museum, Otterlo, the Netherlands www.kmm.nl

Science Avant-Garde

Polyxeni Potter

"To think that the neoimpressionists are painters who cover canvases with little multicolored spots is a rather widespread mistake," wrote Paul Signac in his manifesto of their movement. "This mediocre dot method has nothing to do with the aesthetic of the painters we are defending here, nor with the technique of divisionism they use." Signac was referring to, among others, Georges Seurat, who in trying to systematize the optical discoveries of the impressionists, had taken a scientific approach to painting, one based on color theory. His goal, he once said, was to make "modern people, in their essential traits, move about as if on friezes, and place them on canvases organized by harmonies of color, by directions of the tones in harmony with the lines, and by the directions of the lines."

Signac wholeheartedly adopted Seurat's invention, pointillism or divisionism, though he considered it simply a means of expression, a way to apply paint to the canvas. "The dot is nothing more than a brushstroke, a technique. And like all techniques, it does not matter much." The idea was to render the surface of a painting more vibrant, to maximize the brilliance of color. But the methodical scientific technique alone did not "guarantee luminosity or the intensity of colors or harmony. This is due to the fact that complementary colors are favorable to and intensify each other when they are blended, even optically. A red surface and a green surface, when adjacent, stimulate one another. Red dots blended into green dots produce a gray and colorless whole."

"My family wanted me to become an architect, but I preferred drawing on the banks of the Seine rather than in a workshop of the École des Beaux-Arts," wrote Signac. A visit to an exhibition of Claude Monet in 1880 was a life-changing experience. After a brief stint at the Collège Rollin, he set out to become a painter, which he did, a stellar one and self-taught. His earliest work was filled with energy. "It consisted in pasting reds, greens, blues, and yellows, without much care but with enthusiasm." When he lost his father to tuberculosis, his financially stable and supportive family saw to it he did not have to make ends meet. The same year, at age 17, he bought a painting by Paul Cézanne. His own first painting was dated a year later.

Signac spent most of his life in and around Paris where he was born. He was interested in science, literature, and politics. He was a writer, whose poetic sensitivity found its way into landscape painting. He was an avid traveler. His *Olympia*, a boat named after Édouard Manet's famed nude, took him to Italy, Holland, and Constantinople. He often stopped to paint Mediterranean ports and scenery, immortalizing the French coast in watercolors painted *en plein air*. Although he experimented with oils, pen and ink, etchings, and lithographs, watercolor, "a playful game," was the mainstay of his life's work.

"I have seen Signac, and it has done me quite a lot of good," wrote Vincent van Gogh to his brother Theo. "He was so good and straightforward and simple…. I found Signac very quiet though he is said to be violent; he gave me the impression of someone who has balance and poise." Signac's irrepressible vitality and exuberance, his love of action and the outdoors, and a native combativeness were at times misunderstood, but not by his many friends, an array of artists and anarchists. Unassuming and ragged in his sailor's garb, he was often at sea or at his home in St. Tropez, a meeting place for the exchange and promotion of artistic ideas. Signac equated social revolution with artistic freedom. "The anarchist painter is not the one who will create anarchist pictures, but he who, without desire for recompense, will fight with all his individuality against official bourgeois conventions by means of a personal contribution."

At age 21, Signac became, along with Georges Seurat and others, cofounder of the Société des Artistes Indépendants, a group intended to provide opportunities for exhibiting avant-garde works away from the rigid cultural politics of the Paris Salon. President of the society from 1908 until his death, Signac encouraged young artists by exhibiting controversial works. Meanwhile, with Seurat, he set off to articulate the theories of neoimpressionism. After the untimely death of Seurat from respiratory infection at age 31, Signac became the sole advocate and leader of the movement.

Signac took Seurat's theories to a new level. Armed with watercolor sketches from nature, he moved the stu-

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dio indoors and used mosaic-like squares of pure color to compose large scenes that would influence the works of van Gogh and Gauguin, inspire Matisse, and affect the evolution of future art movements, from fauvism to cubism. Signac was tireless in explaining divisionism. "In order to listen to a symphony, you don't sit in the middle of the orchestra but in the position where the sounds from the various instruments mingle, creating the harmony desired by the composer. Similarly, faced by a 'divided' painting, it is best to first stand at a sufficient distance in order to absorb the whole, before moving closer to study the chromatic effects up close." For the first 20 years of his career, he received little recognition and neoimpressionism received negative criticism, even by those who initially supported it. He died of septicemia in Paris at 72.

La salle à manger, *Breakfast,* on this month's cover, is from a series of views inside contemporary interiors with figures usually posed in stiff profile. Signac valiantly sought art solutions in the scientific process, the precise observation of color tones in close proximity. And, moving away from the subjectivity of impressionism and the passing moment, he searched the small particles of color for truth.

Always interested in human psychology and social justice, he looked for them in home interiors, as he curiously observed the urban middle-class. In *Breakfast*, he spied on them from outside the room, inviting the viewer to do the same. With scant interest in perspective, he placed the human figures on a grid, same as all other objects. Frozen in space and time, these figures were not persons but social types: the retired bourgeois, the maid, the housewife. Uninterested in each other, they played roles, their performance an indictment of society and marriage, which inhibits the development of individual personality.

"By exclusive use of … pure colors, by methodical division and by observing the scientific theory of colors, [the neoimpressionist] guarantees maximal luminosity, color, and harmony to an unprecedented degree," Signac wrote. The painter's vision applies neatly to today's rapid advancement of genome technologies that, by providing tiny bits of data on disease-causing microbes, promise to improve the canvas of clinical and public health laboratory investigations and lower the costs.

The latest genome DNA sequencers generate detailed and robust information for clinical and public health laboratories and could spawn a global system of linked databases of pathogen genomes to ensure more efficient detection, prevention, and control of endemic and emerging diseases and all manner of outbreaks. Even as these new genomic tools enhance diagnosis, they decrease the use of culture and molecular methods that produce data currently critical for epidemiologic investigation. Careful application of current epidemiologic techniques teases apart the dynamic interaction of infectious diseases that drive total illness and death rates up or down, even in outbreaks with universal exposure. New genome-backed epidemiologic approaches will be needed as sequencers replace culture and molecular techniques so this ability is not lost.

"To ensure optical mixture, the neoimpressionists were forced to use brushwork of a small scale so that, when standing back sufficiently, different elements could reconstitute the desired tint and not be perceived in isolation." In genomics approaches, likewise, field epidemiologists must use alternative data sources or original techniques to capture the unique characteristics that tie together the epidemiologically related whole. Without these, the bits provided by the precise genomic tools would only create "industrial art," a canvas without valuable content, aesthetics achieved, in Signac's words, by "empirical formulae and dishonest or silly advice."

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Article Title

Coccidioidomycosis-associated Deaths, United States, 1990–2008

CME Questions

1. You are seeing a 45-year-old man with a 4-week history of cough and fever. He was visiting a cousin in the southwestern United States last month, and you consider whether he might have coccidioidomycosis. What should you consider regarding the epidemiology of coccidioidomycosis?

A. *Coccidioides* spp. are usually found at high elevations
B. *Coccidioides* spp. are usually found in wet climates
C. Up to 50% of people in endemic areas have been exposed to *Coccidioides* spores
D. *Coccidioides* spp. are found on the surface of the soil

2. What should you consider regarding the transmission and clinical course of coccidioidomycosis?

A. Livestock frequently transmit coccidioidomycosis to humans
B. Person-to-person transmission accounts for most cases of coccidioidomycosis
C. One quarter of patients with coccidioidomycosis develop extrapulmonary disease
D. The rate of disseminated disease is higher among men vs women

3. The patient is diagnosed with coccidioidomycosis. What should you consider regarding the demographics of fatal cases of coccidioidomycosis in the current study?

A. There was no sex-based difference in the risk of mortality
B. The highest rate of death was among children and adolescents
C. Race and ethnicity did not affect the risk of mortality
D. Native Americans experienced the highest rate of mortality compared with other racial/ethnic groups

4. The patient has an extensive past medical history. Which of the following comorbid illnesses was most associated with a higher risk of death among patients with coccidioidomycosis in the current study?

A. Rheumatoid arthritis
B. Congestive heart failure
C. Chronic obstructive pulmonary disease
D. Recurrent dental abscesses

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Invasive Pneumococcal Disease and 7-Valent Pneumococcal Conjugate Vaccine, the Netherlands

CME Questions

1. You are seeing a 2-month-old infant whose parents have multiple questions regarding the effects of the 7-valent pneumococcal conjugate vaccine (PCV7). What does previous research demonstrate regarding the efficacy of PCV7?

A. PCV7 primarily reduces the risk of otitis media among children
B. PCV7 has no effect on the rate of invasive pneumococcal disease (IPD) among adults
C. PCV7 has no effect on the rate of IPD among young children
D. The rate of non-PCV7-serotype IPD increased after introduction of PCV7

2. What else should you consider as you discuss PCV7 with these parents?

A. IPD incidence has declined to a greater degree in Europe vs the United States
B. Catch-up vaccination programs for children under 5 have sped up eradication of vaccine serotypes
C. The greatest indirect benefit of PCV7 is found among older adults with comorbid conditions
D. PCV7 contains serotype 1, which is the most common serotype implicated in IPD

3. As you continue your conversation regarding PCV7, which age groups from the current study can you tell these parents experienced the greatest decline in the incidence of IPD?

A. 2 to 4 years of age and 5 to 12 years of age
B. 0 to 2 years of age and middle-aged adults
C. 0 to 2 years of age and 65 years or older
D. 21 to 35 years of age and 50 to 64 years of age

4. What can you tell these parents regarding characteristics of IPD in the current study?

A. IPD incidence due to non–PCV7-serotypes increased among young children but fell among adults
B. Pneumonia was the most common infection related to IPD among children less than 5 years of age
C. PCV-7 appeared particularly effective in reducing IPD among immunocompromised individuals
D. The overall mortality rate associated with IPD declined during the study period

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Nasopharyngeal Bacterial Interactions in Children

CME Questions

1. Based on the observational study by Dr. Xu and colleagues, which of the following statements about patterns of nasopharyngeal bacterial colonization and interaction in healthy young children is most likely correct?

A. *Streptococcus pneumoniae* is positively associated with *Staphylococcus aureus*
B. *S. pneumoniae* is positively associated with *Moraxella catarrhalis*
C. *M. catarrhalis* is positively associated with *S. aureus*
D. Patterns of nasopharyngeal bacterial colonization and interaction in health are likely to predict those at onset of acute otitis media (AOM)

2. Your patient is a 1-year-old, PCV7-vaccinated male with AOM. Based on the observational study by Dr. Xu and colleagues, which of the following statements about the role of *Haemophilus influenzae* is most likely correct?

A. The nasopharyngeal environment is likely to be unfavorable to *H. influenzae* colonization
B. At AOM onset, *H. influenzae* is positively associated with *S. pneumoniae*
C. At AOM onset, *H. influenzae* is positively associated with *M. catarrhalis*
D. *H. influenzae* may become a more important cause of AOM in pneumococcal conjugate vaccinated children

3. Based on the observational study by Dr. Xu and colleagues, which of the following statements about the role of other bacteria in AOM affecting the patient described in question 2 would most likely be correct?

A. During AOM, *S. pneumoniae* colonization is negatively associated with *S. aureus*
B. Viral-bacterial-host interactions in the nasopharynx during AOM are completely characterized
C. Findings of this study support the role of *S. aureus* as a frequent pathogen of AOM
D. Elimination of *S. pneumoniae* PCV7 strains has resulted in the remaining strains competing less effectively with *H. influenzae* in the nasopharynx and as a cause of AOM infection

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Salmonellosis Caused by Bovine-associated Subtypes

Influenza A (H3N2) Variant Virus Infection among Attendees of an Agricultural Fair, Pennsylvania, 2011

Subclinical Influenza Virus A Infections in Pigs Exhibited at Agricultural Fairs, Ohio, 2009–2011

Reservoir Competence of Wildlife Host Species for *Babesia microti*

Borrelia, *Rickettsia*, and *Ehrlichia* spp. in Bat Ticks, France, 2010

Nonprimate Hepaciviruses in Domestic Horses, United Kingdom

Virulent Avian Infectious Bronchitis Virus, People's Republic of China

Transmission Routes for Nipah Virus from Malaysia and Bangladesh

Diagnostic Assays for Crimean-Congo Hemorrhagic Fever

Enterovirus 71–associated Hand, Foot, and Mouth Disease, Vietnam, 2011

Epizootic Spread of Schmallenberg Virus among Wild Cervids, Belgium, 2011

Variant Rabbit Hemorrhagic Disease Virus in Young Rabbits, Spain

Reservoir Competence of Vertebrate Hosts for *Anaplasma phagocytophilum*

MRSA Variant in Companion Animals

Arctic-like Rabies Virus, Bangladesh, 2010

No Evidence of Prolonged Hendra Virus Shedding by 2 Patients, Australia

Porcine Reproductive and Respiratory Syndrome Virus, Thailand, 2010–2011

West Nile Virus Neurologic Disease in Humans, South Africa

Cygnet River Virus, a Novel Orthomyxovirus from Ducks, Australia

Candidatus Neoehrlichia mikurensis in Wild Rodents, France

Human Thelaziosis, Spain

Complete list of articles in the December issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 11–15, 2012
ASTMH 61st Annual Meeting
Atlanta Marriot Marquis
Atlanta, GA, USA
http://www.astmh.org

December 1–7, 2012
ASLM's First International Conference
Cape Town, South Africa
http://www.aslm.org

2013
March 3–7, 2013
The Conference on Retroviruses and Opportunistic Infections (CROI) 2013
Georgia World Congress Center
Atlanta, GA, USA
http://www.retroconference.org

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING INFECTIOUS DISEASES®

September 2012

Maternal and Child Health

Pacific Asia Museum, Pasadena, California, USA, Gift of Eleanor L. Gilmore www.pacificasiamuseum.org

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Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

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Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.