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Nontuberculous Mycobacteria

October 2009

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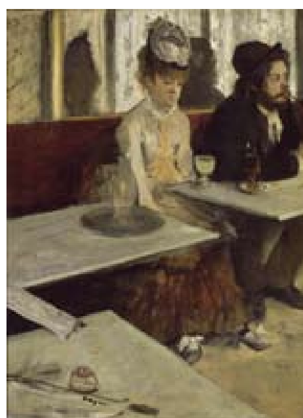

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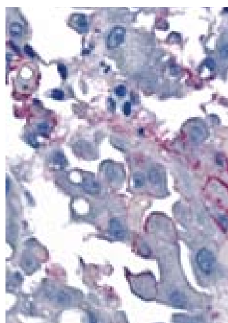

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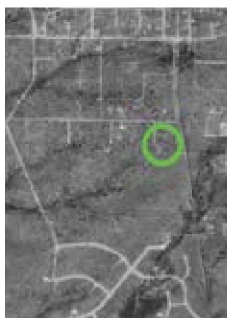

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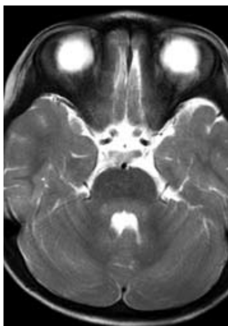

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A Model-based Assessment of Oseltamivir Prophylaxis Strategies to Prevent Influenza in Nursing Homes

Carline van den Dool, Eelko Hak, Marc J.M. Bonten,[1] and Jacco Wallinga[1]

Prophylaxis with neuraminidase inhibitors is important for controlling seasonal influenza outbreaks in long-term care settings. We used a stochastic individual-based model that simulates influenza virus transmission in a long-term care nursing home department to study the protection offered to patients by different strategies of prophylaxis with oseltamivir and determined the effect of emerging resistance. Without resistance, postexposure and continuous prophylaxis reduced the patient infection attack rate from 0.19 to 0.13 (relative risk [RR] 0.67) and 0.05 (RR 0.23), respectively. Postexposure prophylaxis prevented more infections per dose (118 and 323 daily doses needed to prevent 1 infection, respectively) and required fewer doses per season than continuous prophylaxis. If resistance to oseltamivir was increased, both prophylaxis strategies became less efficacious and efficient, but postexposure prophylaxis posed a lower selection pressure for resistant virus strains. Extension of prophylaxis to healthcare workers offered little additional protection to patients.

The prophylactic use of neuraminidase inhibitors is a key component of influenza outbreak control in healthcare institutions (*1*,*2*). Based on its proven efficacy in reducing susceptibility, duration of illness, and infectiousness in household studies (*3*–*6*), oseltamivir is now the antiviral agent recommended for prophylactic use in nursing homes. Although the efficacy of oseltamivir has not been extensively assessed in the elderly, some observational and experimental studies suggest beneficial effects of both continuous and postexposure prophylaxis in containing outbreaks and reducing the number of severe complications among nursing home residents (*2*,*7*–*10*).

During the 2007–08 and 2008–09 influenza seasons, the number of isolated influenza A (H1N1) viruses with resistance to the neuraminidase inhibitor oseltamivir increased considerably (*11*,*12*). Following the emerging resistance against the M2-inhibitors amantadine and rimantadine, the efficacy of this class of neuraminidase inhibitors may also be threatened (*13*). Given the speed at which resistant strains have spread and the large variability of influenza activity, it has been impossible to obtain evidence on how resistance has affected influenza control strategies from randomized controlled trials. This effect can, however, be derived using modeling studies (*14*,*15*). Therefore, we developed a mathematical model of influenza transmission in long-term care facilities to study different scenarios and to perform multiple simulations that minimize the probability of chance outcomes. We primarily determined the effect and efficiency of postexposure and continuous exposure prophylaxis strategies with oseltamivir, as compared with no prophylaxis, on infection attack rates among patients in a long-term care nursing home department. We also determined the influence of increased introduction of resistant virus strains on both strategies and assessed the potential benefits of extending prophylaxis to healthcare workers (HCWs).

Methods

Population and Model

We simulated the occurrence of influenza virus outbreaks during an 80-day period in a typical long-term care

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[1]These authors contributed equally to this article.

nursing home department (30-bed unit with 15 two-bed rooms and a team of 30 HCWs) in the Netherlands. HCWs worked 8-hour shifts; according to a weekly schedule 5, 3, and 1 HCW(s) worked during the day, evening, and night shifts, respectively, which has been observed in some nursing homes in the Netherlands. The average length of stay for a patient was 14 months (*16*,*17*). Because we simulated a small population where chance events can have major effects, we used a stochastic transmission model. The model is described in the online supporting information (online Technical Appendix, available from www.cdc.gov/EID/content/15/10/1547-Techapp.pdf) and has been described in detail in a previous study (*18*). Here, we describe the essential elements of the model's structure for the baseline scenario (parameters for the baseline scenario are shown in Table 1).

Infection Cycle

According to a standard model for infectious disease transmission, persons could be in 1 of several stages of influenza virus infection: susceptible, infected but not yet infectious (exposed), infectious, or recovered/immune (Figure 1) (*19*). The durations of the exposed and infectious periods were exponentially distributed with means of 1.4 days; the resulting generation time equaled 2.8 days, which agrees with observations of generation times during influenza epidemics (*20*,*21*). At the start of the influenza season, 30% of the adult nursing home population was assumed to be immune to infection because of cross protection from earlier infections (*22*). Since the elderly have weakened immune systems (*23*,*24*), but exact estimates are absent, we made the most conservative assumption that their immune systems had no memory of previous infections.

Influenza Vaccination

According to our model, both patients and HCWs could receive influenza vaccine before the influenza season. The average vaccination rate was 75% for nursing home patients (*25*) and 40% for HCWs (*2*). We assumed that for each person vaccination either led to perfect immunity against infection or had no effect (*18*). In a previous study, we showed that this all-or-nothing assumption for vaccine-induced immunity yielded similar results to those of an alternative assumption of incomplete immunity in which vaccinated persons had a lower probability of acquiring infection upon contact with an infectious person (*18*). The assumption of all-or-nothing immunity due to prophylaxis has also been made in other modeling studies (*26*). We assumed the vaccine efficacy against influenza virus infection in healthy adults, and thus HCWs, was 73% (*27*). For elderly nursing home patients, no statistically significant vaccine efficacy against infection has been observed (*28*). However, because other evidence showed that the vaccine protected against influenza disease and complications, we assumed patient efficacy to be 25% (*28*,*29*).

Table 1. Parameter values baseline scenario*

Parameter	Value	Reference
No. beds	30	
No. HCWs	30	
Time step (= shift), h	8	(*18*)
Minimum duration of simulation, d	80	
Discharge/mortality rate, per d	1/425	(*16,17*)
Rate of becoming infectious after infection, per d	1/1.4	(*20,21*)
Infection recovery rate, d	1/1.4	(*20,21*)
Prior immunity HCWs	30%	(*22*)
Prior immunity patients	0	
Vaccine uptake patients	75%	(*25*)
Vaccine uptake HCWs	40%	(*2*)
Vaccine efficacy (against infection)		
Patients	25%	(*28*)
HCWs	73%	(*27*)
Transmission probability per casual contact	0.13	(*18*)
Close/casual transmission probability ratio	2	
Mean visitor frequency/patient/d	0.7	(*31*)
Minimum duration of postexposure prophylaxis, d	14	(*2*)
Minimum duration of postexposure prophylaxis after last detected case, d	8	(*2*)
Parameters in uncertainty analyses		
Probability of disease developing after infection (range)	0.5 (0.30–0.7)	(*4*)
Probability of disease developing after infection, during prophylaxis (range)	0.2 (0.05–0.4)	(*4*)
Oseltamivir efficacy against infection (range)	0.53 (0.2–0.8)	(*4*)
Oseltamivir reduction in infectiousness (range)	0.2 (0–0.5)	(*4*)

*HCW, healthcare worker.

Prophylaxis with Oseltamivir

We compared 2 strategies of prophylaxis with oseltamivir to a control situation in which no neuraminidase inhibitors were used: continuous (seasonal) prophylaxis was given to all patients during 8 weeks (the longest period of prophylaxis described in effectiveness studies) (*30*) around the peak of the influenza season; or postexposure prophylaxis was started for all patients as soon as 1 patient had a laboratory-confirmed influenza virus infection. Because recognition of a possible influenza infection is required before doing a laboratory test, we assumed that only the fraction of infected patients in whom influenza disease developed (the symptomatic patients) could trigger the start of postexposure prophylaxis. We assumed that, for every first symptomatically infected person, the delay between the start of infectiousness and the start of prophylaxis followed a distribution with a mean of 3.5 days. This interval was determined by the time to onset of symptoms, the time to recognition of symptoms, the time to a positive laboratory test, and the delay to start of prophylaxis (online Technical Appendix). Postexposure prophylaxis was given to all patients in the department for at least 2 weeks and was continued until no new cases occurred during a period of 8 days (*2*). Because we did not have data on the efficacy of oseltamivir in elderly persons, we used estimates from household studies (*4*) as the best available evidence. We assumed oseltamivir induced immunity to infection by wild-type strains in 55% of the susceptible patients as soon as it was administered and for the duration of prophylaxis. Immunity did not develop in the other patients, but when they were infected they were considered to become less infectious than persons who did not take oseltamivir (*26*). Based on estimates of the total reduction in infectiousness in persons treated with oseltamivir (*4*), we assumed the probability that the virus was transmitted during contact with a susceptible person was reduced by 20%. In the online Technical Appendix, we describe some uncertainty analyses that we performed for the parameters describing oseltamivir efficacy.

Influenza Disease

On the basis of household studies, we assumed that influenza disease would develop in 50% of patients infected with influenza virus (*4*). For those receiving oseltamivir prophylaxis, this probability was only 20% (*4*).

Contacts

A person's risk of being infected depended on the number and type of contacts with infectious persons. We distinguished between casual and close contacts; casual contact was considered as conversation and close contact occurred with physical contact. We parameterized the contact model; the expected numbers of contacts, specified by type of persons and kind of contact, matched the number of contacts that we observed in 2 nursing home departments in the Netherlands (*18*). The probability of contact between 2 persons, given their type (HCW or patient), as well as the probability that this contact was close (physical contact), is given in Table 2. During the night shift, patients did not have contact with other patients, except for their roommates, who were assumed to be casual contacts. During the day and evening shifts, patients could also have contact with visitors. All contacts with visitors were considered close. The expected number of visitors was based on a study in the Netherlands on nursing home patients and visitors and was estimated to be 0.7 visitors per patient per day (*31*).

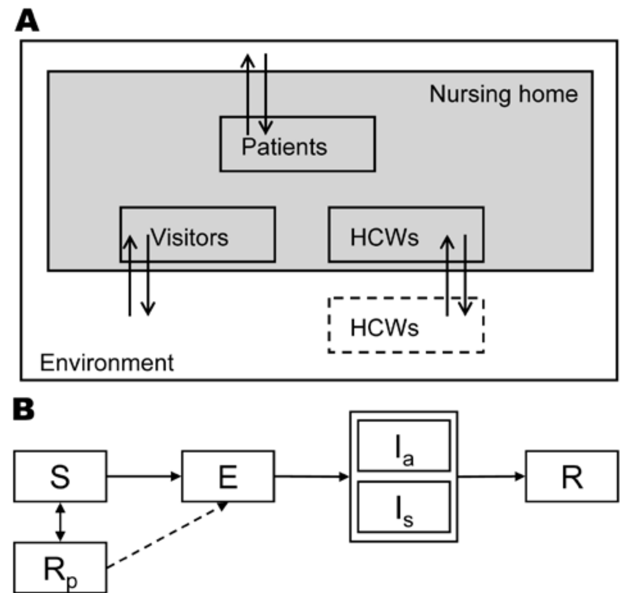

Figure 1. Schematic diagram of our stochastic individual-based model. A) The different types of persons in the nursing home: patients, healthcare workers (HCWs), and visitors. B) The time course of infection: S, susceptible; E, exposed; I_a, infectious and asymptomatic; I_s, infectious and symptomatic; R, recovered/immune; R_p, immune while using prophylaxis. For all patients and HCWs in the model, we kept track of their stage in this infection cycle in time. If the influenza strain that is transmitted is resistant to oseltamivir, persons in the R_p department can still become infected (dashed arrow).

Transmission

For every pair of persons with a casual or close contact, a probability existed that the virus was transmitted if the persons involved in the contact were infectious and susceptible. This probability was determined by sampling from a Bernoulli distribution with mean set equal to the transmission probability. For a casual contact, the transmission probability was 0.13; we chose this probability be-

Table 2. Contact probabilities between persons in a nursing home department*

Person	Contacted person	Probability of contact	Probability of close contact given casual contact
Patient	Patient	0.07	0.06
Patient	HCW	0.52	0.69
HCW	HCW	0.91	0.31

*HCW, healthcare worker.

cause the expected infection attack rate among patients in the absence of HCW vaccination was similar to observed attack rates for influenza-like-illness (*18*,*25*,*32*). For close contacts, the probability of transmission was assumed to be 2× as high as that of casual contacts.

Influenza in the Community

The rate at which influenza virus was introduced into the nursing home by HCWs, visitors, and patients depended on the prevalence of the virus in the community; we used a simulation of an influenza epidemic in a large population (online Technical Appendix). In each simulation, a constant proportion of infections in the community was assumed to be caused by resistant strains.

Oseltamivir Resistance

Resistant viruses were assumed to be completely insensitive to oseltamivir, and therefore prophylaxis had no effect on the susceptibility of a person who was exposed to a resistant strain. We also assumed that use of oseltamivir neither affected the infectiousness nor the development of symptoms in a person infected with a resistant strain. Apart from oseltamivir sensitivity, resistant and nonresistant strains were assumed to be similar. Infection with 1 of the strains conferred cross-protection against infection with other strains during the season.

Outcomes

We defined the infection attack rate and the disease attack rate as the total number of infections or influenza diseases among patients, respectively, divided by the total number of patients in the nursing home department during the study period. We distinguished between infections caused by oseltamivir-sensitive and -resistant strains and compared scenarios with increasing prevalence of oseltamivir resistance. Based on the distribution of infection attack rates in a nursing home in the absence of preventive measures (*18*), we used the proportion of infection attack rates of ≥0.3 as a proxy for the probability of a large outbreak. We calculated the absolute and relative risk reductions for both strategies of prophylaxis (efficacy) and determined the fraction of infections caused by resistant strains. We also computed the number of daily doses of prophylaxis needed to prevent 1 infection or disease (DNP) as the total number of doses administered divided by the number of influenza infections or diseases prevented (the absolute risk difference) (efficiency). Information on the statistical precision of the effect estimates can be found in the online Technical Appendix.

Alternative Scenarios

In addition to the baseline scenario previously described, we considered an alternative scenario in which both patients and HCWs received continuous or postexposure prophylaxis according to the same rules. Postexposure prophylaxis was started after detection of infection in a patient and was given to all patients and all HCWs. We also studied a scenario in which the HCW vaccination rate was only 10%, as was observed in the Netherlands (*33*). Here we considered prophylaxis to patients only and to patients and HCWs.

In the online Technical Appendix, additional scenarios are described for the following circumstances: 1) different delays between the start of infectiousness of the first symptomatic patient and the start of postexposure prophylaxis, 2) different levels of influenza virus activity in the community, 3) higher percentage of HCWs vaccinated, 4) lower patient vaccine uptake, 5) greater percentage of patients with prior immunity, and 6) a 60-bed nursing home department.

Results

Baseline Scenario

In the absence of resistance, the prophylactic use of oseltamivir reduced the number of influenza virus infections among patients during the influenza season. The infection attack rate among patients decreased from 0.19 in the control setting without prophylaxis to 0.13 (relative risk [RR] 0.67) when postexposure prophylaxis was given to all patients (first 2 bars, Figure 2, panel A). The fraction of large outbreaks with an infection attack rate of ≥0.3 decreased from 0.31 to 0.17 (RR 0.55), and outbreaks with attack rates >0.4 rarely occurred (Figure 3). If continuous prophylaxis was given for 8 weeks, the infection attack rate decreased to 0.05 (RR 0.23) (Figure 2, panel B), and the percentage of large outbreaks decreased to 0.03 (RR 0.09). Because of continuous prophylaxis, not only did large outbreaks disappear, but also the percentage of departments without any patient infection increased (Figure 3). Rates of influenza disease decreased from 0.10 to 0.06 (RR 0.60) and 0.01 (RR 0.13), respectively, for the 2 different strategies of prophylaxis (Figure 1, panels C, D). Although the number of infections that could be prevented was higher for continuous prophylaxis, the DNP was ≈3× higher with this strategy than with postexposure strategy (Figure 4). Without resistance, the DNP was 118 for postexposure prophylaxis and 323 for continuous prophylaxis.

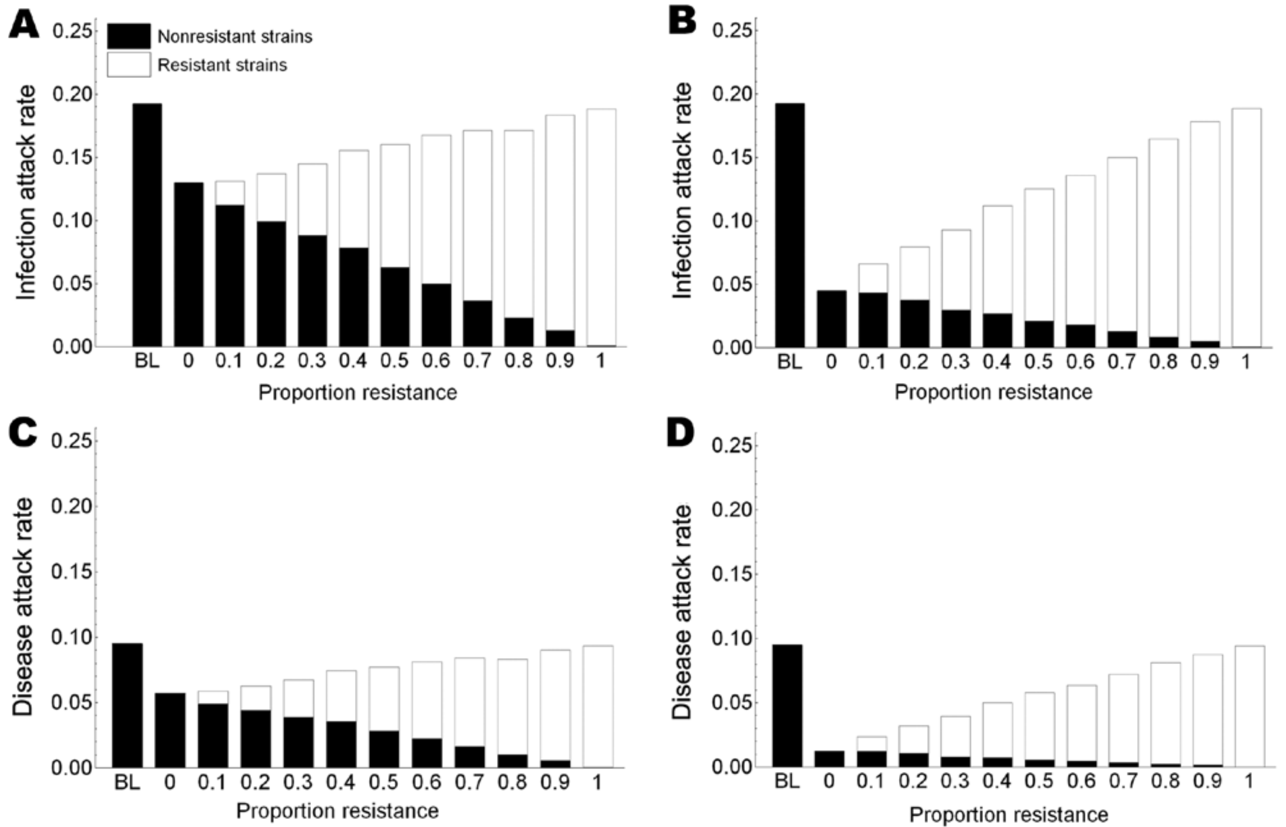

Figure 2. Effects of prophylaxis with oseltamivir on influenza virus infection and disease rates among nursing home patients. The effects of both postexposure and continuous prophylaxis strategies are shown for different proportions of resistant virus strains in the community and compared with a control setting without prophylaxis and resistance. Panels A and C, postexposure prophylaxis given to all patients; panel B and D, continuous prophylaxis for 8 weeks. BL, baseline.

Resistance

An increase in the proportion of oseltamivir-resistant influenza virus strains in the community reduced the efficacy of prophylaxis with oseltamivir against infection and disease (Figure 2). In addition, both prophylaxis strategies became less efficient and the DNP increased rapidly, in particular for the continuous prophylaxis strategy (Figure 4). Prophylaxis caused a selection pressure for resistant strains; the percentage of infections caused by resistant strains in the nursing home was higher than in the community (Figure 5). The selection of resistant strains was most pronounced for continuous prophylaxis strategy.

Alternative Scenario: Prophylaxis Extended to HCWs

Extension of prophylaxis strategies to include both HCWs and patients offered little additional protection to

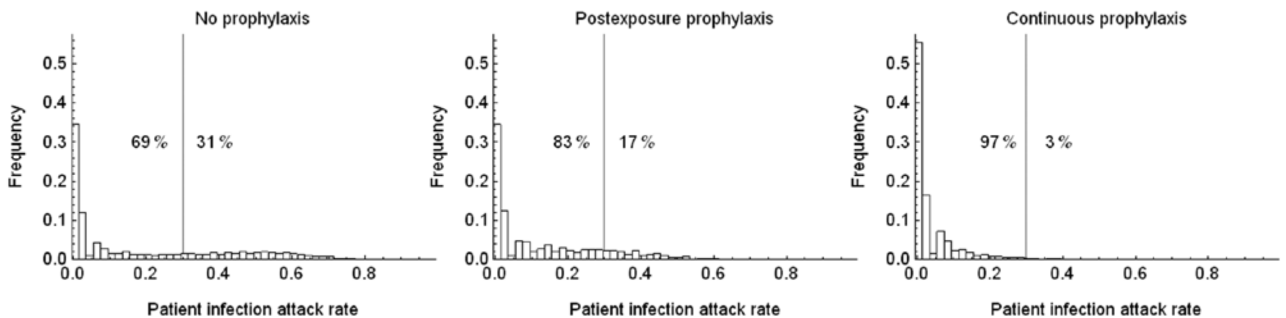

Figure 3. Distribution of influenza virus infection attack rates among patients who received no prophylaxis, postexposure prophylaxis, and continuous prophylaxis, in the absence of resistance.

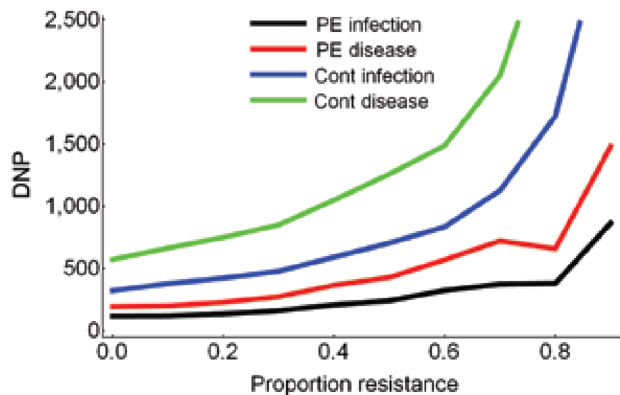

Figure 4. The number of daily doses of oseltamivir needed to prevent 1 influenza virus infection or disease (DNP). Results are shown for both postexposure (PE) prophylaxis and continuous (cont) prophylaxis for increasing proportions of oseltamivir-resistant virus strains in the community.

patients (Figure 6). In the absence of resistance, postexposure and continuous prophylaxis reduced the infection attack rate in HCWs from 0.14 to 0.10 and 0.05, respectively. The attack rate among patients decreased from 0.19 to 0.12 (RR 0.65) and 0.03 (RR 0.15), respectively. Taken together, the DNP for infection (of either patient or HCW) was 140 for postexposure prophylaxis and 366 for continuous prophylaxis; the total number of doses administered was 2 × as high as in the scenario in which only patients received prophylaxis.

When the HCW vaccination rate was 0.1, the infection attack rate among patients without prophylaxis was 0.23. This could be reduced to 0.15 (RR 0.67) when postexposure prophylaxis was given to patients alone and to 0.14 (RR 0.63) when it was given to HCWs as well (Figure 6). Continuous prophylaxis given to patients only or to both patients and HCWs could reduce the infection attack rate to 0.06 (RR 0.26) and 0.04 (RR 0.16), respectively. However, the number of doses required per department was approximately 6 × higher for continuous prophylaxis than for postexposure prophylaxis. Results of other alternative scenarios and the uncertainty analyses are described in the supporting information (online Technical Appendix).

Discussion

Our model predicts that in the absence of resistance, both postexposure prophylaxis and continuous prophylaxis can reduce the number of influenza virus infections in nursing home patients during annual influenza epidemics. Although continuous prophylaxis will prevent more cases, postexposure prophylaxis prevents more cases per dose. If resistance to oseltamivir increases, both prophylaxis strategies become less efficacious and less efficient, with more selection for resistance during continuous prophylaxis. Extension of prophylaxis to HCWs is not expected to have a large effect on the attack rates among patients.

For the results of our modeling study to be correctly interpreted, we must discuss some possible limitations. First, we did not distinguish between different subtypes of influenza circulating in the community. The oseltamivir-resistant strains that dramatically increased in number globally during the last 2 influenza seasons were all influenza A (H1N1) strains and resistance against oseltamivir seemed to be limited to the N1 serotype only. During the 2007–08 season, H1N1 strains were responsible for approximately 60% of influenza virus infections in Europe, which is uncommon when data for the last decade are examined (*34*). The remaining influenza virus infections were caused by A/H3N2 subtype and B type viruses. Thus, even if all influenza A (H1N1) strains acquired resistance against oseltamivir, levels of resistance of ≥60% are not very probable unless resistance develops as well in the other influenza A subtypes and in influenza B. Second, we did not take into account de novo resistance in persons on prophylaxis. We assumed the probability of emergence of resistance was very low (*26*) and, as we studied a small population, the effect on the outcome was assumed to be negligible. Third, we used estimates on the efficacy of oseltamivir prophylaxis from household studies because we did not have data specific for elderly people. More accurate assessment of efficacy and comparison of preventive measures in nursing homes will require new estimates from studies in senior populations. Finally, we studied a 30-bed department instead of an entire nursing home. If an outbreak occurs in 1 department, it might be necessary to start prophylaxis in other nearby departments as well. However, the effects of prophylaxis for individual departments will not be different.

Our model confirmed the beneficial effects of prophylaxis with oseltamivir in reducing the number of infections

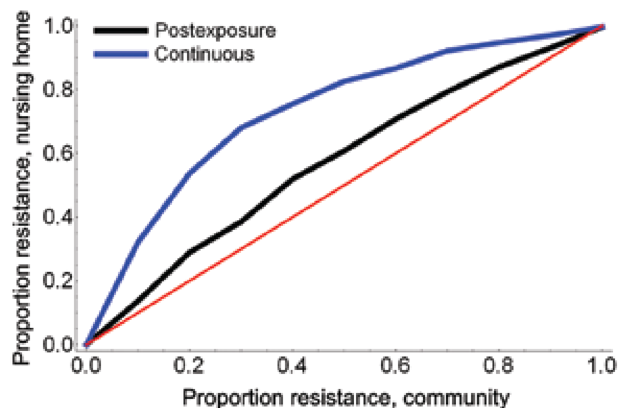

Figure 5. The proportion of infections with oseltamivir-resistant influenza virus strains among nursing home patients for increasing proportions of resistance in the community.

and preventing large outbreaks as has been suggested by some observational and experimental studies (*7–9*). We have not considered the effects of prophylaxis on the number of complications or deaths, but these can be assumed to be somewhat higher than for infection because oseltamivir also prevents complications when taken after infection (*9,35*). Our results suggest a large difference in both efficacy and efficiency between the postexposure and continuous prophylaxis strategies. Although continuous prophylaxis can protect more patients, it also requires large stocks of antiviral drugs and is therefore costly; postexposure prophylaxis might be the preferred strategy. Furthermore, our model suggests that extending prophylaxis to HCWs does not prevent many additional infections among patients when compared with prophylaxis of patients only. Even when the number of infections prevented in HCWs was included, the number of daily doses needed to prevent 1 infection was higher than the number of daily doses needed when prophylaxis was given to patients only. This prediction might be of use for the evaluation of influenza prevention guidelines for nursing homes. Currently, the Dutch guideline for prevention of influenza in nursing homes recommends postexposure prophylaxis for both patients and HCWs (*1*). CDC recommends prophylaxis to nonvaccinated HCWs only, or in case of a mismatch between the vaccine strains and the circulating virus strains, to all HCWs (*2*). Although the latter strategy is expected to be more efficient, the effect on infection attack rates among patients will be less extensive than with prophylaxis of all HCWs. In the postexposure strategy, 1,388 doses of oseltamivir were given to HCWs for every additional prevented infection in a patient. This number was very high compared with the 7 HCW vaccinations needed to prevent 1 infection in patients observed in our previous study (*18*). Therefore, protection of patients by reducing the number of infections in HCWs seems to be more efficiently obtained by increasing vaccine administration among HCWs than by including them in prophylaxis strategies.

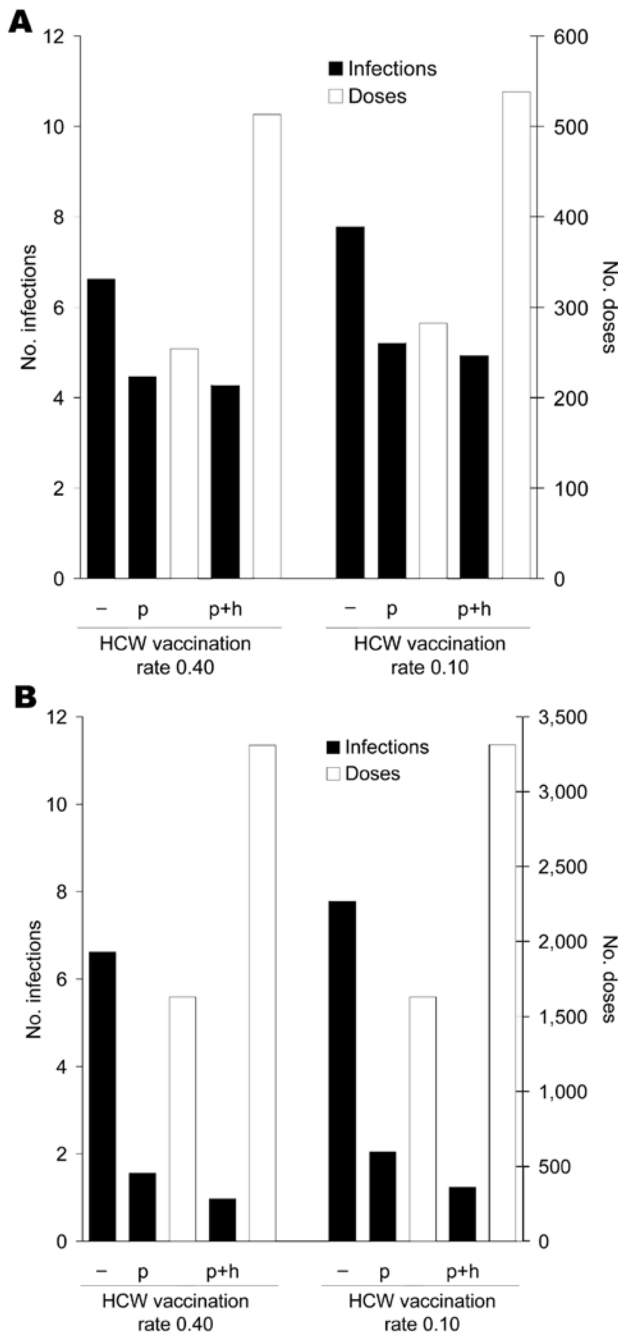

Figure 6. A) Average number of influenza virus infections among patients and B) average number of administered doses of oseltamivir in a 30-bed nursing home department during 1 influenza season. For the postexposure and continuous prophylaxis strategies, results are shown for prophylaxis of patients only (p) and of both patients and healthcare workers (HCWs) (p+h) and compared with a control setting without prophylaxis (–). HCW vaccination rates of 0.4 and 0.1 are considered.

Our study suggests that the selection pressure for resistance is lower for postexposure than for continuous prophylaxis. Moreover, the efficiency of postexposure prophylaxis appears to be less sensitive to the level of resistance than that of continuous prophylaxis. During the 2007–08 influenza season, the prevalence of oseltamivir-resistant influenza A (H1N1) strains in Europe increased from <1% in previous years (*11*) to 25% on average, with a national prevalence ranging from 2.5% in Spain up to 66% in Norway (*36*). During the 2008–09 influenza season almost all influenza A (H1N1) strains were oseltamivir resistant (*12*). Oseltamivir use in Europe was low in both years and, in the absence of an apparent selection pressure for resistance, predicting whether resistance will disappear, persist, or increase next season is difficult. Our findings indicate that increasing resistance should be included in the decision-making process for prevention of influenza in healthcare settings. Use of other antiviral agents that are not as associated with resistance should be considered as an alternative

prevention strategy (*37*). Household studies suggest that prophylaxis with zanamivir, for example, can give similar results as prophylaxis with oseltamivir (*4*). However, zanamir prophylaxis should be studied in more detail in the nursing home population. Future modeling studies should also address other relevant issues such as the use of combination or cycling therapy approaches (*38*) to retain the protection offered by current antiviral drugs.

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Nontuberculous Mycobacteria Infections and Anti–Tumor Necrosis Factor-α Therapy

Kevin L. Winthrop, Eric Chang, Shellie Yamashita, Michael F. Iademarco, and Philip A. LoBue

Patients receiving anti–tumor necrosis factor-α (anti–TNF-α) therapy are at increased risk for tuberculosis and other granulomatous diseases, but little is known about illness caused by nontuberculous mycobacteria (NTM) in this setting. We reviewed the US Food and Drug Administration MedWatch database for reports of NTM disease in patients receiving anti–TNF-α therapy. Of 239 reports collected, 105 (44%) met NTM disease criteria. Median age was 62 years; the majority of patients (66, 65%) were female, and most (73, 70%) had rheumatoid arthritis. NTM infections were associated with infliximab (n = 73), etanercept (n = 25), and adalimumab (n = 7); most patients were taking prednisone (n = 68, 65%) or methotrexate (n = 58, 55%) concurrently. *Mycobacteria avium* (n = 52, 50%) was most commonly implicated, and 9 patients (9%) had died at the time their infections were reported. A high rate of extrapulmonary manifestations (n = 46, 44%) was also reported.

Nontuberculous mycobacteria (NTM) are a large, diverse group of environmental organisms ubiquitous in water and soil (*1*). They cause a variety of diseases in humans, notably severe, protracted lung disease in patients with underlying lung disorders. Conditions such as bronchiectasis, emphysema, previous tuberculosis (TB) or other lung infections, cystic fibrosis, rheumatoid arthritis, and other chronic diseases with pulmonary manifestations can predispose a person to NTM pulmonary disease (*2*). In addition to lung infections, NTM cause skin and soft tissue infections, lymphadenitis (predominantly in young children), and disseminated disease in HIV-infected patients or others with severely compromised immune systems. The immunologic mechanism and related dysfunction that predispose persons to NTM disease are largely unknown, although defects in interleukin-12 or interferon-γ production are known to increase the risk for disseminated NTM disease in humans (*3*).

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Although the epidemiology of NTM disease is not well described, the belief that these infections are increasing in prevalence, particularly among women, is widespread (*2*). Assessment of the epidemiology of these infections may be increasingly useful because newer forms of biologic, immunosuppressive therapies have become widely used for treating patients with rheumatoid arthritis, Crohn disease, and other autoimmune inflammatory conditions. Many of these conditions are associated with lung manifestations known to be associated with NTM pulmonary infections (*2*).

To date, TB and NTM infections and concurrent biologic therapies that inhibit tumor necrosis factor-α (TNF-α) have been reported. These therapies include infliximab (Remicade; Centocor, Malvern, PA, USA), etanercept (Enbrel; Immunex, Seattle, WA, USA), and adalimumab (Humira; Abbott Biotechnology, Abbott Park, IL, USA), which have been approved in the United States and elsewhere to treat patients with rheumatoid arthritis and selected other autoimmune inflammatory diseases (*4*). Because TNF-α is integral to granuloma generation and maintenance (*5*,*6*), patients using these agents are at increased risk for granulomatous infections, including activation of latent TB infection (*7*,*8*).

The US Food and Drug Administration (FDA) postmarketing surveillance system (MedWatch) (www.fda.gov/medwatch) collects voluntary reports of adverse drug events from physicians. The most recent review of this system in 2004 for reports of granulomatous infections that

occurred during TNF-α blockade found that mycobacteria disease was more common than other granulomatous diseases; TB was reported 5–10× more frequently than NTM, dimorphic fungi, and other intracellular infections in this setting (*7*). (Although this program does not specifically target participation outside the United States, it also includes nondomestic case reports.) Subsequently, much attention has been focused on prevention of TB in patients who are using anti–TNF-α agents. To date, little is known regarding the types and relative frequencies of NTM infections that occur in such patients.

We recently conducted a survey among infectious disease physicians within the Emerging Infections Network of the Infectious Diseases Society of America (IDSA). This survey suggested that cases of NTM disease associated with anti–TNF-α therapy occur twice as frequently as cases of TB associated with anti–TNF-α therapy in the United States (*9*). NTM infections are likely underreported to the FDA, relative to TB, for a variety of reasons (*10*). NTM disease is generally insidious, sometimes difficult to diagnose, and is not reportable to health authorities. Accordingly, we reviewed the MedWatch database for NTM reports through January 1, 2007, to evaluate whether these case reports met clinical case criteria, to describe their clinical spectrum and outcome, and to evaluate the relative reporting frequency of cases among the different anti–TNF-α agents now in widespread use.

Methods

At our request, FDA searched its MedWatch database for NTM cases reported among patients using adalimumab, infliximab, or etanercept through January 1, 2007. Using the search terms nontuberculous mycobacteria infections, atypical mycobacteria infections, and leprous infections, FDA compiled all domestic and foreign reports with keywords matching at least 1 of these search terms. The reports were redacted to remove any identifying information and sent to us for review. Because we sought to review only cases involving environmental NTM, cases caused by *Mycobacterium leprae* (leprosy) were excluded (n = 5). We reviewed all reports and extracted the following data: etiologic organism, anti–TNF-α drug, and concomitant immunosuppressive drugs used at the time of infection diagnosis, clinical and radiographic data, death or hospitalization during infection treatment, and time between beginning drug treatment and infection diagnosis.

To define pulmonary disease, we used the American Thoracic Society (ATS)/IDSA case definition in which patients must have ≥2 sputum samples with NTM (or a single isolate in the case of bronchoscopy specimens) coexistent with appropriate radiographic findings and symptoms (*2*). If cases met these criteria they were deemed confirmed. If not enough information was provided for the case definition to be considered, they were deemed probable or unknown based on the consensus opinion of 3 physicians from the Division of Infectious Diseases at Oregon Health and Sciences University. For extrapulmonary disease, patients with NTM isolated from normally sterile sites were considered to have confirmed cases. Disease reports that included infection with *M. tuberculosis* or organisms other than mycobacteria were excluded.

Data Analysis

All data were entered into Epi Info version 3.4.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Two-by-two comparisons among variables were made by using Mantel-Haenszel odds ratios (ORs) and Fisher exact test p values. We did not attempt to calculate or compare NTM incidence rates among different anti–TNF-α products because the MedWatch database does not include drug exposure denominator data.

Results

There were 239 reports of NTM infection in patients who were receiving anti–TNF-α therapy. Most reports were for patients receiving infliximab (n = 174, 75%), followed by etanercept (n = 41, 17%), and adalimumab (n = 19, 8%). One case was reported in 1999 (patient used etanercept); numbers of reported infections among those using each product increased in 2001 and thereafter. Reported cases among those using each of the 3 drugs were highest in 2005 (Figure 1). Of these reports, only 76 (32%) met either ATS/IDSA pulmonary disease criteria or our case definition for extrapulmonary disease. An additional 29 (12%) cases were judged to be probable cases, but the reports did not contain enough clinical or radiographic information to determine whether patients met ATS/IDSA NTM disease criteria. In other instances, the reports were either clearly not of cases of NTM disease (n = 27, 11%) or could not be determined (n = 95, 40%) because of a lack of microbiologic data, unclear reporting, or duplicate reports (n = 12, 5%). Of the 244 reports, 76 (31%) were from outside the United States (Europe, n = 40; Japan, n = 21; Canada, n = 4; Israel, n = 1; South Africa, n = 1; not specified, n = 9). Of patients with confirmed and probable cases (n = 105), a similar proportion (n = 35, 33%) were from outside the United States; most of these were from Europe (n = 15) or Japan (n = 12).

Of the 105 confirmed or probable cases, most were in women (n = 66, 65%), and the median age was 63 years (range 20–90 years). The anti–TNF-α agents reported for these patients included infliximab (n = 73, 69%), etanercept (n = 25, 24%), and adalimumab (n = 7, 7%). *M. avium* was the most common etiologic organism reported (n = 52, 49%), followed by rapidly growing mycobacteria (n = 20, 19%), and *M. marinum* (n = 8, 8%) (Figure 2). Nine patients (9%) had died by the time their case was reported,

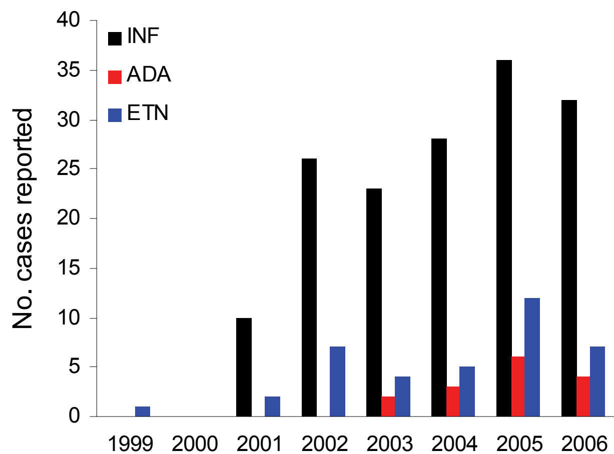

Figure 1. Case reports of nontuberculous mycobacteria in patients using antitumor necrosis factor-α (TNF-α) therapy, US Food and Drug Administration MedWatch database, 1999–2006. Cases are reported by each full year of data reporting for each anti-TNF agent. Reported cases for all agents were most numerous in 2005. INF, infliximab; ADA, adalimumab; ETN, etanercept.

and 64 (61%) had NTM adverse events that resulted in hospitalization. The most common underlying medical indication for anti–TNF-α therapy was rheumatoid arthritis (n = 73, 75%), followed by other inflammatory diseases (Table 1). Sixty-eight (65%) patients received concomitant prednisone, and 58 (55%) received methotrexate at the time of their report. Twenty-five (24%) patients reportedly had ≥1 of the following conditions: bronchiectasis (n = 5, 5%), chronic obstructive pulmonary disease (n = 11, 10%), diabetes mellitus (n = 5, 5%), and rheumatoid lung (n = 4, 4%). Median time between anti–TNF-α agent start date and infection diagnosis was available for only 68 (65%) of the patients. For adalimumab (n = 5), the interval was 18 weeks (range 4–94 weeks), for etanercept (n = 22) it was 35 weeks (range 0–288 weeks), and for infliximab (n = 41) it was 43 weeks (range 2–200 weeks).

The pulmonary region (n = 59, 56%) was the most frequently reported site of disease; the remainder of infections were extrapulmonary or disseminated (Table 2). Compared with patients with extrapulmonary NTM disease, patients with pulmonary NTM disease were more likely to have underlying rheumatoid arthritis (OR 3.6, 95% confidence interval [CI] 1.5–8.8, p<0.01) and more likely to be infected with *M. avium* (OR 11.0, 95% CI 4.4–27.9, p<0.01). Reported cases of pulmonary NTM disease were also more likely to be in female patients (OR 2.3, 95% CI 1.0–5.3, p = 0.04) (Table 3). After we adjusted for differences in sex in a stratified analysis, rheumatoid arthritis remained associated with pulmonary disease (adjusted OR 3.4, 95% CI 1.4–8.3, p = 0.01). There were no significant differences between the proportions of pulmonary disease, disseminated disease, death, or etiologic organisms reported for patients who used infliximab and those who used etanercept. Overall, infliximab users were more likely to be using methotrexate (OR 3.0, 95% CI 1.2–8.0, p = 0.02) than were etanercept users (63% vs. 36%). Prednisone use did not differ between the 2 groups; 50 (68%) of infliximab-treated patients and 14 (56%) of etanercept-treated patients were using prednisone at the time of their report (p = 0.19).

For reported NTM adverse events that did not meet either ATS/IDSA case criteria or our probable case designation, similar analyses were performed. These persons were similar to persons with cases that met confirmed or probable case definitions. They did not differ with regard to sex, underlying medical condition, anti–TNF-α drugs used, mortality rate, or the ratio of pulmonary to extrapulmonary disease manifestations. However, these patients were less likely to be using concomitant prednisone (65% for patients with cases vs. 43% for patients without cases; OR 2.5, 95% CI 1.4–4.2, p<0.01) or concomitant methotrexate (55% for patients with cases vs. 37% for patients without cases; OR 2.1, 95% CI 1.3–3.6, p<0.01).

Discussion

We reviewed the FDA MedWatch database for reports of NTM infections in patients using anti–TNF-α therapies. We found several hundred reported events; most had occurred in the 5 years since the previous review of this database (*7*). Our study scrutinized clinical features of NTM infections reported in the database and characterized the

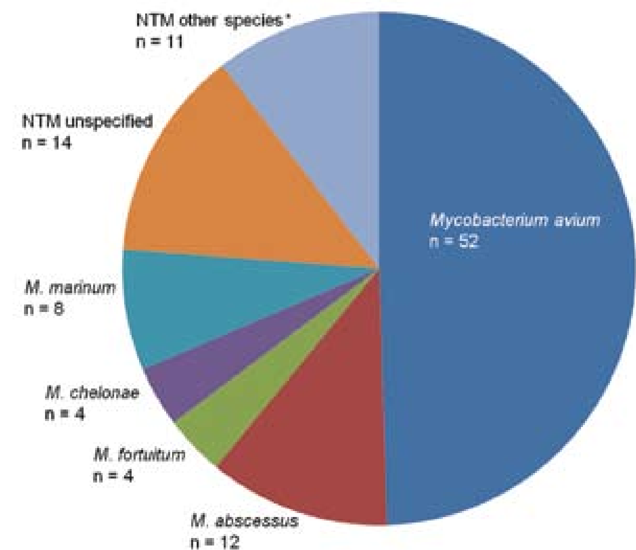

Figure 2. Reported causes of 105 confirmed and probable nontuberculous mycobacteria (NTM) infections associated with antitumor necrosis factor-α agents, US Food and Drug Administration MedWatch database, 1999–2006. *Other species include *Mycobacterium kansasii* (n = 3), *M. xenopi* (n = 3), *M. haemophilum* (n = 2), and *M. mucogenicum* (n = 1).

Table 1. Reported diseases associated with anti–TNF-α therapy and therapy implicated for 105 cases of NTM disease, US Food and Drug Administration MedWatch database, 1999–2006*

Disease	Infliximab	Etanercept	Adalimumab
Rheumatoid arthritis (n = 73)	51	17	5
Ankylosing spondylitis (n = 5)	1	3	1
Psoriasis (n = 4)	2	2	0
Crohn disease (n = 8)	8	0	0
Other (n = 15)†	11	3	1

*TNF-α, tumor necrosis factor-α; NTM, nontuberculous mycobacteria.
†Includes Wegener granulomatosis (n = 2), dermatomyositis (n = 1), uveitis (n = 1), juvenile rheumatoid arthritis (n = 1), and not reported (n = 10).

microbiologic and clinical features of NTM disease in a substantial number of patients who were receiving anti–TNF-α therapy. Similar to patients with anti–TNF-α–associated TB, patients frequently had extrapulmonary disease and most reported use of infliximab. *M. avium* was the most common etiologic agent reported; it was associated with pulmonary disease in elderly, female patients with rheumatoid arthritis.

To date, much of the available information regarding opportunistic infectious complications of anti–TNF-α therapy in the United States has been derived from the passive MedWatch surveillance system, a voluntary system subject to underreporting. The number of NTM reports associated with anti–TNF-α therapy has increased substantially in this database since publication of the initial MedWatch analysis by Wallis et al (*7*), in which the authors reviewed cases reported through September 2002 and before FDA approval of adalimumab (etanercept and infliximab gained FDA approval in the fall of 1998, and adalimumab gained approval at the end of 2002). Unlike that review, we examined clinical details of the cases in these reports to ascertain whether such patients actually met disease criteria. Because the respiratory tract is a nonsterile body site, pulmonary specimens can yield environmental organisms such as NTM in healthy persons. Accordingly, ATS and IDSA collaboratively published clinical criteria that must be met to determine whether a patient with pulmonary NTM isolates actually has NTM disease (*2*). Although we were able to apply this definition in some instances, our experience highlighted one of the limitations of the FDA MedWatch database: frequently a paucity of pertinent clinical details were reported. Accordingly, we found a high percentage of cases in MedWatch reports that did not meet disease criteria either because information was not reported or because some patients simply did not have reported pathologic conditions or other clinical criteria consistent with disease.

In our review, most patients with anti–TNF-α–related NTM were elderly women with rheumatoid arthritis. This finding is probably explained by several factors. First, rheumatoid arthritis is the most prevalent autoimmune inflammatory diseases for which anti–TNF-α therapies are approved (*4*). Approximately 0.5%–1.0% of the US population has rheumatoid arthritis (*11*), and >40% of rheumatoid arthritis patients have been treated with these therapies (*12*). Second, rheumatoid lung disease, which can include bronchiolitis and bronchiectasis, develops in ≈10% of these patients. These and other lung disorders are known to increase the risk for NTM disease. Third, the age and sex distribution of patients in this report mirrors that for patients with pulmonary NTM disease and rheumatoid arthritis independent of anti–TNF-α therapy. NTM disease and rheumatoid arthritis are more common in women >50 years of age (*2*,*11*). Fourth, a high percentage of patients who receive anti–TNF-α therapy are known to have serious medical conditions, some of which might increase the risk for NTM disease (*13*). In our series, ≥15% of patients were reported to have chronic obstructive pulmonary disease or bronchiectasis, which are known risk factors for pulmonary NTM disease.

Nearly half the patients in our series had extrapulmonary disease. In patients with TB, TNF inhibition is known to increase the risk for extrapulmonary and disseminated disease manifestations (*14*,*15*). Similar proportions of such disease were reported in our series of NTM patients. Similar to reports of TB, reports of NTM disease in the database were more numerous for persons who used infliximab. We were not able to access treatment start data for each of these agents because such information is proprietary, although etanercept and infliximab have been used more extensively than the more recently approved adalimumab (*4*).

Although no study has directly compared the risk for NTM disease between users of infliximab and users of other anti–TNF-α agents, use of infliximab may pose greater risk for NTM disease. If true, the risk could be caused by the drug itself or differences in the characteristics of patients given infliximab relative to users of the other anti–TNF-α compounds (*4*,*7*,*8*). For example, in our current series, infliximab users were more likely to be concomitantly using methotrexate at the time of diagnosis. The FDA database is limited because physician case reports are voluntary and

Table 2. Sites of infection for 105 reported anti–TNF-α therapy–associated cases of NTM disease, US Food and Drug Administration MedWatch database, 1999–2006*

Site	No. (%) cases
Pulmonary region	59 (56)
Skin or soft tissue	27 (26)
Bone or joint	10 (9)
Disseminated	8 (8)
Eye	1 (1)

*TNF-α, tumor necrosis factor-α; NTM, nontuberculous mycobacteria.

Table 3. Characteristics of 105 pulmonary and nonpulmonary anti–TNF-α therapy–associated cases of NTM disease, US Food and Drug Administration MedWatch database, 1999–2006*

Characteristic	Pulmonary (n = 59), no. (%)	Extrapulmonary (n = 46), no. (%)
Mycobacterium avium	43 (73)	9 (20)†
RGM	6 (10)	15 (33)†
Age, y	61	63
Female patient	41 (73)	25 (54)†
Rheumatoid arthritis	48 (81)	25 (54)†
Infliximab	40 (68)	33 (72)
Etanercept	13 (22)	12 (26)

*TNF-α, tumor necrosis factor-α; NTM, nontuberculous mycobacteria; RGM, rapidly growing mycobacteria.
†p<0.05 for comparison of pulmonary disease and extrapulmonary disease.

no denominator data with regard to drug exposure are collected. For these reasons, we did not attempt to calculate or compare rates of NTM disease among various anti–TNF-α agents or among reporting years of this study. Thus, firm conclusions cannot be made regarding the comparative risk for use of these agents.

The US Centers for Disease Control and Prevention, the British Thoracic Society, and others have published recommendations describing the role of latent TB screening and treatment before use of anti–TNF-α therapy (*16–19*). Although screening can decrease the risk for TB in such patients (*20*), with the incumbent risk of illness and disease transmission, it is less clear what should be done to prevent NTM disease occurrence or progression in patients who use these compounds. Given the long median periods between the start of drug use and disease diagnosis within this case series, for many of these patients, NTM disease likely had been newly acquired during anti–TNF-α therapy.

Alternately, given the slow progression and insidious nature of pulmonary NTM disease, some of the patients in this series likely had existing but undiagnosed pulmonary NTM disease before starting their anti–TNF-α therapy. This likelihood raises the question whether patients should be screened for NTM disease before initiating anti–TNF-α therapy. According to published guidelines, all patients in the United States should be screened for latent TB infection before receiving therapy, a screening that includes a chest radiograph. Although abnormalities on such a radiograph could trigger sputum evaluation for TB and NTM, a chest radiograph is not sufficiently sensitive for detecting bronchiectasis or other lung abnormalities associated with NTM disease. If not previously obtained, clinicians could consider obtaining a noncontrast chest computed tomography scan before administering therapy to any patient with a history of bronchiectasis or other architectural lung disease, chronic unexplained cough, or abnormalities noted on their screening chest radiograph suggestive of NTM disease (e.g., reticulonodular infiltrate). If chest computed tomography suggests possible NTM disease, further pulmonary testing with sputum or other samples obtained by bronchoscopy would be indicated to rule out active NTM disease before initiating anti–TNF-α therapy (*21*).

Because most physicians would be reluctant to prescribe anti–TNF-α therapy to patients with known or obvious active infections, we suspect that most extrapulmonary infections (primarily soft tissue infections) in our series likely developed in patients after they began anti–TNF-α therapy. It is not clear what risk factors predisposed patients to these infections in our series and unlikely that such complications during therapy can be screened for or prevented.

There are increasing numbers of case reports of pulmonary NTM disease for patients using anti–TNF-α therapy (*9*,*22*,*23*). In some of these patients, pulmonary NTM disease progressed while they were receiving anti–TNF-α therapy, despite aggressive antimycobacterial treatment. Most patients in our series were hospitalized for their infections, and although <10% died by the time their case was reported, we suspect that follow-up beyond the time of the event report would indicate that a greater number of deaths occurred during therapy. Given the serious illnesses and deaths caused by these infections, whether anti–TNF-α therapy can be safely continued during antimycobacterial therapy is not clear. It is also not evident when it would be safe to reinstitute anti–TNF-α therapy in such patients.

We believe that our review of the FDA MedWatch database suggests that NTM represents a serious and severe granulomatous complication that can occur during anti–TNF-α therapy. However, lack of denominators for persons treated with these drugs precludes calculation of rates, and without a control group, it is not possible to definitively conclude that anti–TNF-α therapy causes or is associated with NTM disease. This finding is further complicated by potential confounders such as other immunosuppressive therapy and predisposing conditions among the population being studied. For example, epidemiologic features of patients who use anti–TNF-α drugs are similar to those who are at risk for NTM pulmonary disease in the absence of these drugs (i.e., elderly women, many of whom who have underlying lung disease). Nevertheless, our findings are useful because to date, the possibility of NTM disease has been underreported for patients who use anti–TNF-α therapies. Our findings highlight that these cases are occurring in such patients, often with devastating outcomes. Future population-based studies are necessary to determine

risks for such complications and to define preventive and therapeutic strategies for such patients. For now, clinicians should remain vigilant for these and other types of serious infections that occur in patients using these compounds.

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Nontuberculous Mycobacteria–associated Lung Disease in Hospitalized Persons, United States, 1998–2005

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The prevalence and trends of pulmonary nontuberculous mycobacteria (NTM)–associated hospitalizations in the United States were estimated using national hospital discharge data. Records were extracted for all persons with a pulmonary NTM International Classification of Diseases code (031.0) hospitalized in the 11 states with continuous data available from 1998 through 2005. Prevalence was calculated using US census data. Pulmonary NTM hospitalizations (031.0) increased significantly with age among both sexes: relative prevalence for persons 70–79 years of age compared with those 40–49 years of age was 15/100,000 for women (9.4 vs. 0.6) and 9/100,000 for men (7.6 vs. 0.83). Annual prevalence increased significantly among men and women in Florida (3.2%/year and 6.5%/year, respectively) and among women in New York (4.6%/year) with no significant changes in California. The prevalence of pulmonary NTM–associated hospitalizations is increasing in selected geographic areas of the United States.

Clinic- and laboratory-based studies since the 1980s have shown an increased prevalence of persons with nontuberculous mycobacterial (NTM) pulmonary disease (*1,2*) with a predominance of women >60 years of age who have no underlying risk factors (*3–5*). NTM comprise a multispecies group of environmental organisms living in soil as well as in treated and untreated water sources. These mycobacteria were first identified as human pathogens in the 1950s when 1%–2% of patients in tuberculosis (TB) sanitaria did not respond to traditional TB treatment. Their illnesses were caused by organisms that were not *Mycobacterium tuberculosis*. These patients tended to be older than those having TB, were more likely to be white, and to have underlying lung disease (*6,7*).

The success of TB elimination efforts has resulted in a continued decline in the incidence and prevalence of tuberculosis in the United States. In 2007, the incidence of TB in the United States was 4.4/100,000 population, and 2.1/100,000 among US-born persons, the lowest rates since reporting began in 1953 (*8*). The apparent increase in NTM disease has occurred during the same period that TB has been declining. Although NTM are not transmissible, the diseases they cause may greatly affect public health and medical care resources. In some state health departments, findings of an acid-fast bacilli, indicative of mycobacteria, are reportable (*9*), and may trigger a public health investigation with substantial expenditure of resources until species identification is confirmed.

Population-based surveys conducted during 1981–1983 estimated the prevalence of pulmonary NTM disease at 1–2 cases/100,000 persons in the United States (*10*). A more recent retrospective analysis from Ontario, Canada found an average annual increase of 8.4% for the isolation prevalence of NTM at the Ministry of Health Mycobacterial Laboratory between 1997 and 2003 (*11*). Similar trends have been noted in other areas of the world (*12–16*). However, no current US nationally representative data exist regarding the prevalence of pulmonary disease associated with NTM. Furthermore, information is limited regarding risk factors associated with the disease. Our study describes the prevalence, demographic characteristics, and trends of pulmonary NTM–associated hospitalizations during 1998–2005.

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Methods

Data Source and Study Population

We used data from the Agency for Healthcare Research and Quality's Healthcare Cost and Utilization Project (HCUP), specifically the State Inpatient Databases (SID). The SIDs provide record-level data, without personal identifiers, on nearly 100% of community hospital discharges in participating states. Records were included for hospitalizations that had an International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM), code associated with pulmonary NTM (031.0) as a primary or secondary discharge diagnosis. The study population included all records for persons hospitalized with pulmonary NTM as a primary or secondary diagnosis in the 11 states participating in HCUP (Arizona, California, Colorado, Florida, Illinois, Iowa, Massachusetts, New Jersey, New York, Washington, and Wisconsin) during the years specified (*17*). These states represented 42% of the US population during the study period.

Data Analysis

Data elements available in the HCUP dataset included year of hospitalization, age when hospitalized, sex, state where hospitalization occurred, type of NTM infection (pulmonary, disseminated, cutaneous, unspecified, or other) and up to 29 possible secondary diagnoses. No information on mycobacterial species is available in this dataset. Because NTM is known to be a common opportunistic infection among people with AIDS, particularly before the widespread availability of combination antiretroviral medications (*18*), we limited our analysis to non-AIDS NTM using the code for HIV/AIDS (042), which indicates hospitalizations where AIDS was known to be an underlying illness. Additionally, we restricted our analysis to the 1998–2005 study period to avoid misclassification among types of NTM because the ICD-9-CM code for disseminated NTM was introduced in 1997. Before implementation, hospitalizations associated with disseminated NTM may have been included in the 4 other NTM categories (pulmonary, cutaneous, unspecified, other). We examined prevalence trends in pulmonary NTM by age and sex and described the most frequently associated underlying illnesses. To analyze the most frequent secondary underlying illnesses, we grouped the following conditions/codes as chronic obstructive pulmonary disease (COPD): obstructive chronic bronchitis with and without exacerbation (ICD-9-CM 491.21, 491.22); emphysema not elsewhere classified (492.8); chronic obstructive asthma (493.20); and chronic airway obstruction not elsewhere classified (496).

To estimate prevalence of hospitalizations, we used age- and sex-specific US census data for participating states during the study period; both individual years and midpoint population (average of 2001–2002 census population estimates) were used as appropriate. Although prevalence more often refers to the number of persons with a condition in a population at a determined time, we use it here to describe the number of hospitalizations among persons with NTM. To compare prevalence among states, we calculated age- and sex-adjusted rates using the US census 2000 reference population; χ^2 tests were used to determine significance among groups at a significance level of $p<0.05$. Data analyses were calculated using SAS 8.0 and 9.1 (SAS, Cary, NC, USA) and EpiInfo version 3.4 (Centers for Disease Control and Prevention, Atlanta, GA, USA). The average annual percent increase in prevalence and the significance of these trends were estimated by use of Poisson regression models. Prevalence was modeled as a function of time, with prevalence as the dependent variable and time as the independent variable; Pearson's scale factor was used to account for overdispersion. Model fit was assessed by the value of the scaled Pearson χ^2, which equals the value divided by the degrees of freedom (value/DF); a value of 1 indicates that the model is a good fit. Wald 95% confidence limits were estimated as well. For modeling trends by age and sex for all 11 states combined, separate models were fit for each age and sex group. Prevalence was defined as the number of observed cases in a given age and sex group for each year as the numerator and the estimated annual population for the specified age and sex group for that year as a denominator, modeled in SAS as the observed count data with a log population offset. For estimation of average annual percent change for men and women in 3 states (California, Florida, and New York), age-adjusted prevalence was the dependent variable, modeled as expected number of cases with a log population offset; time (year) was the independent variable. Models were fit separately for men and women. A constant term was included as part of these equations.

Results

From 1998 through 2005, a total of 23,216 pulmonary NTM–associated hospitalizations were identified, of which 16,475 (71%) were non-AIDS related. Of these, 9,439 (57%) were women and 8,997 (55%) were among persons >70 years of age. The proportion of pulmonary NTM hospitalizations among persons >70 years of age varied by sex: 45% of men and 62% of women were >70 years of age. For both sexes, the average annual prevalence of non-AIDS pulmonary NTM-associated hospitalizations increased with age, but among persons >70 years of age, the relative prevalence was higher for women than for men. The relative prevalence for persons 70–79 years of age compared with those 40–49 years of age was 15-fold higher for women (9.4/100,000 vs. 0.6/100,000), and 9-fold higher for men (7.6/100,000 vs. 0.8/100,000); similar relative differences were seen in the ≥80–95-year age group (Figure 1).

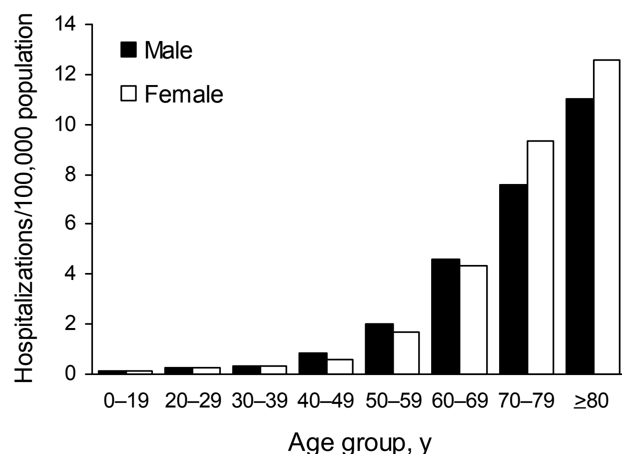

Figure 1. Average annual prevalence of non-AIDS pulmonary nontuberculous mycobacteria–associated hospitalizations by age group and sex, Healthcare Cost and Utilization Project state inpatient databases, USA, 1998–2005.

To study trends within the older age groups over time, we restricted our analysis to the >50-year age group and examined trends during the period 1998–2005 for men and women separately. Among men, the prevalence decreased significantly among the 50–59-year age group (2.7% per year; p = 0.011 by χ^2 test), and increased significantly among men 70–79 years of age (5.3% per year; p = 0.0001 by χ^2 test); no significant changes were evident in the other age groups (Table 1; Figure 2). Among women, the prevalence increased significantly for women 60–79 years of age with an average annual increase of 4.6% (p = 0.0069 by χ^2 test) among women 60–69 years of age and 5.5% (p<0.0001) among women 70–79 years of age (Table 1; Figure 3).

Table 1. Results of Poisson regression modeling for trends in pulmonary NTM, HCUP-SID, USA, 1998–2005*

Group	Sex	Annual % change	Wald 95% CI	p value
State				
California	M	–1.5	–4.0–1.3	0.24
	F	–1.5	–3.3–0.3	0.10
New York	M	–2.7	–5.9–0.54	0.10
	F	4.5	1.1–8.2	0.0097
Florida	M	3.2	0.76–5.7	0.010
	F	6.3	3.1–9.9	0.00010
Age group, y				
50–59	M	–2.7	–4.8 to –0.61	0.0118
	F	0.14	–1.4–1.7	0.8629
60–69	M	–1.5	–3.1–0.01	0.0490
	F	4.6	1.2–8.0	0.0069
70–79	M	5.3	2.5–8.2	0.0001
	F	5.5	2.9–8.2	<0.0001
≥80	M	0.65	–3.0–4.4	0.7327
	F	2.5	–0.62–5.7	0.1177

*NTM, nontuberculous mycobacteria; HCUP, Healthcare Cost and Utilization Project; SID, state inpatient databases; CI, confidence interval. The scaled Pearson χ^2 (value/df) was 1 for all items.

We studied trends by geographic area and chose the 3 states with the greatest numbers of annual observations during the study period to ensure robust trend analysis. California, Florida, and New York represent unique regions in the United States and overall comprised 62% of NTM hospitalizations in the 11 states included in the analysis. To compare prevalence across these states, we calculated age-adjusted prevalence for men and women. Among both sexes, prevalence was highest in Florida; a significant annual increase was seen from 1998 through 2005. Among men, the average annual age-adjusted prevalence in Florida was 2.1/100,000 population, with a significant increase from 2.1 to 2.4 (3.2% increase/year); the average annual prevalence in California was 1.3 and for New York 1.4, with no significant change during the study period (Table 1, Figure 4). Among women, the average annual age-adjusted prevalence in Florida was 2.4/100,000; an increase of 1.8 in 1998 to 2.8 in 2005 (average 6.5%/year) was identified. For women in New York, annual prevalence increased significantly from 1.4/100,000 to 1.9/100,000 (4.6%/year); no significant change was detected in California (Table 1; Figure 5.)

Among the 16,475 non-AIDS pulmonary NTM–associated hospitalizations during 1998–2005, a total of 5,148 (31%) hospitalizations had pulmonary NTM as a primary diagnosis. The other leading primary diagnoses were pneumonia (7%), obstructive chronic bronchitis with acute exacerbation (5%), acute respiratory failure (2%), congestive heart failure (1.4%), and bronchiectasis (1.3%). No other single primary diagnosis comprised >1% of the primary diagnosis (Table 2). We analyzed secondary diagnoses to identify associated underlying illnesses for hospitalizations where pulmonary NTM was the primary diagnosis. Hospitalizations could be associated with combinations of up to

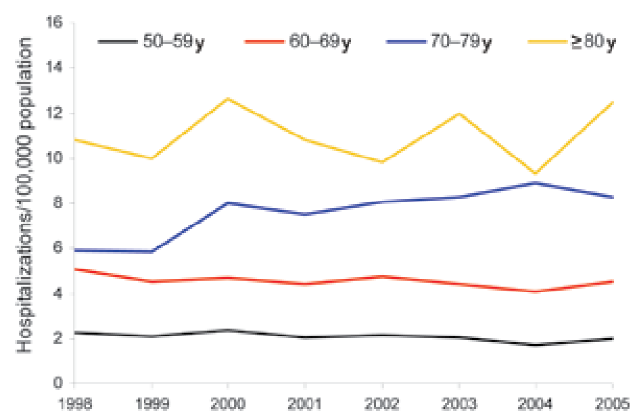

Figure 2. Prevalence of non-AIDS pulmonary nontuberculous mycobacteria–associated hospitalizations among men by age group and year, Healthcare Cost and Utilization Project (HCUP) state inpatient databases, USA, 1998–2005.

Table 2. Primary diagnoses, non-AIDS pulmonary NTM-associated hospitalizations, HCUP-SID, USA, 1998–2005*

ICD-9 code	Primary diagnosis	No. (%)
0310	Pulmonary NTM	5,148 (31.25)
482	Pneumonia	1,156 (7.01)
49121	Obstructive chronic bronchitis with acute exacerbation	821 (4.98)
51881	Acute respiratory failure	392 (2.38)
4280	Congestive heart failure, unspecified	225 (1.37)
4941	Bronchiectasis with acute exacerbation	216 (1.31)
2765	Volume depletion	196 (1.19)
515	Postinflammatory pulmonary fibrosis	186 (1.13)
5070	Aspiration pneumonia caused by inhalation of food/vomitus	176 (1.07)
	Other primary diagnosis <1% of population	7,959 (48.3)

*NTM, nontuberculous mycobacteria; HCUP, Healthcare Cost and Utilization Project; SID, state inpatient databases; ICD-9, International Statistical Classification of Diseases, Revision 9.

29 secondary diagnoses, such that the sum of the underlying illnesses identified in any of those fields could add up to >100%. Of these, preexisting cardiovascular conditions, such as hypertension and atrial fibrillation, were most common (47%). Structural lung diseases, such as COPD (34%) and bronchiectasis (15%), were also common (Table 3).

To identify distinct patterns of underlying illnesses by sex, we analyzed the age and sex distribution for selected underlying illnesses among hospitalizations where non-AIDS pulmonary NTM was the primary diagnosis. For hospitalizations with secondary diagnoses related to COPD, the prevalence of hospitalization was higher for men than for women in all age groups, ranging from 2-fold in the 50–59-year age group, to 1.3× greater in the ≥70-year age group (Figure 6). Among persons hospitalized with bronchiectasis as a secondary diagnosis, the prevalence was consistently higher in women than in men in all age groups, ranging from 3-fold higher in the 50–59-year age group to 4-fold in the 70–79-year age group (Figure 7).

Discussion

We present nationally representative population-based prevalence estimates for pulmonary NTM disease, age-specific prevalence estimates for the United States, and prevalence data available on hospitalizations associated with pulmonary NTM disease. Estimates of this type were reported in 1987 (*10*). In addition, we demonstrate an increasing prevalence of pulmonary NTM-associated hospitalizations among both men and women in Florida, different than that for California and New York, and identify regional differences in disease activity as has been previously suggested (*19*).

The increased prevalence among those >50 years of age indicates a disease process with onset in the fifth or sixth decade of life, either as a result of an underlying genetic susceptibility or onset of underlying illnesses (e.g., COPD). Although our data are derived from hospitalizations associated with NTM rather than outpatient visits, which might be more likely to occur earlier in the disease course, data from outpatient settings show a similarly increased disease effect in the >50 year-old population (*1,3,5*). Because prevalence is a function of disease incidence and duration, the highest prevalence in the oldest age groups likely reflects new cases as well as the accumulation of existing cases, i.e., persons living with the disease. For this reason we cannot draw more specific conclusions regarding age at onset of illness.

Among persons >70 years of age, the higher age-specific prevalence of women relative to men is consistent with prior single site studies showing a predominance of pulmonary NTM diagnosed in women (*1,3–5*), an apparent change from the 1970s and 1980s when men predominated among cases of pulmonary NTM (*10*). Although women aged >70 years have an increased prevalence relative to men in the same age group, the effect among men is still substantial. NTM in women may predominate in more recent clinical studies because women outnumber men in the older age groups; when number of cases relative to their representation in the population are considered (e.g., age-specific disease prevalence), the sex differences are reduced.

The absence of a predominant co-illness is noteworthy, especially in this hospitalized population, and supports the possibility of diverse etiologies for NTM disease. Other than pulmonary NTM, no single diagnosis comprised more than 7% of primary diagnoses. This finding is consistent with observations from recent single-site studies of an increasing proportion of cases having no known risk factors, particularly among women (*1,3–5*). Bronchiectasis, a defining feature for NTM disease (*20*), was identified and

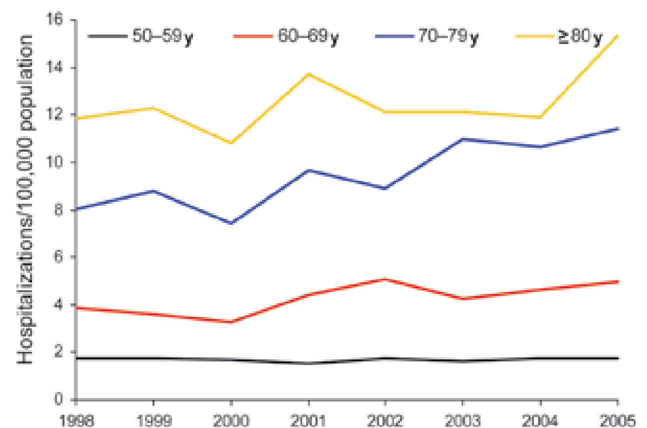

Figure 3. Prevalence of non-AIDS pulmonary nontuberculous mycobacteria–associated hospitalizations among women by age group and year, Healthcare Cost and Utilization Project (HCUP) state inpatient databases, USA, 1998–2005.

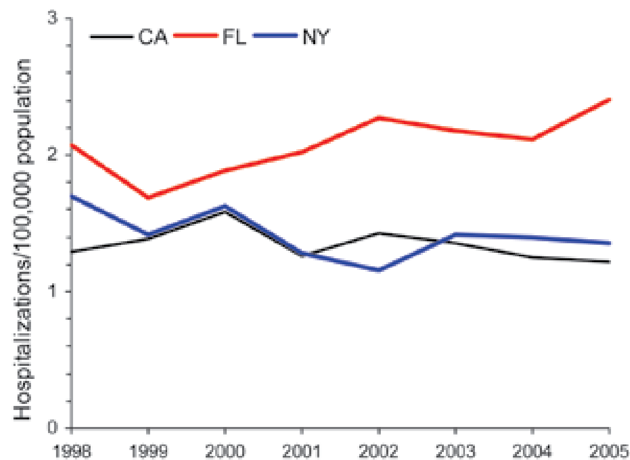

Figure 4. Age-adjusted prevalence of non-AIDS pulmonary nontuberculous mycobacteria–associated hospitalizations among men, California (CA), Florida (FL), and New York (NY), USA, Healthcare Cost and Utilization Project state inpatient databases, 1998–2005.

coded as the primary diagnosis in only 1.3% of hospitalizations caused by NTM. Among discharged patients for whom pulmonary NTM was the primary diagnosis, 15% had bronchiectasis listed as a secondary diagnosis. Because the criteria for defining NTM disease include bronchiectasis, we suspect that a higher proportion of patients than were reported actually had this condition.

The reasons for the low proportion are unclear, but may reflect the relative difficulty of diagnosing bronchiectasis without a computed tomography scan. In this same group having NTM as a primary discharge diagnosis, 47% had cardiac conditions and 33% had COPD/emphysema. The more frequent diagnosis of COPD among men having pulmonary NTM and of bronchiectasis among women with pulmonary NTM is consistent with previous studies (*3,21,22*). Although some of this difference in disease presentation could be related to a gender diagnostic bias (*23*), it may also be related to a number of biologic factors encompassing genetic, immunologic (*24,25*), and anatomic cofactors. Hormonally mediated sex-based responses to inflammation have been postulated as a pathophysiologic mechanism (*23,26*) for pulmonary NTM disease. Even among persons with cystic fibrosis, who have a well characterized genetic predisposition to pulmonary NTM disease, sex differences exist (*23,27*). Finally, a predisposing morphotype of tall, thin white women with underlying illnesses of mitral valve prolapse, scoliosis, and pectus excavatum suggests genetic components to the phenotype (*5,28*).

The overlap between bronchiectasis and pulmonary NTM is extensive but of unclear etiology. Like pulmonary NTM, bronchiectasis is thought to be a common final manifestation of several conditions, including infectious causes as triggers of inflammation (*22*). Current estimates of bronchiectasis are limited, but a recent analysis of a nationally representative nonhospitalized population estimated a prevalence of 272/100,000 persons >75 years of age in 2001; age and sex distribution was strikingly similar to that for pulmonary NTM (*29*). How much of bronchiectasis represents undiagnosed NTM-associated disease is unclear. Among persons >65 years of age in the United States, 26% of patients with chronic heart failure also had COPD and bronchiectasis, and these conditions posed an increased risk for hospitalization (*30*).

The regional differences in prevalence and trends of pulmonary NTM hospitalizations are intriguing. *Mycobacterium avium* complex, the most common group of NTM causing infection in humans, can be acquired through exposure to either soil or water. Whether these geographic differences in prevalence are caused by differential exposure to NTM in certain regions related to human activity or to increased concentrations of mycobacteria in certain environments, or both, is not clear. Heterogeneity in geographic prevalence of disease, NTM isolation, and mycobacterial growth has been demonstrated previously; some of the highest disease and isolation prevalence are found in the southeastern United States, particularly along the coastal regions of the Atlantic and Gulf coasts. A higher prevalence of NTM exposures in these areas, based on skin hypersensitivity tests, was first demonstrated in surveys of Navy recruits using purified protein derivative B (*M. intracellulare*) (*19*).

Subsequent surveys of NTM isolates on the basis of patient isolates referred to state public health laboratories found a greatly elevated prevalence of isolation in Florida (29/100,000 population), relative to California (1.7/100,000

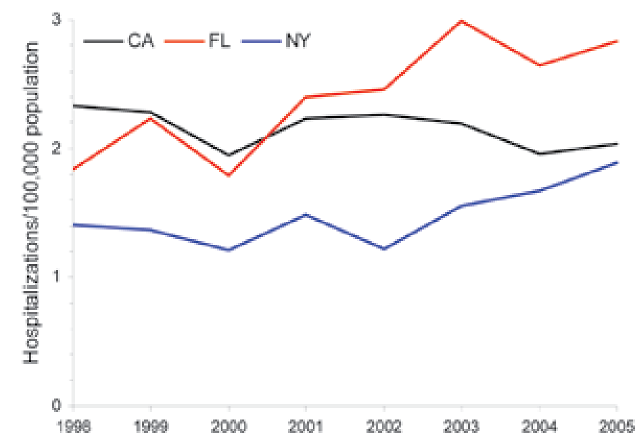

Figure 5. Age-adjusted prevalence of non-AIDS pulmonary nontuberculous mycobacteria–associated hospitalizations among women, California (CA), Florida (FL), and New York (NY), USA, Healthcare Cost and Utilization Project state inpatient databases, 1998–2005.

Table 3. Secondary diagnoses in hospitalizations in which non-AIDS pulmonary NTM is the primary diagnosis, HCUP-SID, USA, 1998–2005*

Secondary diagnosis	Total no.	% Pulmonary NTM as primary diagnosis
Cardiovascular conditions	2,441	47.4
COPD	1,724	33.5
Nutrition/hydration conditions	1,396	27.1
Bronchiectasis	769	14.9
Anemia	536	10.4
Pneumonia	467	9.1
Hemoptysis	438	8.5
Endocrine disorders	393	7.6
Postinflammatory pulmonary fibrosis	388	7.5
Esophageal reflux	295	5.7
Acute respiratory failure	184	3.6

*NTM, nontuberculous mycobacteria; HCUP, Healthcare Cost and Utilization Project; SID, state inpatient databases; COPD, chronic obstructive pulmonary disease.

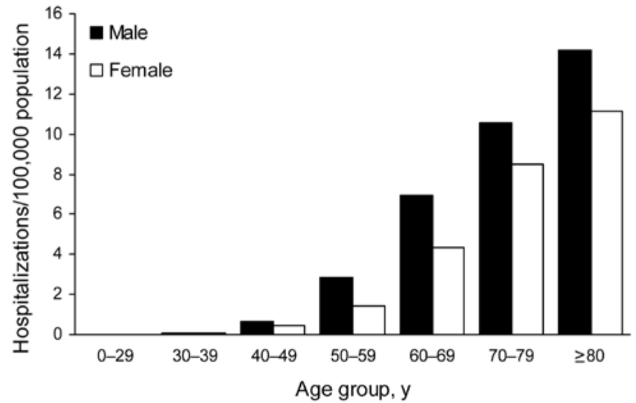

Figure 6. Prevalence of chronic obstructive pulmonary disease as a secondary diagnosis by age group and sex when non-AIDS pulmonary nontuberculous mycobacteria is the primary diagnosis, Healthcare Cost and Utilization Project state inpatient databases, USA, 1998–2005.

population) and New York (2.0/100,000 population) (*31*). More recently, a multisite study of pulmonary NTM prevalence among cystic fibrosis patients found the highest prevalence primarily at sites in the southeastern and southwestern coastal areas (*32*). Higher average temperature and humidity in these areas could favor mycobacterial growth or survival in aerosol droplets. NTM have been isolated and identified in drinking water systems throughout the United States, including those with a variety of water sources (surface/groundwater), water types (hard/soft; high/low organic), and disinfectants used (chlorine/ozone) (*33,34*). The acidic, brown water swamps in the southeastern United States, particularly along the coastal region of the Atlantic and Gulf shores, harbor high numbers of NTM. DNA fingerprinting techniques applied to NTM isolates have shown the identical pattern among isolates obtained from patients and their drinking water supply (*35,36*). Many NTM species have high innate chlorine and biocide resistance, and therefore treatment of municipal water systems with these disinfecting agents may shift the bacterial population towards mycobacteria. Furthermore, some of these species can persist in flowing water distribution systems through their creation of biofilms (*37*).

This study had several limitations. First, these data represent a hospitalized population; most pulmonary NTM diseases are diagnosed and managed in the outpatient setting. Prevalence trends are likely to be different in outpatient populations, depending on the factors influencing hospitalization. Because persons may be more likely to be hospitalized later in the course of the disease, our data could therefore be skewed toward an older population. In a recent case-series of nonhospitalized patients (95% women), the average age at diagnosis was 56 years (*5*). In our study, women >70 years of age predominated. However, until we have better data on outpatients, we cannot definitively know the nature and direction of this bias. Although the populations of some states included in this analysis may have a higher proportion of elderly persons, we accounted for this by estimating age-adjusted or age-specific prevalence.

We cannot know from these data whether the trends in Florida are due to immigration of retirees from other areas, however, geographic differences in exposure have been noted among young Navy recruits who were lifelong residents in their states (*19*). Thus, these differences are unlikely to be explained solely by migration. Awareness of NTM disease may have increased in recent years because of the discovery of new species. Whether this discovery has led to more testing and more frequent diagnosis of NTM along with increased use of commercial molecular probes for the most common species, is uncertain. Also, it is un-

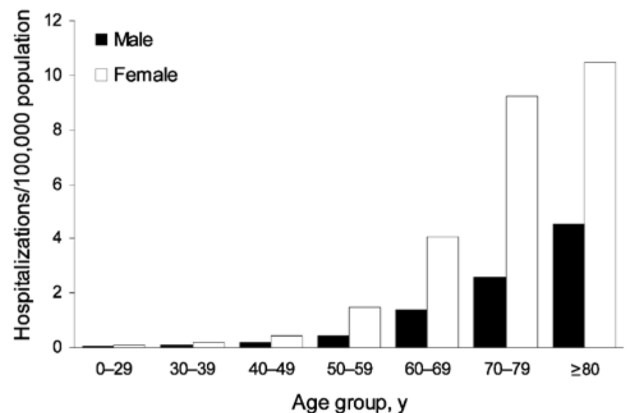

Figure 7. Prevalence of bronchiectasis as a secondary diagnosis by age group and sex when non-AIDS pulmonary nontuberculous mycobacteria is the primary diagnosis, Healthcare Cost and Utilization Project state inpatient databases, USA, 1998–2005.

clear as to whether use of these probes would vary greatly by geographic area. Another limitation is that the validity of the ICD-9-CM codes for NTM is unknown. Because pulmonary NTM is a relatively rare condition, hospitalizations identified by use of these codes likely represent an underestimate of the impact of pulmonary NTM. Because we could not identify multiple hospitalizations for any 1 patient, any given patient could be represented more than once in a given year. However, considering the rarity of this disease it is unlikely that this issue would result in a substantial overestimate of the true impact of pulmonary NTM.

In summary, pulmonary NTM represents an increasing cause of illness in the United States, particularly among women in selected areas. Further research is needed to define the prevalence of disease in nonhospitalized persons in regions throughout the United States and to elucidate risk factors for disease susceptibility as well as environmental exposure.

Acknowledgments

We thank all the states that provided hospital discharge data as part of the Healthcare Cost and Utilization Project, without whom this study would not have been possible.

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Ms Billinger completed this work as part of her master's thesis at the George Washington University School of Public Health. She currently is a nurse in the Inova Health System, Fairfax, Virginia. Her research interests include the epidemiology of infectious diseases.

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Mycobacterium tuberculosis Genotype and Case Notification Rates, Rural Vietnam, 2003–2006

Tran N. Buu, Mai N.T. Huyen, Nguyen N.T. Lan, Hoang T. Quy, Nguyen V. Hen, Matteo Zignol, Martien W. Borgdorff, Dick van Soolingen, and Frank G.J. Cobelens

Tuberculosis case notification rates (CNRs) for young adults in Vietnam are increasing. To determine whether this finding could reflect emergence of *Mycobacterium tuberculosis* Beijing genotype, we studied all new sputum smear–positive pulmonary tuberculosis patients registered for treatment in 3 rural districts in Vietnam during 2003–2006. Beijing strain infections were more frequent in younger patients (15–24 years of age, 53%) than in older patients (31%; p<0.001). The increase in CNRs for youngest patients was larger for disease caused by the Beijing genotype than by other genotypes, but the difference was not significant. For patients 15–24 years of age, 85% of fluctuations in CNRs between years was caused by fluctuations in Beijing genotype infections compared with 53% and 23% in the groups 25–64 and ≥65 years of age, respectively (p<0.001). These findings suggest that young adults may be responsible for introducing Beijing strains into rural Vietnam.

One third of the world's population is infected with *Mycobacterium tuberculosis*, and ≈9 million tuberculosis (TB) cases were diagnosed worldwide in 2006 (*1*). Introduced in the early 1990s, the directly observed treatment, short-course (DOTS) strategy is an essential component of the Global Stop TB Strategy and regarded as a highly cost-effective method for controlling the TB epidemic (*2*). In addition, the DOTS strategy has resulted in decreased numbers of TB cases in Peru, parts of the People's Republic of China, India, and Indonesia (*3–6*) a few years after those countries met the goals of the World Health Organization (WHO), which are to detect ≥70% and cure ≥85% of smear-positive TB cases (*7*).

Conversely, the DOTS strategy has had a limited effect (no decrease in numbers of TB cases) in other regions, such as the former Soviet Union and sub-Saharan Africa (*3,8*). In Vietnam, TB case notification rates (CNRs) have not decreased since 1997 when the National TB Control Program reached WHO goals (*1,9*). This absence of a stable rate decrease reflects a decrease in TB CNRs among middle-age persons, primarily women, which is compensated for by an increase in CNRs in young adults, primarily men (*10*). Several explanations for this phenomenon have been proposed, including the emerging HIV epidemic (*11*), rapid urbanization (*12*), and emergence of the *M. tuberculosis* Beijing genotype (*13*). Studies worldwide indicated that the Beijing genotype is widespread and associated with drug resistance (*14–20*). In Vietnam, a study of isolates from patients located mainly in Ho Chi Minh City showed that the Beijing genotype accounted for 55% of the *M. tuberculosis* isolates and was associated with young age and drug resistance (*21*). Another study in Ho Chi Minh City found that this genotype was more frequent among patients with treatment failure or relapse (*22*). Therefore, emergence of the Beijing genotype, or a higher rate of recurrence of Beijing genotype cases, could explain part of the increase in TB rates among young adults. However, these studies were conducted in large urban areas where rapid urbanization and internal immigration may have confounded these associations. Therefore, we assessed, in a population-based study, the role of the Beijing genotype in the TB epidemic

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in a rural setting in Vietnam. We studied trends in CNRs of new smear-positive TB cases caused by specific genotypes over time by age and sex, and age-specific variations in genotype distribution over time.

Methods

The study was conducted at Pham Ngoc Thach Tuberculosis and Lung Disease Hospital, Ho Chi Minh City, Vietnam, and Tien Giang Provincial Tuberculosis and Lung Disease Hospital, My Tho, Vietnam. The study area consisted of 3 adjacent rural districts in Tien Gang Province, situated in the Mekong River Delta in southern Vietnam. These 3 districts have implemented DOTS strategies since 1994. Each district has a district TB unit that examines sputum smears and treats ambulatory patients with smear-positive results according to the DOTS strategy. HIV testing of TB patients is performed only when HIV infection is suspected on the basis of clinical signs. Details of the study area have been described elsewhere (*13*).

Eligible for inclusion were all patients ≥15 years of age who were residents in the study area and who had registered for treatment of smear-positive pulmonary TB from January 1, 2003, through December 31, 2006, at the participating district TB units or at the provincial TB hospital, and had started treatment for TB ≤2 weeks earlier. Smear-positive TB was diagnosed by microscopic examination of ≥2 Ziehl-Neelsen–stained sputum smears following international guidelines (*23,24*). Eligible patients were included in the study after they provided written informed consent. Scientific and ethical clearance was obtained from the Ethical Health Committee of the Ho Chi Minh City Council. For technical reasons, in 1 of 3 districts, data collection did not start until 2004.

Data Collection and Laboratory Methods

Included patients were asked to submit 2 pretreatment sputum specimens for *Mycobacterium* culture. Specimens were refrigerated and transported to the Mycobacterial Reference Laboratory in Ho Chi Minh City within 72 hours. At the reference laboratory, sputum specimens were decontaminated and liquefied with 1% N-acetyl-L-cysteine, 2% NaOH, placed on modified Ogawa medium, and incubated at 37°C (*23*). Cultures were examined for growth after 1, 2, 4, 6, and 8 weeks of incubation; cultures with no growth after 8 weeks were reported as negative. *M. tuberculosis* was identified by using the niacin and nitrate tests, and isolates were genotyped by spoligotyping by using a standardized method (*25*).

Notification data for new patients with smear-positive results, by age and sex, during 1997–2006 were obtained from routine reports of the district TB units in the study site. Sex- and age-specific population denominators were interpolated and extrapolated from 1999 and 2004 census data; standard exponential population growth was assumed.

Definitions

A new case of new smear-positive TB was defined as a case in a patient who had never had treatment for TB or who had taken drugs for treatment of TB for <1 month (*7*). The Beijing genotype was defined as any isolate without direct repeat spacers 1–34 and with ≥3 spacers 35–43 by spoligotyping (*26*). Other genotypes were defined as described by Brudey et al. (*27*), including the East African–Indian Vietnam genotype (EAI-VNM), which belongs to the EAI genotype family of *M. tuberculosis* and is considered the most common genotype in Vietnam (*21,27*) (Figure 1).

Data Management and Statistical Analyses

Data were entered into EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA, USA). A 20% random sample was double-entered and discrepancies were checked against raw data. Discrepancies were found in <0.5% of the records and <0.1% of the entries. Analyses were performed by using Stata version 8 (StataCorp LP, College Station TX, USA) and Excel 2003 (Microsoft, Redmond, WA, USA). Patients with negative cultures or cultures that grew nontuberculous mycobacteria were excluded from the analyses.

We used the χ^2 test for comparison of proportions. Time trends were assessed by using the Cuzick nonparametric test for trends (*28*). To assess overall trends of CNRs, age- and sex-specific CNRs were standardized by direct standardization using the 1999 census population as the reference and plotted against time. Exponential trend lines were fitted by using the least-squares method. We calculated trends of CNRs for 2003–2006 by sex, age group,

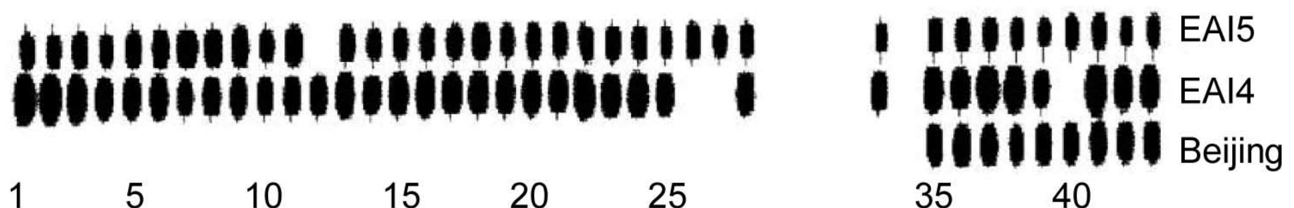

Figure 1. Typical spacer patterns of the *Mycobacterium tuberculosis* spoligotypes most frequently isolated from patients with smear-positive pulmonary tuberculosis, Vietnam, 2003–2006. EAI5 and EAI4 are East African–Indian genotypes.

and genotype by using Poisson regression. Trends over time by genotype and age group were assessed by testing for interactions between these variables and the variable year in Poisson regression models and by using the likelihood ratio χ^2 test for significance testing.

To analyze variation in genotype distribution over time, we first calculated the absolute differences in CNRs between subsequent years and the proportion of these absolute differences for each genotype. Thereafter, averages of these proportions were calculated; to adjust for differences in changes over time, we weighted these averages by absolute variation per year of summed variation for all years. Significance testing was conducted for numbers of cases (i.e., disregarding the population denominator) by χ^2 test comparing genotype against combined strata of year and age group. All tests were conducted at the 5% significance level.

Results

During 1997–2006, CNRs of new smear-positive TB in the study sites decreased by 5.1% per year (95% confidence interval [CI] 4.4%–5.9%), to a lesser extent for men (4.3%, 95% CI 3.4%–5.2%) than for women (7.1%, 95% CI 6.3%–9.1%). Except for patients 15–24 years of age, these decreasing trends were observed for both sexes (Figure 2) and age groups (Figure 3).

During 2003–2006, a total of 2,337 new smear-positive TB patients were registered for treatment in the 3 participating districts; 2,249 (96%) of these patients met inclusion criteria. We excluded 31 patients who did not have data collection forms and 8 other patients whose sputum samples were lost. Patients excluded for this reason did not differ by age or sex from the other patients. A total of 2,210 (94.9%) culture results were available. Of these results, 84 were negative and 29 grew nontuberculous mycobacteria. The remaining 2,097 isolates (89.7%) were genotyped (Table 1).

Of these 2,097 isolates, 682 (32.5%) were of the Beijing genotype, 1,063 (50.7%) were of the Vietnam EAI-VNM genotype, and 353 (16.8%) were of other genotypes. There were no differences in age and sex among the patients from whom the isolates were obtained and subjected to genotyping and all remaining patients registered during the study period. The proportion of isolates with a Beijing genotype was significantly higher for patients 15–24 years of age (52.5%) than for patients of other ages (30.6%; $p<0.001$). The proportion of isolates with the Vietnam genotype was higher among men and among patients >24 years of age; no differences were found for the other genotypes when combined into 1 group (Table 1). No time trends were evident after stratification of the data by age group or sex.

Because the proportion of genotypes other than Beijing and Vietnam was low and was composed of a heterogeneous group, we limited analyses of CNRs by genotype

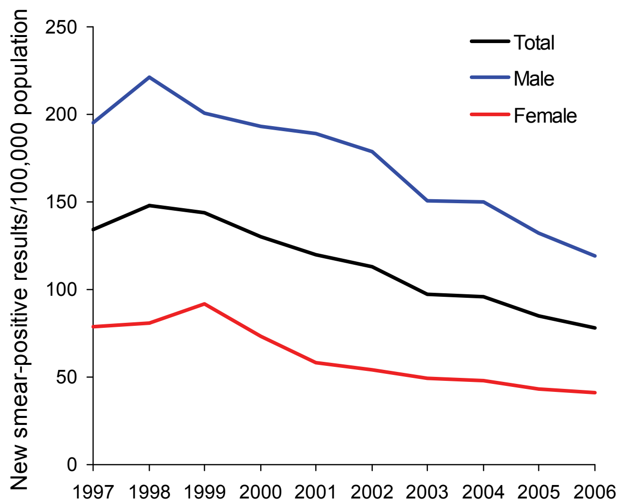

Figure 2. Trends in case notification rates for patients with new smear-positive tuberculosis, by sex, Vietnam, 1997–2006. The annual percentage changes were –4.3% for male patients, –7.7% for female patients, and –5.1% for all persons.

to the isolates of the Vietnam and Beijing genotypes. The age groups 25–34, 35–44, 45–54, and 55–64 years were grouped together because we found no variation in genotype distribution within these subgroups (Table 1).

During 2003–2006, the overall CNR for new smear-positive TB decreased by an average of –4.3% (95% CI –8.0% to –0.3%) per year. Decreasing trends were also seen for patients 25–64 years of age (–5.1% per year, 95%

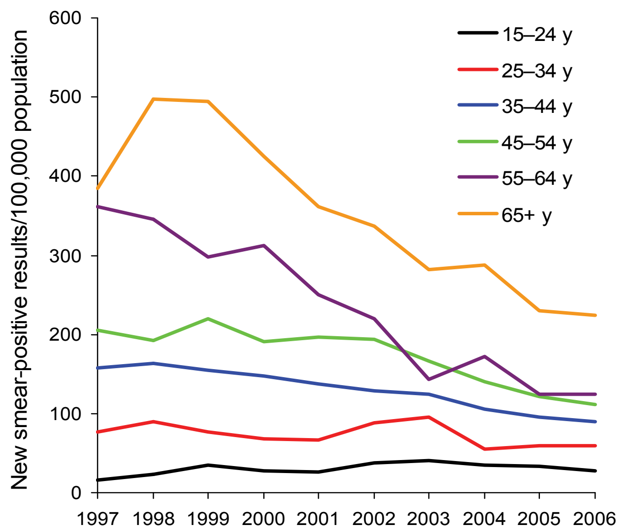

Figure 3. Trends in case notification rates for patients with new smear-positive tuberculosis, by age, Vietnam, 1997–2006. The annual percentage changes were +4.8% for persons 15–24 years of age, –3.3% for those 25–34 years of age, –6.1% for those 35–44 years of age, –6.5% for those 45–54 years of age, –11.5% for those 55–64 years of age, and –7.8% for those ≥65 years of age.

Table 1. Distribution of *Mycobacterium tuberculosis* genotypes among patients, Vietnam, 2003–2006

		Genotype					
		Beijing		Vietnam		Other	
Characteristic	No. patients	No. (%) patients	p value*	No. (%) patients	p value*	No. (%) patients	p value*
Total	2,097	681 (32.5)		1,063 (50.7)		353 (16.8)	
Year							
2003	369	115 (31.2)	0.396	190 (51.5)	0.835	64 (17.3)	0.793
2004	628	207 (33.0)		319 (50.8)		102 (16.2)	
2005	550	192 (34.9)		270 (49.1)		88 (16.0)	
2006	550	167 (30.4)		284 (51.6)		99 (18.0)	
Sex							
M	1,576	494 (31.3)	0.051	826 (52.4)	0.006	256 (16.2)	0.197
F	521	187 (35.9)		237 (45.5)		97 (18.6)	
Age group, y							
15–24	177	93 (52.5)	<0.001	52 (29.4)	<0.001	32 (18.1)	0.854
25–34	277	98 (35.4)		130 (46.9)		49 (17.7)	
35–44	426	137 (32.2)		216 (50.7)		73 (17.1)	
45–54	380	120 (31.6)		205 (53.9)		55 (14.5)	
55–64	266	92 (34.6)		128 (48.1)		46 (17.3)	
≥65	571	141 (24.7)		332 (58.1)		98 (17.2)	

*By χ^2 test for comparison of proportions of each genotype by year, sex, and age.

CI –9.7 % to –0.2%) and ≥65 years of age (–8.0% per year, 95% CI –14.8% to –0.7%). For patients 15–24 years of age, CNRs showed an average annual increase of +5.2% (95% CI –8.4%–20.9%). Rates for women showed an average decrease of –7.9% per year (95% CI –15.2% to –0.2%) and for men, an average decrease of –3.0% per year (95% CI –7.3%–1.6%).

Overall trends in CNRs were similar for patients infected with the Beijing and with the Vietnam genotypes (decreases of –5.9% and –4.4% per year, respectively) (Figure 4). Decreasing trends were observed for middle-age and elderly persons (–6.3% and –5.0% per year for patients 24–64 years of age and –11.3% and –7.1% per year for patients ≥65 years of age).

However, for patients 15–24 years of age, CNRs increased for patients infected with either genotype. This increase was higher for those with the Beijing genotype (9.2%) than for those with the Vietnam genotype (2.0%),

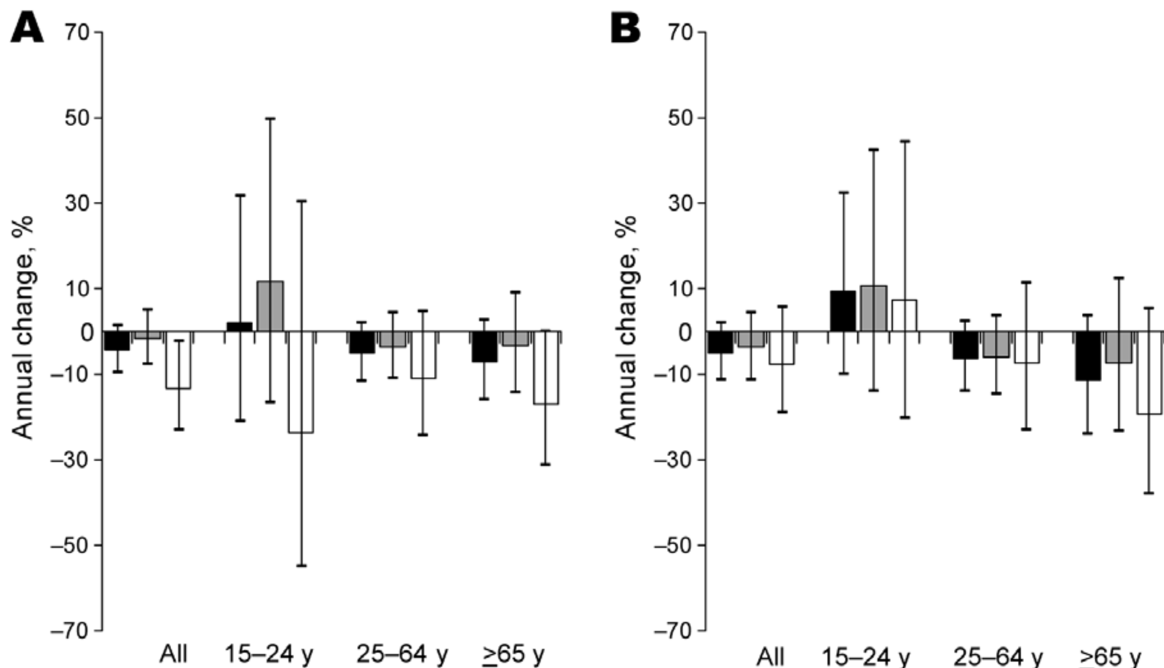

Figure 4. Average annual percentage changes in case notification rates for patients with new smear-positive tuberculosis by age and sex, for the Vietnam genotype (A) and the Beijing genotype (B), Vietnam, 2003–2006. Black columns, total; gray columns, male patients; white columns, female patients. Error bars indicate 95% confidence intervals.

but this difference was not statistically significant (p = 0.860). This difference could be explained by a divergent trend among women: a 7.3% increase per year for those with the Beijing genotype (95% CI –20.2%–44.3%) versus a –13.6% decrease for those with the Vietnam genotype (95% CI –55.1% to –3.5%, p = 0.360) (Figure 4).

Closer inspection of CNRs during 2003–2006 showed an increase, followed by a decrease, which was consistent across age groups but with different patterns over time (Figure 5). The distribution of genotypes over time differed by age group. After correction for differences in absolute changes between years, the proportion of variation that was caused by the Vietnam genotype was ≈50% for patients ≥25 years of age but only 3% for patients 15–24 years of age (Figure 5, Table 2). The proportion of variation caused by the Beijing genotype was 85% for patients 15–24 years of age compared with 53% for patients 25–64 years of age and 23% for patients ≥65 years of age. Differences between the Beijing genotype and remaining genotypes within the youngest and oldest age groups and differences in genotype distribution between the youngest and the oldest age groups were statistically significant (Table 2).

Discussion

During 1997–2006, CNRs for new smear-positive TB for the study site decreased by 4.3% per year likely due to introduction of the DOTS strategy in 1994. As in other studies in Vietnam (*9,10*), we found an underlying pattern of increasing CNRs for young adults, which partially compensated for strongly decreasing CNRs for other age groups.

In the study area, where the Beijing genotype is associated with young age and female sex (*13*), we observed no effect of variations in genotype distribution on the CNRs for all ages and sexes combined. For patients ≥25 years of age, decreasing trends were observed for the Beijing and Vietnam genotypes. There was also no overall difference in CNRs between men and women. However, for patients 15–24 years of age, an increasing trend for both major genotypes was observed. This trend was stronger for the Beijing genotype than for the Vietnam genotype, although the difference was not significant. This difference was most apparent among women in this age group.

Increases in CNRs among young adults are generally considered to reflect recent increases in transmission (*29*).

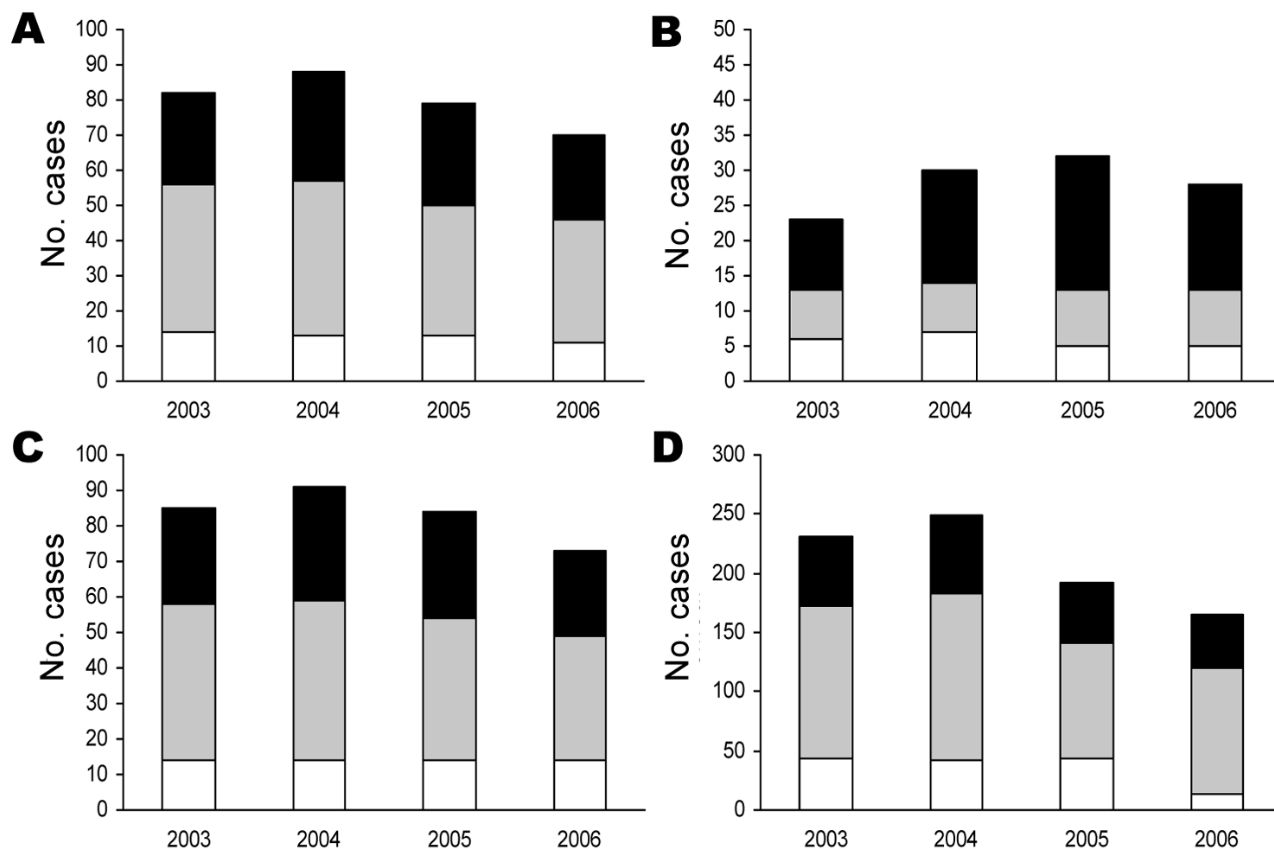

Figure 5. Number of new smear-positive tuberculosis cases, by mycobacterial genotype and patient age, Vietnam, 2003–2006. A), All patients; B) patients 15–24 years of age; C) patients 25–64 years of age; D) patients ≥65 years of age. White columns, other genotypes; gray columns, Vietnam genotype; black columns, Beijing genotype.

Table 2. Annual change in tuberculosis case notification rates, by genotype, Vietnam, 2003–2006*

Age group, y	Absolute change in case rates	Proportion of change caused by: Beijing genotype infections	Vietnam genotype infections	Other genotype infections	p value, within age groups: Beijing genotype†	Vietnam genotype‡	p value, among age groups: Beijing genotype§
All	8.0	0.45	0.44	0.11	<0.001	<0.001	
15–24	4.3	0.85	0.03	0.12	<0.001	<0.001	<0.001
25–64	8.0	0.53	0.47	0.00	0.818	0.907	0.818
≥65	34.0	0.23	0.56	0.21	0.001	<0.05	0.001

*Per 100,000 population. Average weighted for difference in absolute changes.
†By χ^2 test for comparison between Beijing and remaining genotypes in the same age group.
‡By χ^2 test for comparison between Vietnam and remaining genotypes in the same age group.
§By χ^2 test for comparison between each age group with the remaining age groups; Beijing vs. all remaining genotypes.

Therefore, our findings may suggest that the Vietnam genotype is being replaced by the Beijing genotype at the population level in rural Vietnam. The lack of an association between the genotype and the trend in CNRs could then reflect random error or a too-short observation period (4 years). Larger studies of longer duration may be needed to determine whether such an association exists.

The increase in CNRs of new smear-positive TB for persons 15–24 years of age may also be explained by development of the HIV epidemic. HIV increases the risk for progression of *M. tuberculosis* infection to TB and probably increases susceptibility to infection (*1*). In sub-Saharan Africa, 9% of all new TB cases in persons 15–49 years of age were attributable to HIV infection (*8*). In Ho Chi Minh City, the HIV prevalence among TB patients during 1997–2002 increased exponentially from 1.5% to 9% (*11*). This increase in CNRs of new smear-positive TB in patients 15–34 years of age was attributable to HIV, although HIV could not explain the lack of an expected decrease. In our study, HIV data for TB patients were not obtained. The estimated prevalence of HIV infection among persons 15–49 years of age in Tien Giang, Vietnam, in 2005 was much lower than in Ho Chi Minh City (0.5%) (*30*), similar to the national average. The estimated HIV prevalence among TB patients in 30 sentinel provinces was 4.8% in 2004 (D.H. Thanh, Vietnam National Tuberculosis Programme, unpub. data). Thus, it is unlikely that HIV played a role in the observed associations. Furthermore, no association has been found between HIV infection status and isolation of Beijing genotype from pulmonary TB patients elsewhere (*31*).

However, we have alternative explanations for our results. We found that among young adults, nearly all of the variation in CNRs between the years of collection was attributable to variations in CNRs caused by the Beijing genotype; this variation was less apparent for persons in older age groups. This finding suggests that Beijing strains circulate more abundantly among young adults as a consequence of high transmission rates within this age group. Alternatively, the observed fluctuations in numbers of cases caused by Beijing strains may reflect importation from urban areas. Although we did not collect data from these areas, young adults in Tien Giang often travel (70 km) to Ho Chi Minh City for school or work. This explanation is supported by the following findings: the prevalence of infections with the Beijing genotype among persons with TB in this city was higher than the prevalence of comparable patients in rural districts (*13,21*) and our earlier result that infections with the Beijing genotype in Tien Giang were more common in patients living along the main road to Ho Chi Minh City (*13*).

High transmission rates among young adults, particularly in urban areas, may increase emergence of Beijing strain TB infections in Vietnam. Recent data from The Gambia suggest that Beijing strain infections do not show increased secondary attack rates but have shorter incubation periods than other genotypes (*32*). In settings with high transmission rates, such strains may be preferentially selected. Even if their risk for transmission as such is not increased, their faster progression to TB and infectiousness will give them a selective advantage. This hypothesis may also explain the association between the Beijing genotype and a history of imprisonment (e.g., in the former Soviet Union) (*19,33*).

To test this hypothesis, more studies of genotype-specific variations in incubation period and variations in genotype between high-transmission and low-transmission settings are needed. Different sublineages of the Beijing genotype may have different pathogenic characteristics (*34*,*35*). Data from Vietnam suggest that the more recently evolved typical Beijing strains have a higher propensity to evade immunity from *M. bovis* BCG vaccination (*36*). In addition, studies among immigrant populations in the United States, South Africa, and Canada have suggested that the transmission propensity of different genotypes and of different sublineages of the Beijing genotype is dependent on the host population (*19,33,35,37–39*). Such studies (*19,33*) should enable analyses of different sublineages of the Beijing genotype and involve non-East Asian populations.

The association of the Beijing genotype with young adults could threaten the effectiveness of the DOTS strategy in Vietnam and elsewhere. However, this possibility is

unlikely because in our study, although 25% of the elderly patients were infected by Beijing strains, overall CNRs of TB caused by Beijing strains still decreased. Conversely, associations have been found in some studies, including one in the same study area, between the Beijing genotype and drug resistance, particularly for multidrug-resistant TB (MDR TB) (*13,17,18,21,31*). Although until 2001 the prevalence of MDR TB among new patients with smear-positive TB in the study area was relatively low and not increasing (*40*), the effects of genotype-associated risks for increased acquisition or transmission of MDR TB may threaten the effectiveness of long-term TB control.

Our study has several other limitations. First, our data were limited to new patients with smear-positive pulmonary TB and may not be representative of patients with other types of TB, including previously treated patients. In our study area, the Beijing genotype was more prevalent in previously treated patients than in new patients (*13*), which suggested that a stronger effect of genotype on TB trends might be observed if previously treated patients were included in the analyses. Second, we did not include patients who were treated in the private health sector. We collected no data on the proportion of patients treated in the private sector, but local health authorities estimate the proportion of these patients to be <10%. Third, we did not include children <15 years of age because of ethical constraints and because their number was expected to be small. In Vietnam, children account for no more than 0.2% of notified smear-positive TB cases (National Tuberculosis Program, unpub. data). However, pediatric TB may be underdiagnosed if the only test for diagnosis is microscopic examination of sputum smears.

Our data do not prove or refute that the increase of TB in young adults in rural Vietnam is related to emergence of the Beijing genotype. However, they do suggest that the association between the Beijing genotype and young age reflects importation of Beijing strain infections from urban areas into rural areas. This importation may be linked to or driven by high rates of transmission among young adults.

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Lack of Airborne Transmission during Outbreak of Pandemic (H1N1) 2009 among Tour Group Members, China, June 2009

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During June 2–8, 2009, an outbreak of influenza A pandemic (H1N1) 2009 occurred among 31 members of a tour group in China. To identify the mode of transmission and risk factors, we conducted a retrospective cohort investigation. The index case-patient was a female tourist from the United States. Secondary cases developed in 9 (30%) tour group members who had talked with the index case-patient and in 1 airline passenger (not a tour group member) who had sat within 2 rows of her. None of the 14 tour group members who had not talked with the index case-patient became ill. This outbreak was apparently caused by droplet transmission during coughing or talking. That airborne transmission was not a factor is supported by lack of secondary cases among fellow bus and air travelers. Our findings highlight the need to prevent transmission by droplets and fomites during a pandemic.

Since the emergence of a novel influenza A (H1N1) virus (later called influenza A pandemic [H1N1] 2009 virus) in early 2009 in Mexico (*1,2*), the virus has spread to 156 countries, territories, and areas; as of July 27, 2009, a total of 134,503 laboratory-confirmed cases and 816 deaths had been reported (*3*). On June 11, 2009, the World Health Organization declared that the world was experiencing the start of the 2009 influenza pandemic (*4*). Investigations of transmission chains early in the pandemic will add to our understanding of the special characteristics of this new virus, including whether its mode of transmission differs from that of seasonal influenza viruses. This information will be useful for effective control of the spread of this virus.

In the People's Republic of China, the early response strategy has been containment, which includes temperature screening and administration of health questionnaires at international ports of entry, isolation of infected travelers, and quarantine of close contacts of infected persons. During June 2–8, 2009, an outbreak of pandemic (H1N1) 2009 occurred among members of a tour group. We investigated this outbreak to identify the source of infection, mode of transmission, and risk factors for infection.

Methods

The index case-patient was a 40-year-old female US citizen who had traveled from the United States to Jiuzhaigou, a popular tourist spot in southwestern China; she stopped to change planes in Hong Kong and Chengdu. She noticed her first symptom, chills, on June 2, immediately before arriving in Chengdu, ≈23 hours after departure from the United States. After learning that she had traveled on 3 flights and had toured with a group, we obtained the manifests of all flights that she had traveled on and asked all passengers of the 3 flights and all members of the tour group by telephone or in-person interview whether they had had any symptoms from May 27 through June 12, 2009. We also obtained detailed information on the activities of the tour group during the 3-day tour. Health authorities placed members of the tour group under medical observation and

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[1]These authors contributed equally to this article.

isolated those who had clinical signs or symptoms or positive throat swab culture results. Laboratory technicians at the Sichuan Province Center for Disease Control and Prevention collected throat swabs every 24 hours from all members of the tour group and from symptomatic persons who had shared any of the 3 flights with the index case-patient. Real-time reverse transcription–PCR (RT-PCR) was performed to detect nucleic acids specific for the influenza A pandemic (H1N1) 2009 virus by using the primers supplied by the World Health Organization.

We defined a suspected case as onset of ≥1 of 5 symptoms—fever (≥38°C), cough, sore throat, chills, or headache—in a passenger of flight CZ6659 (June 3, Chengdu to Jiuzhaigou) or flight CZ6660 (June 5, Jiuzhaigou to Chengdu) or in a member of the tour group. A confirmed case was a suspected case for which real-time RT-PCR provided laboratory confirmation of the influenza A pandemic (H1N1) 2009 virus infection. A secondary case was a confirmed case for which the patient's signs or symptoms began after 9:00 PM on June 3, i.e., at least 24 hours after the onset of the primary (index) case.

To identify the mode of transmission and risk factors for infection, we conducted a retrospective cohort investigation. We interviewed all members of the tour group by telephone or in-person interview to ascertain details of their contact history with the index case-patient.

Results

During this outbreak, we identified a total of 11 confirmed cases of influenza A pandemic (H1N1) 2009 infection (Figure 1). Average patient age was 36 (range 18–59) years; 2 patients were men and 9 were women. Signs and symptoms were cough (73%), fever (64%), sore throat (64%), headache (27%), chills (27%), runny nose (18%), and myalgia (18%). All 11 case-patients fully recovered; 3 (including the index case-patient) recovered on June 13, 5 on June 15, 1 on June 17, and 2 on June 18. The mean duration of illness was 11 (range 9–14) days.

The index case-patient left New York City, United States, on flight CX841 at 12:00 AM (midnight) June 2 and arrived in Hong Kong at 2:00 PM on the same day. She transferred to flight CA428 (Boeing 757), which departed Hong Kong at 7:25 PM and arrived in Chengdu at 10:00 PM. On June 3, she and her family members joined the tour group at the Chengdu Airport and boarded flight CZ6659 (Boeing 757), which departed Chengdu at 12:25 PM and arrived at Jiuzhaigou (33°15′55″N, 104°13′35″E; average altitude 2,930 m) at 1:10 PM. The group picked up 7 additional members in Jiuzhaigou, where they traveled to various tourist attractions by bus and participated in group activities during the ensuing 3 days. On June 5, the original 24 members of the tour group from Chengdu (without the 7 members who had joined the group in Jiuzhaigou) boarded

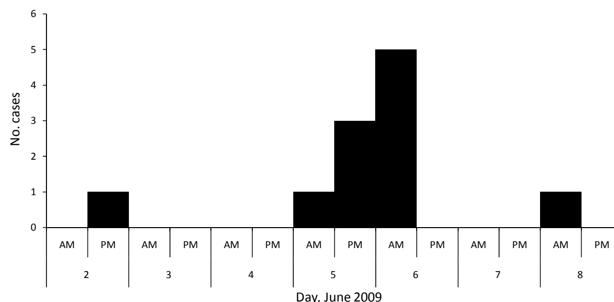

Figure 1. Time of disease onset for persons infected with influenza A pandemic (H1N1) 2009 virus, Sichuan Province, China, June 2009.

flight CZ6660 (Boeing 757), which departed Jiuzhaigou at 1:30 PM and arrived in Chengdu at 2:15 PM, along with 87 other passengers (Figure 2).

All airplanes boarded by the index case-patient had high-efficiency particulate air filters. Less than half of the air in the passenger cabins was recirculated; the rest was from outside. Air in the passenger cabins of the airplanes was recirculated approximately every 3 minutes. The air conditioning system in the tour bus mixed ≈70% recirculated inside air with ≈30% outside air, filtered it, and delivered it into the bus through air outlets above the passenger seats. A vent at the back of the bus continually exhausted air from inside the bus.

The diagnosis of influenza A pandemic (H1N1) 2009 virus infection was made for the index case-patient after she returned to Chengdu on June 5. Subsequently, members of the tour group were placed under involuntary medical observation. No influenza-like illness developed in any of the 91 passengers of flight CA428 (Hong Kong to Chengdu) or in any of the 87 passengers on flight CZ6659 (Chengdu to Jiuzhaigou) who were not members of the tour group.

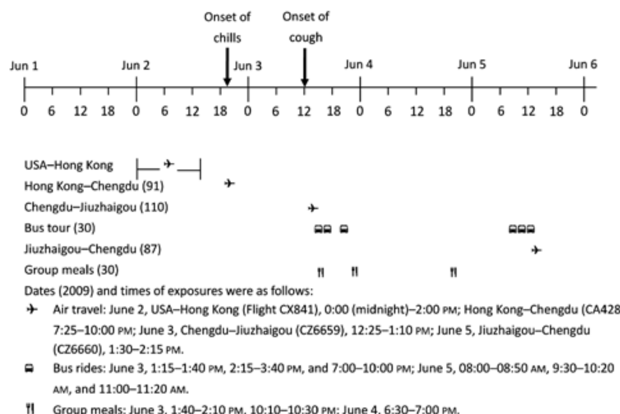

Figure 2. Timeline of exposures to the index case-patient during outbreak of influenza A pandemic (H1N1) 2009, Sichuan Province, China, June 2009. Numbers in parentheses indicate number of persons exposed.

None of the passengers on either flight had donned a mask. However, excluding the index case-patient, 9 (30%) of the 30 members of the tour group became ill with secondary cases of disease. The secondary attack rate did not differ between the members of the tour group who flew from Chengdu to Jiuzhaigou and the members who joined the tour in Jiuzhaigou. Of the 87 passengers on the return flight (Jiuzhaigou to Chengdu) on June 5 who were not members of the tour group, 1 person became ill (Table 1). Her seat (9A) was within 2 rows of seats of the index case-patient (7A) and a secondary case-patient (7B), each of whom was symptomatic during the return flight from Jiuzhaigou to Chengdu on June 5.

Among members of the tour group, the attack rate was higher for women (50%) than for men (13%) (2-tailed Fisher exact test, p = 0.05). The secondary attack rate among persons 18–39 years of age was 41% (7/17; exact 95% confidence interval [CI] 18–67) compared with 21% (3/14; exact 95% CI 4.7–51) for persons 40–63 years of age.

The index case-patient began having chills at ≈9:00 PM during her flight from Hong Kong to Chengdu. She started coughing before she boarded the flight from Chengdu to Jiuzhaigou on June 3 and continued to cough during the entire tour and after she returned to Chengdu. She had extensive interactions with other members of the group, who talked with each other, helped each other take pictures, gave chewing gum to each other, had group meals together, and stayed in the same hotel. During the 3-day trip, the group traveled together in an air-conditioned tour bus; doors were shut and windows were sealed to conserve energy. While traveling among the various tourist attractions, the group was together on bus rides for a total of 6 hours and 50 minutes. When we evaluated the contact patterns of the tour group with the index case-patient, we found that for the 16 tourists who had talked with the index case-patient from close range (<2 m) for ≥2 minutes, the attack rate was 56%, whereas none of the 14 tourists who did not talk with her became ill. Members of the tour group who had talked with the index case-patient for ≥10 minutes were almost 5× as likely to become ill than those who had talked with her for 2–9 minutes (Table 2). The 14 passengers who had not talked with the index case-patient did report other interactions with her, such as dining at the same table, sitting within 2 rows on the same flight or bus ride, and receiving chewing gum from her. Moreover, 3 of these 14 uninfected passengers had sat within 2 seats of the index case-patient during the bus rides but had never talked with her from close range.

Discussion

Since its emergence, pandemic (H1N1) 2009 has spread around the world, including 1,930 confirmed cases in China as of July 27, 2009 (*5*). Of the cases that have occurred in China (excluding Hong Kong, Macao, and Taiwan), >80% have been imported (*6*); however, several outbreaks caused by transmission from imported case-patients have also occurred in China. Our investigation documented 1 such outbreak.

Seasonal influenza A is transmitted directly by large droplets, or indirectly by fomites (*7*). However, the transmission dynamics of the influenza A pandemic (H1N1) 2009 virus have been less well researched. Our data show that this outbreak was caused by talking with the index case-patient at close range, which indicates droplet transmission. Conversely, other kinds of contact, such as dining at the same table and receiving chewing gum from the index case-patient, played no role during this outbreak.

The role of airborne transmission for influenza is debatable (*7–10*). Our investigation did not find evidence of airborne transmission during this outbreak. The lack of cases among 14 tourists who were with the index case-patient in an enclosed bus cabin for nearly 7 hours suggests that airborne transmission was not a factor. The absence of secondary cases among passengers of the flight from Chengdu to Jiuzhaigou also supports this conclusion. Although the case-patient with disease onset on June 8 appeared to have been infected while sharing the flight from Jiuzhaigou to Chengdu, she sat within 2 seats of 2 symptomatic case-patients, which is also consistent with droplet or fomite transmission.

During this outbreak, the index case-patient was febrile while traveling on 3 flights. A secondary case-patient was also febrile while traveling on the return flight (Jiuzhaigou to Chengdu). Neither patient's illness was detected by thermal scanning at the airports. Another secondary case-patient had had a headache during the return

Table 1. Secondary attack rate for influenza A pandemic (H1N1) 2009, by travel history, Sichuan Province, China, June 2009

Group	Total no. persons	No. cases	Attack rate, %
Passengers on flight CA428 (Hong Kong–Chengdu), June 2	91	0	0
Passengers on flight CZ6659 (Chengdu–Jiuzhaigou), June 3	110	7	6.4
Members of the tour group	23	7	30
Not members of the tour group	87	0	0
Members of the tour group, not passengers of flight CZ6659 (Chengdu–Jiuzhaigou), June 3	7	2	29
Passengers on flight CZ6660 (Jiuzhaigou–Chengdu), June 5, not members of the tour group	87	1	1.1

Table 2. Secondary attack rate of influenza A pandemic (H1N1) 2009 among the tour group members, by exposure, Sichuan Province, China, June 2009*

Exposure	Total no. persons	No. cases	Secondary attack rate, %	Rate ratio (95% CI)
Seat proximity to index case-patient during flight CZ6659, Chengdu–Jiuzhaigou, June 3				
>2 rows	19	5	26	Referent
≤2 rows	4	2	50	1.9 (0.35–5.7)
Seat proximity to index case-patient during bus rides				
Never ≤2 rows	8	2	25	Referent
Ever ≤2 rows	22	7	32	1.3 (0.39–6.0)
Talked with index case-patient from <2 m for ≥2 min				
Yes	16	9	56	∞ (2.4–∞)
No	14	0	0	Referent
Length of conversation with index case-patient				
≥10 min	10	8	80	4.8 (1.2–70)
2–9 min	6	1	17	Referent

*CI, confidence interval.

flight. The index case-patient filled out a health questionnaire but did not truthfully inform health authorities of her symptoms. The other 9 case-patients began having symptoms after returning home; hence, they were also not detected by airport screening. These data suggest that thermal scanning and health questionnaires at the airports were not effective for detecting pandemic (H1N1) 2009 infections.

The main limitation of our investigation was the possibility of recall bias; i.e., those who became ill might have more accurately recalled their contact history than those who did not. However, the index case-patient had a highly distinctive hairstyle, which made her easy to remember. Also, interviews about the tourists' exposure to the index case-patient were conducted within 1 week of the completion of their tour. These 2 factors should have helped minimize any potential recall bias.

In conclusion, this outbreak of influenza A pandemic (H1N1) 2009 virus infection was caused by transmission during coughing or vocalization by an imported case-patient. The virus spread by droplet transmission when the index case-patient was talking with her fellow tourists. The findings of our investigation highlight the importance of preventing droplet transmission during a pandemic.

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Community-associated Methicillin-Resistant *Staphylococcus aureus*, Iowa, USA

Philip Van De Griend, Loreen A. Herwaldt, Bret Alvis, Mary DeMartino, Kristopher Heilmann, Gary Doern, Patricia Winokur, Diana DeSalvo Vonstein, and Daniel Diekema

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Learning Objectives

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

- Describe risk factors for healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA) infection
- Define multiresistant MRSA isolates
- Describe characteristics of patients who are likely to be infected by USA300/400 strains of MRSA
- Identify predictive factors for community-associated MRSA infection in 1 US state
- Describe recent patterns of community-associated MRSA infection in the United States

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We performed antimicrobial drug susceptibility testing and molecular typing on invasive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (n = 1,666) submitted to the University of Iowa Hygienic Laboratory during 1999–2006 as part of a statewide surveillance system. All USA300 and USA400 isolates were resistant to ≤3 non–β-lactam antimicrobial drug classes. The proportion of MRSA isolates from invasive infections that were either USA300 or USA400 increased significantly from 1999–2005 through 2006 (p<0.0001). During 2006, the incidence of invasive community-associated (CA)–MRSA infections was highest in the summer (p = 0.0004). Age <69 years was associated with an increased risk for invasive CA-MRSA infection (odds ratio [OR] 5.1, 95% confidence interval [CI] 2.06–12.64), and hospital exposure was associated with decreased risk (OR 0.07, 95% CI 0.01–0.51).

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Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in the 1960s and has since become a major cause of illness and death in the healthcare setting (*1,2*). Risk factors for infection with healthcare-associated MRSA (HA-MRSA) include hospitalization, residence in a long-term care facility, older age, invasive devices (e.g., catheters, feeding tubes), and exposure to antimicrobial agents. HA-MRSA isolates are often resistant to several antimicrobial drug classes in addition to β-lactams (*3*).

In the 1990s, investigators began describing serious MRSA infections among persons who did not have typical risk factors for infections with this organism (*2,4–8*). These community-associated MRSA (CA-MRSA) infections affected young, healthy persons (*4,5,7*) and were associated with factors such as participating in contact sports, sharing towels or athletic equipment, using illegal intravenous drugs, and living in crowded or unsanitary areas (e.g., prisons, hurricane evacuee centers) (*9,10*).

Pulsed-field gel electrophoresis (PFGE) demonstrated that MRSA strains causing these community-associated infections (USA300 and USA400) were different than those causing healthcare-associated infections (USA100 and USA200) (*11*). USA300 and USA400 MRSA strains typically have the staphylococcal cassette chromosome (SCC) *mec* type IV, not the SCC*mec* type II carried by most USA100 and USA200 isolates (*12*). In addition, USA300/400 isolates usually carry the gene that encodes the Panton-Valentine leukocidin (PVL), a bicomponent (*luk*F-PV and *luk*S-PV) pore-forming leukotoxin (*8,13–15*). Currently, the role of PVL in the pathogenesis of infections caused by USA300/400 isolates is controversial. Epidemiologic studies and a study by Labandeira-Rey et al. suggest that PVL is associated with virulence and causes the necrosis characteristic of infections with these strains (*16*). In contrast, a study by Voyich et al. found no difference in virulence between the wild-type parent strains and the isogenic knockout strains that did not produce PVL (*17*).

A recent multicenter study by Moran et al. showed that USA300 MRSA is now the most common cause of skin and soft tissue infections (SSTIs) among adults seeking treatment in emergency departments in 11 large metropolitan areas (*15*). USA300 also causes serious invasive infections such as necrotizing pneumonia, bloodstream infections, and surgical site infections, some of which are acquired in hospitals (*18–22*). Although most USA300 and USA400 isolates are currently resistant to fewer classes of antimicrobial drugs than are HA-MRSA isolates (*13*), a recent paper by Han et al. identified a USA300 subtype that is resistant to erythromycin, clindamycin (constitutive), tetracycline, mupirocin, and fluoroquinolones (*23*).

Most epidemiologic studies of CA-MRSA have examined isolates from SSTIs infections (*7,15,18*), and most studies that evaluated patients with invasive disease have involved single healthcare facilities (*21,24*) or isolates obtained primarily from large urban areas (*22*). We describe the molecular epidemiology of invasive infections caused by USA300 and USA400 in a rural state. We characterized invasive MRSA from 1999–2005 (select isolates) and in 2006 (all isolates) submitted to the statewide surveillance system in Iowa for invasive MRSA infections.

Methods

As part of a statewide surveillance system, the Iowa Department of Public Health has mandated since 1999 that clinical microbiology laboratories submit invasive isolates of MRSA to the University Hygienic Laboratory (UHL), Iowa's public health laboratory (*25,26*). After performing antimicrobial drug susceptibility testing on all isolates, we further characterized (by PFGE, PVL detection, and SCC*mec* typing) all isolates from 1999–2005 that were resistant to ≤3 non–β-lactam antimicrobial drug classes (i.e., consistent with USA300/400) and all 343 isolates from unique patients with invasive infections submitted to the UHL during 2006.

Antimicrobial Drug Susceptibility Testing

All invasive MRSA isolates during 1999–2006 were tested for antimicrobial drug susceptibility by the broth dilution method described by the Clinical and Laboratory Standards Institute (*27*). Invasive isolates were defined as any organism from a normally sterile body site such as blood, cerebrospinal fluid, pleural fluid, joint fluid, or fluid from a liver abscess. Isolates from urine were excluded.

Isolates were tested for susceptibility to tetracycline, erythromycin, clindamycin, trimethoprim/sulfamethoxazole, gentamicin, levofloxacin, moxifloxacin, linezolid, daptomycin, vancomycin, and rifampin. Multidrug-resistant isolates were defined as MRSA isolates that were resistant to more than 3 of 8 representative antimicrobial drug classes: macrolides (erythromycin), lincosamides (clindamycin), quinolones (levofloxacin or moxifloxacin), tetracyclines, sulfa drugs (trimethoprim/sulfamethoxazole), aminoglycosides (gentamicin), glycopeptides (vancomycin), and rifampin.

Molecular Typing and PCR to Assess SCC*mec* Type and Presence of the PVL Gene

PFGE was performed as previously described (*28*). Each gel accommodated bacteriophage Lambda ladders (at 3 places on the gel), DNA from 17 isolates, type strains for USA300 and USA400 from the Centers for Disease Control and Prevention (Atlanta, GA, USA), and *S. aureus* NCTC-8325 (at 3 places on the gel). The gel images were saved as TIFF files and BioNumerics computer software (Biosystematica, Llandysul, Wales, UK) was used to perform cluster analysis. Isolates were classified as the same strain

if cluster analysis indicated that they were ≥80% similar. PFGE patterns for clinical isolates were compared visually and by computer-assisted gel analysis with the type strains for USA300 and USA400. We defined CA-MRSA as MRSA isolates in either the USA300 or the USA400 pulsetypes. Multiplex PCR was performed, as previously described, to type the SCC*mec* A (*29*) and to detect the PVL genes (*30*).

Epidemiologic Data Collection

Epidemiologic data on the isolates were obtained from UHL. These data were age, sex, race/ethnicity, inpatient status, intensive care unit status, long-term-care facility status, hospital admission date, specimen type, specimen collection date, the hospital code number, and the Iowa Reporting Region. Isolates were considered to have been acquired nosocomially if the specimen culture date minus the admission date was ≥2 days.

Statistical Methods

PFGE patterns and antimicrobial drug susceptibility test results were merged with the demographic data. These data were analyzed with SAS version 9.1 (SAS Institute, Cary, NC, USA) to assess trends in the frequency of USA300/400 in Iowa and to identify possible risk factors for invasive infections with these strains. We used χ^2 and adjusted χ^2 tests to analyze categorical data and linear regression and logistic regression to analyze continuous data. Alpha was set at 0.05 and all reported p values were 2-tailed.

Seasonality of infections was analyzed by χ^2 analysis. Winter was defined as December 22 to March 19, spring as March 20 to June 20, summer as June 21 to September 22, and fall as September 23 to December 21.

The relationships between CA-MRSA and potential risk factors were assessed by univariate analysis. Subsequently, stepwise logistic regression was used to identify factors independently associated with invasive CA-MRSA infection.

Results

Patients infected by USA300/400 MRSA were younger than those infected by other strains ($p<0.0001$ for both time periods; Tables 1, 2). During 2006, more males than females were infected with USA300/400 ($p = 0.06$). Most isolates during both time periods were obtained from blood cultures and the distribution of strains did not vary by body site. Most patients were hospitalized for their infections and the proportion of patients admitted to intensive care units did not vary by strain ($p = 0.27$ and $p = 0.35$). However, the proportion of MRSA infections that met the definition of nosocomial decreased significantly from 26.1% from 1999–2005 to 16.6% in 2006 ($p = 0.0003$). During 2006, patients infected with other

Table 1. Descriptive epidemiology of invasive MRSA in Iowa, USA, 1999–2005*

		USA type		
Characteristic†	Total no. (%), N = 1,323	No. (%) USA300/400, n = 26	No. (%) other,‡ n = 1,297	p value
Mean age, y	67.8 (SD = 17.6)	46.0 (SD = 22.0)	68.2 (SD = 17.2)	<0.0001
Female gender	550 (41.6)	9 (34.6)	541 (42.7)	0.549
Inpatient stay	1,124 (85.0)	24 (92.3)	1,100 (84.8)	1.000
ICU admission	221 (16.7)	4 (15.4)	217 (16.7)	0.764
Nosocomial infection	346 (26.2)	5 (19.2)	341 (26.3)	0.306
Specimen type				<0.0001
Blood	1,256 (94.9)	25 (96.2)	1,231 (94.9)	
CSF	9 (0.7)	0	9 (0.7)	
Joint fluid	33 (2.5)	1 (3.9)	32 (2.5)	
Pleural fluid	8 (0.6)	0	8 (0.6)	
Other	6 (0.5)	0 (0.0)	6 (0.5)	
Iowa region				0.054
1	32 (2.4)	1 (3.9)	31 (2.4)	
2	370 (28.0)	10 (38.5)	360 (27.8)	
3	335 (25.3)	2 (7.7)	333 (25.7)	
4	272 (20.6)	4 (15.4)	268 (20.7)	
5	140 (10.6)	5 (19.2)	135 (10.4)	
6	63 (4.8)	4 (15.4)	59 (4.5)	
PVL	ND	ND	ND	ND
SCC*mec* IV	ND	ND	ND	ND

*MRSA, methicillin-resistant *Staphylococcus aureus*; ICU, intensive care unit; CSF, cerebrospinal fluid; PVL, Panton-Valentine leukocidin; SCCmec IV, staphylococcal chromosomal cassette *mec* type IV; ND, not done for all isolates.
†The number of patients missing data on specific variables: age = 13; gender = 11; inpatient = 85; ICU = 356; nosocomial = 358; specimen type = 11; Iowa Department of Public Health Reporting Region = 11.
‡Of the subset of isolates that were typed (N = 180), 173 (96%) were USA100. The remainder clustered with USA200 (3), USA500 (2), or did not match an existing USA type.

Table 2. Descriptive epidemiology of invasive MRSA in Iowa, USA, 2006*

Characteristic†	Total no. (%), N = 343	USA type		p value
		No. (%) USA300/400, n = 54	No. (%) other,‡ n = 289	
Mean age, y	66.3 (SD = 17.0)	50.6 (SD = 21.2)	69.2 (SD = 14.4)	<0.0001
Female gender	135 (39.4)	14 (25.9)	121 (41.9)	0.059
Inpatient stay	278 (81.0)	50 (92.6)	228 (78.9)	0.271
ICU admission	56 (16.3)	8 (14.8)	48 (16.7)	0.348
Nosocomial infection	57 (16.6)	1 (1.9)	56 (19.4)	0.0006
Specimen type				0.0021
Blood	322 (93.9)	45 (83.3)	276 (95.0)	
CSF	0	0	0	
Joint fluid	13 (3.8)	5 (9.3)	8 (2.9)	
Pleural fluid	2 (0.6)	2 (3.7)	0	
Other	6 (1.7)	2 (3.7)	5 (1.4)	
Iowa region				0.268
1	10 (2.9)	0	10 (3.5)	
2	93 (27.0)	13 (24.1)	80 (27.7)	
3	49 (14.2)	12 (22.2)	37 (12.8)	
4	105 (30. 5)	16 (29.6)	88 (30.5)	
6	20 (5.8)	5 (9.3)	15 (5.2)	
PVL	54 (15.7)	52 (96.3)	2 (0.7)§	<0.0001
SCC*mec* IV	68 (19.8)	54 (100.0)	13 (4.5)	<0.0001

*MRSA, methicillin-resistant *Staphylococcus aureus*; ICU, intensive care unit; CSF, cerebrospinal fluid; PVL, Panton-Valentine leukocidin; SCC*mec* IV, staphylococcal chromosomal cassette *mec* type IV.
†The number of patients missing data on specific variables: age = 10; Gender = 12; inpatient = 31; ICU = 122; nosocomial = 101; specimen type = 0; Iowa Department of Public Health Reporting Region = 6; PVL = 3; SCC*mec* IV = 3.
‡Of the subset of isolates that were typed (N = 272) 94% were USA100. The remainder clustered with USA200 (5), USA500 (5), USA600 (1), USA800 (4), or did not match an existing USA type.
§Both isolates clustered with USA100 and were SCC*mec* II.

MRSA strains were more likely than those infected with USA300/400 to have infections that met the definition of nosocomial (p = 0.0006).

The antimicrobial susceptibility of 54 invasive USA300/400 isolates is shown in Table 3. None of the USA300 or USA400 isolates had a multidrug-resistant phenotype (e.g., all were resistant to ≤3 non–β-lactam classes). Specifically, none of the USA300 isolates from Iowa demonstrated the multidrug-resistance pattern described by Diep et al. that is mediated by the multidrug-resistance plasmid pUSA03 (*31*). All isolates were typeable when the DNA was digested with *Sma*I. We did not identify any invasive MRSA infections caused by USA300/400 between 1999 and 2002. USA300 caused 3 (1.5%) of 195 infections in 2003, 14 (5.8%) of 243 infections in 2004, 7 (2.5%) of 275 infections in 2005, and 51 (14.9%) of 343 infections in 2006. USA400 caused 2 (0.7%) of 275 infections in 2005 and 3 (0.9%) of 343 infections in 2006. The proportion of MRSA isolates from invasive infections that were CA-MRSA (either USA300 or USA400) increased significantly from 1999–2005 to 2006 (p<0.0001; Figure 1).

Reporting region 4, which had the third largest population of the 10 regions, submitted the most isolates; region 1, which had the fourth smallest population, submitted the fewest isolates. We did not find significant differences between the type of MRSA causing infections and the reporting region during 2006. Incidence of infections caused by CA-MRSA varied by season during 2006 (p = 0.0004); a total of 47.3% of these infections occurred during the summer (Figure 2).

The full model for predicting invasive infection with CA-MRSA compared with HA-MRSA included age (young vs. old), seasonality, hospital exposure, and specimen type. However, the only significant predictors of CA-MRSA infection compared with HA-MRSA were age <69 years, which was associated with increased risk (odds ratio [OR] 5.1, 95% confidence interval [CI] 2.06–12.64), and hospital exposure (OR 0.07, 95% CI 0.01–0.51), which was associated with decreased risk.

Discussion

The current study was unique because it evaluated invasive MRSA isolates from a statewide surveillance system in a rural area. Most prior studies of the epidemiology of CA-MRSA have focused on SSTI among patients in urban areas.

The published literature documents that incidence of CA-MRSA has increased over time in large urban areas. For example, Kaplan et al. found that incidence of CA-MRSA at Texas Children's Hospital increased each year from August 1, 2001 to July 31, 2004 (*32*). The percentage caused by USA300 increased from ≈50% in 2000 to >90% in 2003. Of these infections, 95.6% were SSTI and 4.4% were invasive. EMERGEncy ID NET reported that USA300 caused 97% of the MRSA SSTIs seen in emergency rooms in 11 US metropolitan areas during August, 2004

Table 3. Antimicrobial drug susceptibility of 54 invasive MRSA USA300/400 isolates, Iowa, USA, 2006*

Antimicrobial agent	% Susceptible
Erythromycin	9
Levofloxacin	57
Clindamycin†	93
Tetracycline	93
Mupirocin	98
Rifampin	98
Trimethoprim/sulfamethoxazole	100
Vancomycin	100
Gentamicin	100
Daptomycin	100
Linezolid	100

*MRSA, methicillin-resistant *Staphylococcus aureus*.
†Includes D-testing of all erythromycin-resistant, clindamycin-susceptible isolates.

(*15*). Seybold et al. demonstrated that by 2004 USA300 had become a common cause of MRSA healthcare-associated bloodstream infections (28%) and of nosocomial MRSA bloodstream infections (20%) at Grady Memorial Hospital in Atlanta (*21*). In contrast, the number of CA-MRSA (primarily USA300) isolates causing invasive infections did not increase substantially in Iowa until 2006.

Klevens et al. published a study of 8,987 invasive MRSA infections reported from the 9 sites in the Active Bacterial Core surveillance (ABCs/Emerging Infections Program Network) from July 2004 through December 2005 (*22*). The investigators conducted PFGE on 864 (11.3%) of the 7,648 isolates submitted from 8 sites. Of these isolates, 29% were USA300 (16% of the healthcare-associated, hospital-onset infections, 22% of the healthcare-associated, community-onset infections, and 67% of the community-associated, community-onset infections); <0.1% were USA400. In our study, 4.5% of all isolates we typed and 14.9% of isolates from 2006 were USA300, which suggested that the incidence of invasive infections caused by USA300 remains lower in Iowa than in the urban centers studied by Klevens et al.

Unlike the findings of Seybold et al. (*21*) and Klevens et al (*22*) from studies conducted in urban areas, USA300/400 rarely caused invasive nosocomial infections in Iowa, a rural state, during the study period. However, unpublished data from the University of Iowa Hospitals and Clinics indicate that these strains are becoming more common causes of invasive nosocomial infections, suggesting that the epidemiology of MRSA may be changing more slowly in rural areas than in large urban areas.

Diep et al. published a follow-up study of previous observations by Han et al. (*23*) about multidrug-resistant USA300 isolates (*31*). These investigators found multidrug-resistant isolates in Boston and in San Francisco and they identified male to male sex, past MRSA infection, and use of clindamycin to be risk factors for multiresistant USA300. A multidrug resistance plasmid (pUSA03) mediated these drug resistances. Fortunately, we did not identify this resistance phenotype among our USA300 isolates from Iowa. However, given the rapidity with which plasmid-mediated antimicrobial drug resistance can spread, and given the epidemic nature of USA300, we will continue surveillance for this and other antimicrobial resistances among USA300 isolates in Iowa.

Investigators in the Netherlands, Denmark, and Canada have found nontypeable MRSA among swine (*33–37*) and persons caring for swine (*33–36*). Strain ST398, which is not typeable by PFGE after DNA is digested with *Sma*I, has been found in all of these countries. Iowa produces ≈25% of the swine in the United States. However, we did not identify this strain among the invasive MRSA isolates submitted to the UHL.

Our data did not include information about preceding influenza infections, but we noted that CA-MRSA was isolated twice from the pleural space; other strains of MRSA were not isolated from this site. This finding suggests that CA-MRSA may have caused serious pulmonary infections in a few persons in Iowa. CA-MRSA, particularly USA300, has caused severe infections after influenza (or influenza-like) infections (*20,24*). During the influenza pandemic of 1918, Chickering and Park noted that many patients acquired severe secondary *S. aureus* pneumonias following influenza infections (*38*). Their observations suggest that coincident outbreaks of pandemic influenza and USA300 pneumonia could be catastrophic.

CA-MRSA, particularly USA300, is a public health concern for several other reasons. As noted previously, USA300 is rapidly replacing other strains of MRSA in the community (*15,31*) and has become an important nosocomial pathogen (*21*). Moreover, the types of infections caused by USA300 and the epidemiology of this strain are changing rapidly (*39*). Currently, most USA300 and USA400 isolates have fewer co-resistances than do HA-MRSA isolates (*13*). However, selective pressures can cause genetic drift in favor of more resistances; papers by Han et al. (*23*) and Diep et al. (*31*) documented that USA300 can

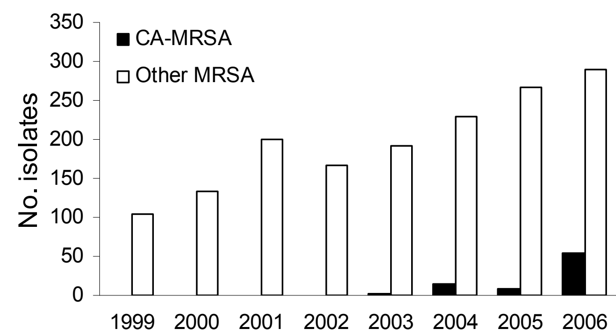

Figure 1. Number of invasive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates submitted in Iowa, USA, 1999–2006. CA-MRSA, community-associated MRSA.

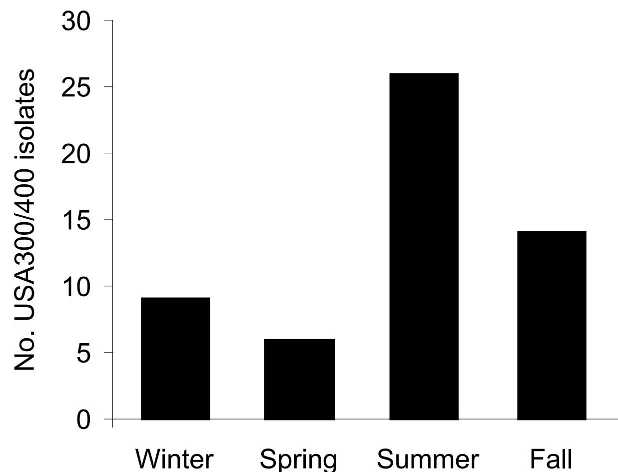

Figure 2. Number of USA300/400 methicillin-resistant *Staphylococcus aureus* isolates submitted by season, Iowa, USA, 2006.

acquire additional drug resistance determinants. If USA300 and USA400 become resistant to oral antimicrobial agents and the proportion of invasive MRSA infections caused by CA-MRSA continues to increase, many more patients will need parenteral vancomycin therapy, which will increase the difficulty and cost of treating these infections. Furthermore, as the incidence of CA-MRSA infections increases, horizontal transmission of these strains could increase in hospitals, making control of MRSA in healthcare settings even more difficult (*6,40*). Nosocomial bloodstream infections, ventilator-associated pneumonia, and surgical site infections caused by these strains could be devastating given the necrotizing nature of the infections.

Our study had several limitations. First, the surveillance system was passive. Consequently, demographic data and data about race/ethnicity and exposure to long-term-care facilities were incomplete and data about prior antimicrobial drug exposure and underlying diseases were not available. Additionally, we could identify the region where the specimen originated but not the specific city or county. Moreover, a validation study in Iowa found that hospital laboratories submit only 37% of the invasive MRSA isolates that they identify (D. Dufficy, pers. comm.). However, underreporting affected all regions equally. Furthermore, we used different selection criteria for typing invasive MRSA isolates submitted from 1999–2005 than we did for those submitted in 2006. We typed isolates from 1999–2005 only if they had $\leq$3 non–β-lactam coresistances, but we typed all invasive MRSA isolates from 2006. Thus, we may have introduced selection bias that would predispose the incidence of USA300/400 during 1999–2005 toward the null hypothesis (i.e., annual proportion of MRSA isolates that were USA300/400 did not change during1999 to 2005) but away from the null hypothesis for the incidence of USA300/400 isolates during 1999–2006 (i.e., H_0: The annual proportion of MRSA isolates that were USA300/400 was the same in 1999–2005 and in 2006). However, given that all the USA300/400 isolates identified during 2006 would have been detected using the 1999–2005 coresistance selection criterion, we believe that we typed all of the invasive USA300/400 isolates obtained during 1999–2005.

Some might argue that the incidence of invasive infections caused by CA-MRSA increased artificially because physicians were more aware of these organisms in 2006 than they were previously. CA-MRSA certainly has become a hot topic. CA-MRSA was initially identified in the mid 1990s, and many articles about these organisms have been published since then. However, the incidence of invasive infections caused by CA-MRSA in Iowa did not begin rising until 2006. Moreover, Iowa requires laboratories to send all invasive MRSA isolates to the UHL, and laboratory personnel are unlikely to know the details of each patient's infection. Thus, laboratories probably would not submit isolates of 1 strain preferentially. Finally, many hospitals submitted isolates to the UHL, which suggests that submission bias was unlikely in 2006.

In conclusion, the number of invasive MRSA infections reported in Iowa and the number of invasive infections caused by CA-MRSA increased in Iowa from 1999–2005 to 2006. The increase of CA-MRSA (particularly USA300) poses a unique public health threat. Our study demonstrated that CA-MRSA no longer causes only SSTIs but now causes an increased proportion of invasive infections in a rural state. This finding is particularly disconcerting in light of the severity of these infections and the reports of necrotizing pneumonia caused by USA300 after influenza or influenza-like illness. Surveillance systems must continue to monitor the number and incidence of infections caused by USA300 and to monitor these isolates for changes in antimicrobial susceptibility. The relationship between seasons and CA-MRSA warrants further study.

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Healthcare Worker Occupation and Immune Response to *Pneumocystis jirovecii*

Renuka Tipirneni, Kieran R. Daly, Leah G. Jarlsberg, Judy V. Koch, Alexandra Swartzman, Brenna M. Roth, Peter D. Walzer, and Laurence Huang

The reservoir and mode of transmission of *Pneumocystis jirovecii* remain uncertain. We conducted a cross-sectional study of 126 San Francisco General Hospital staff in clinical (n = 103) and nonclinical (n = 23) occupations to assess whether occupational exposure was associated with immune responses to *P. jirovecii*. We examined antibody levels by ELISA for 3 overlapping fragments that span the *P. jirovecii* major surface glycoprotein (Msg): MsgA, MsgB, and MsgC1. Clinical occupation participants had higher geometric mean antibody levels to MsgC1 than did nonclinical occupation participants (21.1 vs. 8.2, p = 0.004); clinical occupation was an independent predictor of higher MsgC1 antibody levels (parameter estimate = 0.89, 95% confidence interval 0.29–1.48, p = 0.003). In contrast, occupation was not significantly associated with antibody responses to either MsgA or MsgB. Healthcare workers may have occupational exposure to *P. jirovecii*. Humans may be a reservoir for *P. jirovecii* and may transmit it from person to person.

Although the incidence of *Pneumocystis jirovecii* pneumonia (PCP) has declined in the era of combination antiretroviral therapy, PCP remains the most common serious opportunistic infection among human immunodeficiency virus (HIV)-infected persons in the United States (*1*). The reservoir and mode of transmission of *P. jirovecii* remain uncertain because of an inability to grow the organism in vitro. However, studies of immune responses to *P. jirovecii* have provided important insights into its epidemiology, showing that up to 80%–100% of children have detectable *P. jirovecii* antibodies by 8 years of age (*2–9*). These findings suggest that *P. jirovecii* is ubiquitous, that humans are exposed to *P. jirovecii* early in life, and that PCP that develops later in life results from reactivation of latent infection.

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Emerging evidence suggests that PCP also can result from recent acquisition of *P. jirovecii*, and the organism may be transmitted from person to person (*10,11*). In the hospital or clinic, numerous PCP outbreaks have been reported among immunocompromised patients who shared common healthcare workers (HCWs), hospital rooms, wards, or clinics (*12–21*). In the laboratory, animal-to-animal transmission of *Pneumocystis* spp. has been demonstrated both by immunocompromised and immunocompetent hosts after periods of exposure as short as 1 day (*22,23*). Dumoulin et al. demonstrated that immunocompetent mice became transiently colonized with *Pneumocystis* spp. after contact with *Pneumocystis*-infected mice and then were able to transmit the infection to *Pneumocystis*-free mice that had severe combined immunodeficiency (*23*).

Several studies have found that *P. jirovecii* can colonize immunocompetent humans and suggest that such persons may serve as potential reservoirs (*24*). The question that arises is whether person-to-person transmission occurs through immunocompetent hosts, such as HCWs, who may be transiently colonized with *P. jirovecii* during brief clinical interactions with PCP patients and subsequently transmit the infection to other immunocompromised patients. Prior studies involving HCWs used different specimens (e.g., induced sputum, oropharyngeal wash, nasal rinse, deep nasal swab, blood) and different laboratory methods (i.e., different PCRs and ELISAs) to compare exposed and unexposed groups, making findings difficult to compare

across studies (*25–31*). In addition, these studies compared different groups of HCWs and did not include a control group without patient contact.

Therefore, we performed a cross-sectional study of hospital staff at San Francisco General Hospital (SFGH) in both clinical (exposed) and nonclinical (unexposed) occupations. Our goal was to determine whether HCW occupation was associated with antibody levels to *P. jirovecii*. Finding this association would suggest that HCWs may acquire *P. jirovecii* and potentially be a reservoir in the hospital setting.

Methods

Participants

We recruited participants from the Department of Medicine, the Division of Pulmonary and Critical Care Medicine, and the HIV/AIDS Division because members of these groups provide the most care to patients with HIV infection or PCP, our hypothesized primary reservoirs of *P. jirovecii*. We recruited by word of mouth; emails to departmental listservs; and announcements at medical conferences, staff meetings, and orientations for medical students and residents. From January 2007 through February 2008, we enrolled 126 SFGH staff. We included staff who worked at the hospital during the study period, provided informed consent, and had no clinical evidence of PCP. The University of California, San Francisco, and the University of Cincinnati institutional review boards approved the study.

Questionnaire

We collected information by using a standardized participant-completed questionnaire. In addition to demographic characteristics, we obtained information about occupation, department/division, percentage of time spent seeing patients, and past exposure to patients with PCP. These questions were designed to assess patient contact in general and contact with PCP patients specifically. We also obtained information about cigarette smoking, chronic lung disease (e.g., chronic obstructive pulmonary disease), and immunocompromising conditions (e.g., HIV infection or use of immunosuppressive medications) because these factors have been associated with *P. jirovecii* colonization (*24*). To protect our colleagues' confidentiality regarding their medical history, we asked participants to check yes if they had ≥1 condition from a list of pulmonary or immunocompromising conditions, without requiring them to specify the condition. For example, participants were asked to reply yes or no to the following question: "Do you have any of the following conditions? HIV, cancer including leukemia/lymphoma, organ transplant, bone marrow transplant, steroid medication (e.g., prednisone), chemotherapy medication, immunosuppressive medication (e.g., methotrexate, rituximab, cyclosporine, tacrolimus, azathioprine, cyclophosphamide), pregnancy," but participants were not required to disclose the specific immune disorder (e.g., HIV infection, cancer).

Classification of Participants

We classified staff into 2 groups: those with patient contact (clinical occupation group, n = 103) and those without contact with patients or clinical samples from patients (nonclinical occupation group, n = 23). The clinical group was further subdivided into staff who provide direct patient care (e.g., physicians and nurses) and staff who provide indirect patient care or ancillary services (e.g., medical assistants, social workers, and pharmacists). Because each department/division has defined space on the SFGH campus, people from the same department/division generally have offices in the same location. However, those in the clinical occupation group also work in the hospital wards, clinics, or clinical research units and have contact with patients, whereas those in the nonclinical group have no occupational contact with patients.

Serum ELISA

We collected a serum specimen from each participant and stored it at −80ºC before shipping it to the University of Cincinnati. We used a previously described ELISA (*32*,*33*) to measure immunoglobulin G levels to 3 overlapping fragments that span the length of the *P. jirovecii* major surface glycoprotein (Msg): MsgA, MsgB, and MsgC1 (Figure 1). MsgA is the amino terminus, MsgB is the middle portion, and MsgC is the carboxyl terminus (*34*). MsgC1 is 1 of 4 MsgC variants we have generated and can be used to distinguish between HIV-infected patients with and without PCP on the basis of antigenic recognition (*33*). We tested participant serum specimens and the reference serum against Msg fragments and used phosphate-buffered saline as a negative control. We corrected the reactivity of each serum specimen to Msg by subtracting the reactivity of that serum to phosphate-buffered saline and quantified the results by using the method of Bishop and Kovacs (*35*). A standard curve generated for each Msg construct was used to calculate the units of reactivity. We assigned to each standard serum pool a value of 100 U of reactivity to its target Msg construct in 100 µL of a 1:100 dilution. We assayed test specimens at 1:100–1:200 dilutions to fit the linear portion of the standard curves and calculated units of reactivity. Samples with values below the standard curve were assigned the lowest possible value of 1 U.

Statistical Analysis

The laboratory group was blinded to the clinical data, and the clinical group was blinded to the laboratory results. Prespecified primary predictors of interest were profession-

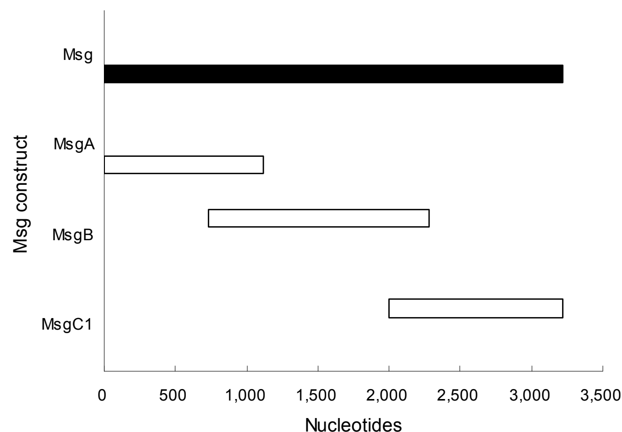

Figure 1. *Pneumocystis jirovecii* major surface glycoprotein (Msg) fragments. Lengths of Msg fragments are expressed on a nucleotide scale. MsgA is the amino terminus, MsgB is the middle portion, and MsgC1 is the carboxyl terminus of the protein.

al and health characteristics. The outcome of interest was Msg antibody level, a continuous variable, which we log transformed to better approximate a normal distribution. We calculated the mean log Msg level for each predictor variable and examined bivariate associations using Student *t* test. The data were then converted to the original scale and presented as geometric means (GMs). For associations with p≤0.1 in bivariate analysis, we performed multivariate linear regression using the natural log of Msg level as the dependent variable and considered a 2-tailed p<0.05 to be statistically significant. All statistics were calculated using SAS software, version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Participants

We enrolled 126 staff. Mean age of participants was 39.6 years (range 22–80 years), 57.1% were female, 60.2% were white/Caucasian, 25.2% were Asian, 16.0% were Hispanic/Latino, and 3.3% were black/African American (Table 1). Forty-two (33.6%) had smoked at least 100 cigarettes in their lifetime, and 16.0% had chronic lung disease, including asthma (n = 17) and interstitial lung disease (n = 1). Overall, 6.4% had an immunocompromising condition. Participants were primarily from the HIV/AIDS Division (44.4%), the Division of Pulmonary and Critical Care Medicine (26.2%), and the Department of Medicine (23.0%). A few participants (6.4%) were from other departments (Obstetrics and Gynecology, Psychiatry, and Radiology) and were involved in the care of HIV-infected or PCP patients. Eighty-five (67.5%) participants reported prior exposure to a PCP patient.

Clinical and Nonclinical Occupation Groups

We classified 103 (81.7%) participants into the clinical occupation group and 23 (18.3%) into the nonclinical occupation group. The clinical group consisted of 27 attending physicians; 17 residents and fellows; 19 medical students; 9 nurse practitioners; 10 nurses; 10 ancillary clinic staff, including medical assistants, social workers, pharmacists, and clinic managers; and 11 clinical research personnel. The nonclinical group consisted of 18 administrative staff and 5 laboratory personnel. These 2 groups did not differ significantly in terms of demographic characteristics (Table 1). However, a significantly greater proportion of the nonclinical group than the clinical group reported having an immunocompromising condition (17.4% vs. 3.9%, p = 0.04), and a significantly greater proportion of the clinical group than the nonclinical group reported prior exposure to a PCP patient (83 [80.6%] of 103 vs. 2 [8.7%] of 23, p<0.001).

Antibody Levels to MsgA, MsgB, and MsgC1

Participants had detectable antibody levels to MsgA (GM 11.8, 95% CI 8.1–17.0), MsgB (GM 2.6, 95% confidence interval [CI] 2.1–3.1), and MsgC1 (GM 17.8, 95% CI 13.8–22.9) (Table 2). Antibody responses were detected in participants from all demographic groups, in smokers and nonsmokers, and in participants with and without chronic lung disease or immunocompromising condition. Responses also were detected in participants with and without exposure to a PCP patient and in both the clinical and nonclinical groups.

Antibody levels to MsgA or MsgB did not differ significantly by age, sex, race, ethnicity, smoking status, presence of chronic lung disease, or presence of immunocompromising condition (Table 2). Similarly, MsgA or MsgB levels did not differ significantly between participants with and without exposure to a PCP patient or between the clinical and nonclinical groups.

In contrast, antibody levels to MsgC1 differed significantly (Table 2). Participants >60 years of age had significantly lower GM antibody levels than all others (4.8 vs. 19.1, p = 0.03); Asians had higher GM antibody levels than non-Asians (27.5 vs. 15.3, p = 0.05); and men had nonsignificantly higher GM antibody levels than women (22.7 vs. 14.8, p = 0.1). In contrast to the findings for MsgA and MsgB, participants in the clinical occupation group had significantly higher GM MsgC1 antibody levels than those in the nonclinical occupation group (21.1 vs. 8.2, p = 0.004).

Predictors of MsgC1 Antibody Levels

To identify independent predictors of antibody levels to MsgC1, we performed multivariate linear regression and included in the model variables that had p≤0.1 in bivariate analysis: older age, Asian race, sex, and clinical occupa-

Table 1. Characteristics of participants in a study of *Pneumocystis jirovecii* antibody levels*

Characteristic	Total, no. (%), N = 126	Clinical, no. (%), n = 103	Nonclinical, no. (%), n = 23	p value
Demographics				
Age group, y				
≤30	33 (26.6)	30 (29.7)	3 (13.0)	Ref
31–40	38 (30.7)	31 (30.7)	7 (30.4)	0.98
41–50	27 (21.8)	20 (19.8)	7 (30.4)	0.35
51–60	21 (16.9)	16 (15.8)	5 (21.7)	0.53
>60	5 (4.0)	4 (4.0)	1 (4.4)	0.92
Sex				
F	72 (57.1)	57 (55.3)	15 (65.2)	0.39
M	54 (42.9)	46 (44.7)	8 (34.8)	Ref
Race				
White/Caucasian	74 (60.2)	60 (60.0)	14 (60.9)	Ref
Asian	31 (25.2)	27 (27.0)	4 (17.4)	0.14
Black/African American	4 (3.3)	2 (2.0)	2 (8.7)	0.14
Other	14 (11.4)	11 (11.0)	3 (13.0)	0.82
Hispanic/Latino ethnicity	20 (16.0)	14 (13.7)	6 (26.1)	0.20
Health conditions				
Ever smoked	42 (33.6)	32 (31.4)	10 (43.5)	0.27
Lung condition	20 (16.0)	14 (13.7)	6 (26.1)	0.20
Immune condition	8 (6.4)	4 (3.9)	4 (17.4)	0.04
Professional characteristics				
Department/division				
HIV/AIDS	56 (44.4)	45 (43.7)	11 (47.8)	Ref
Pulmonary and CCM	33 (26.2)	28 (27.2)	5 (21.7)	0.42
Medicine	29 (23.0)	25 (24.3)	4 (17.4)	0.32
Other	8 (6.4)	5 (4.9)	3 (13.0)	0.14
Ever exposed to PCP patient	85 (67.5)	83 (80.6)	2 (8.7)	<0.001

*Ref, reference category; CCM, critical care medicine; PCP, *P. jirovecii* pneumonia. Participants in this cross-sectional study were healthcare staff recruited during January 2007–February 2008 from San Francisco General Hospital. Mean ages (y ± SD) of participants: total, 39.6 ± 11.7; clinical, 39.1 ± 11.9; nonclinical, 42.0 ± 10.4 (p = 0.29).

tion. Clinical occupation (p = 0.003) and age >60 years (p = 0.03) were the only significant independent predictors of MsgC1 antibody response when we controlled for other factors (Table 3). Because the proportion of persons with immunocompromising conditions differed between the clinical and nonclinical groups, we included this variable in a subsequent model and found that it strengthened the association between clinical occupation and MsgC1 levels (coefficient 0.98, 95% CI 0.38–1.58, p = 0.001).

The GM MsgC1 antibody level was significantly higher in the clinical than in the nonclinical occupation group. Within the clinical group, we examined the association between direct patient care occupations and indirect patient care occupations and MsgC1 antibody levels. We found no significant difference in GM MsgC1 antibody levels between these 2 groups (21.8 for direct care group vs. 18.8 for indirect care group, p = 0.64). Finally, when we examined the association between individual occupations and MsgC1 antibody levels (Figure 2), medical students (28.8) and residents/fellows (32.5) had the highest GM MsgC1 antibody levels of all participants. Laboratory staff had the lowest GM levels (4.2), but these levels did not differ significantly from those of administrative staff (9.9) (p = 0.33).

Discussion

We investigated whether HCW occupation was associated with immune response to *P. jirovecii*. Staff in the clinical occupation group had significantly higher antibody levels to MsgC1 than did those in the nonclinical occupation group, and clinical occupation was the most significant predictor of MsgC1 antibody levels. In contrast, HCW occupation was not significantly associated with antibody levels to either MsgA or MsgB.

Why did we find an association between occupation and MsgC1 but not MsgA or MsgB antibody levels? MsgC1 is the most conserved of the 3 Msg fragments studied (*36*). When a person is repeatedly exposed to *P. jirovecii*, MsgC1 possibly acts as a recall antigen and elicits higher amnestic responses than primary antigens, such as MsgA and MsgB. Previously, we found a significant association between HIV-infected patients with PCP and changes in MsgC1 antibody levels but not with MsgA or MsgB antibody levels (*37*). In PCP patients, antibody levels of MsgC1, but not MsgA or MsgB, increased significantly from baseline to weeks 3–4 (after treatment completion). Furthermore, MsgC1 antibody levels increased only in patients with PCP; levels remained unchanged in patients with non-*P. jirovecii* pneumonia. Thus, increases in

Table 2. Bivariate associations with *Pneumocystis jirovecii* Msg levels*

Characteristic	No. persons	MsgA		MsgB		MsgC1	
		GM (95% CI)	p value	GM (95% CI)	p value	GM (95% CI)	p value
Total	126	11.8 (8.1–17.0)		2.6 (2.1–3.1)		17.8 (13.8–22.9)	
Demographics							
Age group, y							
<30	33	12.0 (5.4–26.8)	Ref	2.5 (1.7–3.7)	Ref	16.3 (9.5–28.0)	Ref
31–40	38	11.0 (5.7–21.3)	0.86	2.7 (1.8–3.9)	0.84	22.6 (14.9–34.4)	0.33
41–50	27	10.1 (4.5–22.4)	0.75	2.4 (1.6–3.7)	0.88	19.8 (11.6–33.7)	0.61
51–60	21	17.2 (6.3–46.4)	0.57	2.4 (1.4–4.2)	0.90	17.4 (8.8–34.3)	0.88
>60	5	12.8 (0.7–243.0)	0.95	3.3 (0.3–32.8)	0.66	4.8 (0.7–34.7)	0.11
Sex							
M	54	14.3 (8.2–25.2)	0.37	2.9 (2.1–4.0)	0.26	22.7 (16.0–32.1)	0.10
F	72	10.2 (6.2–16.7)		2.3 (1.8–3.0)		14.8 (10.4–21.2)	
Race							
Asian	31	6.6 (3.1–14.4)	0.08	2.3 (1.6–3.3)	0.49	27.5 (17.6–42.9)	0.05
Other	92	14.4 (9.4–22.1)		2.7 (2.1–3.5)		15.3 (11.2–20.7)	
Ethnicity							
Hispanic/Latino	20	12.5 (5.5–28.5)	0.92	1.9 (1.3–2.7)	0.17	13.4 (6.2–29.1)	0.35
Non–Hispanic/Latino	105	11.9 (7.9–18.1)		2.7 (2.2–3.5)		18.7 (14.2–24.4)	
Health conditions							
Smoked							
Ever	42	13.9 (7.1–27.1)	0.58	2.8 (1.9–4.1)	0.63	13.2 (8.4–20.9)	0.11
Never	83	11.2 (7.1–17.5)		2.5 (2.0–3.2)		20.5 (15.1–27.8)	
Lung condition							
Yes	20	11.3 (4.4–28.7)	0.88	2.5 (1.5–4.1)	0.83	14.1 (7.2–27.5)	0.45
No	105	12.2 (8.1–18.3)		2.6 (2.1–3.3)		18.5 (14.0–24.4)	
Immune condition							
Yes	8	13.1 (2.6–66.6)	0.90	2.9 (0.8–10.0)	0.76	24.0 (13.6–42.3)	0.26
No	117	11.9 (8.1–17.6)		2.6 (2.1–3.1)		17.3 (13.2–22.7)	
Professional characteristics							
Exposed to PCP patient							
Ever	85	13.1 (8.3–20.7)	0.41	2.6 (2.0–3.3)	0.99	20.8 (15.8–27.3)	0.11
Never	41	9.4 (4.9–18.0)		2.6 (1.8–3.6)		12.9 (7.5–21.9)	
Occupation							
Clinical	103	13.4 (8.9–20.1)	0.14	2.6 (2.1–3.3)	0.80	21.1 (16.3–27.3)	0.004
Nonclinical	23	6.6 (2.6–16.6)		2.4 (1.5–4.0)		8.2 (4.0–17.0)	

*Msg, major surface glycoprotein; GM, geometric mean; CI, confidence interval; Ref, reference category; PCP, *P. jirovecii* pneumonia. Serum antibody levels were calculated from standard curves derived by using a standard serum pool with an assigned value of 100 U; specimens below the curve were assigned the lowest possible value (1 U). Predictor variables were compared with the natural log of Msg levels using Student *t* test, then converted back to the original scale and presented as GM. Participants in this cross-sectional study were healthcare staff recruited during January 2007–February 2008 from San Francisco General Hospital.

MsgC1 levels may indicate recovery from or recent exposure to PCP, and this assay may be a useful epidemiologic tool for studying *P. jirovecii* infection.

The MsgC1 assay may also be useful in the study of *P. jirovecii* exposure and colonization. In the laboratory, *Pneumocystis* colonizes immunocompetent animals after they are exposed to either animals with *Pneumocystis* pneumonia or animals colonized with *Pneumocystis* (*22*,*23*). Colonized animals remain without evidence of pneumonia but can serve as a source of *Pneumocystis* because *Pneumocystis* pneumonia develops in immunocompromised animals subsequently placed in contact with them. These findings suggest that HCWs exposed to patients with PCP (or persons colonized with *P. jirovecii*) can become colonized with *P. jirovecii* and that the MsgC1 ELISA might be used as an indication of this colonization, although the precise relation between antibody levels and *P. jirovecii* colonization has yet to be fully determined.

Prior studies of *P. jirovecii* exposure and colonization in HCWs have yielded mixed results. Girón et al. observed that antibody levels in intensive care unit staff rose after exposure to a PCP patient, whereas those of control staff did not (*25*). Leigh et al. found that antibody levels of HCWs caring for patients with AIDS were significantly higher than those in control workers caring for elderly patients (*26*). Vargas et al. detected *P. jirovecii* DNA in deep nasal swab samples of 2 HCW contacts and 1 family contact of a PCP patient, compared with no detectable DNA in control HCWs who did not enter the patient's room (*29*). Likewise, Miller et al. demonstrated that HCWs in an HIV/AIDS unit were more likely to have detectable *P. jirovecii* DNA in induced sputum and nasal rinse samples than were

Table 3. Factors associated with MsgC1 levels in a multivariate linear regression analysis*

Factor	Estimate (95% CI)	p value
Age >60 y	−1.34 (−2.51 to −0.16)	0.03
Male sex	0.44 (−0.03 to 0.91)	0.07
Asian race	0.42 (−0.12 to 0.95)	0.13
Clinical occupation	0.89 (0.29 to 1.48)	0.003
F value	5.15	<0.001
R^2	0.15	

*Msg, major surface glycoprotein; CI, confidence interval. A multivariate linear regression was generated by using the natural log of Msg levels as the dependent variable and including those predictor variables with $p \leq 0.10$ in bivariate analysis. Participants in this cross-sectional study were healthcare staff recruited during January 2007–February 2008 from San Francisco General Hospital.

those working on a general medical–respiratory unit (*30*). Durand-Joly et al. longitudinally followed HCWs with oropharyngeal washes and found *P. jirovecii* DNA in 10 (6.1%) of 164; duration of carriage ranged from 3 to 10 weeks (*31*).

In contrast, other studies found no evidence of *P. jirovecii* colonization in HCWs. Lundgren et al. found no difference in the frequency or level of *P. jirovecii* Msg antibodies between HCWs caring for PCP patients in an infectious diseases department and control staff from a blood bank and surgical department (*28*). In addition, they were unable to detect *P. jirovecii* DNA in oropharyngeal washes. Similarly, Lidman and colleagues found no evidence of *P. jirovecii* DNA in sputum samples from nurses caring for a PCP patient, and only 2 (8%) of 26 had detectable antibodies to *P. jirovecii* (*27*). The conflicting results from these studies may be due to several factors, including differences in types of staff recruited, specimens collected, or assays used. Staff working in different clinical departments are likely to have differing degrees of exposure to PCP patients. For example, HCWs working on an HIV/AIDS unit (Miller et al. study) may have cared for more PCP patients than did those working in a general infectious diseases department (Lundgren et al. study). This difference in *P. jirovecii* exposure may have contributed to positive findings in the first study and negative findings in the second. In addition, different respiratory specimens have different degrees of invasiveness and different organismal yields, ranging from more invasive bronchoalveolar lavage to less invasive oropharyngeal washes. Similarly, different assays have varying sensitivities for detecting the organism. Among the DNA-based assays, nested PCR is more sensitive than single-round PCR for detecting *P. jirovecii* DNA. For serum assays, ELISA may be more sensitive but Western blot is more specific. Prevalence of the organism also may differ in different geographic regions, as has been seen in various European populations (*4*,*38*,*39*).

Our observation of higher MsgC1 antibody levels in clinical staff than in nonclinical staff is consistent with the possibility of nosocomial transmission of *P. jirovecii*. Moreover, immunocompetent HCWs may serve as reservoirs. *P. jirovecii* may circulate among patients and staff in the hospital, causing colonization in immunocompetent staff and pneumonia in immunocompromised patients. Studies in laboratory animals and outbreaks of PCP within clinical settings support this theory and person-to-person transmission as the mode of transmission. If this pattern does indeed occur, it would have clinically relevant implications for hospital infection control policies.

Our study is limited by an imprecise measure of *P. jirovecii* exposure. Most staff can recall direct contact with PCP patients but cannot recall transient exposure or indirect contact. In addition, asymptomatic persons colonized with *P. jirovecii* may be another source of exposure not recognized by HCWs. Our cross-sectional study design and method of data collection (participant recall) limit our ability to accurately capture information about *P. jirovecii* exposure and intensity of exposure over time. Comparing hospital staff with patient contact to those without patient contact allowed us to evaluate whether clinical occupation was independently associated with *P. jirovecii* antibody levels. Although the nonclinical group had a higher proportion of immunocompromising conditions than did the clinical group, this difference most likely did not influence the results. Whether an immunocompromising condition would be associated with lower antibody levels caused by impaired antibody production or higher antibody levels caused by permissive colonization is unclear. Our results suggest that the presence of an immunocompromising condition may be associated with higher levels. Regardless, including this predictor in the multivariate analysis only strengthened the association of clinical occupation and MsgC1 levels. Norris and colleagues showed that a rise in antibody levels is associated with colonization in nonhuman primates (*40*), and although antibody levels may not correlate directly with colonization, a similar pattern might occur in humans.

Early serologic studies of *P. jirovecii* led investigators to conclude that PCP results from reactivation of latent in-

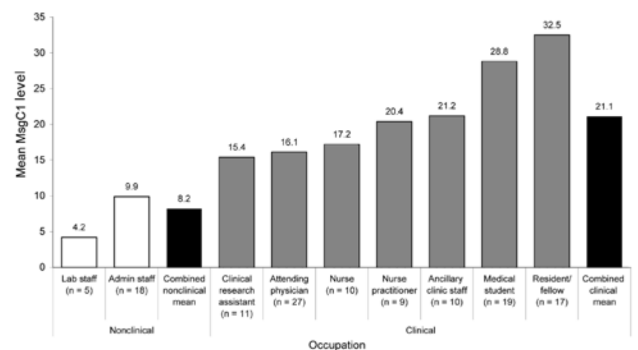

Figure 2. Major surface glycoprotein C1 (MsgC1) levels by occupation. Geometric mean MsgC1 levels are shown for nonclinical and clinical staff, by job title.

fection; however, our data add to more recent studies suggesting that PCP may be acquired from recent exposure. Higher antibody levels in clinical staff than in nonclinical staff may be evidence of patient-to-provider transmission of *P. jirovecii*. Furthermore, evidence from animal models suggests that parallel provider-to-patient transmission could occur with immunocompromised patients. Future studies should use serologic assays, such as the MsgC1 assay, as epidemiologic tools to assess *P. jirovecii* exposure in HCWs longitudinally, in addition to examining direct measures of colonization such as *P. jirovecii* DNA in respiratory specimens. If further data support the pattern observed here, clinicians may consider respiratory precautions and respiratory isolation when caring for patients with PCP. Preventing *P. jirovecii* transmission may further decrease the incidence of this grave disease.

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Dr Tipirneni was a medical student and research fellow at the University of California, San Francisco, during this study. She is now an intern in internal medicine at Massachusetts General Hospital. Her research interests include the basic epidemiology of *P. jirovecii*.

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etymologia

Kobuvirus

The Japanese term *kobu* (kō′ bü), meaning hump or knob, was chosen to designate this genus in the family *Picornaviridae* because of the distinctive icosahedral structure of the viruses in this group. The genus contains the species *Aichi virus* and *Bovine kobuvirus*, and a candidate porcine kobuvirus has been identified. *Aichi virus*, the type species, was first recognized in 1989 as the cause of oyster-associated nonbacterial gastroenteritis in persons in the Aichi Prefecture in Japan.

Source: Mahy B. The dictionary of virology, 4th edition. London: Elsevier; 2009; Pringle CR. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. Arch Virol 1999; 144:2066–70.

Nosocomial Outbreak of Novel Arenavirus Infection, Southern Africa

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A nosocomial outbreak of disease involving 5 patients, 4 of whom died, occurred in South Africa during September–October 2008. The first patient had been transferred from Zambia to South Africa for medical management. Three cases involved secondary spread of infection from the first patient, and 1 was a tertiary infection. A novel arenavirus was identified. The source of the first patient's infection remains undetermined.

Arenaviruses associated with rodents are known to cause fatal hemorrhagic fevers in humans in South America and West Africa. We describe a nosocomial outbreak of infection with a novel arenavirus involving 5 patients, 4 of whom died, which occurred in South Africa in September–October 2008. The first patient was transferred from Zambia to South Africa for medical management. The source of her infection remains undetermined. Three cases involved secondary spread of infection from the first patient, and 1 tertiary infection occurred.

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The Outbreak

Patient 1 (Figure 1) was a travel agent who lived on an agricultural smallholding on the outskirts of Lusaka, Zambia, where she kept horses, dogs, and cats. She occasionally encountered dormice in her home, and evidence of rodent activity was found in the stables. She had visited a tourist camp on the Zambezi River on July 30 and 31, 2008, and participated in a polocrosse tournament on August 1 and 2 (but neither event occurred within the known incubation period of arenavirus infections from the date of onset of her illness). On August 30, she was cut on the shin by broken glass from a dropped bottle. Severe headache and malaise developed in the patient on September 2. On September 4, she traveled by air to attend a wedding in South Africa and was taking medication for suspected influenza. Diarrhea and vomiting developed in 4 of 110 guests who attended the wedding on September 6. Patient 1 was not affected, but she reported feeling cold and took breaks from dancing to warm herself at a fireplace. On returning to Lusaka on September 7, she was exhausted and experienced an attack of diarrhea and vomiting.

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She remained ill in bed on September 8. The following day she had fever, severe chest pain, and sore throat and was treated for food poisoning and influenza with antiemetic, antipyretic, and analgesic medications and a cephalosporin antimicrobial drug. On September 10, she felt slightly better, but overnight a rash developed that extended from her toes to her cheeks, as did severe myalgia and facial swelling. She was treated at a clinic on September 11 for a presumed allergic reaction to the cephalosporin, was discharged, and then readmitted that evening with severe sore throat. She was moved to a hospital for stabilization on September 12 and then evacuated by air to South Africa. During the flight, the attending physician and paramedic were potentially exposed to infection through nebulization, suctioning, and manual ventilation of the patient.

When she arrived at a private hospital in Johannesburg, her pupillary and corneal reflexes were absent, and she had cerebral edema (confirmed by computed tomography scan), acute respiratory distress syndrome, and deteriorating renal function. She had thrombocytopenia, granulocytosis, and raised serum alanine and aspartate transaminase levels. The observation of an apparent eschar on her right foot prompted treatment for rickettsiosis. Despite intensive care, including hemodialysis, she died on September 14. No autopsy was performed, and the body was returned to Zambia for cremation.

Patient 2 (Figure 1) was the paramedic who attended patient 1 during the air evacuation to Johannesburg. He had onset of illness on September 21 with headache, myalgia, and fever, and on September 24 he was admitted to a hospital in Lusaka. On September 27, he was evacuated to the same hospital in Johannesburg as patient 1, and on the following day the link between the 2 was identified and a presumptive diagnosis of viral hemorrhagic fever was made. Patient 2 died October 2. Tracing of contacts of the 2 patients was instituted, and it was found that a nurse, patient 3 (Figure 1), who had attended and cleaned the body of patient 1, became ill on September 23 while on leave. She was admitted to a private hospital west of Johannesburg on October 1, where she died October 5.

Blood samples were collected from patient 2 on September 29 and 30 and from patient 3 on October 3. The samples were screened at the National Institute for Communicable Diseases (NICD) for evidence of infection with known agents of the viral hemorrhagic fevers of Africa by reverse transcription–PCR (RT-PCR) for presence of viral nucleic acid, including 2 procedures with primers designed to detect all known Old World arenaviruses (*1*,*2*). Samples were also tested by ELISA for antibodies, without positive results, and injected into Vero cell cultures for isolation of virus. For administrative reasons, the taking of liver samples with biopsy needles and the taking of skin punch samples from the bodies of patients 2 and 3 was delayed

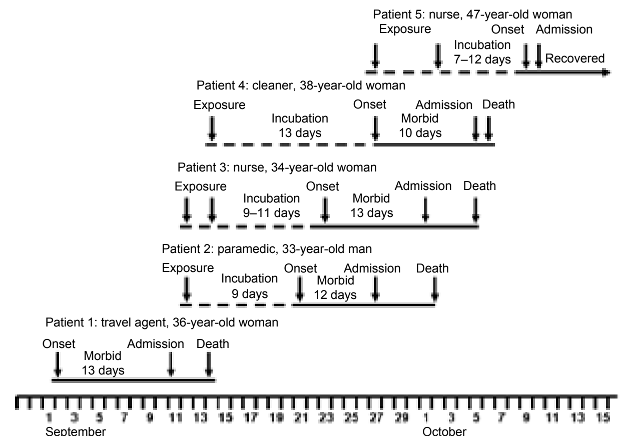

Figure 1. Epidemic curve showing, as appropriate, dates of exposure to infection, onset of illness, admission to hospital, and death or recovery of 5 patients involved in an outbreak of infection with a novel arenavirus, southern Africa, 2008.

until October 9, and by the next morning the Department of Anatomical Pathology at the University of the Witwatersrand reported observing hepatocyte necrosis and skin vasculitis compatible with viral hemorrhagic fever (Figure 2, panel A).

Diagnosis and Contact Tracing

Blood, liver, and skin samples sent to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) arrived on October 10. The following day the Infectious Disease Pathology Branch reported that immunohistochemical staining of liver and skin sections resulted in the detection of antigen with monoclonal antibody that was broadly cross-reactive for Old World arenaviruses, the first indication of an etiologic diagnosis (Figure 2, panel B). Consequently, the same arenavirus RT-PCR procedures as above were applied to liver extracts and produced positive results at NICD and the Special Pathogens Branch, CDC. One RT-PCR procedure yielded ≈300-bp glycoprotein gene product (*1*), and the other yielded ≈1,000-bp nucleoprotein gene product (*2*). Nucleotide sequencing of the PCR products and phylogenetic analysis performed as described (*3*) but using MEGA 4.0 (*4*) showed that a novel arenavirus, since named Lujo virus (*5*), was involved (Figure 3).

Replicate nucleic acid extracts from liver samples that were sent to Columbia University (New York, NY, USA) were subjected to 454 pyrosequencing to produce full-length genome sequences of the arenavirus, extending the preliminary findings based on partial sequence data to indicate that the new virus is related to, but distinct from, known Old World arenaviruses (*5*). The novel arenavirus was isolated in culture from the blood and liver samples of patients 2 and 3, as confirmed by immunofluorescence with

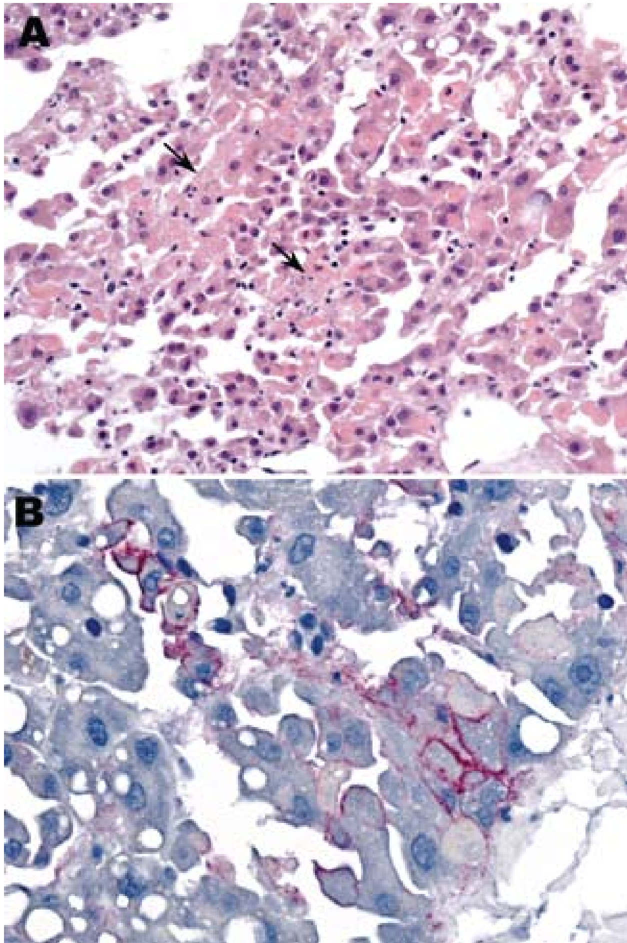

Figure 2. Liver biopsy specimen from patient 2 showing focal hepatocyte necrosis (arrows) without prominent inflammatory cell infiltrates (A) and Lujo virus antigens (red) distributed predominantly in a membranous pattern around infected hepatocytes (B). Hematoxylin and eosin staining in panel A and immunoalkaline phosphatase staining with naphthol fast-red stain and monoclonal antibody against GP2 Lassa virus diluted 1:1,000 in panel B. Original magnifications ×50 (A) and ×100 (B).

polyvalent Old World arenavirus antiserum and sequencing of PCR products. However, the isolates from the blood samples took a minimum of 7 days to become detectable in culture, as compared with 3–5 days for liver samples and other blood samples tested during the investigation. Thus, it can be surmised that the viral loads were likely too low in the blood samples of patients 2 and 3 for detection of viral nucleic acid with the generic primers.

Through contact tracing, we found that a cleaner who worked at the hospital where patient 1 was treated, patient 4 (Figure 1), became ill on September 27, was admitted to a provincial hospital on October 5, and was transferred the same day to a tertiary academic hospital. She had a chronic underlying disease and died on the day after admission. Her only potential exposure to infection occurred September 14 when she cleaned the cubicle where patient 1 was treated. On October 9, a second nurse, patient 5 (Figure 1) became sick and was admitted to the hospital the following day. She had attended patient 2 and been involved in the insertion of a central venous line on September 27 before barrier nursing was instituted.

Treatment of patient 5 with the oral form of the antiviral drug ribavirin was started on October 11 with a loading dose of 30 mg/kg (2 g), followed by a projected schedule of 15 mg/kg every 6 h (1 g) for 4 days and 7.5 mg/kg every 8 h for 6 days. However, after the patient was intubated on October 12, treatment was continued through nasogastric tube until the intravenous formulation of the drug was obtained on October 17 (*6*). The patient reported improvement beginning October 27 and was discharged from the hospital on December 2 after RT-PCR did not detect viral RNA in blood and urine on 3 consecutive occasions.

The first 4 patients were initially managed without special infection control precautions, apart from the donning of surgical gloves for intubation or the taking of blood samples, and the use of impervious aprons in wards. Consequently, healthcare workers and maintenance staff such as cleaners were all potentially exposed to contaminated bedding, eating utensils, and bedpans which contained excreta and vomitus. On the other hand, no incidents occurred that could be construed as constituting specific exposure to infection, for example, needlesticks. After the possible

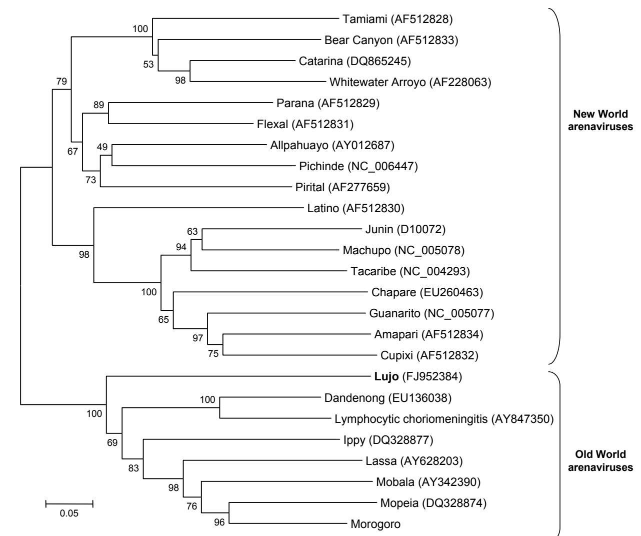

Figure 3. Neighbor-joining tree reconstructed by using bootstrap analysis with 1,000 pseudoreplicate datasets showing the phylogenetic relationship of known arenaviruses (data derived from GenBank) to the novel Lujo arenavirus from southern Africa (**boldface**), inferred from a 619-nt region of the 5′ end of the nucleoprotein gene. GenBank accession numbers for nucleotide sequence data are shown on the tree. Scale bar indicates 5% divergence.

involvement of a viral hemorrhagic fever was recognized on September 28 following the admission of patient 2 to the hospital in Johannesburg, barrier nursing procedures were instituted as successive patients were identified, but only patient 5 was managed with full precautions throughout her illness. The full precautions consisted of the donning of double surgical gloves, protective oversuits, impervious overshoes, disposable balaclavas, and N95 masks plus goggles or visors. Alternatively, the protective oversuits were replaced with surgical scrub-suits and impervious disposable gowns. No infections appeared to have occurred after the adoption of the full precautions.

The putative incubation periods for patients 2 to 5 ranged from 7 to 13 days, and the periods of illness for the 4 patients with fatal infections ranged from 10 to 13 days (Figure 1). All patients sought treatment for nonspecific febrile illness with headache and myalgia, which increased in severity over 7 days with the development of diarrhea and pharyngitis. A morbilliform rash became evident on the face and trunk in 3 Caucasian patients on day 6–8 of illness, but not in 2 African patients, and neck and facial swelling occurred in 3 patients. Four patients exhibited transient subjective improvement after ≈1 week of illness; this brief improvement was followed by rapid deterioration with respiratory distress, neurologic signs, and circulatory collapse, the terminal features in patients who died. Bleeding was not a prominent feature. However, 1 patient had a petechial rash, 1 had gingival bleeding, and another experienced the oozing of blood from venipuncture sites. Chest pain was a prominent symptom for 2 patients. All patients had thrombocytopenia on admission to the hospital (platelet count range 20–104 × 10^9 cells/L). Three patients had leukocyte counts in the normal range, and 2 had leukopenia on admission, whereas leukocytosis developed in 4 patients during the illness. The maximum aspartate aminotransferase values recorded in the 4 patients who died from the disease ranged from 549 IU/L to 2,486 IU/L, compared with 240 IU/L in the survivor who was treated with ribavirin.

Blood samples from patients 4 and 5 were positive by RT-PCR for arenavirus nucleic acid at NICD and yielded virus in culture. Blood samples taken from patient 1 on September 12 and from patient 2 on September 25 were traced to a hospital laboratory in Lusaka and sent to CDC late in the course of the investigation. The sample from patient 1 was RT-PCR positive, and both samples yielded isolates of the novel virus in cell culture. In summary, isolation of virus was achieved from the blood samples of all 5 patients, plus liver samples from patients 2 and 3, at NICD, CDC, or both (Table).

Tracing and monitoring of all contacts of known patients for 21 days from last date of contact with a patient or fomites in Zambia and South Africa failed to identify cases additional to those reported here. Antibody surveys still need to be conducted to assess the occurrence of less severe infections and to determine the distribution and prevalence of the virus. Rodent studies are also indicated, particularly in the areas frequented by patient 1, as the original source of the infection remains unknown.

Discussion

Arenaviruses are negative-sense single-stranded RNA viruses, most of which cause chronic infection of rodents, with excretion of virus in urine. Humans become infected from contaminated food or household items. Several New World arenaviruses are associated with hemorrhagic fevers in South America, including Junin, Machupo, Sabia, Guanarito, and Chapare viruses. Old World arenaviruses include the prototype member of the family, lymphocytic choriomeningitis virus, which has a worldwide distribution and is often associated with pet rodents. Clinical manifestations range from inapparent infection to severe meningoencephalomyelitis in humans. Dandenong virus is an arenavirus related to lymphocytic choriomeningitis virus (Figure 3) that was recently isolated in Australia from patients who had received organ transplants from a deceased donor who had traveled in eastern Europe (*7*). Lassa fever virus is an Old World arenavirus associated with the multimammate mouse (*Mastomys natalensis*) that causes hemorrhagic fever, which affects large numbers of persons in West Africa. Imported cases of the disease have occurred in Europe,

Table. Summary of diagnostic RT-PCR and virus isolation studies on 5 novel arena virus–infected patients, southern Africa, 2008*

Patient no.	Onset of illness	Date sampled	Day of illness sampled	Sample type	RT-PCR	Virus isolation
1	Sep 2	Sep 12	11	Blood	+	+
2	Sep 21	Sep 25	5	Blood	–	+
2	Sep 21	Sep 29	9	Blood	–	+
2	Sep 21	Sep 30	10	Blood	–	+
2	Sep 21	Oct 9	12†	Liver	+	+
3	Sep 23	Oct 3	11	Blood	–	+
3	Sep 23	Oct 9	13†	Liver	+	+
4	Sep 27	Oct 6	10	Blood	+	+
5	Oct 9	Oct 10	2	Blood	+	+

*Chronology of testing samples differs from the order in which they were collected from patients. RT-PCR, reverse transcription–PCR; +, positive; –, negative.
†Refers to day of illness on which the patient died.

North America, Asia, and South Africa (*8;* NICD, unpub. data). The distribution of the multimammate mouse and related species extends across much of sub-Saharan Africa. Old World arenaviruses not known to be pathogenic for humans include Ippy and Mobala viruses from the Central African Republic and Morogoro virus from Tanzania (*9*). Arenaviruses have also been found in rodents in Mozambique (Mopeia virus) and Zimbabwe (*10*,*11*) as well as South Africa (NICD, unpub. data), but phylogenetic analysis using partial nucleotide sequence data indicate that these are distinct from the new virus described here, and none of these viruses have been associated with human disease in these 3 countries, despite sustained monitoring over 3 decades.

As described elsewhere (*5*), this is the first highly pathogenic arenavirus to be identified in Africa in 4 decades. This outbreak serves as a warning that pathogenic arenaviruses could be more widely prevalent in Africa than presently realized and reinforces the need for strict screening of internationally transferred patients to ensure early recognition of infectious diseases and the maintenance of appropriate infection control precautions at all times.

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We thank S. Günther and H. Leirs for furnishing partial nucleotide sequence data for the Morogoro arenavirus isolate from Tanzania and are also grateful to D. Bausch for advice on clinical management of arenavirus infection. This manuscript is dedicated to those persons with the infection, and the healthcare workers who continued to tend patients despite the threat to themselves.

Dr Paweska is head of the Special Pathogens Unit, National Institute for Communicable Diseases, South Africa. His research interests include viral hemorrhagic fevers, arboviruses, rabies, and rabies-related viruses.

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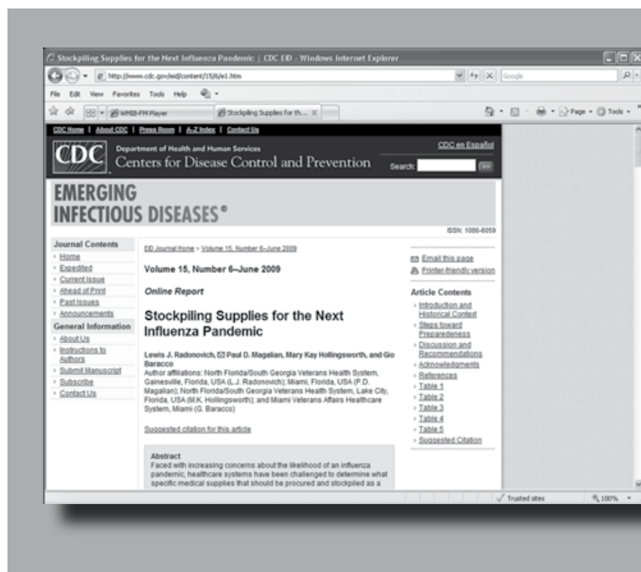

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Review of an Influenza Surveillance System, Beijing, People's Republic of China

Peng Yang, Wei Duan, Min Lv, Weixian Shi, Xiaoming Peng, Xiaomei Wang, Yanning Lu, Huijie Liang, Holly Seale, Xinghuo Pang, and Quanyi Wang

In 2007, a surveillance system for influenza-like illness (ILI) and virologic data was established in Beijing, China. The system tracked ILI and laboratory-confirmed influenza in 153 general hospitals from September 1, 2007, through April 30, 2008. To analyze the ILI surveillance data (weekly ILI rates and counts) and the effectiveness of the system, we used the US Centers for Disease Control and Prevention Early Aberration Reporting System. The data indicated that the highest rate of influenza isolation and the highest ILI count occurred in the first week of 2008. The system enabled us to detect the onset and peak of an epidemic.

Surveillance systems in Beijing, People's Republic of China, play a pivotal role in the detection of seasonal influenza. They enable the onset and the peak of an influenza epidemic to be reported in a timely and accurate manner. These systems may be critical to monitoring future emerging aberrant situations, such as an influenza pandemic.

Since 1997, >400 human cases of infection with avian influenza virus A (H5N1) have been documented worldwide, with death rates of ≈60% (*1*). Of concern is that these influenza A viruses might undergo the genetic changes of antigenic drift into novel pathogenic forms (*2*), triggering human influenza pandemics (*3*). Recently, the World Health Organization (WHO) raised the influenza pandemic alert to level 6 because of the emergence of the influenza A pandemic (H1N1) 2009 virus (*4*). Experts at WHO believe that "the world is now closer to another influenza pandemic than at any time since 1968" (*5*).

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An influenza surveillance program, consisting of disease and virologic data collection, aims to assist in the early detection of influenza, help define the distribution of influenza in the community, and provide timely information about circulating strains. These data, in turn, can be used to analyze geographic, temporal, and biologic differences in circulating influenza strains and assist in monitoring for emerging unusual or critical situations, such as a pandemic (*6*–*8*). This information can guide the crucial process of strain selection for vaccine development and other prevention and control strategies (*7*), as well as aid influenza diagnosis and enhance patient care (*9*–*12*).

To use data from a surveillance system efficiently, however, public health professionals need suitable and robust aberration detection methods. The Early Aberration Reporting System (EARS) pioneered by the US Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) was initially a method for monitoring bioterrorism events, but it has evolved into a tool that also can be used to monitor naturally occurring outbreaks and seasonal diseases. Nonhistorical methods based on a positive 1-sided cumulative sum (CUSUM) calculation in EARS can analyze the data without long-term background data (*13*,*14*).

In 2007, a surveillance system for influenza-like illness (ILI) and virologic data was established in Beijing. This system tracks ILI and laboratory-confirmed influenza in 153 general hospitals throughout Beijing. We describe the surveillance system, the surveillance data accumulated during the 2007–08 influenza season, and the performance of the early warning system.

Methods

Beijing is located in the temperate zone of the Northern Hemisphere, where influenza typically peaks seasonally once each year (*15*). Hospitals in Beijing are classified into 3 levels, depending on their size and the techniques, equip-

ment, and staff available (Table 1) (*16*). In Beijing, patients with ILI traditionally seek medical attention at their local hospitals rather than at private clinics.

Table 1. Three levels of hospitals in Beijing, People's Republic of China

Level	Description
1	Services include medical treatment, prevention, healthcare, and rehabilitation for a community with a population <100,000 persons
2	Services include medical treatment, prevention, healthcare, and rehabilitation for multiple communities (population >100,000 persons
3	Regional healthcare facility with specialized high-level healthcare services for several districts

Surveillance for ILI and Virologic Data

Influenza surveillance was performed from September 1, 2007, through April 30, 2008. In this system, ILI surveillance was conducted in the outpatient and emergency clinics of internal medicine and pediatric wards of 153 hospitals: 29 were level 1, 71 were level 2, and 53 were level 3.

Under the system, participating referral doctors were required to diagnose ILI by using a strict ILI definition (fever ≥38°C, either cough or sore throat, and no other laboratory-confirmed evidence) (*7*) and to record the number of ILI consultations by age group (i.e., 0–4 years, 5–14 years, 15–24 years, 25–59 years, and ≥60 years) on a fixed form daily. These data were entered daily into the Beijing Monitoring and Early Warning System for Infectious Diseases in Hospitals by designated hospital staff.

Fourteen hospitals from 6 districts were selected as sites for collecting specimens. Pharyngeal swab specimens from the ILI case-patients (within 3 days of symptom onset from patients who had not received antiviral drugs) were collected from the hospitals by district CDC staff. The specimens were transported to the correspondent laboratories in viral transport medium at 4°C for subsequent isolation and identification. Six independent laboratories in different districts participated in the collaborative laboratory network.

Weekly laboratory surveillance data were used as the approved standard estimate to measure the onset of an influenza epidemic. By monitoring the rate of positive isolations, changes in the activity of influenza virus were tracked. The positive isolation rate and the maximum weekly positive isolation rate were compared to determine the week the epidemic began. Because these rates change yearly, a fixed rate could not be regarded as the threshold. We used the following standard to ascertain the onset week: if the positive isolation rate in any given week exceeded 40% of the maximum weekly positive isolation rate in the overall influenza season, this week was then considered the onset week of the influenza epidemic (*17*).

EARS and Early Aberration Detection of ILI Surveillance Data in Beijing

We used EARS-X v2.8 (*18*) to analyze the ILI surveillance data. The methods used in EARS are described elsewhere (*13*,*14*,*18*). Both the weekly ILI rates and ILI counts were analyzed by EARS-X. The nonhistorical method used in EARS consists of 3 algorithms, called C1-mild (C1), C2-medium (C2), and C3-ultra (C3). The terms mild, medium, and ultra refer to the level of sensitivity of the 3 alternative statistical methods, with C1 being the least sensitive and C3 the most sensitive. The thresholds for C1, C2, and C3 are based on a 1-sided positive CUSUM calculation (*13*,*14*,*18*). For C1 and C2, a warning is generated when the current count is greater than the baseline mean plus 3 SD. C1 uses data from 1 to 7 days before the current day for calculating the mean and SD, whereas C2 and C3 use data from 3 to 9 days in the past for calculating the mean and SD. For C3, the algorithm is based on a CUSUM calculation with an average run length time of 3 days. If the calculated value is >2, a C3 warning is produced. C1, C2, and C3 methods were simultaneously adopted for comprehensive analysis.

To determine the onset of the influenza epidemic, we established a standard based on warings in EARS (C1, C2, and C3). According to the difference of the sensitivities of C1, C2, and C3, the standard incorporated the following situations: 1) when a C1 warning was produced in a given week and the value of the following week was greater than that of the selected week, the selected week was the week of onset; or 2) when a C2 warning was produced in a given week and a C1 or C2 warning was generated in the following week, the following week was the week of onset; or 3) when a C3 warning was produced in a given week, and the following consecutive 2 warnings of C1, C2, or C3 were generated in the following 2 weeks, and the above 2 situations did not occur, the third week of the warning occurring was the week of onset.

Results

ILI Surveillance

During the 2007–08 influenza season, the peak in ILI was identified on the basis of the weekly ILI surveillance data. For the 153 hospitals in the surveillance system, the highest weekly ILI rate during this season was 40.1 cases per 1,000 consultations in the week 53 of 2007 (Figure 1), 1 week earlier than when the ILI count peaked (18,203 cases in the first week of 2008, Figure 2). The highest weekly ILI rate and count are 1.7× and 2.0× as high as the average ILI rate (23.8/1,000) and count (9,154 cases), respectively. After stratification by hospital levels, the trends for level 1, 2, and 3 hospitals were similar. The highest ILI count for all age groups except the ≥60 years group occurred in the

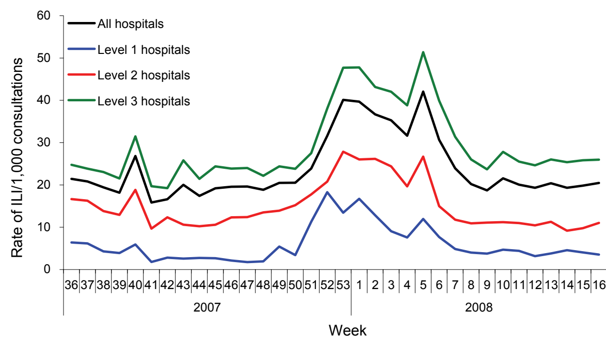

Figure 1. Weekly influenza-like illness (ILI) rates during the 2007–08 influenza season, Beijing, People's Republic of China.

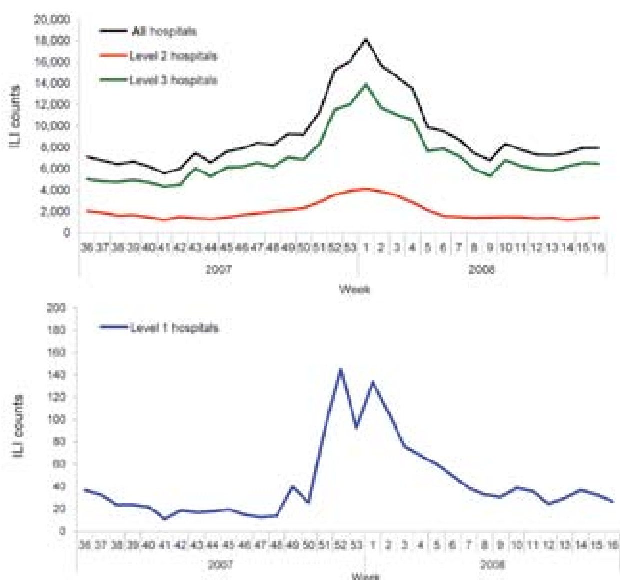

Figure 2. Weekly influenza-like illness (ILI) counts during the 2007–08 influenza season, Beijing, People's Republic of China.

first week of 2008. For the ≥60 years group the ILI count peaked in the third week of 2008 (Figure 3).

Virologic Surveillance

Pharyngeal swab samples were collected from 2,057 ILI case-patients during the influenza surveillance period. Thirty percent (n = 611) of these patients tested positive for influenza (type A, 151; type B, 450; untyped, 10). Overall, influenza B (Yamataga-lineage) was the dominant strain detected; however, late in the season, influenza A was isolated more frequently (Figure 4). On the basis of the 40% maximum weekly isolation rate (the gold standard indicating the onset of the influenza epidemic), week 49 of 2007 was considered the onset week (Figure 4).

Early Warning by EARS of the Onset of an Influenza Epidemic

According to the ILI rate data (Figure 5), a warning was first produced in week 51 of 2007, with C1, C2, and C3 warnings generated simultaneously. During week 52, the rate of ILI was greater than in the previous week; therefore, week 51 was set as the onset of the influenza epidemic, according to the standard described in the methods. From the ILI count data (Figure 6), 3 consecutive C3 warnings were produced between week 46 and week 48. According to these data, the influenza epidemic could have begun during week 48.

After stratifying the data by hospital level and age group, we determined the onset week from the ILI rate data for levels 1, 2, and 3 hospitals was week 51 of 2007 (Table 2). On the basis of the ILI count data, the onset week for level 1 hospitals was week 51, whereas for the level 2 and 3 hospitals, it was week 49. According to the ILI count data, onset weeks varied for different age groups.

Discussion

The 2007–08 influenza season in Beijing was mild, without a large documented epidemic or an outbreak. Influenza B predominated during the season in Beijing, which differed from the situations recorded in other countries and regions, including Europe and North America, where influenza A predominated (*19*). Influenza activity in Beijing may have been mild because of the epidemic characteristics of influenza B (*20*). From the data, we found that the

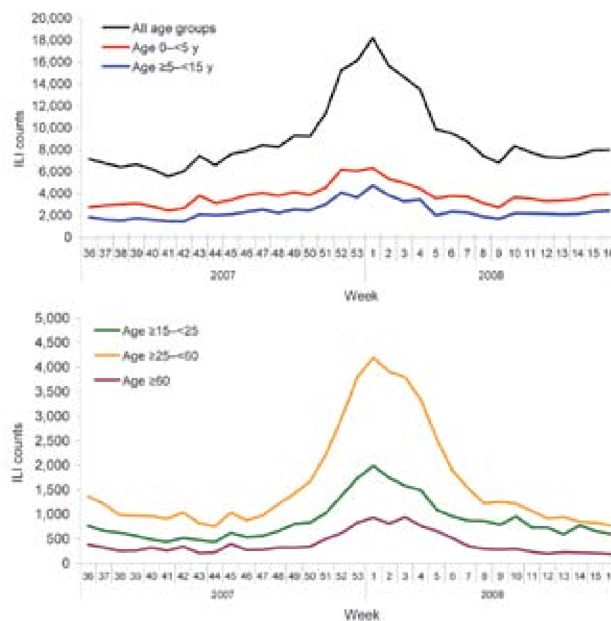

Figure 3. Weekly influenza-like illness (ILI) counts by age group during the 2007–08 influenza season, Beijing, People's Republic of China.

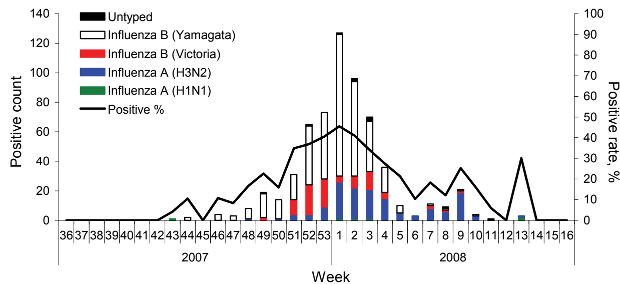

Figure 4. Weekly distribution of influenza isolates during the 2007–08 influenza season, Beijing, People's Republic of China.

highest rate of influenza isolation occurred in the first week of 2008, and the highest ILI rate (except the false high ILI rate in the fifth week of 2008, the Chinese Spring Festival week) occurred 1 week earlier (week 53 of 2007). However, on closer examination, we found that the week of the peak influenza isolation rate was actually almost identical to that of the highest ILI rate. Occurrence of the highest ILI rate in week 53 of 2007 resulted from the New Year's holidays, which could have caused a decline in consultations during this period.

To understand the differences between the efficiencies of ILI rates and ILI counts in detecting the onset of an influenza epidemic, we analyzed both by using EARS. The onset week determined by the ILI rate (week 51) was 2 weeks later than the onset week (week 49) determined by a standard. However, in comparison, the onset week as determined by the ILI count was week 48, which still was close to the onset week determined by the reference standard but 3 weeks earlier than that determined by the ILI rate. When the data were stratified by hospital level and by age group, the onset weeks (week 46–week 49) as determined by the ILI count data were earlier than the week determined by the ILI rates (week 51), except for level 1 hospitals and the age group ≥60 years of age. One possible reason for the earlier onset detected by the ILI counts than by the ILI rates was the dependence of ILI rates on ILI counts and total consultations to the hospitals. Because humans are at high risk for many diseases during winter, consultations about other diseases also will increase. In this situation, it is optimal to consider the results for both the ILI rates and ILI counts simultaneously before any public health decisions are made.

Assessment of the timeliness and accuracy of determining the onset by hospital level showed that ILI count data from level 2 and 3 hospitals were more timely and accurate than were data from level 1 hospitals (week 49 rather than week 51). This result may be due to the tendency for level 2 and 3 hospitals to be larger and able to afford more comprehensive and reliable data than level 1 hospitals. Fluctuations in consultations could affect the accuracy of determining the onset of the influenza epidemic.

The inclusion of level 1 hospitals in the surveillance system may play a substantial role in the surveillance for human avian influenza and future pandemic activity. These hospitals were selected from smaller towns that have more poultry workers. These hospitals are likely to be the first destination for poultry workers who have ILI symptoms. If an atypical increase in the number of ILI consultations in these hospitals occurs in a short period, this increase could signal a need for further investigations and the institution of possible control measures. Poultry workers are a high-risk population for avian influenza (*21*,*22*), and ILI symptoms are the precursory symptoms of avian influenza (*23*). Earlier detection of ILI cluster cases in poultry workers may be helpful for finding clustered cases of avian influenza, human-to-human transmission cases, and the early stages of a pandemic. Therefore, ILI surveillance in these level 1 hospitals still should be continuously conducted.

When we stratified ILI count data by age group, we found the accuracy and timeliness of determining the onset of the influenza epidemic by the ILI count data were most efficient when using data in the age groups 15–24 years and 25–59 years. Although the onset weeks determined by the ILI data were earlier than week 49 for those 0–4-years and 5–14 years, this finding may be more likely to have resulted from respiratory syncytial virus circulation and infection. This virus can increase the number of emergency department visits and hospitalization in young children, and its season usually occurs before the annual influenza season (*24*,*25*). Another reason might be that the infection or reinfection rate in children by predominating influenza B virus was higher than that in adults (*26*), and thus more children were brought to doctors for treatment than adults. ILI data from the those ≥60 years of age were the last to give a warning for the epidemic onset (week 51) from the 5 age groups, perhaps because

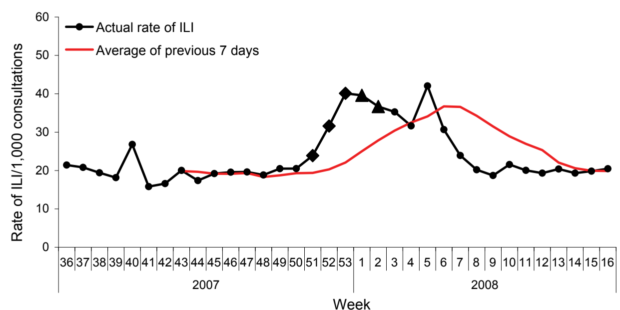

Figure 5. Weekly influenza-like illness (ILI) rates by Early Aberration Reporting System to detect the onset of the influenza epidemic during the 2007–08 season, Beijing, People's Republic of China. Squares and triangles represent different alert situations—C1-mild (C1), C2-medium (C2), and C3-ultra (C3)—automatically generated by the reporting system: diamond, C1C3; triangle, C2C3.

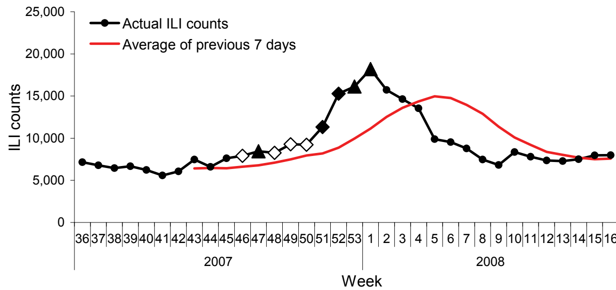

Figure 6. Weekly illness-like illness (ILI) counts by Early Aberration Response System to detect the onset of the influenza epidemic during the 2007–08 season, Beijing, People's Republic of China. Triangles and diamonds represent different alert situations—C1-mild (C1), C2-medium (C2), and C3-ultra (C3)—automatically generated by the reporting system: triangle, C2C3; open diamond, C3; and solid diamond, C1C2C3.

the ILI definition used may have been too strict to screen the influenza cases in the elderly. In elderly persons, fever and cough are relatively less common than they are in younger persons (*27*). In this system, monitoring ILI data in the young age groups is more efficient than monitoring influenza activity in the older age groups.

The ILI data obtained were the only means used to evaluate the efficacy of the surveillance system in detecting the onset of the influenza epidemic. We did not use the site-specific or age group–specific virologic data to ascertain whether true differences existed in the timing of influenza virus circulation between these specific groups. This omission is a limitation of this study.

In many developing countries, such as the People's Republic of China, surveillance systems have only recently been implemented; therefore, classic statistical methods that require detailed historical data are not suitable (*28–31*). In these countries, programs such as EARS provide a suitable tool for detecting the aberration of data in surveillance systems without the need for historical data. It provides the most optimal and accessible tool, not only for seasonal surveillance but also for outbreak surveillance in developing countries. Furthermore, EARS is simple to grasp and practice and is freely downloadable from the US CDC website (http://emergency.cdc.gov/surveillance/ears)

The influenza surveillance system introduced in Beijing provided timely and accurate surveillance information that was consistent with data obtained from virologic surveillance for influenza. The system enabled us to detect the onset and peak of the epidemic. The ILI data from the larger hospitals may have afforded more valuable information for monitoring the onset of the epidemic than the data from smaller hospitals. However, given the current climate with avian influenza, it is crucial that the small provincial hospitals remain. In this situation, EARS was useful in the analysis of disease surveillance data, giving us the opportunity to undertake the surveillance without any historical data.

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Table 2. Onset of influenza epidemic as determined by the various ILI data during the 2007–08 influenza season, Beijing, People's Republic of China*

	ILI rate		ILI count	
Data	Warnings meeting the standard	Onset week	Warnings meeting the standard	Onset week
Hospital level				
1	C1 in a given week and the greater following value	51	C1 in a given week and the greater following value	51
2	Consecutive 2 C2	51	Consecutive 2 C2	49†
3	C1 in a given week and the greater following value	51	Consecutive 3 C3	49†
Age group, y				
0–4	NA	NA	Consecutive 3 C3	48
5–14	NA	NA	Consecutive 2 C2	46
15–24	NA	NA	C1 in a given week and the greater following value	49†
25–59	NA	NA	C1 in a given week and the greater following value	49†
≥60	NA	NA	C1 in a given week and the greater following value	51

*ILI, influenza-like illness; C1, C1-mild algorithm; C2, C2-medium algorithm; C3, C3-ultra algorithm; NA, not available.
†Onset week, determined by the accepted standard based on 40% of the maximum weekly isolation rate, is the threshold

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Discriminatory Ability of Hypervariable Variable Number Tandem Repeat Loci in Population-based Analysis of *Mycobacterium tuberculosis* Strains, London, UK

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To address conflicting results about the stability of variable number tandem repeat (VNTR) loci and their value in prospective molecular epidemiology of *Mycobacterium tuberculosis*, we conducted a large prospective population-based analysis of all *M. tuberculosis* strains in a metropolitan setting. Optimal and reproducible conditions for reliable PCR and fragment analysis, comprising enzymes, denaturing conditions, and capillary temperature, were identified for a panel of hypervariable loci, including 3232, 2163a, 1982, and 4052. A total of 2,261 individual *M. tuberculosis* isolates and 265 sets of serial isolates were analyzed by using a standardized 15-loci VNTR panel, then an optimized hypervariable loci panel. The discriminative ability of loci varied substantially; locus VNTR 3232 varied the most, with 19 allelic variants and Hunter-Gaston index value of 0.909 . Hypervariable loci should be included in standardized panels because they can provide consistent comparable results at multiple settings, provided the proposed conditions are adhered to.

Globally, tuberculosis (TB) accounts for almost 2 million deaths each year (*1*). Although TB notification rates in the United Kingdom (13.8/100,000 in 2007) remain low, rates differ substantially by region: London (43.2/100,000) accounts for ≈40% of all TB cases registered in the United Kingdom, and ≈75% of TB patients in London were born abroad (*2*). Rates of drug resistance also are higher in London than in the rest of the United Kingdom: 8.6% of isolates are isoniazid resistant, and 1.2% are multidrug resistant (UK Health Protection Agency; www.hpa.org.uk).

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In settings where incidence of TB is low or moderate, molecular genotyping is used to investigate suspected TB outbreaks, laboratory cross-contamination, and reactivation and (at a population level) to identify clustered cases that are not apparently linked; for the latter purpose, the highest possible level of discrimination is required (*3*). For these purposes, insertion sequence (IS) *6110* restriction fragment length polymorphism (RFLP) analysis—often supplemented with spoligotyping and, more recently, with variable number tandem repeat (VNTR) typing—is used routinely.

The highest levels of epidemiologic discrimination of strains of the *Mycobacterium tuberculosis* complex (MTBC) can be achieved by using multilocus VNTR typing, but these results depend on the number and loci used, particularly for homogenous strain groups such as the Beijing family (*3–5*). This approach overcomes technical difficulties associated with IS*6110*-RFLP and is amenable to automation that results in a high throughput (*6–10*). A standardized panel of 15 + 9 VNTR loci (24 loci) has been proposed (*7*,*11*), but it is unclear whether sufficient discrimination would be seen when the panel is used in populations with a substantial prevalence of homogenous MTBC families (*4*,*5*,*12*). In addition, the discriminative power of VNTR loci may vary markedly among genetic families (*7*,*13*). Recent studies evaluating the discriminative power of VNTR typing have produced conflicting results that were generated by using convenience samples (small populations with low diversity or populations confined to a single geographic setting). These studies highlighted a need for larger population-based studies to identify discrimina-

[1]These authors contributed equally to this article.

tive VNTR loci and ascertain their applicability for various genetic groups.

Concerns about the stability and reproducibility of particularly useful hypervariable loci, such as 3232, 2163a, 3336, and 1982 (*3–5*,*14*), have been raised (*7*,*15*). As a result, they have been excluded from the proposed international panels for VNTR typing. For these reasons, we conducted a study to examine the stability of hypervariable loci and the parameters associated with reproducibility, to select loci suitable for prospective molecular epidemiologic studies, and to evaluate the discriminatory power of these loci at a population level in a metropolitan setting.

Materials and Methods

Bacterial Isolates

A total of 2,261 individual MTBC isolates (1 per patient) were included in this prospectively designed population study. These isolates represented 95.7% of the bacteriologically confirmed TB cases reported from the 30 London hospitals in the 12 months from April 2005 through March 2006. These isolates had been characterized by using spoligotyping, and all but 4 were assigned to 1 of 36 spoligotype families (*16*,*17*). Multiple isolates were available from 265 patients (11.7%), resulting in serial isolate sets of 2–6 isolates, which had been sampled at intervals of 3 days to 11 months (N = 632).

Multilocus VNTR Analysis

All extracts were typed by using 15 mycobacterial interspersed repetitive unit (MIRU)-VNTR loci as previously described (*3*). Isolates clustered when the 15 MIRU- Hunter-Gaston index value VNTR profiles we used were reanalyzed with an additional panel of VNTR loci 2163b, 2347, 3232, 2163a, 1982, 3336, and 4052 as previously described (*3*,*5*) after optimization of factors affecting reproducibility (see Hypervariable Loci Optimization). Variability or discrimination at a locus was assessed by using the Hunter-Gaston Discriminative Index (HGDI) (*18*). Loci with HGDI values <0.3, 0.3–0.6, and >0.6 were considered poorly, moderately, and highly discriminative, respectively (*19*).

Hypervariable Loci Optimization

We selected 16 previously characterized MTBC isolates to cover the complete range of repeat sizes at control loci MIRU 26 and exact tandem repeat (ETR)–B and experimental hypervariable loci VNTRs 1982 and 3232 (except 0 repeats for the locus 3232). For each of the 16 extracts, four 10-µL PCRs were conducted for each of the primer mixes in duplicate. Of these 4 reactions, the first was performed as described previously with BIOTAQ polymerase (Bioline, London, UK) (any enzyme in the given context means enzyme in conjunction with the buffer recommended and supplied by a manufacturer). Three other sets of PCRs were conducted under different amplification conditions (*1*).

Method 1

Diamond DNA polymerase (Bioline) was used (*9*). The PCR amplification cycle was 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 2 min at 72°C, and 1 final cycle of 5 min at 72°C (*2*).

Method 2

HotStartTaq DNA polymerase (QIAGEN, Hilden Germany) was used. Each 10-µL reaction contained 1× PCR buffer (QIAGEN), 0.25 U/µL of the relevant polymerase, 0.2 µmol/L dNTPs, 0.125 µmol/L of relevant primer, and 5% dimethylsulfoxide. The DNA amplification cycle was 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and a final cycle of 10 min at 72°C (*3*).

Method 3

HotStartTaq Plus DNA polymerase (QIAGEN) was used. The PCR mixture was the same as in method 2, and the amplification cycle was the same, except that the initial 95°C activation time was reduced to 5 min.

We manually calculated the number of repeats within each PCR product by resolving 4 µLof each product on a 1.2% (wt/vol) agarose gel (Agarose LE Analytical grade; Promega, Southampton, UK) against a 2,000-bp Hyper-Ladder II standard (Bioline). The number of repeats at each locus also was calculated by sizing in a denaturing capillary electrophoresis system using a CEQ 8000 instrument with a DNA Size Standard 600 (Beckman Coulter, High Wycombe, UK) and MapMarker D1 labeled 640–1000 (BioVentures, Inc., Murfreesboro, TN, USA) because fragments were expected to be >600 bp. Three parameter sets (Table 1) were used to analyze all fragments. The different parameters examined were capillary temperature (60°C for methods 1 and 2 and 50°C for method 3, respectively), denaturation time (120 s for method 1 and 180 s for methods 2 and 3, respectively) and separation time (60 min for methods 1 and 2 and 70 min for method 3, respectively). Fragment data traces were automatically analyzed by using the scheme shown in Table 1. For locus 3232, we accounted for offset values (i.e., difference among actual sizes of PCR fragments and apparent sizes indicated by electrophoresis) when calculating number of repeats in Table 1.

Assessing Stability and Reproducibility of VNTR Loci

All isolates were grouped into 265 sets of serial isolates (2–6 isolates each) and typed at all 22 loci. Primer sequences for all loci were as described previously (*3*,*9*,*20*,*21*). PCR was set up by using BIOTAQ polymerase for amplifying 12 MIRU and 3 ETR loci and Diamond polymerase for the

Table 1. Expected molecular weights of *Mycobacterium tuberculosis* of fragments at each locus, with different numbers of copies, London, UK, 2005–2006*

	Length of expected fragments for each locus, bp			
No. repeats	MIRU 26	ETR-B	VNTR 1982	VNTR 3232†
0	244	121	178	
1	295	174	256	242
2	344	227	334	286
3	393	280	412	330
4	442	333	490	372
5	491	386	568	415
6	540	439	646	458
7	589	492	727	501
8	638	545	802	546
9	687	598	880	587
10	736	651	958	630
11	785		1,038	673
12	834		1,116	716
13	883		1,194	759
14	932			802
15				845
16				888
17				931
18				974
19				1,017
20				1,060

*MIRU, mycobacterial interspersed repetitive unit; ETR, exact tandem repeat; VNTR, variable number tandem repeats.
†No isolates had 0 repeats in locus 3232 in our population.

additional 7 VNTR loci. Capillary electrophoresis was performed by using the parameters described in method 1.

Results

Optimization of Hypervariable Loci

We evaluated factors that potentially affect the reproducibility of hypervariable VNTR loci by using various PCR and capillary and manual electrophoresis separation conditions as described in the Materials and Methods. The ability to correctly amplify different VNTR loci depended on the enzyme used (Table 2); all polymerases efficiently amplified MIRU 26 and ETR-B, as indicated by the presence of PCR fragments on agarose gels and capillary electrophoresis peaks. However, locus VNTR 3232 was amplified effectively only with Bioline Diamond (15/16 strains, 93.8%). Although all polymerases except Bioline BIOTAQ were able to amplify DNA at locus VNTR 1982, longer fragments were amplified more efficiently by QIAGEN and Bioline Diamond polymerases. Therefore, Diamond polymerase was selected for the amplification of additional VNTR loci.

We assessed 3 methods for capillary electrophoresis. For each locus, apparent fragment sizes were plotted against expected fragment sizes for each method (Figure 1).

MIRU 26 fragments sizes were as expected for all allelic variants (except for the variant with 2 repeats) when BIOTAQ and Diamond polymerases were used, but sizes were larger than expected with QIAGEN polymerases. The smaller ETR-B fragments with 1 and 2 repeats all gave expected sizes with methods 1 and 2 but were less than expected with method 3 (where the capillary temperature was decreased). These results did not affect overall interpretation. For the higher number of repeats (4–6 repeats), all polymerases generated fragments that, when analyzed by using method 3, gave apparent sizes lower than expected. In some cases, this result affected the interpretation. The apparent sizes of VNTR 1982 fragments were all similar to the expected values independent of the polymerase used and the method used for capillary electrophoresis.

Serial Isolates

Amplification was performed by using BIOTAQ polymerase for 12 MIRU and 3 ETR loci and Diamond

Table 2. Number of DNA extracts (from n = 16) for which peaks were detected by different conditions for capillary electrophoresis of *Mycobaterium tuberculosis* after amplifying the loci with different polymerases, London, UK, 2005–2006*

		Bioline polymerases‡		QIAGEN polymerases‡	
Locus	Method†	BIOTAQ	Diamond	HotStartTaq	HotStartTaq Plus
MIRU 26	1	16	16	16 (1)	16 (1)
	2	15	16	16 (1)	16 (1)
	3	16	16	16	16
ETR-B	1	16	16	16 (1)	16 (1)
	2	15	16	16 (1)	16 (1)
	3	16	16	16 (2)	16 (2)
VNTR 1982	1	8	13	14	14
	2	9	13	12	14
	3	6	11	12	14
VNTR 3232	1	11	15	13	14
	2	10	15	14	14
	3	11 (3)	15 (7)	13 (6)	13 (4)

*MIRU, mycobacterial interspersed repetitive unit; ETR, exact tandem repeat; VNTR, variable number tandem repeats.
†Refer to Table 1.
‡Numbers in parentheses represent number of extracts whose calculated number of repeats were higher and lower than the expected value on the basis of that produced by the standard procedure (method 1).

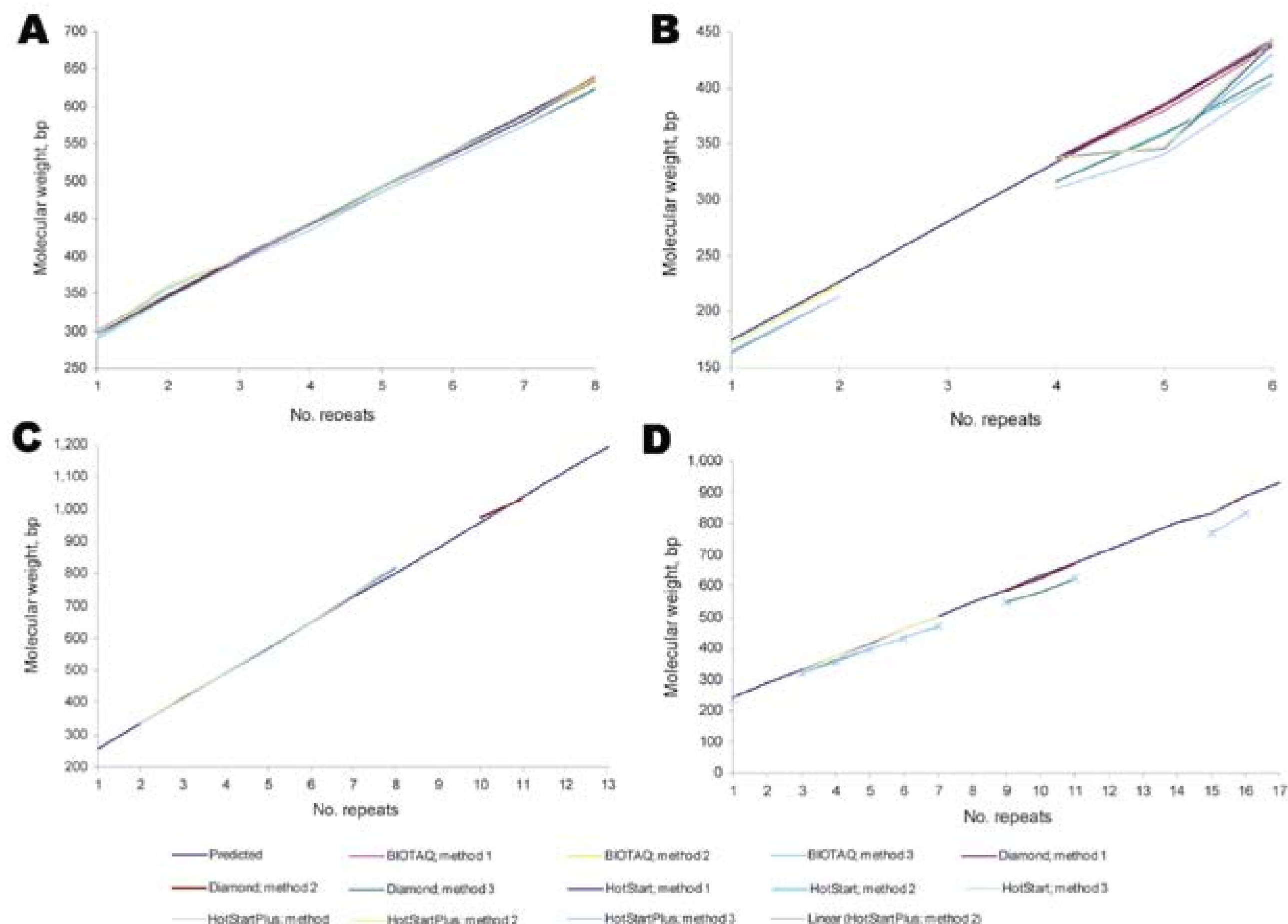

Figure 1. Effect of various enzymes and separation conditions on amplification and detectable molecular weights of PCR fragments for 4 variable number tandem repeat (VNTR) loci. A) Mycobacterial interspersed repetitive unit locus 26; B) locus exact tandem repeat; C) locus 1982; D) locus 3232.

polymerase for 7 VNTR loci with the optimized parameters in method 1. Analysis was blinded. No disagreements occurred in the interpretation of VNTR repeat numbers among isolates in a set. In a proportion of isolates (N = 124), genotyping results were validated by using both capillary electrophoresis and manual electrophoresis for PCR fragment separation, and again, no discrepancies were found between VNTR loci copy numbers in strains isolated from the same patient at different time points (Figure 2).

Population Genotyping in Metropolitan Setting with 2 Panels of VNTR Loci

A total of 2,261 MTBC isolates circulating in London with known spoligotypes were genotyped by using a defined set of 15 loci (12 MIRU and 3 ETR); all known spoligotyping families were represented in the test population (online Technical Appendix, available from www.cdc.gov/EID/content/15/10/1609-Techapp.pdf). Complete 15-loci profiles were obtained for 2,046 strains (90.5% of all strains). Data for the remaining profiles were incomplete for $\geq$1 locus. Overall PCR failure rate was 1.6%, with the highest number of failures (n = 72) at locus ETR-A and the lowest number of failures (n = 4) at locus ETR-C. When PCR failed, DNA was reextracted from original cultures, and genotyping was attempted again. If the second attempt was unsuccessful, the results for the locus were marked as missing.

Genotyping of MTBC isolates by using 15 MIRU-ETR loci yielded 1,036 unique profiles and 235 clusters containing 2–53 isolates (Table 3). Clustered profiles were shared by 1,225 isolates, giving a clustering rate of 54.2%.

Subsequently, 1,196 (97.6%) of 1,225 isolates (15 MIRU-ETR clustered isolates) were subjected to secondary typing by using VNTR loci 2163b, 2347, 3232, 2163a, 1982, 3336, and 4052. Resolution improved because strains that had been clustered initially were subdivided into new groups: 1,730 isolates now had unique genotyping patterns, and the remaining 502 isolates were grouped into 158 clusters, giving a new, substantially lower, clustering rate of 22.2% (Table 3).

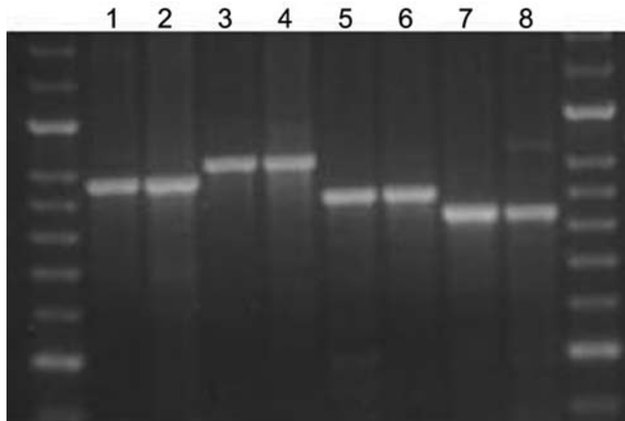

Figure 2. Agarose gel showing the stability of amplified fragments of variable number tandem repeat (VNTR) 3336 from 2 serial isolates isolated from 4 patients. Lane 1, patient A, isolate 1, isolated 2005 Jun 20, 8 copies; lane 2, patient A, isolate 2, isolated 2005 Jul 11, 8 copies; lane 3, patient B, isolate 1, isolated 2005 Jul 8, 9 copies; lane 4, patient B, isolate 2, isolated 2005 Aug 8, 9 copies; lane 5, patient C, isolate 1, isolated 2005 Nov 11, 7 copies; lane 6, patient C, isolate 2, isolated 2005 Nov 15, 7 copies; lane 7, patient D, isolate 1, isolated 2005 May 16, 6 copies; lane 8, patient D, isolate 2, isolated 2005 May 25, 6 copies.

Variability and Discriminative Power of VNTR Loci

The discriminative ability of VNTR loci varied markedly among the 22 VNTR loci and among spoligotyping families (online Technical Appendix) with locus VNTR 3232 showing the greatest variation (HGDI = 0.909 and 19 allelic variants) and loci MIRU 2 and 20, the least (HGDI = 0.134 and 0.196; number of allelic variants 4 and 3, respectively). Twelve loci each had ≥10 allelic variants. MIRU 4 showed moderate discriminative power, and MIRU 10, MIRU 16, MIRU 23, MIRU 26, MIRU 40, ETR-A, ETR-C, and VNTR 2163B, 2163A, 1982, 3232, 3336, and 4052 showed high discriminative power with HGDI values varying from 0.524 to 0.909. None of the 22 loci were monomorphic in the current study. With the exception of VNTR 2347, all loci included in the additional VNTR panel displayed higher variability than the primary panel of 15 MIRU-ETR loci used for UK national typing, which indicates their potential for increasing the power of prospective molecular genotyping.

The discriminative power of VNTR loci also varied among spoligotype families. The mean 15 MIRU-ETR HGDI value for the Beijing family was low (0.163), which indicates that this family is relatively homogeneous, even within the diverse London population settings. Notably, mean 15 MIRU-ETR HGDI values for genetic families within the Euro-American lineage (T, Haarlem, S, X, Latin American–Mediterranean) were generally higher (0.307–0.378) than those for Beijing and Central Asian (CAS) (0.235). Within spoligotype families, the additional 7 VNTR increased variability in all cases, except for *M. bovis*. The highest HGDI were seen in the Latin American–Mediterranean family with locus 2163B; in Beijing, Haarlem, and *M. africanum* with VNTR 3232; in East African–Indian with VNTR 2163A; in X with VNTR 1982; in T with VNTR 3336; and in CAS with VNTR 4052. Within the East African–Indian family, the hypervariable loci VNTR 3232 varied little, with 93.7% isolates having a single copy. A small proportion of strains (Table 4) analyzed by using more discriminative loci, including VNTR 3232, 1982, 2163A, and 3336, generated PCR products that were too large for automated analysis but were resolved manually.

Discussion

Polymorphisms in rapidly evolving repetitive sequences, such as minisatellite VNTR, are a valuable tool for prospective epidemiologic analyses and provide a high degree of discrimination in situations in which few a priori epidemiologic data are available. In this population-based study, we genotyped 2,261 individual MTBC isolates obtained from patients residing in London by using 22 VNTR-MIRU loci.

Conflicting views on the use of hypervariable loci for typing have been reported, even when loci such as VNTR 3232 have been shown to have high discriminatory power (*3*,*5*,*14*). Some studies have demonstrated difficulty in amplification of multiple alleles, absence of PCR amplification products, varying data interpretation, and lack of reproducibility among laboratories (*7*). Similar problems were found with another potentially valuable hypervariable locus, VNTR 1982 (*5*,*7*). Therefore, we believed that by identifying the conditions that provided good, reproducible discrimination, we would be able to define the optimal conditions that would enable molecular epidemiologists to use VNTR 1982 and 3232. We addressed variability and reproducibility for these 2 loci using MIRU 26 and ETR-B as

Table 3. Discriminatory power of VNTR typing used in the study in establishing true minimum cluster size as marker of real transmission rate*

Genotyping method	No. distinct profiles (variety of types)	No. clusters	Size of clusters, no. isolates	Clustering rate, % (n/N)	Recent transmission rate, % ([*n* – *c*]/*N*)	No. unique isolates
MIRU15 (n = 2261)	1,271	235	2–53	54.2	44.0	1,036
MIRU15 + Spoligotyping	1,619	196	2–48	37.1	29.0	1,423
MIRU15 + VNTR7	1,888	158	2–35	22.2	17.0	1,730

*MIRU, mycobacterial interspersed repetitive unit; VNTR, variable number tandem repeats; n, no. clustered cases; N, total no. of strains; c, no. of clusters.

Table 4. Allelic variants of additional hypervariable VNTR loci that cannot be resolved with the CEQ automated sequencer*†

Locus	Maximum no. repeats suitable for automated analysis	Fragment size, bp	Proportion of strains with allelic variants beyond the automated system resolution, %
3232	15	830	4.1
1982	9	880	11.9
2163A	11	876	10.8
3336	11	875	21.1

*Beckman Coulter, Fullerton, CA, USA.
†VNTR, variable number tandem repeats.

controls that give stable comparable results in both agarose gel and capillary electrophoresis and have been used previously in a multilaboratory comparative study (*7*).

In all cases, identical data were produced for MIRU 26 and ETR-B irrespective of the DNA polymerase used. Amplification of VNTR 1982 and 3232 varied with different DNA polymerases, particularly when expected fragments were long.

The differing performances of polymerases for amplifying different loci can be explained by their varying properties. BIOTAQ polymerase is a basic Taq that can be used for a wide range of templates, whereas Diamond polymerase has been modified by a point mutation at the active site of the enzyme, enabling it to read through regions of secondary structure, microsatellites, and guanine cytosine–rich templates, such as those found in the *M. tuberculosis* genome. The QIAGEN polymerases are chemically modified polymerases with a high specificity similar to that of Diamond polymerase; thus they showed similar capabilities in amplifying VNTR 1982 and 3232. In addition, the buffer used with the QIAGEN polymerases is designed to increase the specificity of primer binding, making these polymerases suitable for dealing with complex genomic DNA.

Conditions that affect the denaturation of PCR products, and therefore their linearity before fragment sizing by electrophoresis, would be expected to influence apparent sizes of PCR fragments and copy number enumeration. We investigated the influence of DNA denaturation time and capillary separation temperature. As expected, we found that lowering the separation rate increased the discrimination of fragments >1,000 bp.

A marked difference was observed when the capillary temperature was decreased (method 3), which was independent of the polymerase used and locus investigated and demonstrated that separation conditions are critical for the correct interpretation of the VNTR typing results. In method 3, apparent fragment sizes were smaller and offset values were markedly larger, to the point that in some cases the calculated copy number was different from that expected.

Taking all the data together, we used BIOTAQ for amplifying MIRU and ETR loci, and Diamond polymerase for amplifying the extra 7 hypervariable VNTR loci, using the separation conditions detailed in method 1. We also demonstrated the reproducibility and stability of the extra 7 VNTR loci by comparing 22 MIRU-VNTR profiles from serial isolates. The resulting profiles of serial isolates from the same patients were identical, indicating that the conditions used for fragment amplification, detection, and analysis were ideal for typing of these loci and that these loci could be used for routine genotyping.

Clustering rates seen by using 15 MIRU-ETR loci far exceeded those previously reported when IS*6110* RFLP was used in a London population study (*22*,*23*). We concluded that 15-MIRU-ETR genotyping was insufficiently discriminative and was producing so-called false clustering. This view was supported by the spoligotyping results in which 38 (16%) of 235 isolates of 15 MIRU-ETR clusters contained isolates that belonged to $\geq$2 spoligo families (Table 3).

Applying all 22 loci gave the lowest clustering rate (22.2%) in MTBC strains obtained over 1 year from a single metropolitan setting (London), a rate almost identical to the proportion established in previous studies conducted in London in 1993 and 1995–1997 (*22*,*23*) and similar to previously reported rates in population-based studies in low- to-middle TB incidence settings where RFLP and PCR-based genotyping methods were used (*11*,*24*–*26*). These findings suggest, from the public health viewpoint, that TB transmission in London has remained stable over the past decade. Our study provides strong evidence that PCR-based methods, especially VNTR-MIRU, can replace IS*6110* RFLP typing for prospective analysis and that 12 MIRU (*27*), and 15 MIRU-ETR loci panels alone are insufficiently discriminating for evaluation of TB transmission.

The recently proposed VNTR panel (*3*,*5*,*7*,*11*) provides similar degrees of discrimination (comparable to that achieved by IS*6110* RFLP), although discrimination of individual VNTR loci is not equal for different MTBC genetic families (*13*). Inclusion of highly polymorphic VNTR loci effectively differentiates strains within highly conserved groups and is vital for prospective genotyping. Our study demonstrated that even in settings of low TB incidence and relatively low TB transmission rates, TB families, such as Beijing and CAS, remain more conserved than others, and hypervariable loci (e.g., VNTR 3232, 2163A, 4052) provide much higher discrimination than MIRU and ETR loci either alone or in combination.

Our current results agree with the preliminary results of our earlier studies about the applicability of hypervariable VNTR loci (VNTR 3232, VNTR 3336; VNTR 2163a,

and VNTR1982, in particular) and recent reports (*28–30*) demonstrating their effectiveness for discrimination among Beijing strains. This agreement suggests that these loci are discriminating and reproducible, especially where Beijing strains are dominant (e.g., China, Russia, Baltic countries) (*28*) and should be included in standardized VNTR panels. They can be used successfully at multiple laboratories with consistent results, provided the conditions for proposed reaction and PCR fragment separation are adhered to and specific DNA polymerases are used.

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Excess Deaths and Immunoprotection during 1918–1920 Influenza Pandemic, Taiwan

Ying-Hen Hsieh

To determine the difference in age-specific immunoprotection during waves of influenza epidemics, we analyzed excess monthly death data for the 1918–1920 influenza pandemic in Taiwan. For persons 10–19 years of age, percentage of excess deaths was lowest in 1918 and significantly higher in 1920, perhaps indicating lack of immunoprotection from the first wave.

Recent studies have focused on quantifying the global effects of the influenza pandemic of 1918–1920 (*1*–*3*). This pandemic swept through Taiwan in 2 waves, at the end of 1918 and again in early 1920, causing devastating loss of human life. A report about the devastation brought by the first wave of the influenza epidemic, published in February 1920 (*4*), indicated that as of December 15, 1918, a total of 779,522 persons (20.8% of the population) had been infected and 25,394 persons had died from influenza; case-fatality rate was 3.26% (*4*,*5*). Although the number of infections decreased dramatically in early 1919, a second wave of the epidemic at the end of that year created another severe death toll.

Previous studies have shown that excess deaths, similar to those noted in the temperate zones, were also observed in Taiwan, which is in a tropical–subtropical zone, during periods of previously recognized influenza epidemics (*6*,*7*). A recent study has also shown Taiwan to be an evolutionarily leading region for global circulation of influenza virus A (H3N2) (*8*). Therefore, we analyzed the 1918–1920 pandemic in Taiwan to contribute to understanding of and preparation for possible future pandemic events.

The Study

Using data from the 1895–1945 Statistical Abstract of Taiwan (*9*), we compared monthly deaths during the 2 waves of epidemics in 1918 and 1920 with deaths during corresponding nonpandemic periods of the adjacent years. For example, we compared monthly deaths for November and December 1918 with the mean deaths for November and December 1916–1917 and 1919–1922. Statistically significant excess deaths were computed by detecting the data points at which the all-cause deaths exceeded the mean of the adjacent years +2 SDs (*6*,*10*). Excess deaths, computed from the mean number of deaths at these data points, were then used to ascertain the effect of the pandemic on deaths during these periods. During 1918–1920, population data were divided into 3 major groups: Taiwanese (95.2%), Mainland Chinese (0.57%), and Japanese–Korean (4.2%). However, only records of all-cause deaths for Taiwanese and Japanese were available and used in our analysis.

Figure 1 gives the mean monthly number of all-cause deaths and 95% confidence intervals (CIs) for each month during 1916–1922, excluding the known anomaly months (the 2 epidemic waves) of November–December 1918 and January–February 1920. The number of deaths increased markedly during the anomaly months. When we plotted the anomaly points against the actual number of deaths, we noted that the anomaly points were significantly >2 SDs above the means and that substantial excess deaths had indeed occurred. Moreover, we estimated the excess deaths during these 2 waves by subtracting the mean number of all-cause deaths from the number of deaths during the anomaly periods (Table 1). Because the 2 waves of epidemics had overlapped the adjacent months, we also included increases during these months.

We used age-specific data on deaths to quantify the effect of the 1918–1920 influenza pandemic on each age group. Because monthly age-specific death data were not available (*9*), we used yearly age-specific all-cause death data to quantify age-specific excess deaths during 1918 and 1920. Figure 2, panel A, gives the age-specific percentages of all-cause deaths of Taiwanese persons during 1917–1921 only. The percentages of all-cause deaths by age group were computed for 1918 and 1920 and compared

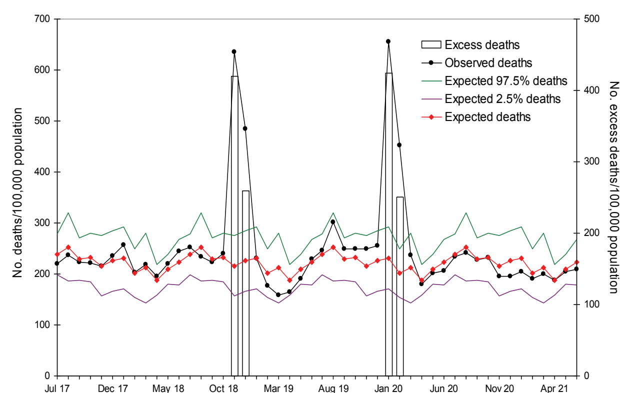

Figure 1. Monthly all-cause and excess death rates, Taiwan, July 1916 through June 1922. Monthly averages for excess deaths exclude those of the pandemic years 1918 and 1920. Bars indicate excess deaths per 100,000 population during the pandemic months of November–December 1918 and January–February 1920.

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Table 1. Excess all-cause deaths of Taiwanese and Japanese persons during 2 influenza epidemic waves in Taiwan, 1918–1920*

Time period	Observed no. deaths	Expected no. deaths†	SD	Excess no. deaths (95% CI)	Excess no. deaths, by wave‡ (95% CI)
First wave					
1918 Oct§	8,725	8,366	489	359 (0–1,337)¶	24,907 (21,426–29,008)
1918 Nov	23,156	8,042	768	15,114 (13,578–16,650)	
1918 Dec	17,658	8,224	793	9,434 (7,848–11,021)	
Second wave					
1919 Dec§	9,319	8,224	793	1,095 (0–2,682)¶	26,141 (20,572–32,845)
1920 Jan	23,906	8,478	973	15,429 (13,482–17,375)	
1920 Feb	16,466	7,466	955	9,001 (7,090–10,911)	
1920 Mar§	8,625	8,009	630	616 (0–1,877)¶	

*CI, confidence interval.
†Expected monthly no. deaths was computed from mean no. deaths for that month during 1916–1922, excluding the epidemic month. Total excess no. deaths for these 2 periods = 51,048 (95% CI 41,998–61,853).
‡Including the excess no. deaths in adjacent months.
§Excess no. deaths for these months are not statistically significant (i.e., not >2 SD above mean).
¶Max (0, lower bound).

with the respective averages of percentages for the adjacent years (1917, 1919, and 1921).

For persons 5–39 years of age, percentages of all-cause deaths for 1918 and 1920 were clearly higher than those for the average of adjacent years; for persons ≥55 years of age, they were lower. In addition, deaths were higher in 1918 than in 1920 for persons 25–39 years of age, but deaths were higher in 1920 for those 5–24 years of age. The excess percentages of deaths for 1918 and 1920 in age groups 10–19, 20–29, and 30–39 years were computed by subtracting the average percentages of deaths in these age groups during the adjacent years from the respective true percentages of deaths in these age groups during 1918 and 1920 (Table 2).

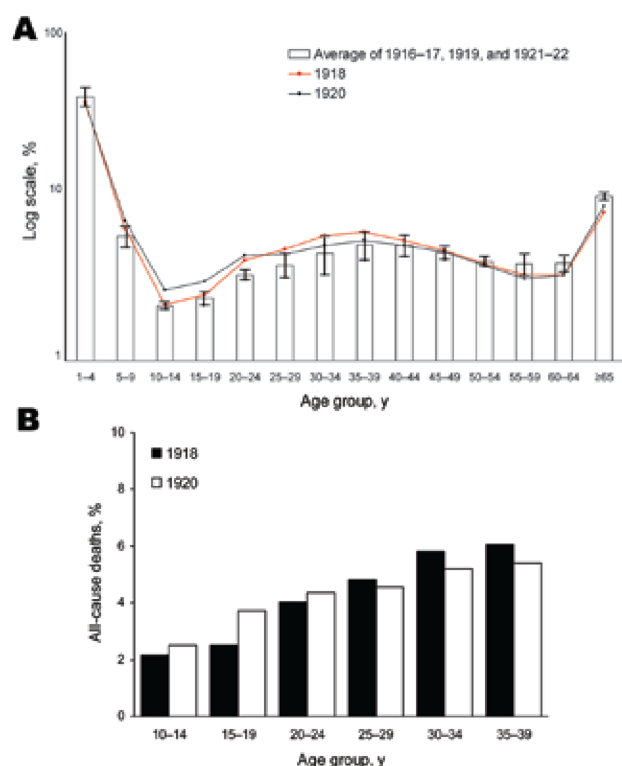

Figure 2. A) Percentages (in log scale) of all-cause deaths in Taiwan, by age group. Error bars indicate 95% confidence intervals. B) Percentages of all-cause deaths for persons 10–39 years of age in 1918, grouped by 5-year age groups.

The percentages of excess deaths were most significant for persons 20–39 years of age in 1918 and 10–29 years of age in 1920. When we compared the 1918 and 1920 waves, the percentage of excess deaths decreased during the second wave for persons 30–39 years of age, was almost identical for those 20–29 years of age, and was significantly higher for those 10–19 years of age. Even for those 5–9 years of age, the percentage of deaths was higher during 1920 than either during 1918 or for the average of adjacent years (Figure 2, panel A).

Our estimate of 1.38% (95% CI 1.14–1.68) excess deaths for Taiwan is close to the estimate of 1.44% (95% CI 1.40–1.48) by Murray et al. (*3*). However, their estimate was based on calculations of 3-year excess deaths for 1918–1920 over the preceding 3 (1915–1917) and the following 3 years (1921–1923). During 1919–1920, a cholera outbreak caused 2,693 deaths in 1919 and 1,675 deaths in 1920, which might have skewed their estimate of excess deaths for Taiwan. In contrast, our 1920 estimate, obtained by using higher resolution monthly data (more precise than yearly data), accounted for only the first 3 months of the year. Although the exact months of the cholera outbreak are unknown, we can reasonably assume that the excess deaths caused by cholera during these 3 months were substantially fewer than excess deaths from the entire cholera outbreak during 1919–20.

Ample literature describes the unusual age-specific death patterns for the 1918–1920 pandemic (*11*–*13*). To compare the age-specific differences between the 2 waves, we compared the percentages of all-cause deaths of persons 10–39 years of age in 1918 with the corresponding percentages of all-cause deaths for the same groups of per-

Table 2. All-cause deaths for Taiwanese persons during 1916–1922, by age group, and excess percentages of age-specific deaths for 1918 and 1920*

		Age group, y					
		10–19		20–29		30–39	
Year	Total no. deaths	No. (%) deaths	Excess % deaths (95% CI)	No. (%) deaths	Excess % deaths (95% CI)	No. (%) deaths	Excess % deaths (95% CI)
1918	124,677	5,836 (4.68)	0.19 (0–0.48)‡	11,028 (8.85)	1.88† (1.1–2.67)	14,804 (11.87)	2.78† (0.02–5.54)
1920	119,477	6,888 (5.77)	1.27† (0.09–0.56)	10,579 (8.85)	1.89† (1.11–2.68)	12,305 (10.30)	1.20 (0–3.96)‡

*CI, confidence interval.
†The excess percentages for these age groups are statistically significant.
‡Max (0, lower bound).

sons, who 2 years later were 12–41 years of age (Figure 2, panel B).

Within the 10–39-year age group in 1918, the age groups with the lowest percentage of excess all-cause deaths in 1918 (10–19 years) had markedly increased deaths in 1920, and the age groups with a higher percentage of excess all-cause deaths in 1918 (30–39 years) had noticeably decreased deaths in 1920 (Table 2; Figure 2, panel B). This finding could be explained in part by acquired immunoprotection by those age groups during the first wave, thus giving credence to the belief that the 2 waves were caused by the same virus strain.

Conclusions

In 1918, the epidemic swept through all 12 administrative districts on the island in <1 month. Given that the population of Taiwan has increased >6-fold since then and that the current population is much more mobile and travels more, a future outbreak of a similarly virulent influenza virus strain could conceivably spread through the island in a few days. The total estimated number of excess deaths is 51,048 (95% CI 41,998–61,853). Given the average Taiwan population size during 1918–1920, the percentage of excess deaths was 1.38% (95% CI 1.14–1.68). Given the population of Taiwan at the end of 2007, the number of persons killed by an epidemic of similar magnitude today would be ≈315,000 (95% CI 259,900–384,000). Pandemic readiness planning for Taiwan, or any other country, must consider the potential magnitude of a similar-sized pandemic.

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Rabies in Foxes, Aegean Region, Turkey

Ad Vos, Conrad Freuling, Seza Eskiizmirliler, Hikmet Ün, Orhan Aylan, Nicholas Johnson, Semra Gürbüz, Winfried Müller, Necdet Akkoca, Thomas Müller, Anthony R. Fooks, and Haluk Askaroglu

At the end of the 1990s in the Aegean region of Turkey, rabies rapidly spread among foxes. This spread likely resulted from spillover infection from dogs and led to increased rabies cases among cattle. To control this outbreak, oral rabies vaccination of foxes has been used.

In Turkey, dog-mediated (spread by dogs as host species) rabies dominates the epidemiology of rabies (*1*). During 1990–2000, a total of 2,856 rabies cases were reported from Turkey; dogs (*Canis lupus familiaris*) accounted for 78% of reported cases, whereas wildlife accounted for only 1.6% (data from 44 issues of Rabies Bulletin Europe, available from www.who-rabies-bulletin.org). In the past decade (1998–2007), however, an increasing number of rabies cases in foxes (*Vulpes vulpes*) have been reported from the Aegean region in western Turkey. Rabies in foxes has been reported incidentally from other regions in Turkey, especially from the central and eastern parts. Rabies cases in foxes have been considered to be rare, dead-end, spillover events from rabid dogs and to have no epidemiologic significance. However, surveillance data from most of these regions are limited; therefore, whether rabies in wildlife occurs independently from rabies in dogs is unknown. Sufficient data are available for the Aegean region, and phylogenetic studies have concluded that rabies recently spilled over from domestic dogs to foxes in this area (*2*).

The Aegean region is characterized by mountain ranges, except for the coastal plains, where most of the human population is concentrated and where ≈3.5 million persons live in Turkey's third largest city, Izmir. This economic and industrial center lies in a predominantly agricultural area. Before 1999, rabies in the Aegean region was predominantly mediated by dogs, and no clear movement from an urban focus was noted. Most cases were observed in and around the city of Izmir. The number of rabies cases had decreased notably in the Aegean region, from 137 cases in 1988 to only 2 cases in 1995, after which the number started to increase again (*1*).

To determine more about the epidemiology of this disease, we analyzed the spatial and temporal incidence of rabies in 8 provinces of the Aegean region (Figure 1) during 1998–2007. We emphasized the shift from dog-mediated to fox-mediated rabies and the consequences to the disease profile in this area.

The Study

During the study period, animals from the Aegean region suspected of having rabies were submitted for rabies diagnosis to the Bornova Veterinary Control and Research Institute in Izmir. Routine rabies diagnosis was based on

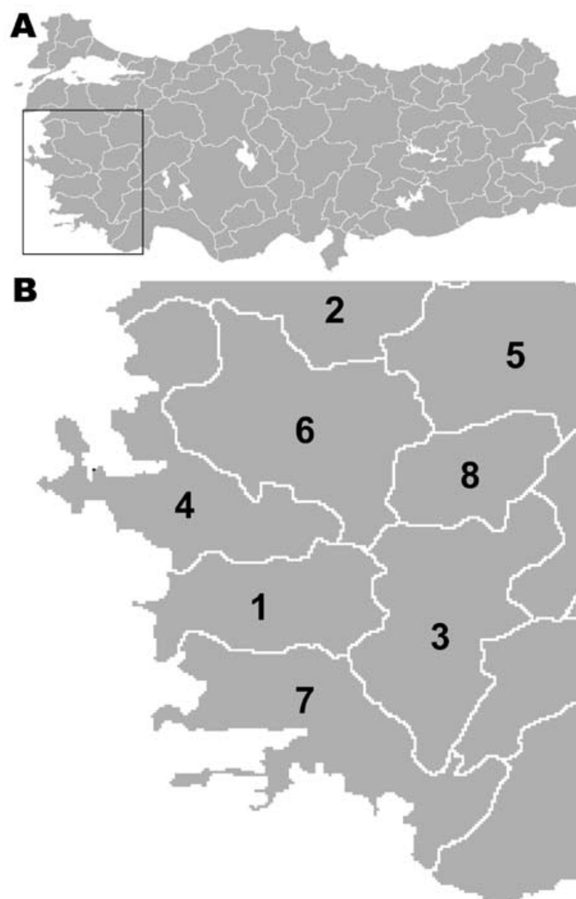

Figure 1. A) Map of Turkey showing location of Aegean region (box). B) The 8 provinces in the Aegean region of Turkey that were studied for spatial and temporal incidence of rabies during 1998–2007. 1, Aydin; 2, Balikesir; 3, Denizli; 4, Izmir; 5, Kütahya; 6, Manisa; 7, Mugla; 8, Usak.

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detection of Negri bodies, followed by fluorescent antibody test results; for negative samples, the mouse inoculation test was also performed (*3*). From 1998 through 2007, a total of 3,737 specimens were submitted; of these, 118 samples could not be examined because the samples were of poor quality (Table 1). Analysis of the data for the 3 major animal species submitted (cattle, dogs, and foxes) during this period showed dramatic changes in the number of rabies cases for each of these species (Table 2). In 1998, no rabies in foxes was reported, and dogs clearly dominated rabies submissions. However, in subsequent years, rabies emerged in foxes; this emergence coincided with an increased number of rabid cattle reported and a decreased number of rabid dogs. The annual number of rabid dogs correlated only weakly with total number of dogs submitted for testing; coefficient of determination (R^2) was 0.56. However, this correlation was markedly higher for cattle and foxes ($R^2 = 0.99$ for both species). This finding indicates that the lower number of rabid dogs did not result only from decreased surveillance for this species.

From 1998 through 2000, almost all cases in the 3 most affected species were reported from the area between the cities of Izmir and Manisa (Figure 2), and the epizootic progressed in a wave-like fashion southward; in 3 years, the area of rabies cases moved ≈150 km. In 2003, the southeastward movement of the rabies epidemic slowed and, in 2004, appeared to halt. In 2005, cases again increased in the northern part of Aydin Province, which borders Izmir Province. In 2006, no clear movement of this outbreak was observed.

The northern Aegean region may not be a suitable habitat for foxes and therefore may not have supported a sufficient number of foxes for an epizootic. Reliable data on fox density or even the number of foxes shot are lacking. Several cases of rabies in foxes were reported in 2006 from the area where the fox outbreak had started 7 years earlier. In 2007, rabies in foxes was still reported from this area, and the outbreak in Aydin had moved eastward and established itself in the neighboring province of Denizli.

Table 1. Rabies testing results for specimens submitted to Bornova Veterinary Control and Research Institute, Izmir, Turkey, 1998–2007

Species	No. positive/total no. tested (% positive)
Horses	5/17 (29)
Donkeys	4/14 (29)
Goats	29/46 (63)
Sheep	27/49 (55)
Cattle	605/758 (80)
Cats	46/782 (6)
Dogs	327/1,581 (21)
Hamsters	0/49 (0)
Rabbits	1/18 (6)
Mice and rats	0/67 (0)
Squirrels	0/11 (0)
Foxes	165/174 (95)
Jackals	2/2 (100)
Wolves	0/2 (0)
Badgers	1/2 (50)
Mustelids	13/18 (72)
Wild cats	0/1 (0)
Bats	0/5 (0)
Humans	6/8 (75)
Other	0/15 (0)
Total	1,231/3,619 (34)

Conclusions

The high number of free-roaming dogs and the low vaccination coverage of the dog population would have provided ample opportunities for infected dogs to transmit the virus to foxes. Hence, foxes (or a fox) in the Izmir area are assumed to have become infected, and rabies then spread in the fox population. The close association of the sequences of rabies virus isolates from dogs and foxes in this area supports a recent transfer (*2*). However, the phylogenetic data provide no indication of the direction of virus transmission. The lack of reported rabies in a fox

Table 2. Rabies testing results for 3 most commonly affected species, Aegean region, Turkey, 1998–2007*

	Species						Total†	
	Dogs		Cattle		Foxes			
Year	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
1998	67	102	3	4	0	1	76	177
1999	78	104	13	5	3	1	106	193
2000	39	158	7	5	10	1	72	281
2001	30	129	66	16	22	0	139	252
2002	30	125	236	24	44	0	339	247
2003	11	142	100	13	24	0	154	251
2004	12	124	49	24	9	1	74	248
2005	17	126	55	18	14	1	95	234
2006	17	114	39	10	20	1	89	229
2007	26	130	37	34	19	3	87	277

*Pos, positive; Neg, negative.
†Includes all species tested.

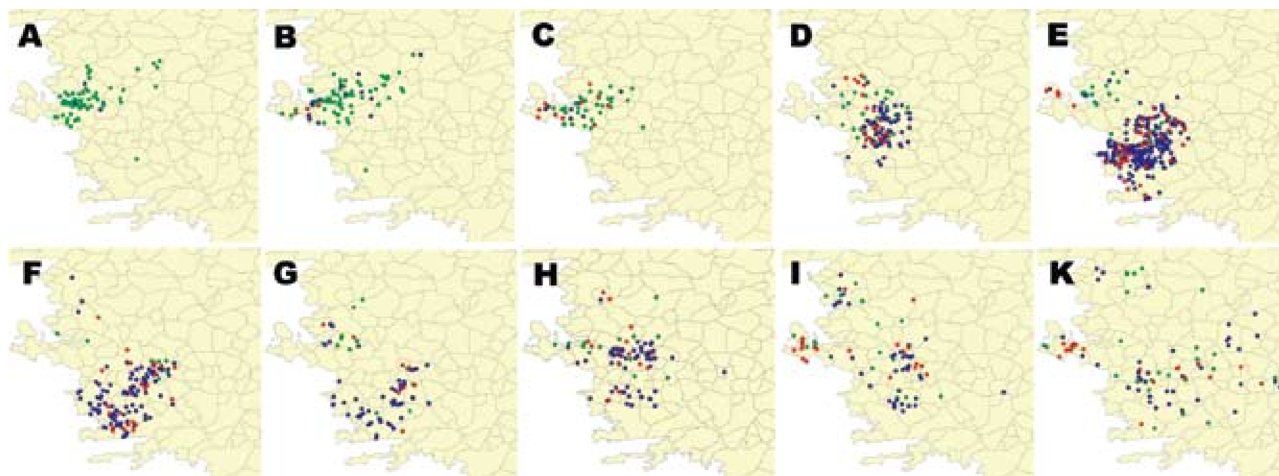

Figure 2. Location of rabies cases in the 3 most affected species in Aegean region by year. Red, red foxes; green, dogs; blue, cattle. A) 1998; B) 1999; C) 2000; D) 2001; E) 2002; F) 2003; G) 2004; H) 2005; I) 2006; K) 2007.

in this area since 1986 suggests that dogs transferred the virus to foxes, leading to the present rabies outbreak, not the reverse.

Since 1999, rabies has moved predominantly south and southeast at ≈40–50 km per year from the area where the first cases in foxes were reported. This movement was associated with increases in the number of rabies cases in foxes and cattle until 2003, when the number of cases sharply declined after mass vaccination of cattle. Since 2003, the numbers of rabid dogs and rabid foxes reported each year has remained approximately the same. However, the number of dogs submitted for rabies diagnosis is ≈9× higher than the number of foxes submitted. Furthermore, only 20% of the dogs tested were rabies virus–positive, compared with almost all (95%) of the foxes; therefore, the true number of rabid foxes can be assumed to exceed the number of rabid dogs.

Although during the 10 years of the study period, 6 cases in humans in the study area were reported, none were linked to foxes. Hence, the public health risks associated with rabid foxes are relatively small compared with those associated with rabid dogs. However, if not eliminated, rabies in foxes will form a reservoir for disease persistence. A high number of rabies cases in cattle causes an economic loss. Mass vaccination of cattle reduces these losses but does not solve the problem. After the initial epizootic, the disease seems to have become endemic to the Aegean region. Preventing the spread of rabies to foxes in unaffected areas is crucial. Therefore, to control the present outbreak, a campaign to orally vaccinate foxes against rabies in the affected provinces of Turkey was initiated during February 2008 and conducted again in 2009.

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Fine-scale Identification of the Most Likely Source of a Human Plague Infection

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We describe an analytic approach to provide fine-scale discrimination among multiple infection source hypotheses. This approach uses mutation-rate data for rapidly evolving multiple locus variable-number tandem repeat loci in probabilistic models to identify the most likely source. We illustrate the utility of this approach using data from a North American human plague investigation.

Linking human disease events to likely sources of infection has been advanced by molecular epidemiology. However, isolates from several potential infection sources often are similar, and none may exactly match the clinical isolate genotype, especially if the methods used provide high discrimination (*1*). Conclusions from partial-match genotypes are problematic but may provide the only data for weighing the relative importance of similar source genotypes. Even perfect-match genotypes do not preclude partial-match sources as likely infection sources (*2*). We present a probabilistic approach based on mutation rates that can be used to identify the most likely source of infection. Our example is human plague, but the approach could be applied to other diseases for which data on marker mutation rates are available (*3*).

Plague is caused by the bacterium *Yersinia pestis*. Because *Y. pestis* is an obligate pathogen that continuously cycles between rodents and fleas, mutations are generated regularly and can be observed among even closely related isolates (*1*). Human contact with infected fleas or rodents can result in human plague (*4*). Plague is rare in the United States, with <20 cases in 2006 (*5*) but is of concern because of the potential use of *Y. pestis* as a biological weapon (*6*). Thus, the ability to link a human plague isolate to a likely source has implications for investigating both natural disease and bioterrorism events.

Multiple locus variable-number tandem repeat (VNTR) analysis (MLVA) is useful for molecular epidemiologic studies of *Y. pestis* because of its discrimination power (*1*,*7*,*8*). We previously used MLVA to genotype the human isolate described below and queried the resulting genotype against a database containing genotypes from hundreds of *Y. pestis* isolates (*9*). This statistical approach identified isolates that most closely matched the human isolate and confirmed its most likely coarse geographic origin (northern New Mexico). However, this set of near matches from the database query included isolates representing several different potential local infection sources, leaving the most likely fine-scale source unclear. The human and environmental isolates were indistinguishable with pulsed-field gel electrophoresis (PFGE); thus, the most likely fine-scale source could not be identified (*10*).

The Study

In November 2002, while visiting New York, New York, USA, 2 persons from Santa Fe County, New Mexico, USA, became ill with fever and unilateral inguinal adenopathy; clinicians subsequently identified the illness as bubonic plague. Investigation by the New Mexico Department of Health and the Centers for Disease Control and Prevention indicated the patients were infected in New Mexico because *Y. pestis*–positive fleas were collected near the patients' home (*10*). However, because plague is endemic to the region, and flea samples from which isolates were obtained were collected at the home and along a local trail on which the patients hiked, either location could be the source. To identify the most likely fine-scale source of their infections, we examined specific mutations separating the human isolate from closely related environmental isolates.

We examined 5 *Y. pestis* isolates (Table 1) to develop a fine-scale spatial analysis of the infection. The reference isolate was obtained from 1 patient, 3 isolates were obtained from fleas collected in the patients' yard (*9*) (2 were collected before their illness as part of a long-term investigation), and 1 isolate was obtained from the trail flea samples a short time later as part of the same long-term study (Figure 1). Other isolates were collected and examined but were excluded from this fine-scale analysis because they were more distinct from the human isolate, differing at ≥4 VNTR loci. DNA extracts were prepared from each isolate (*11*,*12*) and analyzed using a 43-loci MLVA system as previously described (*1*,*8*).

We observed 3 MLVA genotypes (A–C) among the 5 samples (Table 1, Figure 2). The human isolate was assigned

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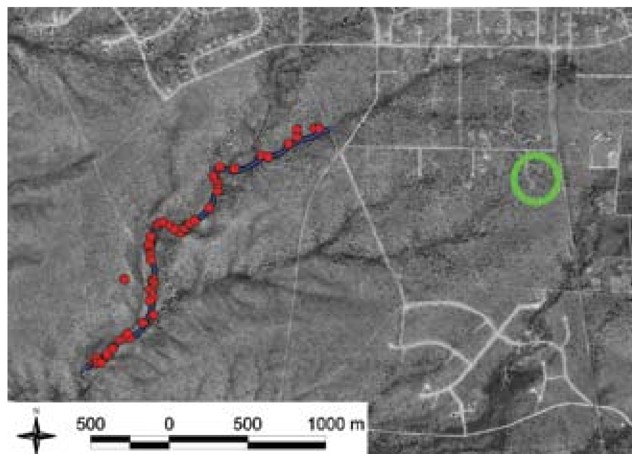

Figure 1. Distribution of rodent trapping stations along a hiking trail in Santa Fe County, New Mexico, USA. Each red circle indicates a single trapping site that had 3 traps. Trap stations (not shown) also were placed throughout the patients' yard (green circle).

genotype A. Genotype B, observed in 3 isolates obtained from the yard, differed from the reference by single-repeat mutations at 2 VNTR loci (M25 and M34; Figure 2, panel A). Genotype C, observed in 1 isolate from a flea obtained along the trail, also differed from the reference isolate at loci M25 and M34. However, the mutation at M25 was a double-repeat mutation that could be explained 2 ways: as a single 2-repeat mutational event (Figure 2, panel B) or as 2 sequential single-repeat mutations at the same locus (Figure 2, panel C). Although all 43 VNTR loci are useful for identifying the coarse geographic origin of an unknown isolate by using a database approach (*9*), our analysis examined only polymorphic loci because monomorphic loci provided no additional information. The molecular epidemiologic goal was to identify the environmental isolate most closely related to the human isolate and thus the most likely fine-scale geographic source of the infection.

To this end, we examined the relative probability of each mutation (Table 2) using published mutation rate data (*1*,*8*). We used mutation rate estimates for specific mutational events to judge relative probabilities of different scenarios. This approach assumes 1) there is an intrinsic mutation rate at each loci for each event, 2) we have accurately estimated these rates (*1*,*8*), and 3) we can use intrinsic rates to judge the relative likelihood of ≥2 hypotheses. We multiplied individual probabilities of mutations within a scenario to calculate the overall relative probability (ORP) that an environmental isolate was related to the infection source (Table 2; Figure 2). To select the most likely source, we compared the ORP of each scenario with the others in a pairwise fashion (odds ratios, Table 2). In practice, only the most likely source needs to be compared with all other sources.

Conclusions

The patients most likely were infected from a source in their yard. Genotype B was observed in isolates from the yard, and this scenario had the highest ORP (7.9×10^{-9}; hypothesis B→A; Table 2). The first scenario for genotype C (C1→A; Table 2) is second most likely (ORP 1.0×10^{-9}). The odds ratio shows the most likely scenario (B→A) is just 7.9× more likely than this scenario (C1→A). These 2 near matches illustrate the power of this approach: one is the most likely source, but the other is statistically possible because this odds ratio difference would not be significant

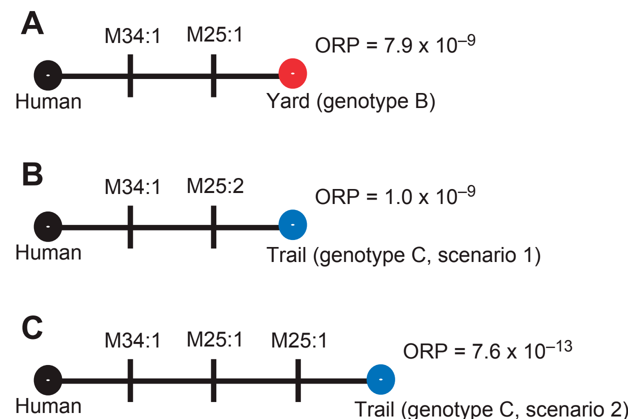

Figure 2. Alternate infection source hypotheses for the plague cases in the persons who visited New York, New York, USA. Closed circles indicate genotypes; black, red, and blue circles indicate genotypes A, B, and C, respectively. Individual mutations are indicated as vertical lines on the comparisons and are labeled with the locus that mutated and the number of repeats involved in the mutations. Overall relative probabilities (ORP) based on *Yersinia pestis* mutation rates are presented for each comparison.

Table 1. Five *Yersinia pestis* isolates examined to determine the source of a human plague infection in New Mexico, USA*

CDC isolate ID	Collection date	Collection source	MLVA genotype†	Flea source of *Y. pestis* isolate	Rodent source of flea
NM024452	2002 Nov 5	Human	A	NA (human)	NA (human)
NM02-1852-138	2002 Jul 17	Yard	B	*Orchopeas sexdentatus*	*Neotoma micropus*
NM02-1856-140	2002 Jul 18	Yard	B	*O. neotomae*	*N. micropus*
NM02-4477-309	2002 Nov 9	Yard	B	*Peromyscopsylla hesperomys*	*Peromyscus leucopus*
ED425	2003 Apr 4	Trail	C	*O. sexdentatus*	*N. micropus*

*CDC, Centers for Disease Control and Prevention; ID, identification number; MLVA, multiple locus variable-number tandem repeat analysis; NA, not applicable.
†See Figure 2.

Table 2. Overall relative probabilities of isolates with genotypes B or C as the source of a human plague infection in New Mexico, USA*

MLVA genotype	Rates of specific mutations between each genotype and genotype of the human isolate (A)†			Hypothesis	Overall relative probability	OR‡
	M34:1	M25:1	M25:2			
B (yard)	8.2×10^{-5}	9.7×10^{-5}	–	B→A	7.9×10^{-9}	–
C (trail, scenario 1)	8.2×10^{-5}	–	1.3×10^{-5}	C1→A	1.0×10^{-9}	7.9
C (trail, scenario 2)	8.2×10^{-5}	$(9.7 \times 10^{-5})^2$	–	C2→A	7.6×10^{-13}	1.0×10^4

*MLVA, multiple locus variable-number tandem repeat analysis; OR, odds ratio.
†Values generated using data and approaches described in (*8*).
‡The overall relative probability for each subsequent hypothesis is compared with the most likely hypothesis (B→A).

at $\alpha<0.05$ (odds ratio >20). However, the ORP (1.0×10^4) for the second scenario for genotype C (C2→A; Table 2) would be statistically significant, enabling it to be rejected.

When a high-resolution typing approach based on loci with fast mutation rates, such as MLVA, is used, near matches should be the rule rather than the exception. After transmission, the pathogen will continue to propagate in environmental sources and in the patient, leading to additional mutations before investigators obtain isolates. Mutations may also occur during routine laboratory procedures (e.g., culturing) before genotypic comparisons. Thus, perfect matches are rarely observed during phylogenetic analysis. Rather, the common ancestor (i.e., genotype of the source strain at time of infection) of the human isolate and each potential source isolate will need to be hypothesized. MLVA and probabilistic modeling provide a rigorous means to identify the most likely fine-scale environmental source. The same principles can be applied to other subtyping approaches used in investigations, including those with slower evolution patterns such as PFGE. In these cases, matches and near matches also should be judged by their relative evolutionary rates. Applying evolutionary probabilistic modeling to subtyping will generate stronger conclusions by evaluating the relative strengths of alternative hypotheses regardless of the subtyping approach.

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Borrelia hispanica Relapsing Fever, Morocco

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We found that 20.5% of patients with an unexplained fever in northwestern Morocco had tick-borne relapsing fever. Molecular detection specific for the 16S rRNA gene identified *Borrelia hispanica*. The noncoding intergenic spacer sequence domain showed high sensitivity and good resolution for this species.

Tick-borne relapsing fever (TBRF) is caused by *Borrelia* species transmitted to humans by infected ticks. This condition is frequently undiagnosed and its true incidence is underestimated (*1*,*2*). TBRF is endemic to sub-Saharan Africa, and the most prevalent *Borrelia* species in this region are *B. duttonii* in the eastern region and *B. crocidurae* in the western region (*3–5*). The disease is rarely detected in northern Africa and Mediterranean countries (*3*,*6*,*7*). *B. hispanica* and *B. crocidurae* infections have been detected in northern and southern Morocco, respectively, along with the tick vectors responsible for their transmission (*Ornithodoros erraticus* and *O. sonrai*, respectively) (*8*). However, local transmission has not been detected in Morocco since the reports of Baltazard et al. in 1954 (*9*) and Rodhain in 1976 (*10*), except for 1 traveler who returned from Spain and Morocco in 2005 with a *B. hispanica* infection (*7*).

Conventional diagnosis of TBRF is based on detection of spirochetes in blood smears sampled during the acute febrile phase. However, molecular methods have been shown to be more reliable for diagnosis (*6*,*7*,*11–13*). We conducted retrospective and prospective surveys of patients with unexplained fever (suspected TBRF) in northwestern Morocco during 2000–2006 and used 2 genomic regions of *Borrelia* spp. to test blood samples from these patients.

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The Study

We conducted a preliminary retrospective study during 2000–2004. Results for *Borrelia* spp. screening, which was performed at the same time as diagnostic tests for malaria, were compiled retrospectively from 10 medical centers in the Kenitra District of northwestern Morocco. *Borrelia* spp. spirochetes were identified by observing 200 microscope fields of Giemsa-stained thin blood smears under an oil-immersion objective (magnification ×1,000). Of 75,950 patients, 84 had *Borrelia* spp. infections. On the basis of these results, we conducted active prospective surveillance in 6 medical centers that reported previous TBRF cases (Figure 1).

From January 2005 through December 2006, we investigated all patients with unexplained fever who did not have malaria. All patients were screened for *Borrelia* spp. infection by microscopy and molecular methods in 2005 and only by molecular methods in 2006. Our study design conformed to directives concerning the conduct of clinical trials in Morocco.

DNA was extracted from whole blood sampled during the febrile phase of the patients by using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany). Two PCR methods were used. First, a seminested protocol specific for the entire 16S rRNA gene that used outer primers fD3 and T50 was conducted as described (*11*). The inner primers were REC4, as described (*11*), and RF16SR (5′-pos 867-AGGCGCCACACTTAACACGT-3′-pos 847). REC4 and RF16SR were paired with T50 and fD3, respectively, to obtain 2 amplicons with a 208-bp overlap. These 2 contigs were aligned to obtain the sequence of the entire 16S rRNA gene (1.5 kb). We then conducted a nested protocol

Figure 1. Locations in the Kenitra District of Morocco where tick-borne relapsing fever was diagnosed. A, Sidi Mohamed Lahmar; B, Had Ouled Jelloum; C, Idrissi Kenitra; D, Lalla Mimouna; E, Mnasra; F, Sidi Taybi.

specific for the noncoding intergenic spacer region (IGS) as described (*14*). Two negative controls were included in all experiments: a negative extraction control and the PCR mixture without DNA. *B. hermsii* DNA was used as a positive control.

PCR products were sequenced on both strands by using Genome Express (Meylan, France) and the same primers as for amplification. All sequences determined in this study were submitted to GenBank (accession nos. FJ827568–FJ827590 for IGS sequences and GQ202254–GQ202265 for 16S rRNA sequences). Multiple sequence alignments were generated with the ClustalW program (www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis was conducted with MEGA software (www.megasoftware.net) as described (*11*).

We included 127 patients in the prospective study. *Borrelia* spp. were detected in 10 (17.5%) of 57 patients in 2005 and 16 (22.9%) of 70 patients in 2006 (mean prevalence rate 20.5%). The patients had not traveled outside Morocco. The most common signs and symptoms associated with fever were chills (88%), myalgia (61%), and gastrointestinal disorders, such as diarrhea and vomiting (54%). Fifteen percent of the patients reported ≥1 relapse. Patients did not report any tick bites. All patients were successfully treated with doxycycline (100 mg/day for 7 days). Only 2 patients had a diagnosis of TBRF on the basis of microscopy; the diagnosis was confirmed by both PCR methods.

Results for molecular detection are shown in the Table. All blood samples positive according to the 16S rRNA PCR assay were also positive according to the IGS PCR assay. The 26 patients positive for *Borrelia* spp. were from Had Ouled Jelloum and Sidi Mohamed Lahmar; 99 (78%) of the 127 patients were from these 2 sites. At Sidi Mohamed Lahmar, the difference in PCR results for 16S rRNA and IGS remains unexplained. *B. hispanica* was identified by BLAST analyses (www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html) of the entire 16S rRNA gene sequences from the 12 patients from whom we were able to amplify this gene. Ten of the 12 sequences were identical to the *B. hispanica* DQ057988 sequence from GenBank. The 2 remaining sequences (GQ202254 and GQ202257) differed from this sequence by 1 nt (99.92% identity). Levels of similarity between the 12 sequences from Morocco and other relapsing fever species sequences from Africa ranged from 99.4% (2-nt difference) when compared with *B. crocidurae* DQ0057990 sequences to 99.35% (7-nt difference) when compared with *B. recurrentis* AF107362 sequences.

Phylogenetic analysis of 16S sequences showed single clusters for each relapsing fever species, with small differences between African species, which verified previous results (Figure 2, panel A) (*7*,*15*). Although the IGS locus is not a coding sequence, the level of polymorphism among sequences from Morocco was low. Sequence identity ranged from 99.1% to 100%, with no more than 4-nt differences. We found 6 alleles among 23 IGS sequences. The shallow division between the 2 close branches of the IGS phylogenetic tree did not correspond to the 2 sites at which patients with *B. hispanica* relapsing fever were located (Figure 2, panel B).

All IGS sequences obtained from our patients were grouped in a single cluster separate from other *Borrelia* spp. responsible for TBRF. The sequences from Morocco differed from those of other species from Africa, such as *B. crocidurae* (77.6% identity with *B. crocidurae* DQ000287 sequence) and the *B. duttonii*/*B. recurrentis* complex (75.5% identity with *B. duttonii* DQ000279/*B. recurrentis* DQ000277 sequences). Our results verify those of previous studies, which showed that IGS sequences cannot be used to differentiate *B. recurrentis* and *B. duttonii* from East Africa (*15*). Because the single IGS cluster, which included all sequences from Morocco, included the 12 *B. hispanica* identified by their 16S sequences, we conclude that all 23 *Borrelia* spp. DNA samples identified in this study were *B. hispanica*.

Conclusions

The prevalence of *B. hispanica* TBRF was high in the Kenitra District of northwestern Morocco. *B. hispanica* was detected in 20.5% of patients with unexplained fever. This result may be explained by use of molecular methods for detection, selection of patients with unexplained fever, and living conditions in this region, in which persons live in traditional mud huts and grow groundnuts, particularly at Had Ouled Jelloum, where the highest frequency was observed (*3*,*10*).

Our series highlights the endemicity of TBRF in Morocco, but investigations in other districts are needed. Pa-

Table. Results of PCR assays for detection of *Borrelia* spp. in 127 patients with unexplained fever in Kenitra, northwestern Morocco

Location	No. patients with positive results/total no. patients (%)	
	16S rRNA seminested PCR	Intergenic spacer sequence nested PCR
Sidi Mohamed Lahmar	2/73 (2.7)	15/73 (20.0)
Had Ouled Jelloum	10/26 (38.5)	11/26 (42.3)
Idrissi Kenitra	0/8	0/8
Lalla Mimouna	0/12	0/12
Mnasra	0/3	0/3
Sidi Taybi	0/5	0/5
All	12/127 (9.4)	26/127 (20.5)

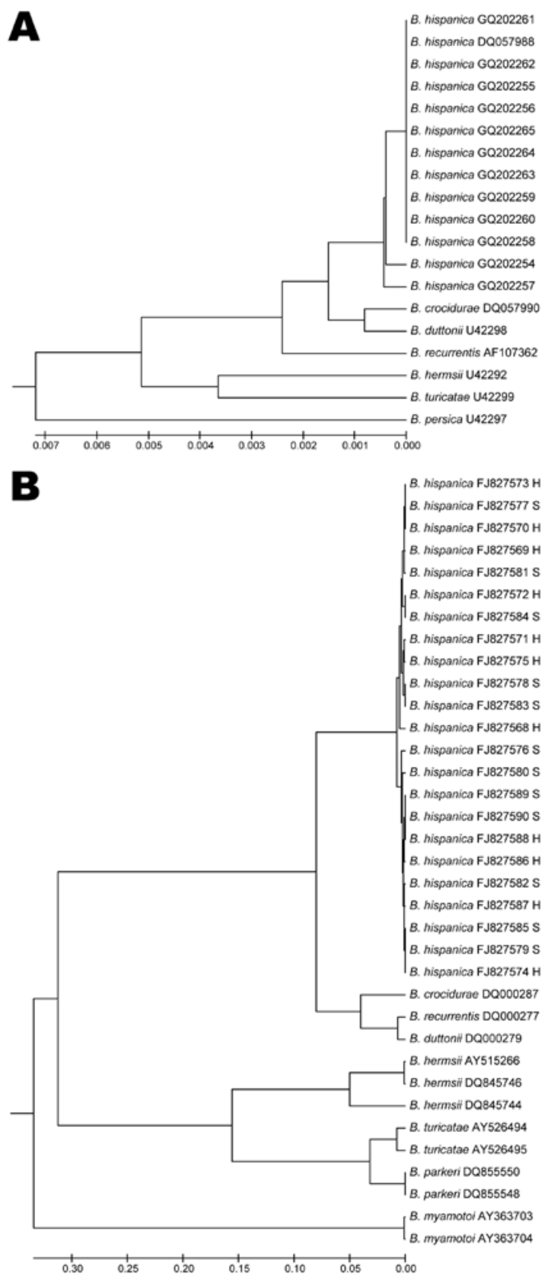

Figure 2. Phylogenetic trees constructed by the unweighted pair group method with arithmetic mean method, by using a pairwise deletion procedure. Distances were calculated by using the Jukes and Cantor method (www.tau.ac.il/~doronadi/jc.pdf). Sequences from GenBank are indicated by accession numbers. A) Phylogenetic tree based on the 16S rRNA sequences of 12 *Borrelia hispanica* DNA samples from patients in Morocco. Sequences from this study submitted to GenBank are accession nos. GQ202254–GQ202265. B) Phylogenetic tree based on noncoding 16S–23S intergenic spacer sequences of 23 *B. hispanica* DNA samples from patients in Morocco. Sequences from this study submitted to GenBank are accession nos. FJ827568–FJ827590. The letters S or H indicate the location at which the sample was obtained: S, Sidi Mohamed Lahmar; H, Had Ouled Jelloum. Scale bars indicate genetic distances between DNA sequences.

tients living in Morocco and travelers returning from this country with unexplained fever should be tested for relapsing fever caused by *Borrelia* spp. by using molecular methods.

We have shown that PCR amplification and sequencing of the IGS domain is a sensitive method with a high resolution level for detection of *B. hispanica*. This domain may also be useful for detection of other relapsing fever *Borrelia* spp., such as *B. crocidurae*, *B. hermsii*, *B. turicatae*, *B. parkeri*, and *B. myamotoi*. However, this method is not reliable for detection of *B. recurrentis* and *B. duttonii*, which cannot be differentiated by their IGS sequences (*15*).

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EMERGING INFECTIOUS DISEASES

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Persistent Reemergence of Dengue

Diversity and Origin of Dengue Virus Serotypes 1, 2, and 3, Bhutan

Tandin Dorji, In-Kyu Yoon, Edward C. Holmes, Sonam Wangchuk, Tashi Tobgay, Ananda Nisalak, Piyawan Chinnawirotpisan, Kanittha Sangkachantaranon, Robert V. Gibbons, and Richard G. Jarman

To determine the serotype and genotype of dengue virus (DENV) in Bhutan, we conducted phylogenetic analyses of complete envelope gene sequences. DENV-2 (Cosmopolitan genotype) predominated in 2004, and DENV-3 (genotype III) predominated in 2005–2006; these viruses were imported from India. Primary dengue infections outnumbered secondary infections, suggesting recent emergence.

Dengue infections have increased worldwide in recent decades. Before 1970, only 9 countries had experienced epidemics of dengue hemorrhagic fever; by 1996, this number had increased to 102 (*1*). Dengue is endemic to most of Southeast Asia; high numbers of cases are reported each year in Laos, Cambodia, Vietnam, Singapore, and Thailand. Dengue was first reported in Nepal in 2004 (*1*). Serologic testing of a group of febrile patients in Nepal showed that 8% had immunoglobulin (Ig) M against dengue, and a recent report noted 11 serologically confirmed dengue cases in 2006 (*2*,*3*). The Armed Forces Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, recently confirmed the presence of all 4 dengue serotypes in Nepal (*4*).

In another Himalayan nation, Bhutan, dengue was first suspected in the summer of 2004. Bhutan is an extremely rugged and mountainous country of 38,394 km^2 with an altitude ranging from 150 m on the southern border with India to >7,000 m in the mountains bordering Tibet (online Appendix Figure, available from www.cdc.gov/EID/content/15/10/1630-appF.htm). The dengue outbreak resulted in 2,579 cases (*5*), almost all of which occurred in and around Phuntsholing district (2005 census population 20,537) in southern Bhutan on the border with India. Fifty-two serum samples from this outbreak were tested by using dengue enzyme immunoassay (EIA) at the National Institute of Communicable Diseases, New Delhi, India. Twelve (23%) samples were positive for antidengue IgM. Thirty-five (67%) of the 52 samples also were tested at Suraksha Hospital, Kolkata, India, of which 5 were positive.

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Since the initial report in 2004, fewer clinical cases were reported from Bhutan in 2005 and 2006; dengue virus 3 (DENV)-3 was the dominant serotype (*5*). Although some serologic analysis is available, serum samples from Bhutan have not been evaluated for dengue by using molecular techniques. In particular, circulating DENV in Bhutan has not been genetically characterized, so from where and how frequently DENV is imported into Bhutan are unclear.

The Study

Acute-phase blood samples were collected from persons with suspected dengue in and around Phuntsholing during 3 periods in 2004–2006 (June 15–August 2, 2004; September 8–December 2, 2005; and July 19–November 30, 2006). All samples were collected from hospitalized patients in whom dengue infection was diagnosed on the basis of clinical signs and symptoms. Convalescent-phase samples were not available. Because all samples were collected by the Bhutan Ministry of Health as part of a public health effort, the study did not require institutional review board–approved human-use protocol.

A total of 168 samples (53 from 2004, 19 from 2005, and 96 from 2006) were delivered to AFRIMS for confirmatory dengue testing. Samples were tested by using reverse transcription–PCR/nested PCR modified from published methods (*6*) and the in-house dengue IgM/IgG EIA (*7*). In addition, the complete envelope (E) gene was sequenced for samples that were dengue positive by PCR. Two DENV-2–positive samples from 2004 and 19 DENV-3–positive samples from 2005–2006 underwent E gene sequencing. Sequencing also was attempted on additional PCR-positive samples but was not successful because of insufficient genetic material. All dengue E gene sequences from the study have been submitted to GenBank and assigned accession nos. FJ606692–FJ606712.

The E gene sequences generated here were combined with homologous sequences from 262 isolates of DENV-2 (total data set of 264 sequences, 1,485 nt) and 245 isolates of DENV-3 (total data set of 264 sequences, 1,479 nt), representing the full phylogenetic spectrum of these viruses in humans. Maximum-likelihood (ML) phylogenetic trees were estimated for both datasets using the method implemented in the PAUP* package (*8*). The general time reversible + I + Γ_4 model of nucleotide substitution was the best

Table. Laboratory-confirmed dengue cases, Bhutan, 2004–2006*

Year	Dengue cases		PCR serologic results	
	Primary	Secondary	Positive	Negative
2004	15	13 (1 DENV-2)	6 (DENV-2)	19
2005	8 (2 DENV-3)	2	2 (DENV-3)	7
2006	23 (13 DENV-3)	16 (11 DENV-3)	17 (16 DENV-3, 1 DENV-1)	40

*DENV, dengue virus.

fit to DENV-2 and DENV-4 as determined by MODELTEST (*9*). A neighbor-joining bootstrap resampling analysis (1,000 replications) also was performed to assess support for specific nodes, again by using the ML substitution model.

A total of 168 samples from persons suspected to have dengue were tested at AFRIMS. DENV infection was confirmed by PCR or EIA for 34 (64%) of 53 samples from 2004, 12 (63%) of 19 samples from 2005, and 57 (59%) of 96 samples from 2006 (combined 103 [61%] of 168 positive). PCR testing showed 7 samples from 2004 were DENV-2; 4 samples from 2005 were DENV-3; and from 2006, 40 were DENV-3 and 1 was DENV-1 (combined 52 [31%] of 168 PCR positive).

Serologic testing indicated that 28 samples from 2004 were positive for dengue by EIA; 15 were primary infections, and 13 were secondary infections according to published criteria (*7*). Ten samples from 2005 were EIA positive; 8 were primary infections, and 2 were secondary infections. Thirty-nine samples from 2006 were EIA positive; 23 were primary infections, and 16 were secondary infections (Table).

Clinical information for these dengue-positive samples was limited. However, mean age of the 103 patients with laboratory-confirmed dengue was 31 years (32 years in 2004, 28 years in 2005, and 30 years in 2006) (range 2–69 years). Mean age of the 46 patients with primary dengue infection was 29 years; that of the 31 patients with secondary dengue infections was 32 years.

Phylogenetic analysis of complete E gene sequences indicated that the 2 DENV-2 isolates sampled in 2004 fell into the Cosmopolitan genotype, which is widely distributed throughout the tropical and subtropical world (Figure 1). These viruses were closely related to those sampled in a similar period in the Indian subcontinent (India and Sri Lanka), suggesting these DENV-2 isolates were imported into Bhutan from the Indian subcontinent. A remarkably similar picture was seen for DENV-3 (Figure 2). The 19 DENV-3 isolates sampled from 2005 and 2006 clustered closely within viral genotype III, which is found in Africa, the Western Hemisphere, and the Indian subcontinent. Again, the DENV-3 phylogeny is compatible with the migration of this virus northward through India to Bhutan.

Conclusions

Our genetic characterization of DENV in Bhutan demonstrates that at least 3 DENV serotypes have circulated there. The E gene sequence data for the 2 DENV-2 strains from 2004 and the 19 DENV-3 strains from 2005 and 2006 indicate that the DENV strains in Bhutan are similar to those circulating regionally and thus were most likely introduced (or reintroduced) from neighboring areas. That such emergence in Bhutan has occurred only recently is further supported by the observation of more primary than secondary dengue infections for all 3 years, as well as the relatively advanced mean age of patients.

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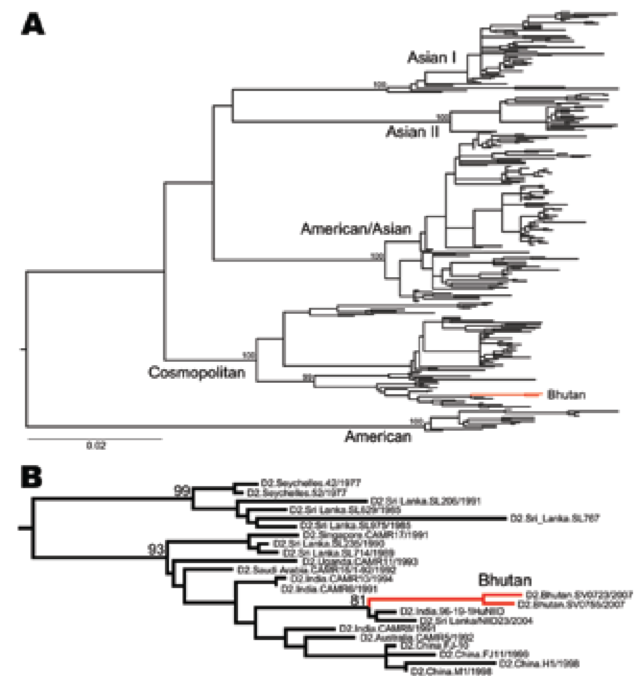

Figure 1. A) Maximum likelihood phylogenetic tree of 264 complete envelope gene sequences of dengue virus serotype 2 (DENV-2). The different genotypes of DENV-2 and the isolates from Bhutan (red) are indicated. Scale bar indicates number of substitutions per site. B) Magnification of the part of the phylogeny where the Bhutan sequences (red) fall. The tree is midpoint rooted for clarity only, and all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. Bootstrap support values are shown for key nodes only.

Figure 2. A) Maximum-likelihood phylogenetic tree of 264 complete envelope gene sequences of dengue virus serotype 3 (DENV-3). The different genotypes of DENV-3 and the isolates from Bhutan (red) are indicated. Scale bar indicates number of substitutions per site. B) Magnification of the part of the phylogeny where the Bhutan sequences (red) fall. The tree is midpoint rooted for clarity only, and all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. Bootstrap support values are shown for key nodes only.

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Ducks as Sentinels for Avian Influenza in Wild Birds

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To determine the effectiveness of ducks as sentinels for avian influenza virus (AIV) infection, we placed mallards in contact with wild birds at resting sites in Germany, Austria, and Switzerland. Infections of sentinel birds with different AIV subtypes confirmed the value of such surveillance for AIV monitoring.

As a consequence of infections of wild birds and poultry with highly pathogenic avian influenza virus (HPAIV) subtype H5N1, surveillance of wild birds for avian influenza viruses (AIVs) has intensified in Europe since 2005. Reporting of results is compulsory in the European Union (*1*,*2*). HPAIV surveillance includes investigation of dead or sick wild birds (*3*) with the aim of early detection of HPAIV (H5N1) complemented by sampling of healthy wild birds to monitor for low pathogenicity (LP) AIV strains (*4*). Previously, sentinel birds were used successfully to obtain information about AIV subtypes circulating in wild birds (*5*), but results of those studies are now outdated. Also, the effectiveness of sentinel birds has not yet been documented for AIV strains that emerged during the past decade.

We evaluated a sentinel approach to monitor the prevalence of HPAIV and LPAIV within an ecosystem, obtain information about seroconversion and duration of immunity after infection with AIV, and serve as an early warning system for the introduction of HPAIV (H5N1) and other notifiable AIVs (subtypes H5 and H7) to wild bird populations. Here we summarize results from a 2-year period of 3 international sentinel projects ongoing since 2006.

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The Study

In 2006, multiple introductions and spread of HPAIV (H5N1) occurred in Europe, including the wetlands in Austria, Germany, and Switzerland (*3*,*4*,*6*,*7*). For our study, we selected 5 locations with substantial and heterogeneous wild bird populations on the basis of HPAIV (H5N1) subtype detected during 2006. Sentinel stations were located around Lake Constance and in 2 other wetlands in Germany. The sentinel flocks at Lake Constance were situated in 1) Radolfzell (Möggingen), Germany (47°45′58″N, 8°59′45″E); 2) Altenrhein, Switzerland (47°29′25″N, 9°32′45″E); and 3) Bregenz-Thal (Rheindelta), Austria (47°30′60″N, 9°38′55″E). The 2 other stations were situated 4) on the Isle of Koos close to the Island of Rügen in Mecklenburg–Western Pomerania, Germany (54°10′13″N, 13°24′11″E) and 5) near the Oder Valley at Lake Felchow (Brandenburg), Germany, (53°03′09″N, 14°08′06″E) (Figure 1). After their wing feathers were clipped, 10–20 hand-bred adult mallard ducks (*Anas plathyrhynchos*) <1 year of age were placed in pens in natural water bodies, allowing continuous direct contact with wild water birds as previously described (*5*). Cloacal and oropharyngeal swabs and blood were taken from the mallards and tested negatively by using 1-step TaqMan real-time reverse transcription–PCR (RT-PCR) or competitive nucleoprotein antibody ELISA (cNP ELISA) (ID-Vet, Montpellier, France; Anigen, MegaCorGmbH, Hörbanz, Austria) before their use as sentinels.

At all sentinel stations in Germany, we collected oropharyngeal and cloacal swab samples from the sentinels every 14 days starting in autumn 2006. Sampling at the station in Austria started in February 2007 and at the station in Switzerland in October 2007. Laboratory tests were conducted in accordance with the Diagnostic Manual for Avian Influenza of the European Union (*8*). RNA was isolated from swabs by using viral RNA kits (QIAGEN, Hilden, Germany; Macherey-Nagel, Oensingen, Switzerland) and analyzed by real-time RT-PCR for influenza virus matrix (M) or nucleocapsid protein (NP) gene fragments. In positive samples, H5-, N1- and H7-specific real-time RT-PCRs were used to identify or exclude respective subtypes (*9*,*10*). H5 and H7 isolates were pathotyped following the European Union directive (*8*). Direct hemagglutinin (HA) typing or sequencing of positive samples was carried out as previously described (*7*,*11*–*13*). The neuraminidase (NA) subtype was identified molecularly, by following the method of Fereidouni et al. (*14*). Simultaneously, we attempted

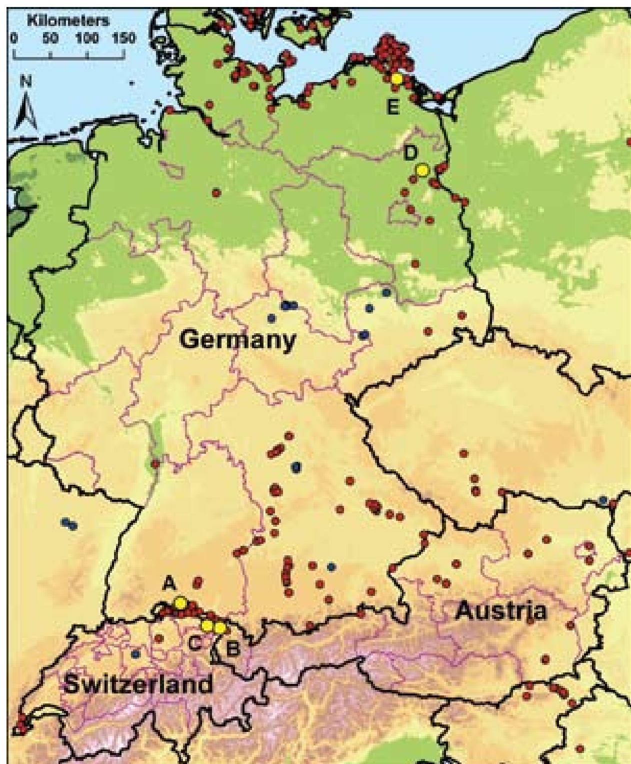

Figure 1. Locations of sentinel duck flocks at 5 locations in Germany, Switzerland, and Austria. A–C) Sites at Lake Constance: Radolfzell, Germany (A); Bregenz-Thal, Austria (B); and Altenrhein, Switzerland (C). D–E) Additional sentinel stations at Lake Felchow, Brandenburg, Germany (D), and Isle of Koos, Mecklenburg–Western Pomerania, Germany (E). Yellow dots mark the location of sentinel stations. Red dots mark detections of highly pathogenic avian influenza virus (HPAIV) (H5N1) in dead wild birds in 2006, and blue dots in 2007. In 2008, HPAIV (H5N1) was not found in dead wild birds in any of the 3 countries but was found in a live pochard (*Aythya ferina*) from Switzerland/Lake Sempach (blue dot in Switzerland).

virus isolation in embryonated chicken eggs from positive samples (*8*).

From October 2006 through September 2008, at least 23 specifiable AIV infections were detected at the sentinel stations by the fortnightly swabbing. After initial AIV introduction, virus was excreted during the following 1–3 sampling dates (Figure 2). All ducks at all sites tested positive at least once. Infections caused by AIV of 8 HA subtypes, including H5 and H7, and 6 NA subtypes, were found in clinically healthy sentinel birds (Tables 1, 2). Viral RNA and, in 44% of AIV cases, infectious virus also were recovered both from cloacal and oropharyngeal swabs. AIVs were subtyped as H1N1, H1Nx, H2N2, H2N5, H3N2, H3Nx, H3N8, H4N6, H5Nx, H6N5, H6N8, H7N3, H7Nx, and H9N2. Cycle threshold values ranged from 24 to 40. Pathotyping of H5 and H7 subtypes showed the exclusive presence of LP viruses. Additional AIVs were detected but could neither be isolated nor sequenced for subtype identification because of low loads of viral RNA. However, we did not detect H5, N1, and H7 subtypes by using real-time RT-PCR. Infections occurred most frequently from August through January (Figure 2; Table 1). Reinfection of the sentinels with the same subtype occurred in 2 of the sentinel flocks in Germany (Table 1).

Blood samples were collected from the ducks once a month, and serum was tested in a cNP-ELISA after heat inactivation at 56°C for 30 min. After each natural infection, sentinel animals seroconverted, and serum scored positive in the cNP-ELISA within 2–4 weeks. By hemagglutination inhibition test using homosubtypic but not autologous antigen, HA-specific antibodies were detected only rarely and at low titers.

Detection rates of AIV in sentinel ducks were compared with data from monitoring of healthy, trapped wild birds. From October 2006 through September 2008, a total of 1,953 wild birds were investigated for AIV within a radius of 30 km of Lake Constance, resulting in 47 (2.4%) AIV detections of subtypes H3Nx, LP H5N2, H6N8, LP H7Nx, H1N1, HxN1, H1Nx, and H9N2. During January 2007–May 2008, a total of 8 (0.4%) of 2,028 investigated wild bird samples from Brandenburg tested positive (subtypes H3N6, H6). In Mecklenburg–Western Pomerania, 8,066 birds were tested; 23 (0.3%) AIV infections (subtypes H1Nx, LP H5N2, H6Nx, H12Nx, H16Nx) were found.

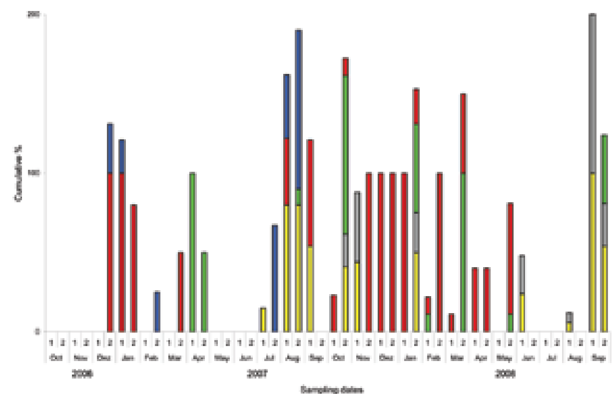

Figure 2. Months with positive results for sentinel birds over a 2-year period at 5 locations in Germany, Switzerland, and Austria. Sites at Lake Constance: Radolfzell, Germany (yellow); Bregenz-Thal, Austria (gray); and Altenrhein, Switzerland (green). Additional sentinel stations at Lake Felchow, Brandenburg, Germany (red), and Isle of Koos, Mecklenburg–Western Pomerania, Germany (blue). Bars indicate the cumulative percentage of sentinel birds tested positive at each of the 5 locations at the time of sampling (maximum 500% at all 5 stations). For example, in December 2006, all sentinel ducks at station 4, but only 30% of sentinels at station 5, were positive at the date of sampling. 1, days 1–15 of month; 2, day 16 through end of month.

Table 1. Detection of AIV by sampling of sentinel mallard ducks (*Anas plathyrhynchos*) at 5 locations in Germany, Switzerland, and Austria*†

Sentinel location	Date sampled	Ct range‡	Duration virus excretion, d	HA subtype	NA subtype	Isolate	Sequence of HA-cleavage site (H5, H7)
Radolfzell, Germany	2007 Aug	29–37	Min 28, max 42	H6	N8	Yes	
	2007 Oct	31–39	Min 14, max 28	H2	N5	Yes	
				H3	N2	Yes	
	2008 Jan	33–38	Punctual	H3	N2	Yes	
	2008 Jun	33–39	Punctual	H3	N2	No	
	2008 Sep	33–39	Min 14, max 28	H3	N8	Yes	
Altenrhein, Switzerland	2007 Oct	27–39	Min 15, max 39	ND§	N2	No	
	2007 Dec	24–37	Punctual	H2	N2	No	
	2008 Aug	29–34	Punctual	H9	N2	No	
Bregenz-Thal, Austria	2007 Apr	28–38	Min 23, max 38	H3	ND¶	No	
	2007 Oct	22–40	Min 12, max 26	H9	N2	Yes	
	2008 Jan	27–38	Min 14, max 42	H1	N1	No	
	2008 Mar	25–35	Min 14, max 42	H7	ND¶	No	PEIPKGR GLF
Lake Felchow, Brandenburg, Germany	2006 Dec	27–35	Min 22, max 36	H6	N2	Yes	
	2007 Jan	29–38	Min 14, max 34	H5	N3?	No	PQRETR GLF
	2007 Mar	35–38	Punctual	H5	ND¶	No	
	2007 Sep	31–39	Punctual	H6	N5	Yes	
	2007 Dec	27–37	Min 42, max 56	H9	ND¶	No	
				H1?	ND¶	No	
				H11?	ND¶	No	
	2008 Feb/Mar	30–38	Min 56, max 70	H9	ND¶	No	
Isle of Koos, Mecklenburg–Western Pomerania, Germany	2006 Dec	29–35	Min 14, max 28	H4	N6	Yes	
	2007 Aug	32–38	Min 35, max 49	H7	N3	Yes	PEIPKGR GLF

*Radolfzell, Altenrhein, and Bregenz-Thal are located along Lake Constance. AIV, avian influenza virus; Ct, cycle threshold value; HA, hemagglutinin; NA, neuraminidase; min, minimum; max, maximum; ND, not determined.
†Each sentinel flock comprised 10–20 birds. Only the initial detection of each AIV introduction and determined HA or NA subtypes are presented.
‡Results of matrix and nucleocapsid protein gene fragments by real-time reverse transcription–PCR.
§AIV-positive, HA subtype not determined, non-H5, non-H7.
¶AIV-positive, NA subtype not determined, non-N1.

Conclusions

In practice, AIV surveillance of live wild birds is difficult and involves substantial labor and costs, particularly for purchase and maintenance of trapping equipment, salary of trapping staff, and laboratory analysis. Trapping of wild birds also can be biased by season and by bird species that are easier to catch. Low proportions of AIV-positive results (<3%) indicate the low cost:benefit ratio of surveillance based on trapping wild birds (*2*). In contrast, our findings demonstrate that the use of sentinel birds in regions with substantial wild bird populations achieves a high rate of AIV detection and, therefore, is an efficient supplement to active AIV monitoring. The detection of different AIVs among the sentinel ducks reflects the natural ecology of AIV at discrete locations. Recently, all duck species, especially dabbling ducks, have been assessed as high-risk species for possibly contributing to the transmission of HPAIV (H5N1) (*15*). Therefore, mallards as sentinel species ensure a high probability of detecting AIV if kept in direct contact with wild water birds. In addition, sites for sentinel stations need to be selected carefully to achieve spatial representation.

Although our study was conducted in areas where HPAIV (H5N1) had circulated in wild birds in 2006, this subtype was not found by screening live wild birds or by using sentinel birds during the study period. Therefore,

Table 2. Frequency of sentinel duck sampling and frequency of AIV detection at 5 locations in Germany, Switzerland, and Austria*

Sentinel location	Investigation period	No. samplings of sentinel flock†	No. AIV detections	AIV subtypes
Radolfzell, Germany	2006 Oct–2008 Sep	53	11	H6N8, H2N5, H3N2, H3N8
Altenrhein, Switzerland	2007 Oct–2008 Sep	24	7	H2N2, H9N2, HxN2
Bregenz-Thal, Austria	2007 Feb–2008 Sep	44	9	H9N2, H3Nx, H1N1, H1Nx, LP H7Nx
Brandenburg, Germany	2006 Oct–2008 May	41	20	H1?, LP H5Nx, H6N2, H6N5, H9Nx, H11?
Mecklenburg–Western Pomerania, Germany	2006 Oct– 2008 Jun	40	6	H4N6, LP H7N3

*Radolfzell, Altenrhein, and Bregenz-Thal are located along Lake Constance. AIV, avian influenza virus; LP, low pathogenicity.
†Each flock consisted of 10–20 birds.

persistent circulation of HPAIV (H5N1) in the wild bird populations is unlikely for the area of Lake Constance, the coastal area of Mecklenburg–Western Pomerania, and the region of the Oder Valley in Brandenburg. However, because of the limited sample sizes, a low prevalence cannot be excluded. Although HPAIV (H5N1) was found only rarely in apparently healthy birds, e.g., in a pochard (*Aythya ferina*) in Switzerland in 2008 (Figure 1), regular testing of sentinel birds could increase the probability of detecting sporadic transmission of HPAIV in healthy wild water birds even in the absence of detectable deaths.

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Immunologic Response of Unvaccinated Workers Exposed to Anthrax, Belgium

Pierre Wattiau, Marc Govaerts, Dimitrios Frangoulidis, David Fretin, Esther Kissling, Mieke Van Hessche, Bernard China, Martine Poncin, Yvo Pirenne, and Germaine Hanquet

To determine immunologic reactivity to *Bacillus anthrax* antigens, we conducted serologic testing of workers in a factory that performed scouring of wool and goat hair. Of 66 workers, ≈10% had circulating antibodies or T lymphocytes that reacted with anthrax protective antigen. Individual immunity varied from undetectable to high.

Industrial anthrax, also known as woolsorter's disease, was a serious threat in the 19th and early 20th centuries when the wool industry was flourishing. The causal agent, *Bacillus anthracis*, was brought into factories in sporulated form with the organic matter that was contaminating the animal fibers. The pathogen provoked the characteristic necrotic lesions on the skin of the wool workers (cutaneous anthrax), but it could also cause a respiratory disease through airborne transmission (inhalational anthrax). In 1950, 90% of those with the latter form died, although the proportion of deaths could be lowered to 50% with the aggressive therapy that was later used to treat the victims of the deliberate release of anthrax in the United States in 2001 (*1*,*2*).

Today, industrial processing of wool and goat hair has almost disappeared from Western industrialized countries. Cases of human anthrax have become rare in Europe (*3,4*), but they can sometimes result from contact with imported contaminated material (*6*,*6*). Apart from the 2001 attacks (*7*), the most recent human anthrax epidemic in the United States was reported in 1957 in a large goat hair–processing mill in Manchester, New Hampshire (*1*). In a recent study, we investigated the microbiologic flora of a Belgian factory (in operation since 1880) that processes and scours wool and goat hair from all over the world. Living anthrax spores were demonstrated in goat hair fibers, air dust, and unprocessed wastewater produced from goat hair scouring (*8*). No clinical case of anthrax was recorded among the employees of this company except for a possible cutaneous lesion, reported by a worker in 2002, the cause of which remained unconfirmed. In the current study, we investigated the immunity of the factory workers. Since none of these workers had been vaccinated against anthrax, we assumed that immunologic reactivity to anthrax antigens, if any, would very likely demonstrate exposure to *B. anthracis*.

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The Study

Blood samples were obtained from 66 of the 67 factory workers, including the administrative employees. Serologic testing was carried out at 2 time points (December 2006 and December 2007) by using a commercial ELISA (Serion, Würzburg, Germany) based on plates coated with purified anthrax protective antigen (PA) (online Technical Appendix, available from www.cdc.gov/EID/content/15/10/1637-Techapp.xls). The first year, 3 workers had immunoglobulin (Ig) G titers above the threshold recommended by the manufacturer for vaccine protection (>15 IU/mL), and titers for another worker were considered borderline (10–15 IU/mL). All 4 workers had positive results by Western blot or dot blot analysis with pure recombinant anthrax PA and lethal factor (LF). One year later, 54 workers were sampled (2 were new employees). The second round of testing gave similar results, except for 3 additional borderline cases which could also be confirmed by Western blot/dot blot analysis (Table). Lymphocyte proliferation assays were performed concurrently by using fresh, heparinized, whole blood samples to evaluate the cell-mediated immunity of the workers (*9*). This technique measures the ability of lymphocytes placed in short-term tissue culture to undergo clonal proliferation when stimulated in vitro by a foreign antigen. Cell proliferation was determined by measuring the incorporation of ^{3}H-thymidine into chromosomal DNA. The release of interferon-gamma (IFN-γ) in the course of lymphocyte stimulation was also measured to assess antigen-specific, cell-mediated reactivity. The antigens used here were pure recombinant PA and LF, along with positive control (concanavalin A) and negative control (phosphate buffer) stimulants. As shown in the Table, 2 cultures were positive in proliferation assays. Of these 2 cultures, 1 reacted with PA and LF, and 1 reacted with PA only. When added together, PA and LF suppressed the proliferative effect of the individual antigens, consecutive to the probable cytotoxicity induced by the 2 assembled antigens (porin +

Table. Number of workers showing immunoreactivity against *Bacillus anthracis* antigens, as assayed by 3 different methods*

Status	Anti-PA ELISA†		Western blot/dot blot‡		L_T proliferation§			
	Year 1	Year 2	Year 1	Year 2	Ag = PA	Ag = LF	Ag = PA + LF	Ag = conA
Negative	62	48	–	–	52	53	54	13
Positive	3	2	4	6	2¶	1¶#	0	41
Borderline	1	4	–	–	0	0	0	0

*PA, protective antigen; LT, lymphocyte; Ag, antigen; LF, lethal factor; conA, concanavalin A.
†Performed on serum samples according to the manufacturer's instructions and thresholds (Serion, Würzburg, Germany). Note: 1 worker tested positive at year 1 was not enrolled at year 2, and 3 workers tested negative at year 1 seroconverted to borderline at year 2.
‡Conducted only on serum samples found positive or borderline by anti-PA ELISA together with negative controls (n = 25). Dot blots were spotted with 1,000, 100, 10, and 1 ng of each purified PA and LF Ag purchased from Quadratech Ltd. (Epson, UK). Western blot antigens consisted in supernatant proteins derived from the culture of a reference *B. anthracis* strain.
§Assayed on blood samples from year 2 as described earlier (9) by using a proliferative index threshold set to 3× the index of a negative control stimulant (phosphate buffer). Stimulating Ag was used at a final concentration of 4 mg/mL (PA, LF, conA). ConA was used as positive control stimulant and was purchased from Sigma (St. Louis, MO, USA). Lymphocyte cultures found activatable by PA were confirmed by quantifying IFN-γ release by ELISA, according to the manufacturer's instructions (Pierce, Rockford, IL, USA)
¶Serum samples from these workers tested positive by both Western blot and dot blot analysis.
#This culture was also stimulated by PA.

toxin). Typical examples of lymphocyte proliferation results are shown in Figure 1. The lymphocyte cultures found to be responsive to pure anthrax antigens originated from workers who had little circulating anti-PA IgG (<15 IU/mL), as tested by ELISA (Figure 2). However, their serum tested positive by Western blot analysis (Table). Moreover, supernatants of PA-stimulated lymphocyte cultures derived from the blood of these workers with positive results by determining counts per minute, were confirmed by IFN-γ release assay.

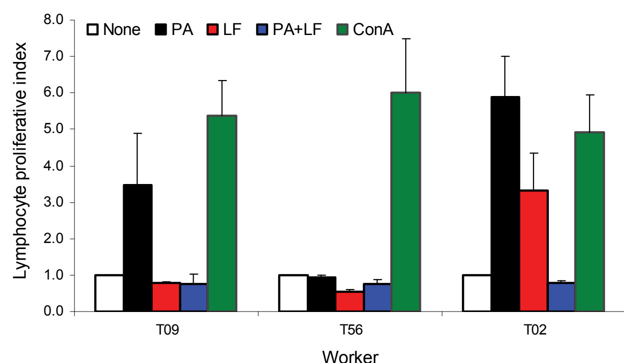

Figure 1. Representative examples of lymphocyte proliferation results. Proliferation was assayed by measuring ^{3}H-Thymidin incorporation (counts per minute [cpm]) of culture lymphocytes stimulated with different antigens and by determining the respective proliferative Indexes. The latter were calculated by dividing the cpm induced by a given antigen by the cpm induced by a negative control antigen (phosphate-buffered saline (PBS), white boxes). The proliferative index is a parameter that reflects the reactivity of a lymphocyte culture toward a given antigen. It is indicative of the cellular immunity of a person toward this antigen. The antigens used in this experiment are listed in the Table. The figure shows 3 representative culture profiles that react either with protective antigen (PA) and lethal factor (LF) (1 sample, T2), with PA only (1 sample, T9), or with none of them (41 samples, exemplified by T56). Each value is the mean of 3 independent experiments and is shown with the standard deviation (error bar).

Conclusions

Although some progress made in improving the biologic safety of the industrial processing of wool and goat hair (for example, systematic disinfection, air filtering, and protective gear for employees working in closed areas), this study shows that *B. anthracis* still poses a health risk to modern wool workers. Handling nondisinfected, raw animal fibers from areas where anthrax is endemic, such as the southern Caucasus region and the Middle East, has been and remains an at-risk activity. The presence of circulating antibodies and T lymphocytes that react with antigens expressed only by vegetative cells of *B. anthracis* in unvaccinated wool workers confirms several previous findings. First, these findings support the conclusions that anthrax spores are able to germinate into vegetative cells at the sites of exposure (skin, mucosa, respiratory tract) and cause asymptomatic infection (*10*,*11*). Second, the extent to which the human immune system responds to exposure to anthrax spores from the surrounding environment as well as the nature of this response varies tremendously from person to person. This conclusion was well exemplified by the situations of 2 workers. Results from 1 worker (T29) displayed a high IgG titer (>100 U/mL) but little or no cell-mediated reactivity. Results from the other worker (T2) showed significant lymphocyte reactivity (^{3}H-Thymidin counts >700 counts per minute, which corresponds to a proliferative index of 6, $p>0.01$), but a low IgG titer (Figure 2), which reflects reciprocal T- and B-cell responses. None of the persons whose samples tested positive by ELISA reported a past episode of anthrax (according to face-to-face interviews conducted when blood was sampled). Hence, their seroconversion most likely resulted from asymptomatic *B. anthracis* infection. One worker reported having had a skin lesion possibly compatible with cutaneous anthrax 4 years before the study. That worker's samples tested positive by lymphocyte proliferation assay, Western blot, and dot blot, but not by anti-PA ELISA.

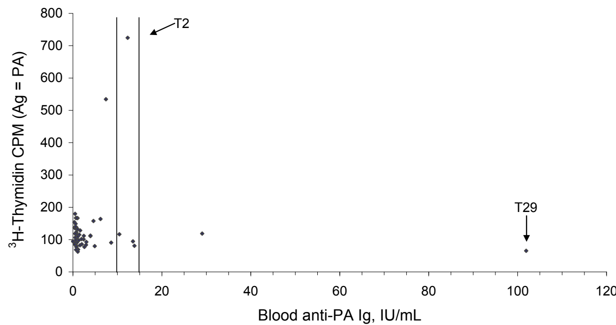

Figure 2. Graph showing anti–protective antigen (PA) immunoglobulin G (IgG) titers plotted against ^{3}H-thymidin counts per minute (cpm) derived from PA-stimulated blood cell cultures conducted in year 2. The vertical lines define the ELISA borderline and upper thresholds (10 IU/mL and 15 IU/mL, respectively), which were defined as PA titers by the ELISA kit manufacturer, i.e., titers supposed to confer protection after vaccination. Samples testing below the borderline threshold are considered negative. T29 and T2 refer to workers whose samples had a remarkably high antibody titer or lymphocyte proliferation count, respectively.

Notably, samples from many workers from the same factory, who had been exposed to goat hair for years in similar conditions, did not display positive serologic results when tested by ELISA. During our study, however, we noticed that serum samples from 3 workers had seroconverted from negative to partially protective (borderline) IgG titers at some point between the 2 blood samplings as determined by anti-PA ELISA. Given the long history of these workers at the company, the apparent lack of anti-PA antibodies at the first blood sampling may have been misestimated due to the high threshold defined for seropositivity by the commercial ELISA used in the study. This commercial kit is indeed primarily aimed at evaluating the efficacy of anthrax vaccination rather than at detecting antibody responses after exposure to subinfectious doses of anthrax spores (*12*). Accordingly, we noticed that of the 3 workers who seroconverted, 2 tested positive by Western blot, and 1 tested positive by dot blot when tested retrospectively at year 1. Blotting techniques seem thus more sensitive than the presently used ELISA seropositivity threshold for detecting low anti-PA antibody titers. The low sensitivity of the method used in this work to assess cell-mediated immunity (whole blood proliferation assay) may have also underestimated the actual number of workers who exhibited cell-mediated immunity against *B. anthracis*, and the results should be regarded as indicative rather than representative.

PA-based anthrax vaccines are available to protect professionally exposed people, such as the US anthrax vaccine adsorbed or the UK anthrax vaccine. These vaccines are efficient and elicit humoral responses that protect the vaccinees against toxin-associated death (*13)*. They do require long clinical protocols and yearly boosters (*14*) and are not officially licensed in European Union member states (except the United Kingdom). According to some authors, these vaccines might not protect wool-workers efficiently against subclinical infection, spore germination, or bacteremia (*13*,*15*). Anthrax vaccines that confer long-term immunity of both the humoral and cellular type are not yet available for the general public. Vaccines with such characteristics would be highly desirable to better protect persons who work with animal products that are possibly contaminated with anthrax spores.

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Molecular Epidemiology of Clade 1 Influenza A Viruses (H5N1), Southern Indochina Peninsula, 2004–2007

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To determine the origin of influenza A virus (H5N1) epizootics in Cambodia, we used maximum-likelihood and Bayesian methods to analyze the genetic sequences of subtype H5N1 strains from Cambodia and neighboring areas. Poultry movements, rather than repeated reintroduction of subtype H5N1 viruses by wild birds, appear to explain virus circulation and perpetuation.

From 2004 through 2007, a total of 26 outbreaks of influenza A virus (H5N1) infection have occurred in poultry in Cambodia, and 7 human cases have been reported. Subtype H5N1 infections were observed primarily during the dry season, from January through April. The origin of these epizootics in Cambodia remains unclear. Here we have used maximum likelihood and Bayesian methods to analyze the Cambodian virus genetic sequences, together with those obtained from other H5N1 viruses isolated in neighboring countries since 2004, to understand that patterns of virus transmission.

The Study

We analyzed the sequences of all 8 genomic segments of 33 subtype H5N1 viruses sampled in Cambodia from 2004 through 2007, together with publicly available sequences from 116 isolates from Southeast Asia obtained from GenBank. We included all subtype H5N1 viruses sequences from southern Vietnam. In Cambodia, we sequenced systematically at least 2 strains from each infected flock and obtained a total of 137 sequences. When sequences obtained from viruses isolated during the same outbreak site were identical, we used only one for the analyses. After alignment, phylogenetic analyses were conducted by using maximum-likelihood and Bayesian methods (*1*,*2*).

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All H5N1 viruses that were detected in Cambodia from 2004 through 2007 belong exclusively to clade 1, genotype Z (Figure 1, panel A) (*3*,*4*). The hemagluttinin (HA) phylogenetic tree shows that viruses isolated from Yunnan in 2002 and 2003 are at the origin of all clade 1 viruses from Thailand, Cambodia, and Vietnam (Figure 1, panel A). Sequences obtained from viruses first isolated in Cambodia in January 2004 cluster with a large number of sequences of H5N1 viruses isolated in 2004 and 2005 in Thailand (Figure 1). Subsequent Cambodian subtype H5N1 isolates are grouped into closely related phylogenetic sublineages and are denoted by sublineage number (arbitrarily numbered I–VII for purposes of molecular epidemiology), and these sublineages are also denoted in the map (Figure 2) to show their geographic locations and relationships to likely virus introduction routes. All of these viruses are within the World Health Organization (WHO) clade 1, and these sublineage numbers are not to be confused with the WHO clade/subclade nomenclature.

In 2005, all 4 reported human cases were identified in a limited area of Kampot Province, near the Vietnamese border (Figure 2). Sequences of A/Cambodia/JP52a/2005 detected in January 2005 cluster with strains isolated in 2004 in Southeast Vietnam. The HA gene sequences fall within a cluster of viruses, closely related phylogenetically within clade 1, which we arbitrarily named sublineage I (Figure 1, panel A; Figure 2). Similarly, the strains A/chicken/Cambodia/013LC2b/2005, A/Cambodia/P0322095/2005, and A/Cambodia/408008/2005 detected during March–April 2005 in Kampot and Kandal provinces form separates clusters with sequences of South Vietnamese strains isolated in 2004–2005, namedand named sublineages II, III, and IV, respectively (Figures 1 and 2).

When all the major outbreak sites were plotted on a map of Cambodia, especially sites where viruses from sublineage V (Figure 1, panel A; Figure 2) were isolated in February and early and late March 2006, respectively, it appears that subtype H5N1 virus spread north to south following a main road (Figure 2). Virus A/Cambodia/Q0405047/2006 detected in March 2006 (sublineage VI) is phylogenetically closely related to viruses detected in 2 Vietnamese provinces between August and December 2005 (Figure 1, panel A). We observed 2 virus strains, A/duck/Cambodia/D2KC/2006 and A/duck/Cambodia/D1KC/2006, which were isolated in poultry at the same time and location (February 2006 in Kampong Cham Province), but belong to different sublineages, V and VI,

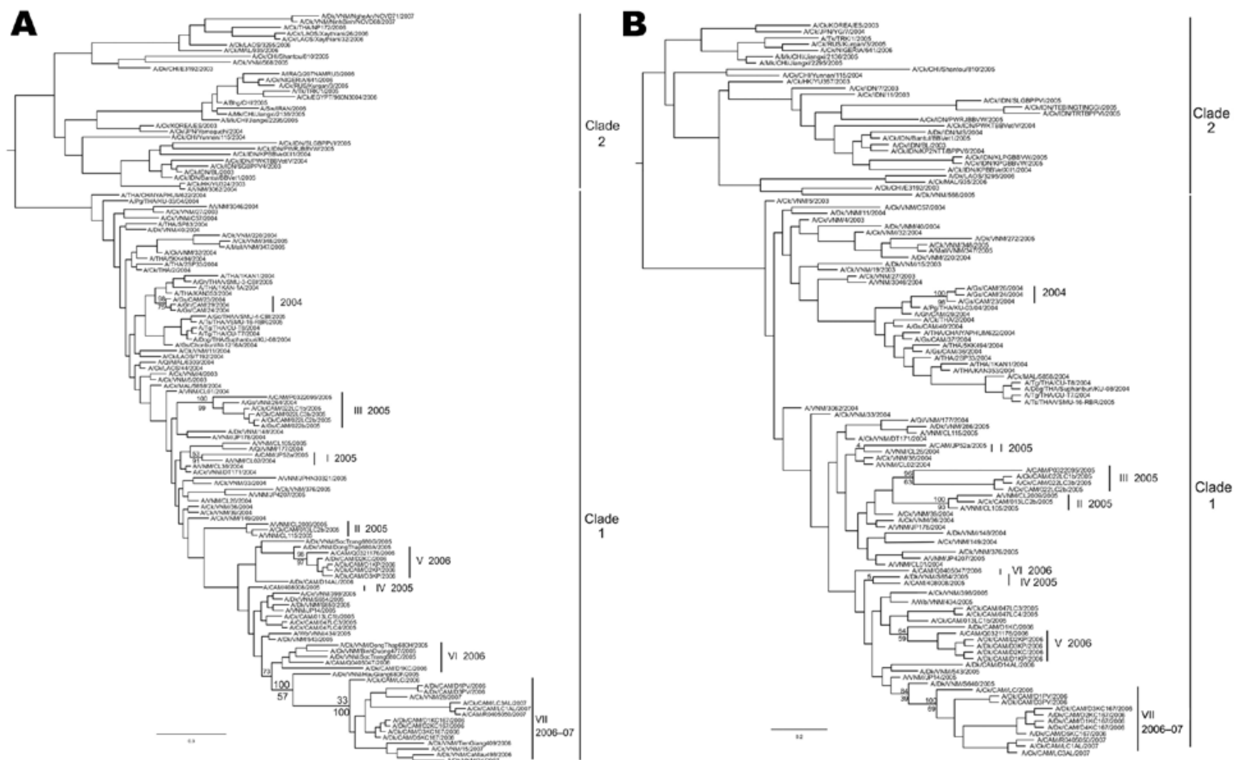

Figure 1. Phylogenetic relationships of the hemagluttinin (HA) (A) and neuraminidase (NA) (B) genes of 33 Cambodian strains and of representative influenza A viruses (H5N1). Trees were generated by Bayesian analysis using MrBayes v3.1 software (*2*). Numbers above and below branches indicate Bayesian posterior probability and maximum likelihood bootstrap values (PHYML v2.4 software, www.atgc-montpellier.fr/phyml), respectively. Analysis was based on nucleotides 28–1578 of the HA gene and 67 to 1248 of the NA gene. Both trees were rooted to A/goose/Guangdong/6/96. Scale bar indicates 0.3 and 0.2 substitutions per site for HA and NA genes, respectively. Dk, duck; Ck, chicken; Gs, goose; Bhg, bar headed goose; Pg, pigeon; Gh, gray heron; Ql, quail; Mall, Mallard duck; Tg, tiger; CAM, Cambodia, VNM, Vietnam, THA, Thailand, MAL, Malaysia, CHI, People's Republic of China; TRK, Turkey; RUS, Russia, JPN, Japan. Cambodian subtype H5N1 viruses that are grouped in closely related phylogenetic sublineages and are denoted by sublineage number (arbitrarily numbered I–VII for purposes of molecular epidemiology) and these sublineages are also denoted in the map (Figure 2) to show where these viruses have found and the likely virus introduction routes. It is to be emphasized that all these viruses are within the World Health Organization (WHO) clade 1, and these sublineage numbers are not to be confused with the WHO clade/subclade nomenclature.

respectively. The HA sequences of the 2 viruses differ by 2 nonsynonymous substitutions that lead to amino acid changes at positions 184 and 195, and by 23 synonymous substitutions. The neuraminidase (NA) gene sequences of both viruses are phylogenetically closely related to the same NA sublineage V, which suggests that a reassortment event occurred (Figure 1, panel B).

Sublineage VII contains 5 separate branches (Figure 1, panel A), which correspond viruses isolated from different geographic locations at different times: Kampong Speu in March 2006 (LC/2006), Kampong Cham in August 2006 (e.g., D1KC167/2006), Prey Veng in August 2006 (e.g., D1PV/2006, which also clusters with a 2007 Vietnamese virus), Kampong Cham in April 2007 (e.g., R0405050/2007) and Vietnam 2006/2007 (Figure 1, panel A). A/Chicken/Vietnam/29/2007 is closely related phylogenetically to Cambodian viruses detected in August 2006 and not closely related to the group of Vietnamese strains from 2006/2007. This suggests that the 2007 Vietnamese strains also have various parental origins, probably related to geographically distinct circulation areas.

For all gene segments, the percentage of homology varied from 98% (for the HA segment) to 99.8% (for the M1 coding gene) between sequences of the first and the latest virus for which complete genome sequences were obtained. Mutations S123P and S129A, R to G substitution in the HA cleavage site, and S155N and K189N mutations at antigenic site B (which could explain the antigenic drift measured) are characteristics that seem to have become established in the latest Cambodian isolates (online Appendix Table, available from www.cdc.gov/EID/content/15/10/1641-appT.htm).

Conclusions

Cambodia is essentially a poultry-importing country. The first poultry deaths were observed in semi-industrial

chicken farms that imported broiler and layer parental stocks from a sister company in Thailand, where outbreaks occurred contemporaneously. Whether the viruses were introduced from Vietnam into Cambodia or vice versa cannot be ascertained from the phylogenetic evidence. However, given the known direction of poultry imports from Vietnam to Cambodia, multiple introductions of subtype H5N1 viruses most likely occurred through illegal trading in poultry from Vietnam; this hypothesis would explain how several sublineages emerged in Cambodia in 2005 and 2006. The national veterinary surveillance system in Cambodia is probably not sufficiently sensitive to detect outbreaks resulting from a low prevalence of circulating subtype H5N1. The silent circulation of subtype H5N1 virus in vaccinated poultry (e.g., in ducks) in Vietnam in October 2005 could explain why Vietnam appeared free of subtype H5N1 infections from December 2005 through October 2006, while viruses were still able to cross the border and to infect nonimmune (nonvaccinated) Cambodian poultry (*5*). Similar to the situation in Vietnam (*6*), Cambodian farmers tend to release ducks in the paddy fields, particularly after harvesting periods in which duck flocks could feed on the harvest's leftovers. Seasonality could then be explained by higher duck density (*7*,*8*). Viruses in Cambodia very likely represent multiple external introductions rather than becoming established within Cambodia on a continuous basis. This may be related to the fact that the poultry density in Cambodia is lower (average 94 heads/km^2) (*9*) compared with that in neighboring countries such as Thailand and Vietnam (average 640 heads/km^2) (*10*), and one may speculate that a minimum density of poultry is required for maintaining an endemic virus.

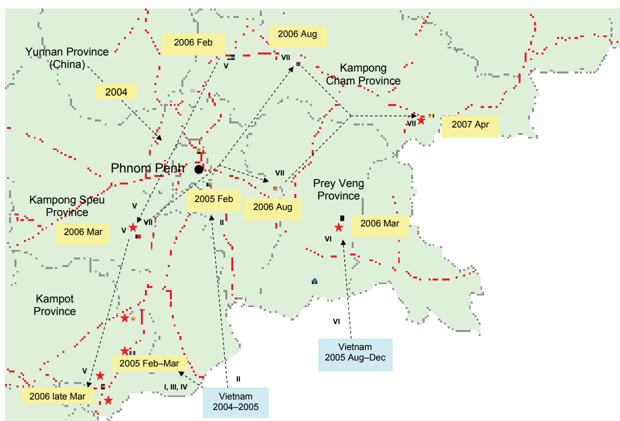

Figure 2. Map of Cambodia showing the locations of influenza A (H5N1) outbreaks in poultry (circles) and human cases (stars) detected since 2005. Arrows are proposed to illustrate the hypothetical paths of introduction of H5N1 virus sublineages (see Figure 1) in Cambodia from its neighboring countries. A sublineage number adjacent to the arrow implies that the respective sublineage viruses are found at the start and the end of the arrow with the years of the detection noted (online Technical Appendix Table, available from www.cdc.gov/EID/content/15/10/1641-appT.htm). Molecular characteristics of hemagluttinin (HA) sequence and hemagglutination inhibition (HI) tests of Cambodian viruses and some reference subtype H5N1 strains. A) H5 aa numbering. RBS, receptor binding site; DEL, deletion. B) BHG, bar-headed goose; VNM, Vietnam; CAM, Cambodia; INDO, Indonesia. Numbers I–VII refer to sublineages; within sublineage VII, PV06: group of viruses represented by the strain A/Duck/Cambodia/D1PV/2006; KC06: group of viruses represented by the strain A/Duck/Cambodia/D1KC1672006; CAM07: group of viruses represented by the strain A/Cambodia/R0405050/07; VNM06–07: group of viruses isolated in South Vietnam in 2006 and 2007. C) Antigenic characterization was performed by using the HI assay with ferret antisera raised to World Health Organization reference subtype H5N1 viruses. Numbers are the results of the differences between the $\log_2$(HI titer/10) of the reference virus and the virus tested. D) For HI tests, the virus A/BHG/Qinghai Lake/1A/2005 was tested against this homologous serum, while ferret serum against A/Turkey/15/2005 was tested against the reassortant virus NIBRG-23 derived from A/Turkey/15/2005. A/BHG/Qinghai Lake/1A/2005 and A/Turkey/15/05 are both clade 2.2 viruses.

Lake Tonle Sap (16,000 km^2 surface) and other lakes and wetlands host numerous wild birds, mostly resident or regional migratory birds (M. Gilbert, pers.comm.), which could be involved in the spread of subtype H5N1 virus. Should this mode of transmission play a role, it would probably be limited to local or regional transmission, especially since viruses from other clades (i.e., clade 2.2) that are imported to neighboring countries by migratory birds from the People's Republic of China, have never been detected in Cambodia.

Sublineage VII–specific mutations (i.e., R325G, S123P) in strains from Cambodia and Vietnam suggest that this new sublineage became endemic in the southern Cambodia–Mekong Delta region during 2006. The phenomenon of virus spread through poultry trading, particularly along the roads, accounts for maintenance and extension of virus within a given region and sustains the risks of transmission to humans.

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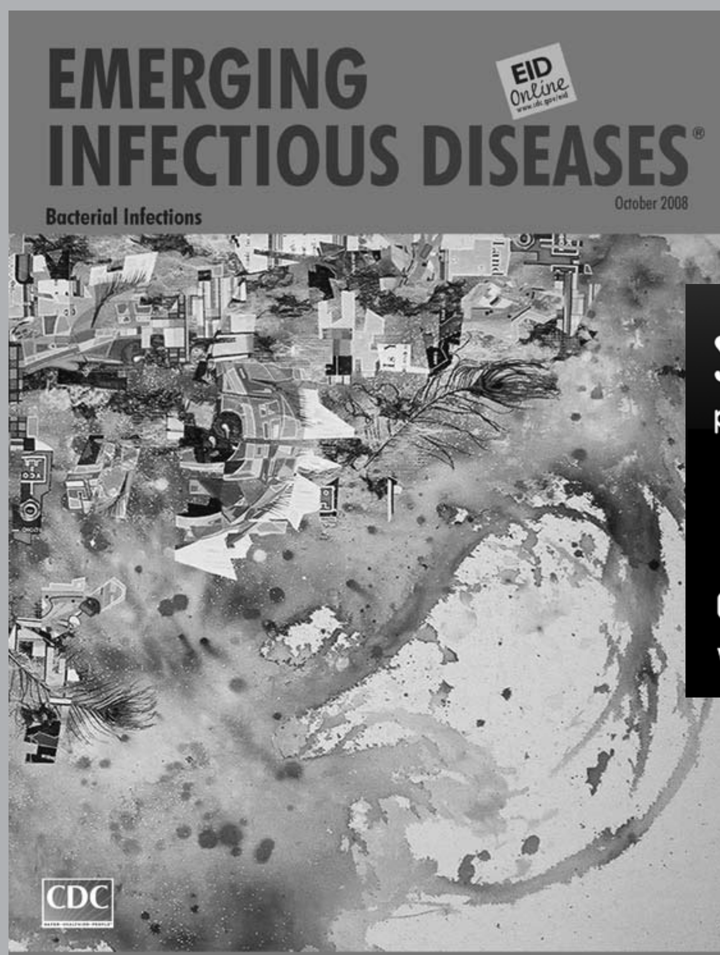

Melioidosis in a Tropical City State, Singapore

Tong Jen Lo, Li Wei Ang, Lyn James, and Kee Tai Goh

The incidence of melioidosis in Singapore decreased during 1998–2007, with the exception of the first quarter of 2004. After heavy rainfalls, an increase in pneumonic cases with a high case-fatality rate was detected. We show that melioidosis has the potential to reemerge following adverse climate events.

Melioidosis is a tropical infectious disease caused by a gram-negative bacillus, *Burkholderia pseudomallei*. It is endemic to southeast Asia and northern Australia, and cases are increasingly being reported in countries elsewhere in Asia, the Pacific, the Americas, the Caribbean, Africa, and the Middle East, and in travelers returning from tropical countries (*1*).

B. pseudomallei is a saprophytic bacterium that can be found in soil and water samples in melioidosis-endemic countries. Transmission is generally by direct inoculation from exposure to soil or water, or through inhalation of aerosolized particles. The disease often affects persons with underlying conditions such as diabetes mellitus (*1*,*2*). Clinical manifestations are protean and may range from chronic abscesses to fulminant pneumonia and septicemia with high death rates (*1*).

Singapore is a tropical island city state in southeast Asia. More than 80% of the population lives in high-rise public housing estates. Although the first case of melioidosis in Singapore was reported in 1920 (*3*), little is known about the incidence of the disease before it was made notifiable in 1989 when 3 apparently healthy young men died from melioidosis (*4*). We studied the epidemiology and clinical features of melioidosis in Singapore over a 10-year period (1998–2007). Our study assessed the trends in the epidemiology of the disease, clinical features, case-fatality rates, and risk factors associated with death.

The Study

We analyzed the epidemiologic data of all cases of melioidosis reported by registered medical practitioners and laboratories to the Singapore Ministry of Health during 1998–2007. Clinical and laboratory criteria for notification were based on guidelines disseminated by the Ministry (*5*). Upon notification, trained public health officers carried out epidemiologic investigations by using a standardized form. Investigations included interviews with the patient or family members and a review of hospital records and laboratory results. Data obtained included age, gender, date of onset of the illness, travel history, possible occupational or recreational exposure to contaminated soil or water, concurrent medical conditions, laboratory and microbiologic results, and clinical outcome.

Clinical specimens from patients with melioidosis were sent to clinical laboratories in the admitting hospitals for culture of *B. pseudomallei* (*6*) and, in some cases, for serology. Isolates were tested for antimicrobial sensitivity (*7*). A definitive case of melioidosis was defined as a clinically compatible case in which *B. pseudomallei* was isolated from a clinical specimen. If the diagnosis was based on an indirect hemagglutination test that showed a titer >16 (*8*), the case was considered presumptive. Both definitive and presumptive cases were included in the analysis.

The estimated midyear population used for the calculation of the incidence rate was obtained from the Singapore Department of Statistics. Data on rainfall was obtained from the Meteorological Services Division of the National Environment Agency. Foreigners seeking medical treatment for melioidosis were excluded from the data analysis. Statistical analyses were performed by using SPSS Software version 15.0 (SPSS Inc., Chicago, IL, USA). Biivariate analysis was performed by using χ^2 test for categorical data. A p value ≤0.05 was considered statistically significant.

A total of 693 cases of melioidosis were reported during 1998–2007; of these, 83% were diagnosed by culture and 17% by serologic analysis. We observed a decreasing trend in the annual incidence rate, with the exception of an increase in 2004 (Figure 1). Patients ranged in age from 1 month to 97 years. The highest age-specific incidence rate of melioidosis was for adults >45 years of age. The annual incidence rate for male patients was 2.8–7.2× that for female. An increase in the number of cases in March and April 2004 was preceded by heavy rainfall (Figure 2), strong winds, and flash floods. A total of 23 cases of melioidosis were reported with onset of illness during the 5-week period between March 7 and April 10 (epidemiologic weeks 10–14). The proportion of cases with the pneumonic form of melioidosis during this period was 82.6%, compared to 47.8% for the remainder of 2004. The case-fatality rate was 52.6% and 36.4%, respectively (*9*). Only 4 of the 23 case-patients (17.3%) reported occupations that had exposure to soil, e.g., construction workers or gardeners.

A total of 112 deaths were reported during the 10-year period; overall case-fatality rate was 16.2% (range 8.8%–27.1%). Of the reported cases, 75.5% had co-existing diseases, with diabetes (47.9%), hypertension (26.4%), renal impairment (13.3%), and ischemic heart disease (12.0%)

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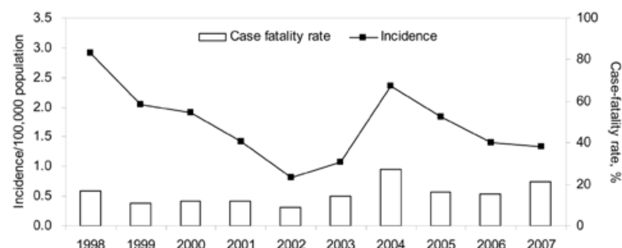

Figure 1. Incidence (per 100,000 population) and case-fatality rate (%) of melioidosis cases, Singapore, 1998–2007.

being the most common. Patients with co-illnesses had a significantly higher case-fatality rate (19.3%) compared to those without (6.5%) (p<0.0005). Approximately half (50.4%) of the melioidosis cases were associated with bacteremia. Patients with bacteremic melioidosis had a significantly higher case-fatality rate (25.8%) than those without bacteremia (5.5%) (p<0.0005). Clinical isolates of *B. pseudomallei* demonstrated antimicrobial sensitivity to imipenem (100.0%), ceftazidime (99.1%), doxycycline (99.0%), amoxicillin/clavulanate (94.2%), and chloramphenicol (96.1%).

Conclusions

Our study showed that male gender, old age, and diabetes mellitus were risk factors for melioidosis. The presence of bacteremia and co-illnesses were risk factors for death in patients with melioidosis, consistent with findings in other endemic countries. The overall case-fatality rate in this study was much lower compared to cases during 1989–1996 (39.5%) (*10*). This may be due to greater awareness among medical practitioners, earlier recognition of the disease, better intensive care, and an appropriate antimicrobial drug regimen.

Unlike patients in Australia or Thailand, most of the case-patients in our study could not recall any occupational or recreational exposure to wet soil. The only reported episodes of percutaneous inoculation in Singapore were a few young adults with localized cutaneous infections and abscesses caused by occupational exposure to soil (*4*). *B. pseudomallei* was isolated from only 1.8% of soil samples and from none of the water samples in Singapore collected during epidemiologic investigations of reported cases (*11*). Tan et al. have suggested that in an urban setting excessive soil excavations could contribute to aerosolization of the bacterium (*12*).

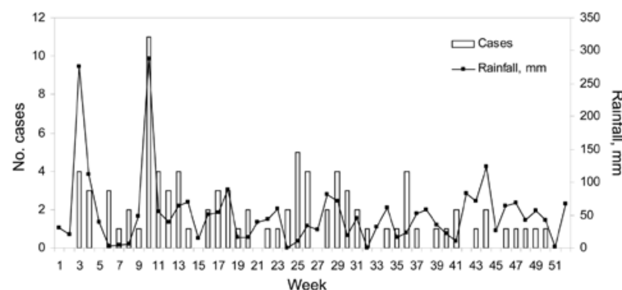

Figure 2. Weekly melioidosis cases by onset date and rainfall totals, Singapore, January 4, 2004–January 1, 2005

Rapid molecular typing of the bacteria during the outbreak in 2004 showed that the isolates were genetically heterogeneous, thus excluding the possibility of a common source (*13*). Moreover, the cases were distributed in different parts of the island without any particular geographic predilection. An epidemiologic investigation of cases in the first half of 2004 demonstrated a relationship between incidence of melioidosis and cumulative rainfall 7 days before onset of illness (*14*). This finding is consistent with other studies that demonstrated an association between incidence of melioidosis and intensity of rainfall. Researchers have posited that heavy rainfall causes movement of the bacteria to the surface with the rising water table (*15*). Severe climatic events may cause aerosolization of the bacteria and increase the risk for inhalation. Infection following inhalation of *B. pseudomallei* may result in more fulminant disease and a higher case-fatality rate (*15*).

Melioidosis is emerging as a serious public health problem in many countries. Although the incidence and case-fatality rate of melioidosis in Singapore has decreased, it has the potential to resurface with adverse climate events such as heavy rainfall and flash floods.

Dr Lo is an assistant director with the Ministry of Health, Singapore. His research interests include epidemiology of infectious diseases, public health policy, and health services.

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Escherichia coli as Reservoir for Macrolide Resistance Genes

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The plasmid-borne *mph*(A) gene that confers resistance to azithromycin and has recently emerged in *Shigella sonnei* is present in multidrug- and non–multidrug-resistant *Escherichia coli* isolates from 4 continents. Further spread of *mph*(A) to *Shigella* and *Salmonella* spp. may be expected.

Macrolides have been regarded for many decades as having good activity and safety for the treatment of infections caused by gram-positive cocci. In general, macrolides show modest potency against *Enterobacteriaceae*. Most *Shigella* and *Salmonella* spp. pathogens display MICs of azithromycin, a macrolide antimicrobial drug, ranging from 2 mg/L to 8 mg/L (*1*). Despite these relatively high MICs, azithromycin is an attractive option for several reasons. It can be given once a day and attains high intracellular concentrations and sufficient concentrations in the colon of patients to inhibit *Shigella* and *Salmonella* spp. Azithromycin is recommended by the American Academy of Pediatrics for treatment of shigellosis in children (*2*) and by the World Health Organization as a second-line treatment for adults (*3*). It has also been proposed for short-course treatment of typhoid fever (*4*).

We recently reported an outbreak of shigellosis in Paris, France; failure of azithromycin treatment was related to emergence of plasmid-mediated resistance to macrolides (*5*). Resistance was related to the expression of a macrolide 2′-phosphotransferase encoded by the *mph*(A) gene. Because shigellosis remains a common gastrointestinal disease in both developing and industrialized countries, emergence of macrolide resistance may have major public health consequences.

Since the early reports by Ochiai (*6*) and Akiba (*7*) at the end of the 1950s, plasmid-mediated transfer of resistance genes between *Escherichia coli* and *Shigella* spp. has been reported in several instances (*8*). Therefore, we hypothesized that *E. coli* might constitute a major reservoir for macrolide resistance genes that could be subsequently transferred to *Shigella sonnei*.

Acquired resistance to macrolides may result from a variety of mechanisms of resistance, several of which have already been reported in *Enterobacteriaceae* (*9*,*10*). These mechanisms include target site modification by methylases encoded by *erm* genes, in particular *erm*(A), *erm*(B), and *erm*(C). Macrolides may be inactivated by modifying enzymes first reported in *Enterobacteriaceae* (*11*,*12*), e.g., esterases encoded by *ere*(A) or *ere*(B) genes or phosphotransferases encoded by *mph*(A), *mph*(B), and *mph*(D) genes. The third mechanism is acquisition of efflux pumps, *mef*(A) and *msr*(A), that have been found essentially in gram-positive organisms, although *mef*(A) has been identified in gram-negative organisms (*10*). All of these genes confer full cross-resistance between erythromycin and azithromycin (*9*). We aimed to assess the prevalence of acquired resistance to macrolides in commensal and clinical isolates of *E. coli* from various geographic origins and to characterize the mechanisms underlying *E. coli* resistance to macrolides.

The Study

A total of 190 *E. coli* isolates were collected from 5 countries on 4 continents. Some of these isolates were obtained from populations exposed to low antimicrobial selective pressure; 45 commensal isolates were from feces of healthy Wayampi Amerindians in French Guiana, 20 from feces of children living in a remote village of Senegal, and 49 from feces of healthy nurses working in a hospital in Paris. Other isolates were obtained from populations having received multiple antimicrobial drug treatments; 29 isolates were from feces of children from Niger hosted in a center for nutritional rehabilitation, and 47 isolates were producers of extended-spectrum β-lactamase (ESBL) obtained from various clinical samples in hospitalized patients in Vietnam (n = 37) and France (Hospital of Caen) (n = 10).

Susceptibility to 16 antimicrobial drugs was determined by the disk-diffusion method. MICs of erythromycin were determined by the agar dilution technique, and ESBLs were detected by the double-disk synergy test, as recommended by the French Society for Microbiology (www.sfm.asso.fr).

E. coli isolates from French Guiana, Senegal, and Paris were susceptible to quinolones, gentamicin, and third-generation cephalosporins. Resistance to amoxicillin-ticarcillin (by penicillinase production) was detected for 22.2%, 20.4%, and 40.0% of the isolates obtained from nurses in Guiana, Paris, and Senegal, respectively. Coresistance to amoxicillin and cotrimoxazole was found for 13%, 14%, and 35% of isolates, respectively.

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Table 1. MICs of erythromycin and distribution of macrolide resistance genes among 190 *Escherichia coli* isolates from 5 countries*

E. coli origin (no. isolates)	MIC of erythromycin, mg/L			Gene, no. (%)†		
	Range	MIC_{50}	MIC_{90}	*erm*(B)	*mph*(A)	*mph*(B)
French Guiana, Amerindians (45)	32–1,024	64	128	0	1 (2)	0
Senegal, remote village (20)	64–128	128	128	0	0	0
Niger, children (29)	64–>1,024	256	>1,024	0	9 (31)	0
France, healthy nurses (49)	16–256	64	128	0	2 (4)	1 (2)
France, ESBL isolates (10)	64–1,024	128	1,024	0	3 (30)	1 (10)
Vietnam, ESBL isolates (37)	32–>1,024	512	>1,024	5 (13.5)	19 (51)	0
France, hospital isolates resistant to ampicillin and cotrimoxazole (100)	32–>1,024	64	>1,024	1 (1)	13 (13)	0

*ESBL, extended-spectrum β-lactamase; MIC_{50}, MIC at which 50% of isolates are inhibited; MIC_{90}, MIC at which 90% of isolates are inhibited.
†No isolate contained the *erm*(A), *erm*(C), *ere*(A), *ere*(B), *msr*(A), or *mef*(A) genes.

Multidrug-resistant isolates were commonly obtained from Niger natives; 34.4% were resistant to both cefotaxime (mostly by ESBL production) and ciprofloxacin, and 58.6% to gentamicin. ESBL producers from Vietnam and Caen hospital displayed resistance to ciprofloxacin for 86.5% and 60.0% and resistance to gentamicin for 86.4% and 50.0% of isolates, respectively. MICs of erythromycin ranged from 16 mg/L to >1,024 mg/L (Table 1; Figure). Distribution of MICs of erythromycin was bimodal; mode = 64 mg/L for 1 population with low MICs and ≥1,024 mg/L for the other population with high MICs (Figure).

MICs differed according to the origin of the isolates. Multiple resistance was associated with MICs of erythromycin >256 mg/L with 1 exception: an isolate from Guiana was resistant only to amoxicillin and cotrimoxazole (MIC of erythromycin, 1,024 mg/L).

We screened for macrolide resistance genes by using oligonucleotide primers and PCR conditions (Table 2). PCR reactions were performed as follows: an initial denaturation step (95°C, 3 min) followed by 30 cycles consisting of denaturation (95°C, 30 s), annealing at a temperature depending on the primers used (30 s), elongation (72°C, 30 s) and a final extension step (72°C, 10 min). Positive and negative controls were included in each run.

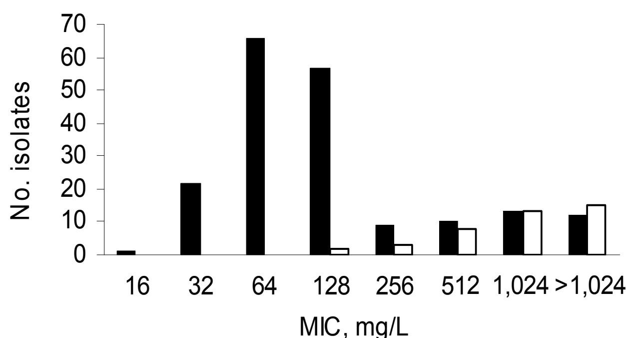

Figure. Distribution of MICs of erythromycin for *Escherichia coli* isolates according to the presence of genes resistant to macrolides. MIC distribution is shown for all strains (black bars). Solid white bars indicate strains containing a macrolide resistance gene: *erm*(B), *mph*(A), or *mph*(B). Some isolates may contain 2 genes resistant to macrolides.

The *mph*(A) gene was commonly present in 34 isolates (MICs 256 mg/L to >1,024 mg/L). The gene was mostly detected in isolates resistant to cefotaxime (27 isolates) but also in 4 (21%) of 19 isolates resistant to only amoxicillin and cotrimoxazole in different countries. To confirm this latter association, we searched for the *mph*(A) gene in 100 clinical isolates of *E. coli* from the Caen hospital coresistant to amoxicillin and cotrimoxazole but susceptible to cefotaxime, which is a common phenotype present in ≈25% of *E. coli* isolates from this hospital. The gene was detected in 13 isolates (MIC >256 mg/L), confirming the presence of the gene in non–multidrug-resistant *E. coli* (Table 1). In a previous study on the distribution of 7 macrolide resistance genes in gram-negative isolates from the urine and oral cavity of healthy children in Portugal, Ojo et al. detected the *mph*(A) gene in 15 of 26 studied *E. coli* isolates (*10*). However, the profile of resistance to other antimicrobial drugs was not determined.

The other macrolide resistance genes were more scarce. The *erm*(B) gene was detected in 2 isolates (MICs >1,024 mg/L) and *mph*(B) in 2 others (MICs 128 mg/L). In 4 isolates (MICs >1,024 mg/L), both *mph*(A) and *erm*(B) were amplified. The 6 other genes, *erm*(A), *erm*(C), *ere*(A), *ere*(B), *mef*(A), and *msr*(A), were not detected. In 6 isolates with MICs of erythromycin equal to 256 mg/L and 2 with MICs of erythromycin equal to 512 mg/L, no resistance gene could be amplified, suggesting the presence of other macrolide resistance determinants. Distribution of the resistance genes *mph*(A), *erm*(B), and *mph*(B) is shown in Table 1 and in the Figure.

Conclusions

The plasmid-borne *mph*(A) gene detected in *S. sonnei* resistant to azithromycin was the most common macrolide resistance gene detected in *E. coli* collected in 5 countries on 4 continents. The gene was mostly detected in isolates from patients who had received antimicrobial drugs or had been hospitalized, in particular in ESBL producers, but was also detected in *E. coli* isolates coresistant to amoxicillin and cotrimoxazole, which are common worldwide. Because *E. coli* and *Shigella* spp. are phylogenetically closely

Table 2. Oligonucleotide primers used for detection of *Escherichia coli* macrolide resistance genes

Target gene	Primer	Sequence, 5′ → 3′	Product size, bp	Annealing temperature, °C
mph(A)	mphAF	GTGAGGAGGAGCTTCGCGAG	403	60
	mphAR	TGCCGCAGGACTCGGAGGTC		
mph(B)	mphBF	GATATTAAACAAGTAATCAGAATAG	494	58
	mphBR	GCTCTTACTGCATCCATACG		
erm(A)	ermAF	TCTAAAAAGCATGTAAAAGAAA	533	52
	ermAR	CGATACTTTTTGTAGTCCTTC		
erm(B)	ermBF	GAAAAAGTACTCAACCAAATA	639	45
	ermBR	AATTTAAGTACCGTTACT		
erm(C)	ermCF	TCAAAACATAATATAGATAAA	642	45
	ermCR	GCTAATATTGTTTAAATCGTCAAT		
ere(A)	ereAF	GCCGGTGCTCATGAACTTGAG	420	60
	ereAR	CGACTCTATTCGATCAGAGGC		
ere(B)	ereBF	TTGGAGATACCCAGATTGTAG	537	55
	ereBR	GAGCCATAGCTTCAACGC		
mef(A)	mefAF	AGTATCATTAATCACTAGTGC	345	54
	mefAR	TTCTTCTGGTACTAAAAGTGG		
msr(A)	msrAF	GCACTTATTGGGGGTAATGG	384	58
	msrAR	GTCTATAAGTGCTCTATCGTG		

related species that easily exchange plasmids, further dissemination of resistance to macrolides in the latter species may be predicted. Also, plasmid-mediated resistance to macrolides may emerge in *Salmonella* spp., which is also a target of azithromycin.

Ms Nguyen is pursuing a master's degree at the University of Caen. The focus of her work is the identification of reservoirs of macrolide resistance genes.

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West Nile Virus Antibodies in Wild Birds, Morocco, 2008

Jordi Figuerola, Riad E. Baouab, Ramon Soriguer, Ouafaa Fassi-Fihri, Francisco Llorente, and Miguel Angel Jímenez-Clavero

To determine circulation of West Nile virus (WNV) during nonepidemic times, we serosurveyed wild birds of Morocco in 2008. We found antibodies against WNV in 12 (3.5%) birds, against Usutu virus in 1 (0.3%), and against both in 2 (0.6%). High WNV prevalence among juvenile birds suggests local virus circulation among resident birds.

In the Mediterranean basin, West Nile virus (WNV) causes sporadic disease outbreaks, which usually affect a low number of humans and animals, after which long periods without virus circulation occur. This pattern has occurred in France (outbreaks in 2000, 2003, 2004, and 2006), Italy (1998 and 2008), Algeria (1994), Tunisia (1997 and 2003), Morocco (1996 and 2003), Romania (1996–2000), and Israel (1998–2000) (*1*; www.oie.int/wahis/public.php?page=home). This finding has led to the hypothesis that the virus is absent from Europe and North Africa and periodically seeded into different places by infected migratory birds. An alternative hypothesis is that the virus can remain silent, circulating in a sylvatic enzootic bird–mosquito cycle and only under appropriate conditions causing new outbreaks in humans and horses (*2*). To test these 2 hypotheses, research under nonepidemic conditions is needed.

During the summer of 1996, WNV outbreaks caused the death of 42 horses and 1 human (*3*); during 2003, a total of 5 horses died from WNV infection (*4*). To determine circulation of the virus during a nonepidemic year, we conducted a serosurvey of wild birds in Morocco in 2008.

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The Study

From June through July 2008, we captured wild birds during 2 periods of 6 days each in Sidi Allal Tazi, Sidi Kacem Province (34°31′8″N, 6°14′48″W), ≈40 km northeast of Kenitra. The area is dominated by rice fields flooded from regulated channels from the Sebou River. Each captured bird was marked with a numbered metal ring; when possible, age was determined according to plumage characteristics. Blood was taken from the jugular vein and allowed to clot at ambient temperature. The blood was then centrifuged (10 min at 6,000 rpm), and the serum was stored in liquid nitrogen and transported to a deep freezer (–80°C) in the laboratory.

Neutralizing antibody titers against WNV (strain Spain/2007 GE-1B/b) and Usutu virus (USUV) (SAAR1776) were determined by using a micro virus-neutralization test as described previously (*5*). We used USUV as a control for WNV antibody specificity. Serum samples were inactivated at 56°C for 30 min before analysis. Dilutions of test serum (25 μL) were incubated with one hundred 50% tissue culture infective doses of the virus in the same volume (25 μL) for 1 h at 37°C in modified Eagle medium (*5*), after which 50 μL of a suspension (2×10^4 cells/mL) of Vero cells plus fetal calf serum was added to the same medium to reach a final concentration of 5%. The mixture was further incubated for 6–7 days at 37°C until cytopathic effects were observed in control wells containing ten 50% tissue culture infective doses of virus. Samples were titrated by analyzing serial serum dilutions from 1:10 to 1:640. Only samples that showed neutralization (absence of cytopathic effect) at dilutions 1:≥20 were considered positive. Samples that showed neutralization at the same dilutions were scored as positive for flavivirus but not conclusive for WNV and USUV. Controls for cytotoxicity in the absence of virus were included for every sample at a dilution of 1:10. Cytotoxicity prevented determination of neutralizing antibodies in 1 sample, which was therefore excluded from the analysis. A bird was considered to have seroconverted if it was seronegative at the time of first capture and seropositive (titer increase by at least 4-fold) at the time of recapture (*6*).

We analyzed 360 samples from 346 birds (Table). Neutralizing antibodies against WNV were found in 12 (3.5%) newly captured birds, against USUV in 1 (0.3%), and against both in 2 (0.6%). Positive results were obtained for 3 species. The highest prevalence was found among blackbirds (*Turdus merula*); neutralizing antibodies against WNV were found in 6 (19.3%) blackbirds, against flavivirus in 2 (6.5%), and against USUV in 1 (3.5%). Prevalence of WNV neutralizing antibodies among house sparrows (*Passer domesticus*) was much lower (2.2%). Additionally, 1 Cetti's warbler (*Cettia cetti*) was negative for WNV neutralizing antibodies in June but had seroconverted by the

Table. Number of wild birds with antibody titers against West Nile virus, by species, Morocco, 2008*

Species	Titer, no. birds (no. juveniles)						
	0	20	40	80	160	320	640
Acrocephalus scirpaceus	1 (1)	0	0	0	0	0	0
Alcedo atthis	4 (1)	0	0	0	0	0	0
Asio otus	1 (1)	0	0	0	0	0	0
Carduelis carduelis	9 (3)	0	0	0	0	0	0
Carduelis chloris	12 (2)	0	0	0	0	0	0
Cettia cetti	19 (4)	0	0	0	0	0	0
Hippolais pallida	49 (17)	0	0	0	0	0	0
Hippolais polyglotta	5 (2)	0	0	0	0	0	0
Luscinia megarhynchos	4 (2)	0	0	0	0	0	0
Oriolus oriolus	1	0	0	0	0	0	0
Passer domesticus	175 (109)	1	1	2 (1)	0	0	0
Pycnonotus barbatus	3	0	0	0	0	0	0
Serinus serinus	9 (3)	0	0	0	0	0	0
Sturnus vulgaris	1 (1)	0	0	0	0	0	0
Sylvia melanocephala	18 (7)	0	0	0	0	0	0
Turdus merula	23 (18)	4 (1)	1	1	0	0	2

*Captured in June (n = 197) and July (n = 149 + 14 recaptured birds first sampled in June); only data for first capture are shown.

time of recapture in July. Of the 13 additional birds sampled twice, 10 were negative for antibodies in both samples and 3 were positive in both samples. Prevalence of antibodies was significantly higher among adult than among juvenile (<1 year of age) blackbirds ($\chi^2 = 8.22$, 1 df, $p = 0.004$) but not among house sparrows ($\chi^2 = 0.99$, 1 df, $p = 0.32$).

Conclusions

Antibodies in juvenile birds provide evidence of circulation of WNV and USUV in 2008 in the study area (maximum titers from neutralization tests were 80 for WNV and 160 for USUV). Because antibodies are maternally transmitted for only 2–4 weeks after birth (*7*,*8*), these high antibody titers suggest direct exposure to the virus during the summer of 2008. In addition, high titers (640) in 2 adult birds suggest recent circulation of WNV in the area. Blackbirds and house sparrows have short life spans; annual survival is estimated to be <50% (*9*). Consequently, antibodies in 58.3% of adult blackbirds cannot be explained by the exposure of these individual birds to WNV in 2003 (of 100 alive in 2003, <3 would be expected to be alive in 2008). All birds with neutralizing antibodies were members of nonmigratory (resident) species. These results suggest that WNV was present in Morocco in 2008 without resulting in disease outbreaks among humans or horses, as opposed to in 1996 and 2003, when cases did occur. The high prevalence of antibodies among adult birds also suggests that substantial circulation of the virus may have occurred during the previous season or seasons.

Although in recent years USUV circulation has been restricted to western Europe, especially in blackbirds (*10*,*11*), we did find 1 USUV-seropositive bird in Morocco. A strain of USUV was isolated from mosquitoes in Spain in 2006 (*12*), and serologic evidence of its circulation in Italy in 2007 has been found (*13*), indicating that the virus may have been circulating undetected in southern Europe. Our results suggest similar circulation in Morocco.

As evidence for silent circulation of WNV and USUV in the Mediterranean area (e.g., Spain, Czech Republic, or Italy) accumulates, the need to understand the ecologic factors related to virus circulation and the conditions leading to disease outbreaks increases (*5*,*11*,*14*). Factors may include strain characteristics, environmental conditions favoring virus amplification, or ecologic conditions favoring spillover to humans or horses, e.g., changes in mosquito feeding behavior increasing virus transmission from birds to humans (*15*). Long-term active monitoring programs that facilitate the understanding of virus circulation under nonepidemic conditions are needed.

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Novel Rickettsia in Ticks, Tasmania, Australia

Leonard Izzard, Stephen Graves, Erika Cox, Stan Fenwick, Nathan Unsworth, and John Stenos

A novel rickettsia was detected in *Ixodes tasmani* ticks collected from Tasmanian devils. A total of 55% were positive for the citrate synthase gene by quantitative PCR. According to current criteria for rickettsia speciation, this new rickettsia qualifies as *Candidatus* Rickettsia tasmanensis, named after the location of its detection.

In Australia, 4 rickettsial species are known to cause disease in humans; none of these species has been identified in Tasmania. However, 3 cases of human rickettsial infections in Tasmania have been documented (*1–3*). *Ixodes tasmani* ticks are of particular interest because they are known to be vectors for other rickettsial species in Australia (*4*) and are also the most common tick species in Tasmania (*5*). In addition, because these ticks bite humans, they are candidates for rickettsial transmission in Tasmania.

Although *Candidatus* Rickettsia tasmanensis, a proposed new species of rickettsiae, has not been associated with human disease, the possible virulence of this rickettsia cannot be disregarded. Some initially identified rickettsiae were later found to cause disease in humans. For example, *R. parkeri* was discovered in 1939 (*6*) but was only confirmed as a human pathogen in 2004 (*7*). To investigate infections in Tasmania, we collected ticks from Tasmanian devils (*Sacrophilus harrissi*) and analyzed them for rickettsial species.

The Study

Forty-four *I. tasmani* ticks were collected from Tasmanian devils from various sites in Tasmania during 2005–2006; 36 were engorged females, 5 were nymphs, and 3 were males. Each tick was washed in 70% ethanol, rinsed in sterile phosphate-buffered saline, and homogenized. Homogenates were then subjected to DNA extraction by using a QIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). The presence of a rickettsial agent was detected by real-time PCR (*8*). Characterization of novel rickettsial species was achieved by comparing sequences of genes as described (*9*).

Amplification and sequencing of 1,096-, 3,005-, 588-, and 4,918-bp products for the citrate synthase (*gltA*), surface cell antigen (*sca4*), outer membrane protein A (*ompA*), and *ompB* genes, respectively, were conducted by using primers previously described (*9*). The 16S rRNA (*rrs*) gene was not amplified because cell culture isolation was not performed. Amplicons were cloned by using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and extracted by using a QuickLyse Mini Prep Kit (QIAGEN).

Big Dye sequencing was performed by using a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA, USA). Resulting products were analyzed at the Australian Genomic Research Facility by using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems).

Sequences were assembled and edited by using the SeqMan Pro program (DNASTAR, Inc., Madison, WI, USA) and evaluated by using neighbor-joining and maximum-parsimony methods in MEGA 4 (*10*) and the maximum-likelihood method in PHYLIP (*11*). Results were confirmed by using BLAST analysis software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences have been deposited in GenBank (Table).

Rickettsial DNA was detected in 24 (55%) of 44 *I. tasmani* ticks by using a *gltA*-specific quantitative PCR (qPCR) assay. Because most ticks were engorged females, no statistical correlation was found between the sex of ticks and presence of rickettsiae. Distribution of the ticks collected and degree of positivity are shown in Figure 1.

Sequences from *I. tasmani* ticks were compared with reported sequences (*12*). Results showed that the closest phylogenetic relative for 3 of the genes was *R. raoultii* strain Khabarovsk, with sequence similarities of 99.1% (1,086 bp/1,096 bp), 96.9% (570 bp/588 bp), and 97.7% (4,782 bp/4,895 bp) for the *gltA*, *ompA*, and *ompB* genes, respectively, and 98.1% (2,930 bp/2,988 bp) to *R. japonica* strain YM for the *sca4* gene.

Comparison of our sequences with that of a partially sequenced rickettsia (*R. tasmanensis* strain T120) previously detected in an *I. tasmani* tick removed from a child near Underwood, Tasmania (N. Unsworth, unpub. data) found homology levels to be within the species threshold. No data on the clinical state of the child were obtained.

Sequences closely matched genes of a second partially sequenced rickettsia (*Rickettsia* sp. 518) from an *I. tasmani* tick removed from a Tasmanian devil in Tasmania by researchers at Macquarie University (Sydney, New South Wales, Australia) (*13*). Of the 3 partial gene sequences reported, *ompB* and *gltA* gene sequences matched to the spe-

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Table. GenBank accession numbers of additional rickettsia sequences used in this study*

Strain	*rrs*	*gltA*	*ompA*	*ompB*	*sca4*
Rickettsia sp. 518	–	EU430246	EU430247	EU430242	–
Candidatus R. tasmanensis T120	–	GQ223395	–	GQ223396	GQ223397
Candidatus R. tasmanensis T152-E	–	GQ223391	GQ223392	GQ223393	GQ223394

**rrs*, 16S rRNA; *gltA*, citrate synthase; *omp*, outer membrane protein; *sca*, surface cell antigen.

cies level with *Candidatus* R. tasmanensis; however, *ompA* gene sequences did not. Their isolate could be another new species, although it is difficult to draw conclusions with sequences of small fragments.

Results of sequence analysis of the *ompB* gene by using the neighbor-joining algorithm are shown in Figure 2. Although all selected genes were analyzed, the *ompB* gene tree had the strongest bootstrap values and the largest compared fragment size.

Conclusions

All 44 ticks were collected from northeastern and eastern Tasmania. The number of positive samples (55%) contrasts with the small number of reported SFG rickettsial human infections in Tasmania because *I. tasmani*, which is known to opportunistically bite humans (*5*), has a high density in Tasmania. Clinical cases of infection may be missed because physicians are not aware of human rickettsial disease in Tasmania.

A recent study showed a high level of exposure to SFG rickettsia in cats and dogs near the city of Launceston, Tasmania (L. Izzard, unpub. data); in the Ravenswood area 10 of 16 tick samples were positive for SFG rickettsia by qPCR. However, the species of SFG rickettsiae could not be determined in this study because only serologic analysis was conducted. Because *I. tasmani* ticks are common in Tasmania and parasitize cats and dogs, *Candidatus* R. tasmanensis is likely to be the causative agent in some of the cases.

When gene sequences of *Candidatus* R. tasmanensis were compared with those of validated species (*12*), they did not closely match either of the 2 validated SFG rickettsia in Australia (*R. australis* or *R. honei*). Similarly, *Candidatus* R. tasmanensis sequences were divergent from 2 *Candidatus* species (*Candidatus* R. gravesii and *Candidatus* R. antechini) in Australia, which are currently being characterized. *Candidatus* R. tasmanensis had the highest phylogenetic similarity to *R. raoultii* strain Khabarovsk for 3 of 4 gene sequences. This rickettsial species was isolated in the Russian Far East (>10,000 km north of Tasmania) from a *Dermacentor silvarum* tick and is a known human pathogen (*12*). However, the similarities between the gene sequences of these 2 organisms were well below the threshold defined by Fournier et al. (*9*). On the basis of these results, we propose to give this *Rickettsia* sp. a *Candidatus* status and formally name it *Candidatus* R. tasmanensis after the location from which it was originally isolated. To validate *Candidatus* R. tasmanensis as a novel species, isolation and subsequent sequencing of its *rrs* gene are needed. Multigene sequencing of 4 other qPCR-positive *I. tasmani* ticks will also be useful.

Because the range of this study was limited to eastern Tasmania, *I. tasmani* ticks from western Tasmania and other parts of Australia should also be examined for this rickettsial agent. This analysis would help determine its true range. Testing the blood of animals infested with *I. tasmani* ticks for evidence of SFG rickettsial exposure may also provide data on the pathogenesis and range of this rickettsia.

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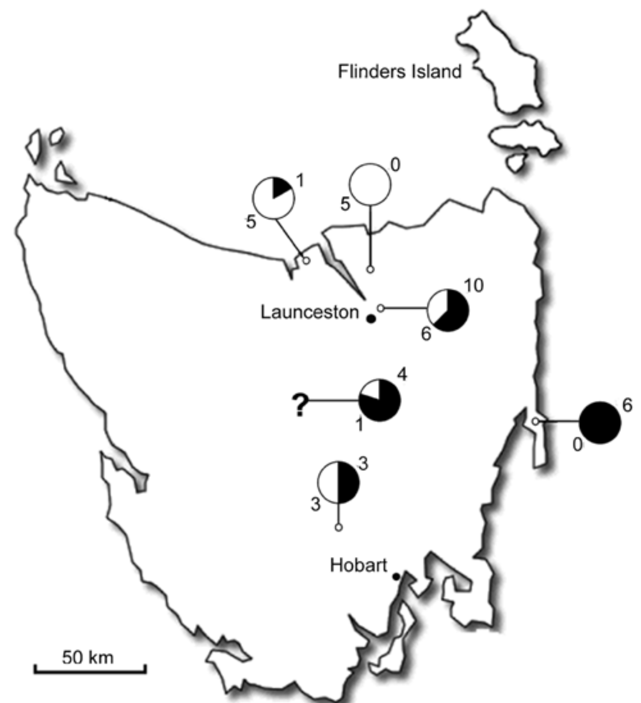

Figure 1. Map of Tasmania, Australia, showing number of positive (black) and negative (white) ticks and their locations. The question mark indicates unknown locations. A total of 55% of the ticks were positive for a spotted fever group rickettsia.

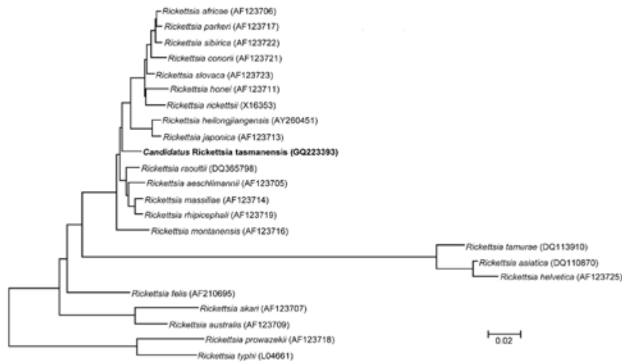

Figure 2. Phylogenetic tree showing the relationship of a 4,834-bp fragment of the outer membrane protein B gene of *Candidatus* Rickettsia tasmanensis (in **boldface**) among all validated rickettsia species. The tree was prepared by using the neighbor-joining algorithm within the MEGA 4 software (*10*). Bootstrap values are indicated at each node. Scale bar indicates 2% nucleotide divergence.

of novel rickettsial species and molecular diagnostics of rickettsial pathogens in Australia.

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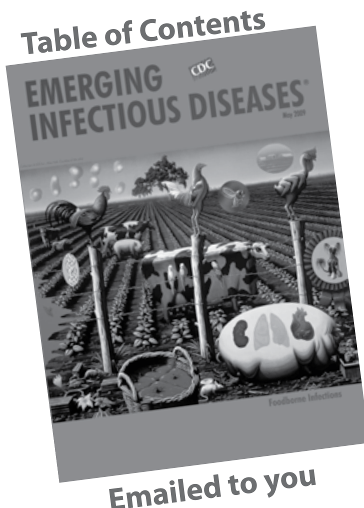

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Orangutans Not Infected with *Plasmodium vivax* or *P. cynomolgi*, Indonesia

Balbir Singh and Paul Cliff Simon Divis

After orangutans in Indonesia were reported as infected with *Plasmodium cynomolgi* and *P. vivax*, we conducted phylogenetic analyses of small subunit ribosomal RNA gene sequences of *Plasmodium* spp. We found that these orangutans are not hosts of *P. cynomolgi* and *P. vivax*. Analysis of ≥1 genes is needed to identify *Plasmodium* spp. infecting orangutans.

Parasites belonging to the genus *Plasmodium* cause malaria and are usually host specific. For example, humans are natural hosts for *P. falciparum, P. vivax, P. malaria,* and *P. ovale*, and orangutans are naturally infected with *P. pitheci* and *P. silvaticum* (*1*,*2*). However, simian malaria parasites can infect humans (*1*); for example, *P. knowlesi*, normally associated with infections in long-tailed and pig-tailed macaques, has recently been found to have caused malaria in humans in several countries in Southeast Asia (*3–8*). This finding raises the possibility that other zoonotic malaria parasites may emerge in humans.

Malaria parasites have distinct small subunit ribosomal RNA (SSU rRNA) genes that are developmentally regulated (*9*). Each *Plasmodium* species has at least 2 types of SSU rRNA genes, and the stage-specific expression of these genes varies among species. In general, the A-type genes are transcribed or expressed mainly during the asexual stages, and the S-type genes are transcribed mainly during the sporozoite stage. *P. vivax* also has O-type genes, which are expressed during ookinete and oocyst development. Phylogenetic analysis of the *P. vivax* and *P. cynomolgi* SSU rRNA genes has indicated that the genes appear to have evolved as a result of 2 gene duplication events (*10*). A more recent study, involving SSU rRNA sequence data from a much larger number of *Plasmodium* spp., demonstrated that gene duplication events giving rise to the A-type and S-type sequences took place independently at least 3 times during the evolution of *Plasmodium* spp. (*11*).

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Reid et al. (*12*) analyzed the DNA sequences of SSU rRNA genes of *Plasmodium* spp. from blood of orangutans in Kalimantan, Indonesia. Using phylogenetic analysis, they concluded that, in addition to *P. pitheci* and *P. silvaticum*, the orangutans were infected with the human malaria parasite *P. vivax* and the macaque malaria parasite *P. cynomolgi*. Their report implies that human and macaque malaria parasites could be transmitted to orangutans and that orangutans could act as reservoir hosts for at least 1 of the human malaria parasites.

When taxonomic inferences of species within a genus are made from phylogenetic trees, trees must be reconstructed by using orthologous genes and must include as many species sequences as possible. However, Reid et al. used sequence data of only the S-type SSU rRNA genes for *P. vivax, P. cynomolgi,* and *P. knowlesi* and data of only the A-type genes for *P. inui* and *P. fragile*. Furthermore, they analyzed sequence data from only 4 simian malaria parasites. Nishimoto et al. recently included data from 10 simian malaria parasites (*11*). We therefore reanalyzed the SSU rRNA sequence data of malaria parasites of orangutans together with the A-type, S-type, and O-type SSUrRNA gene sequence data for various *Plasmodium* spp.

The Study

We used the neighbor-joining method, as described previously, to reconstruct the phylogenetic tree (*3*). Our phylogenetic analyses showed that SSU rRNA gene sequences VM88, VM82, and VM40 from orangutans (*12*) represent A-type SSU rRNA genes and that the VS63 sequence represents an S-type gene of *Plasmodium* spp. (Figure). No morphologic features of the malaria parasite stages in the blood were described for the Kalimantan orangutans by Reid et al. (*12*). Therefore, on the basis of SSU rRNA sequence data available for VM82 and VM88, whether these represent *P. pitheci* or *P. silvaticum*, previously described malaria parasites of orangutans, or some other species of *Plasmodium* cannot be determined with certainty.

The VS63 sequence is clearly not *P. vivax,* as previously reported by Reid et al. (*12*); it represents a *Plasmodium* sp. that is closely related to *P. inui*. It is most probably the S-type gene for either VM82 or VM88, which are A-type genes of *P. pitheci* and/or *P. silvaticum*. Furthermore, the VM40 sequence from orangutans represents a *Plasmodium* sp. closely related to the gibbon malaria parasite, *P. hylobati (1)*, and is not the macaque malaria parasite, *P. cynomolgi,* as previously reported (*12*).

Conclusions

Phylogenetic analyses of the SSU rRNA genes indicate that none of the *Plasmodium* spp. isolated from orangutans in Kalimantan, Indonesia, are *P. cynomolgi* or *P. vivax*, as previously reported by Reid et al. (*12*). Before any

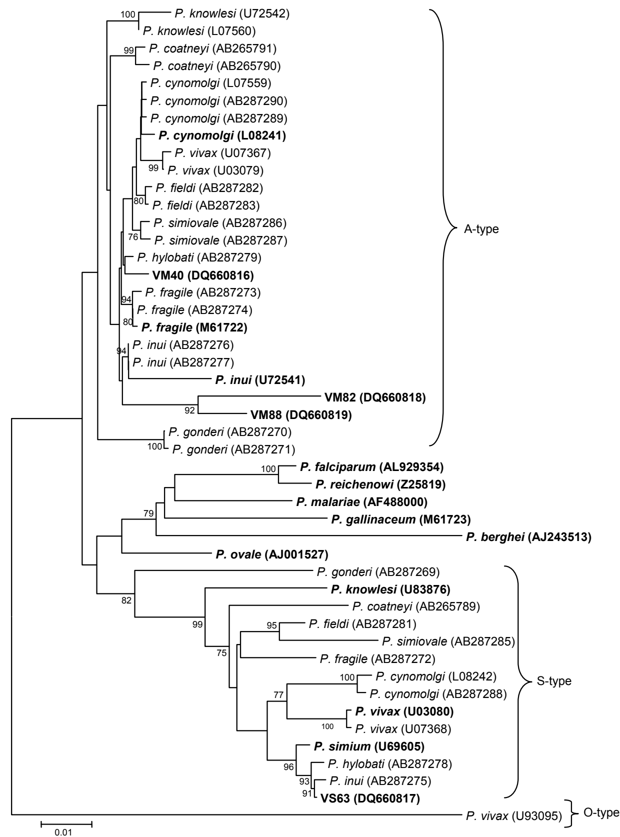

Figure. Phylogenetic relationship of *Plasmodium* spp. inferred from small subunit ribosomal RNA sequences. Tree was reconstructed by using the neighbor-joining method. **Boldface** indicates those sequences derived from orangutans (VM40, VM82, VM88, and VS63) and those used by Reid et al. (*12*) in their phylogenetic analysis. Numerals on the branches are bootstrap percentages based on 1,000 replicates; only those >70% are shown. GenBank accession numbers are in brackets. Scale bar indicates nucleotide substitutions per site.

conclusion about the identity of the malaria parasites infecting orangutans and their corresponding SSU rRNA gene sequences can be derived, a second or third gene of malaria parasites from these orangutans needs to be analyzed and the morphology of the corresponding blood stages needs to be described. Our study underscores the importance of using orthologous genes and sequence data from as many species as possible when inferring species within a genus from phylogenetic trees.

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Acute Q Fever and Scrub Typhus, Southern Taiwan

Chung-Hsu Lai, Yen-Hsu Chen, Jiun-Nong Lin, Lin-Li Chang, Wei-Fang Chen, and Hsi-Hsun Lin

Acute Q fever and scrub typhus are zoonoses endemic to southern Taiwan. Among the 137 patients with acute Q fever (89, 65.0%) or scrub typhus (43, 31.4%), we identified 5 patients (3.6%) who were co-infected with *Coxiella burnetii* and *Orientia tsutsugamushi*.

Q fever is a worldwide zoonosis in humans caused by *Coxiella burnetii* infection. Ticks are the main arthropod vectors of *C. burnetii*; the major animal reservoirs include goats, sheep, cattle, and domestic cats. Humans are infected mainly by inhaling organism-contaminated aerosols (*1*). Scrub typhus, caused by *Orientia tsutsugamushi* infection, is endemic to eastern Asia and the western Pacific region. *O. tsutsugamushi* is transmitted vertically in mites (particularly *Leptotrombidium* species) by the transovarial route, and horizontally in rodents through trombiculid larval (chigger) bites. Humans contract scrub typhus by being bitten by chiggers infected with *O. tsutsugamushi;* such bites occur accidentally during agriculture or field recreational activities (*2*).

Although the major arthropod vectors, animal reservoirs, and routes of transmission to humans are different for *C. burnetii* and *O. tsutsugamushi*, co-infection may occur when humans have been exposed to an environment where arthropod vectors and animal reservoirs are prevalent. In southern Taiwan, acute Q fever and scrub typhus are endemic zoonoses (*3–5*), and co-infection with the 2 pathogens may occur. We report 5 cases of co-infection with the agents of acute Q fever and scrub typhus.

The Study

This study was conducted at E-Da Hospital, a teaching hospital located in Kaohsiung County in southern Taiwan, and approved by its Institute Ethics Committee (E-MRP-096-065) in 2006. Rickettsial diseases are notifiable diseases in Taiwan, and suspected cases with appropriate clinical characteristics are reported to the Center for Disease Control, Taipei, Taiwan for confirmation. Because acute Q fever, scrub typhus, and murine typhus (caused by flea-borne *Rickettsia typhi*) are the most common rickettsial diseases in Taiwan (*3–5*), we requested tests for the etiologic agents of the 3 diseases simultaneously in patients who sought treatment, regardless of which disease we suspected on the basis of clinical features.

Serologic assessments for specific antibodies to *C. burnetii* and *O. tsutsugamushi* were performed by using indirect immunofluorescence antibody assay as previously described (*5*). Acute Q fever was diagnosed by either an antiphase II antigen immunoglobulin (Ig) G titer $\geq$320 and antiphase II antigen IgM titer $\geq$80 in a single serum sample, or a $\geq$4-fold rise of antiphase II antigen IgG titer in paired serum samples. Antigens of 3 major strains of *O. tsutsugamushi* (Karp, Kato, and Gilliam strains) were used to diagnose scrub typhus: either an IgM titer $\geq$80 or a $\geq$4-fold rise in IgG titer in paired serum samples for Karp, Kato, and Gilliam strains of *O. tsutsugamushi*. Murine typhus was diagnosed by an IgM titer $\geq$80 or a $\geq$4-fold rise in IgG titer against *R. typhi* in paired sera. Co-infection with *C. burnetii* and *O. tsutsugamushi* was diagnosed if the serologic results fulfilled the diagnostic criteria for both infections.

From April 15, 2004, through June 30, 2008, we identified 12 cases of murine typhus, 89 cases of acute Q fever, and 43 cases of scrub typhus; 5 persons had both acute Q fever and scrub typhus. All 5 patients with co-infections denied having fever within 3 months before admission. The demographic data and clinical manifestations of the 5 case-patients co-infected with *C. burnetii* and *O. tsutsugamushi* are listed in the online Appendix Table (available from www.cdc.gov/EID/content/15/10/1659-appT.htm). Thrombocytopenia and elevated liver enzyme levels improved after antimicrobial drug treatment.

Conclusions

In this study, we found that 5.3% (5/94) and 10.4% (5/48) patients who fulfilled the serologic diagnosis of acute Q fever and scrub typhus, respectively, were co-infected with *C. burnetii* and *O. tsutsugamushi*. Although the major arthropod vectors, animal reservoirs, and route of transmission to humans are different for *C. burnetii* and *O. tsutsugamushi*, co-infection with these 2 organisms can occur, particularly in regions where both Q fever and scrub typhus are endemic, such as southern Taiwan. Identification of co-infection improves understanding of the epidemiology of each disease and reminds clinicians to monitor patients for whom the development of chronic Q fever after acute Q fever is a high risk; such follow-up is not needed for the cases of scrub typhus alone.

Few cases of concurrent Q fever and other rickettsioses have been reported in the literature (*6,7*). Rolain et al.

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found that 6 patients with tick-borne rickettsioses had concomitant or consecutive infection with *C. burnetii*; antibodies were routinely tested against antigens of *C. burnetii, Rickettsia* spp., *Anaplasma phagocytophylum, Francisella tularensis,* and *Borrelia burgdorferi* (*6*). Five and 2 of their 6 patients had an eschar and skin rash, respectively, and none had a history or clinical signs suggestive of acute Q fever. In contrast, none of our patients had an eschar, the characteristic manifestation of scrub typhus, and only 1 had a skin rash, which made a presumptive clinical diagnosis of scrub typhus difficult.

For scrub typhus, co-infection with *Leptospira* spp. (*8–12*) and *R. typhi* (*13*) has been reported. Co-infection with *O. tsutsugamushi* and *Leptospira* spp. tends to be associated with severe illness and death (*8–12*), and cases are mainly reported in Southeast Asia, particularly in Thailand (*8*) and Taiwan (*9–12*). However, we did not routinely test for leptospirosis in this study. Cases of *O. tsutsugamushi* and *R. typhi* co-infection had been found in a surveillance of patients with fever of unknown causes, which were possibly cases of scrub typhus in China (*13*). No co-infection of *O. tsutsugamushi* and *R. typhi* was identified in our study, which might be because relatively fewer cases of murine typhus were identified.

In the 6 patients with tick-borne rickettsioses and *C. burnetii* coinfection reported by Rolain et al., 3 patients likely had concomitant infection caused by tick bites and the other 3 were possibly consecutive infections (*6*). It was difficult to identify concomitant or consecutive infection in our patients because we could not determine a definite time when patients were bitten by arthropod vectors, had contact with animal reservoirs, or were exposed to the environments abundant in *C. burnetii* and *O. tsutsugamushi*. With the assessment of serologic results, however, case-patients 3, 4, and 5 might have acquired scrub typhus first and later been infected with *C. burnetii*; titers of IgM and IgG against *O. tsutsugamushi* were high, and antibodies against *C. burnetii* phase II antigen were negative on first tests in case-patients 4 and 5 (online Appendix Table). In case-patient 3 who sought treatment for fever 20 days before admission, the antibody titers for *C. burnetii* phase II antigen and *O. tsutsugamushi* were rising and declining, respectively, in paired serum samples tested. Case-patient 2 might have had Q fever first and scrub typhus later because the first serum tests were positive for *C. burnetii* phase II IgM and IgG, but negative for *O. tsutsugamushi* antibodies. For case-patient 1, the rising antibody titers to both pathogens in paired serum specimens suggest a concomitant infection. Because chiggers were rarely reported as arthropod reservoirs of *C. burnetii*, we believe that our patients might have acquired dual infection through different routes.

The possibility of serologic cross-reaction between Q fever and scrub typhus that results in misdiagnosis of co-infection is low. Cross-reactivity of serologic test results for *C. burnetii* and *Bartonella* spp., *Legionella pneumophila*, *L. micdadei*, and *Ehrlichia chaffeensis* have been reported, but there were no data available on cross-reactivity for *O. tsutsugamushi* (*6,14*). In conclusion, although major arthropod vectors, animal reservoirs, and routes of transmission to humans are different for *C. burnetii* and *O. tsutsugamushi*, co-infection with these 2 organisms may occur, particularly in regions where both Q fever and scrub typhus are endemic.

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Poor Clinical Sensitivity of Rapid Antigen Test for Influenza A Pandemic (H1N1) 2009 Virus

Jan Felix Drexler, Angelika Helmer, Heike Kirberg, Ulrike Reber, Marcus Panning, Marcel Müller, Katja Höfling, Bertfried Matz, Christian Drosten, and Anna Maria Eis-Hübinger

Influenza A pandemic (H1N1) 2009 virus RNA was detected by reverse transcription–PCR in 144 clinical samples from Bonn, Germany. A common rapid antigen–based test detected the virus in only 11.1% of these samples. The paramount feature of rapid test–positive samples was high virus concentration. Antigen-based rapid tests appear unsuitable for virologic diagnostics in the current pandemic.

In April 2009, a novel human influenza virus A (H1N1) variant, influenza A pandemic (H1N1) 2009 virus, was identified in Mexico and the United States (*1*). Efficient human-to-human transmission facilitated global spread of this virus. On June 11, 2009, the World Health Organization (WHO) raised its pandemic alert level to Phase 6, indicating ongoing pandemic transmission. By July 27, WHO had registered 134,503 laboratory-confirmed cases and 816 confirmed deaths caused by pandemic (H1N1) 2009 virus infection worldwide (*2*).

In Germany, 5,324 cases were confirmed by July 30 (*3*). Almost 50% (n = 2,184) of these cases occurred in the federal state of North Rhine-Westphalia in western Germany, where our institution is located. As of July 30, we had tested 1,838 suspected cases and confirmed 221. All testing was based on real-time reverse transcription–PCR (RT-PCR) specific for the hemagglutinin (HA) gene of pandemic (H1N1) 2009 virus in clinical specimens. Although the real-time RT-PCR format provides considerably decreased turnaround times in molecular diagnostics, delays associated with shipping of samples and laboratory-based testing are a concern when many patients have to be seen in short time. Antigen-based rapid assays can be used as bedside tests and have been successfully applied in studies of influenza caused by the seasonal strains A (H1N1) and A (H3N2) (*4*).

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The Study

To evaluate the clinical applicability of a widely distributed rapid test in patients with pandemic (H1N1) 2009 virus, we retrospectively tested 144 PCR-positive clinical specimens from 144 different patients with the BinaxNOW Influenza A&B Rapid Test (Inverness Medical, Cologne, Germany). The assay uses monoclonal antibodies directed against the influenza A and B nucleoproteins (NP).

During preanalytical preparation, all of our samples (nasal and throat swabs) had been eluted from the swab in 500 μL of 0.9% sodium chloride solution and used for RNA extraction. The remaining part of the suspension was immediately stored at −70°C until use in this study. Before inclusion in the study, presence of pandemic (H1N1) 2009 virus in RNA extracts of all 144 samples was confirmed with a second pandemic (H1N1) 2009 virus–specific real-time RT-PCR targeting the matrix gene. Virus concentrations in samples were determined from the RNA extract without thawing the original sample, using real-time RT-PCR for the HA gene. Absolute quantification was done using photometrically quantified RNA in vitro transcripts, according to methodology described earlier (*5*).

The BinaxNOW assay was used exactly according to the manufacturer's instructions on 100 μL of freshly thawed original sample. Of 144 PCR-confirmed cases, only 16 could be detected by using the rapid antigen–based test, corresponding to a sensitivity of merely 11.1% (95% confidence interval 6.7–17.7). Samples that yielded positive results in the rapid test had a median concentration of 4,570,880 RNA copies/mL of suspension (range 5,370–74-,131,020) (Figure). This contrasted with 20,089 (range 120–64,565,420) median viral RNA copies in the rapid test–negative group. This difference was highly significant at $p<0.001$ (1-way analysis of variance [ANOVA], $F = 38.824$, done on logarithmic RNA concentrations).

The BinaxNOW rapid antigen–based assay was used in our institution during the preceding 2 influenza seasons (2007–08 and 2008–09). The 2007–08 epidemic was driven almost exclusively by seasonal influenza virus A (H1N1) in Germany, whereas the 2008–09 epidemic was mostly caused by influenza virus A (H3N2) (*6*). The sensitivity of BinaxNOW in comparison with a standard RT-PCR assay (*7*) was 37.5% (6 rapid test positives in 16 PCR positives) and 51.9% (14 rapid test positives in 27 PCR positives) in the influenza seasons 2007–08 and 2008–09, respectively. These results agree with published data on the application of BinaxNow and other rapid antigen–based tests (*8*–*10*). Similar to pandemic (H1N1) 2009 virus, virus concentration was the main determinant for a positive rapid test during both earlier influenza seasons, although slightly less marked than in the current pandemic (2007–08, median RNA copies/mL 74,131,020 [range 3,981,070–8,709,635,900] and 346,740 [range 2,450–5,495,410] in

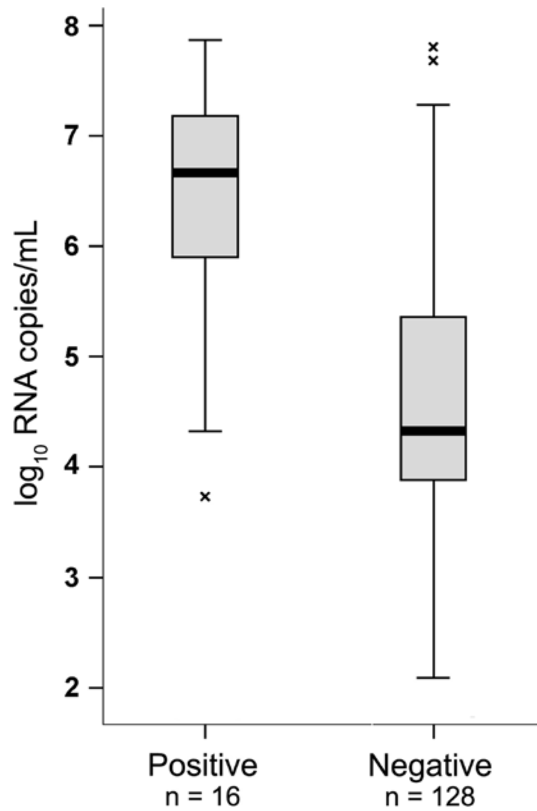

Figure. Influenza A pandemic (H1N1) 2009 virus variant RNA concentrations in rapid test–positive and –negative patients, Germany, 2009. Viral RNA concentration is compared between patients yielding positive and negative results in the BinaxNOW (Inverness Medical, Cologne, Germany) antigen-based rapid test. Boxplots were produced using SPSS, version 13.0 (SPSS, Chicago, IL, USA). The box shows the median and interquartile range (box length). The whiskers represent an extension of the 25th or 75th percentiles by 1.5 × interquartile range. Data points beyond the whisker range are considered as outliers and marked as crosses.

rapid test–positive vs. –negative patients, respectively [1-way ANOVA, p<0.001, F = 21.127]; 2008–09, median RNA copies 19,498,446 [range 138,040–1,737,800,830] and 120,230 [range 38,900–100,000,000] in rapid test–positive versus –negative patients, respectively [1-way ANOVA, p = 0.001, F = 15.659]).

Conclusions

Because children tend to have higher influenza virus shedding than adults, the overall better sensitivity of the antigen-based rapid test in preceding influenza A seasons might be due to sample selection effects because the current pandemic leads to higher percentages of adult samples than does regular seasons. The median age in our cohort was 18 years (range 1–59 years), with no significant age difference between rapid test–positive and –negative patients (1-way ANOVA, p = 0.246, F = 1.356). Most of the samples from earlier influenza A seasons originated from children <7 years of age (2008, median 3 years, range 1–17 years; 2009, median 4 years, range 1–67 years).

In agreement with our study, a recent report by Faux at al. on the detection of pandemic (H1N1) 2009 virus by rapid antigen test found low clinical sensitivity for the QuickVue Influenza assay (Quidel, San Diego, CA, USA) in comparison to RT-PCR (*11*). These authors found clinical sensitivity of QuickVue to be ≈51%. Notably, earlier studies on seasonal influenza have identified 27% clinical sensitivity for the same assay in comparison to RT-PCR (*12*), and the higher sensitivity for pandemic (H1N1) 2009 virus observed by Faux et al. might be due either to a rather small sample size in this early study (39 patients) or a different composition of the cohort (military personnel) as opposed to our study. Nevertheless, our clinical observations are supported by recent reports suggesting low analytical sensitivity of antigen-based assays on cultured pandemic (H1N1) 2009 virus (*13*,*14*) and clinical material analyzed using 2 different antigen-based rapid tests, virus culture, and a Luminex-based multiplex assay (*15*). A synopsis of data suggests clearly that testing of patients suspected of pandemic (H1N1) 2009 infection with antigen-based assays may produce misleading results in clinical practice. Application of such assays should be discouraged in favor of continued molecular diagnostics.

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Dr Drexler is a physician and clinical virologist affiliated with the University of Bonn. He is working on the implementation of methods for affordable viral load monitoring and the characterization of novel human and zoonotic viruses.

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Human Rickettsialpox, Southeastern Mexico

Jorge E. Zavala-Castro, Jorge E. Zavala-Velázquez, Gaspar F. Peniche-Lara, and Justo E. Sulú Uicab

The detection of *Rickettsia akari* in 2 human patients increased the diversity of rickettsioses affecting the public health in the southeast of Mexico. Rickettsialpox should be considered in the differential diagnosis with other febrile illnesses for the correct diagnosis and accurate treatment of this potential threat to human health.

Rickettsialpox is an illness characterized by fever, headache, papulovesicular rash over the trunk and extremities, and, in 80% of cases, appearance of an eschar. *Rickettsia akari*, the etiologic agent of rickettsialpox, is commonly transmitted by the bite of the house-mouse mite, *Liponyssoides sanguineus*. Human cases of rickettsialpox, as well as infected mites and potential reservoirs of *R. akari*, have been found in several countries, including the United States, Turkey, Croatia, and Ukraine (*1–5*). Despite the presence of the house mouse (*Mus musculus*) around the world, in Latin America human cases caused by *R. akari* have not been reported, and rickettsial diseases caused by antigenically related rickettsiae have been confined to *R. rickettsii*, *R. felis*, *R. prowasekii*, *R. typhi*, and *R. parkeri* (*6–11*). We report 2 human cases of *R. akari* infection in the Yucatan Peninsula of Mexico.

The Study

Patient 1 was a 9-year-old girl who came to the public hospital in Merida, Yucatan, in May 2008. Her illness had started abruptly with high fever and headache, then evolved over a 12-day period to include nausea, vomiting, hemorrhagic conjunctivitis, excessive lacrimation, and epistaxis. She was treated empirically with antipyretic drugs and had a slight improvement; 3 days after beginning treatment, fever and epistaxis returned with myalgia; irritability; papulovesicular rash involving the extremities, thorax, and oral mucosa; vaginal and gingival bleeding; and disseminated ecchymoses. Clinical laboratory studies showed hemoglobin 9.9 g/dL and hematocrit 29.0% (reference ranges 12–18 g/dL and 31%–51%, respectively), thrombocytopenia (45 $\times 10^3$ platelets/mL [reference range 140–440 $\times 10^3$ platelets/mL]), prolonged prothrombin and thromboplastine times (20 s and 64 s [reference range 10–15 s and 25–35 s, respectively]), neutrophilia, and elevated transaminase levels (aspartate transaminase 100 mU/mL [reference range 14–36 mU/mL], alanine transaminase 148 mU/mL [reference range 9–52 mU/mL]). The girl was hospitalized in the intensive care unit with a preliminary diagnosis of shock from dengue hemorrhagic fever.

Patient 2 was a 32-year-old woman in whom rickettsialpox was diagnosed in July 2008. She reported visiting a suburban area and being bitten by an unidentified arthropod. Her illness started abruptly with fever, headache, myalgia, and arthralgia in her extremities. The patient showed signs of dengue fever and was treated symptomatically. Three days after the first symptoms, a papulovesicular rash appeared on her extremities and thorax. Clinical laboratory results showed thrombocytopenia (100 $\times 10^3$ platelets/mL [reference range 140–440 $\times 10^3$ platelets/mL]) with slightly prolonged clotting times of thrombin and prothrombin, and neutrophilia.

Rickettsiosis was diagnosed on the basis of PCR amplification and sequencing of bacterial genes; immunofluorescent assay (IFA) and restriction fragment length polymorphism (RFLP) analyses confirmed the diagnosis and identified the *Rickettsia* species. Blood was collected in 3.8% sodium citrate as anticoagulant, and DNA was extracted immediately by QIAamp DNA kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. PCR amplification was performed by using genus-specific primers for the rickettsial 17-kDa protein gene (5′-GCTCTTGCAACTTCTATGTT-3′ and 5′-CATTGTTCGTCAGGTTGGCG-3′) (434-bp PCR fragment) and the outer membrane protein B (*omp*B) primers (5′-ATGGCTCAAAAACCAAATTTTCTAA-3′ and 5′-GCTCTACCTGCTCCATTATCTGTACC-3′) (996-bp PCR fragment). The positive controls used were DNA of *R. felis*, *R. rickettsii*, *R. akari*, *R. typhi*, *R. conorii*, and *R. honei*, provided by the Rickettsial and Ehrlichial Diseases Research Laboratory (University of Texas Medical Branch, Galveston, TX, USA); 1 reaction without DNA was used as a negative control. To avoid contamination, DNA of the positive controls and the patients was handled separately.

PCR products from the 17-kDa PCR were digested with the *Alu*I (Invitrogen, Carlsbad, CA, USA) restriction enzyme (Figure). Sequences of the *omp*B PCR and 17-kDa products were compared at the National Center for Biotechnology Information by using the BLAST software (*12*). The *omp*B sequence showed 100% identity with *R. akari* (GenBank accession no. CP000847), 92% with *R. australis* (GenBank accession no. AF123709.1), 90%

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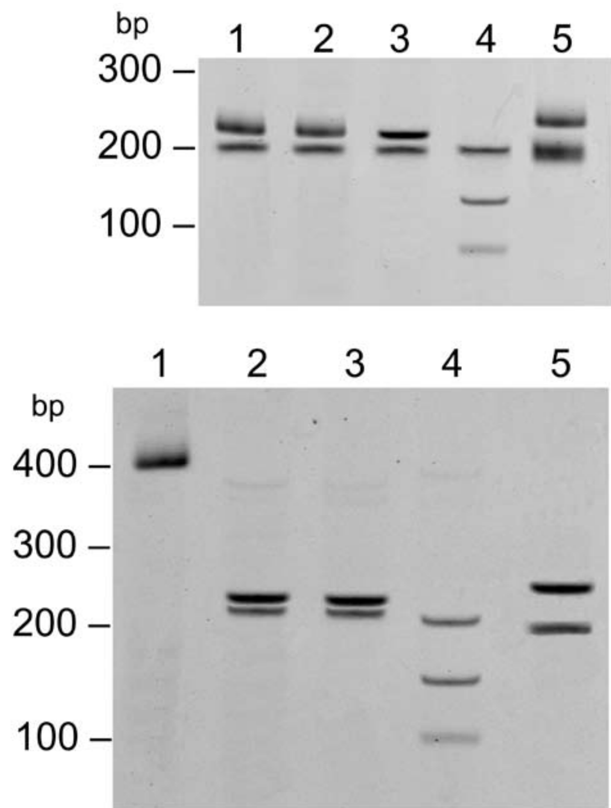

Figure. Restriction fragment length polymorphism of the 17-kDa PCR product (434 bp) digested with *Alu*I. Top: lane 1, 32-year-old woman; lane 2, 9-year-old girl; lane 3, *Rickettsia akari*–positive control; lane 4, *R. rickettsii*–positive control; lane 5, *R. typhi*–positive control. Bottom: lane 1, undigested 17-kDa gene PCR amplicon; lane 2, 9-year-old girl; lane 3, 32-year-old woman; lane 4, *R. conorii*–positive control; lane 5, *R. honei*–positive control.

with *R. felis* (GenBank accession no. CP000053.1), and <90% with other *Rickettsia* spp. The 17-kDa sequence showed 100% identity with *R. akari* (GenBank accession no. CP000847.1), and ≤95% with *R. rickettsii* (GenBank accession no. CP000848.1), *R. conorii* (GenBank accession no. AE006914.1), and other *Rickettsia* spp.

IFA showed that both patients had moderate antibody titers reactive with *R. akari* (patient 1, 256; patient 2, 128), low antibody titers reactive with *R. rickettsii* (patient 1, 64; patient 2, 32), and no antibodies to *R. typhi* antigens. A positive human serum control and IFA slides were provided by Rickettsial and Ehrlichial Diseases Research Laboratory. We detected immunoglobulin M by using a μ heavy chain–specific conjugate. Only an acute serum sample was collected from each patient during the 12–21 days after illness onset. Positive serum samples were serially diluted to 1:4,096 to determine the endpoint titer.

Patient 1 was treated with intravenous chloramphenicol, 75 mg/kg 1× per day for 7 days; clinical signs were reduced in 36 hours. Both patients were treated with 100 mg of oral doxycycline 2× per day for 7 days; symptoms were reduced in 48–72 hours.

Conclusions

Human rickettsioses have tremendously affected public health in the Americas. In the past decade, several Latin American countries have reported infected vectors, potential reservoirs, and human cases of rickettsial infections. Human cases have been limited to infections with *R. rickettsii*, *R. felis*, *R. prowasekii*, *R. typhi,* and *R. parkeri* (*6–11*). In southeastern Mexico, *R. rickettsii* and *R. felis* have been the only rickettsiae detected for many years; several cases of human illness and even deaths have occurred (*6,8*).

Rickettsialpox is a benign, self-limiting disease that usually resolves within 14–21 days; no deaths from rickettsialpox have been reported. However, for 1 of our patients, hemorrhage was the most prominent sign; the severity of the clinical features could have caused death had the correct treatment not been instituted promptly. Although hemorrhages have not been described for rickettsialpox, dissimilarities in the clinical features of rickettsial disease among countries have been reported (*13*). Organ infections caused by increased vascular permeability have been described in rickettsial infections with different degrees of severity. Rickettsialpox has even been associated with hepatitis, a not well-documented complication of rickettsialpox (*14*).

The similarity among symptoms of rickettsial infections and other febrile illnesses endemic to Yucatan, such as dengue fever, and the continuous environmental exposures of rural inhabitants to vectors of rickettsial diseases encouraged us to implement epidemiologic surveillance. Our 2 patients were detected by this surveillance. Both patients came from the relatively close rural areas of Yucatan and Campeche, Mexico, and reported mice near their homes.

Although our patients did not have eschars, we diagnosed the infection as *R. akari* serologically and molecularly. However, if IFA gives inconclusive results, PCR amplification of the bacterial genome is the decisive parameter for diagnosis. Our report of these 2 human rickettsialpox cases in Mexico provides a new rickettsial infection to consider in the differential diagnosis of febrile illnesses.

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This work was conducted at the Facultad de Medicina, Unidad Interinstitucional de Investigación Clínica y Epidemiológica, Universidad Autónoma de Yucatán, Mérida Yucatán, México.

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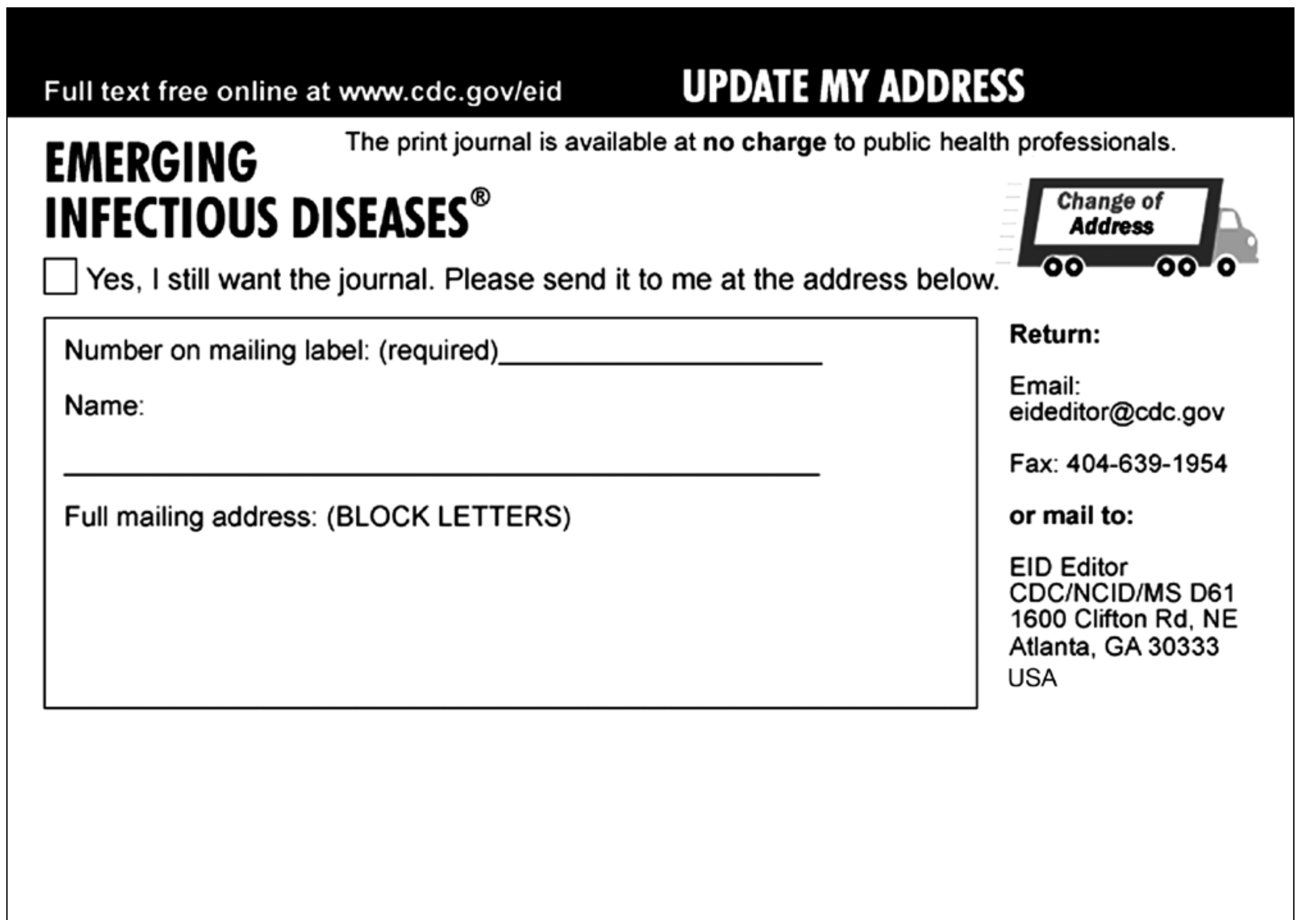

West Nile Virus Infection in Plasma of Blood and Plasma Donors, United States

Christina B. Planitzer, Jens Modrof, Mei-ying W. Yu, and Thomas R. Kreil

This study investigated the association of ongoing West Nile virus (WNV) infections with neutralizing antibody titers in US plasma-derived intravenous immune globulin released during 2003–2008. Titers correlated closely with the prevalence of past WNV infection in blood donors, with 2008 lots indicating a prevalence of 1%.

West Nile virus (WNV) is a flavivirus endemic to the United States; typically, hundreds of clinical cases of infection occur each year. The observed number of clinical WNV infections as collated by ArboNET (www.cdc.gov) and the incidence of asymptomatic WNV infections as shown by nucleic acid testing (NAT) of the US blood supply (*1*) indicate that ≈3 million WNV infections occurred in humans during 1999– 2008.

Because the immune system elicits WNV neutralizing antibodies in response to WNV infection, detectable levels of WNV neutralizing antibodies in the blood of persons with previous WNV infection is expected. Consequently, lots of immune globulin-intravenous (human) (IGIV) manufactured from plasma collected in the United States contain WNV neutralizing antibodies (*2*). Those IGIV lots, each prepared from several thousand plasma donations to ensure a broad spectrum of antibodies, can be used as an epidemiologic tool that enables the surveillance of thousands of persons in a community through analysis of comparatively few samples. In this study, we demonstrated the increasing trend of WNV-neutralizing antibody titers in lots of IGIV.

Comparing these titers with those of persons with confirmed past WNV infection provides an independent measure of the percentage of the US population previously infected with WNV. Several WNV vaccine trials are ongoing or imminent, so information about the prevalence of past WNV infection in the United States is valuable for planning the demonstration of vaccine efficacy. Low incidence and lack of highly WNV-endemic areas in the United States preclude classic vaccine field trials because of study size requirements and cost-logistics difficulties.

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The Study

The WNV neutralization titers of several US plasma–derived IGIV products (Gammagard Liquid/KIOVIG; Gammagard S/D/ Polygam S/D; Iveegam EN [Baxter Healthcare Corporation, Westlake Village, CA, USA]) and plasma samples obtained from US blood donors after a NAT-confirmed WNV infection were determined by an infectivity assay as earlier described (*2*), adapted to a classical microneutralization format (*3*). WNV neutralization titers (i.e., the reciprocal dilution of a 1:2 series resulting in 50% neutralization [NT_{50}; detection limits <0.8 for undiluted IGIVs and <7.7 for 1:10 prediluted serum]) are reported as the mean ± SEM. An unpaired *t* test was used to evaluate whether titer differences between 2 groups were statistically significant.

Using an extrapolation derived from screening the US blood supply for WNV (*1*), we calculated the average annual number of WNV infections in the United States for 1999–2008. The total number of neuroinvasive cases reported for those years to the US Centers for Disease Control and Prevention (CDC) through ArboNET was multiplied by 256 (i.e., the factor between all WNV infections and neuroinvasive cases). The cumulative infection rate for each year during 1999–2008 was then calculated by dividing the infections occurring up to a specific year by the US population for that year (determined by US Census Bureau estimates [www.census.gov/popest/states/NST-ann-est.html]).

Although WNV was first introduced into the United States in 1999, only in 2003 did the mean WNV neutralization titers of IGIV lots released to the market start to increase markedly (Figure 1). According to extrapolations from the WNV screening of the US blood supply (*1*), by 2003, an estimated 0.5% of the US population had been infected with WNV, although most infections were asymptomatic.

A delay of ≈1 year occurs between the collection of plasma and the release of IGIV lots to the market; thus, the WNV-positive IGIV lots in 2003 reflect the larger number of WNV infections occurring in 2002. Using the same extrapolations from the US blood supply (*1*), we found that the ≈0.1% annual increments in the proportion of the US population with past WNV infection follow a straight line ($r^2 = 0.9996$), generally paralleled by the mean WNV neutralization titers of IGIV lots. During 2005–2008, when large numbers of lots of a single IGIV product (Gammagard Liquid) could be analyzed, the WNV neutralization titer increased by 3.6 per year ($r^2 = 0.9793$).

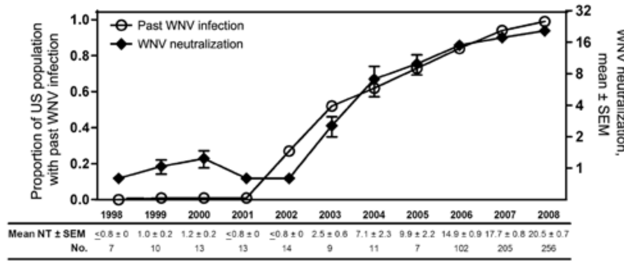

Figure 1. West Nile virus (WNV) neutralization titers of US plasma-derived immune globulin intravenous (human) (IGIV) lots by year of production and estimated percentage of the US population with past WNV infection by year. WNV neutralization titers were determined either for retention or lot release samples of 3 IGIV products produced during 1998–2005 or for a considerable proportion of Gammagard Liquid/KIOVIG lots produced during 2006–2008. Results are shown as mean ± SEM (limit of detection <0.8) by year of product release. For 5% of IGIV samples, titers were multiplied by 2 for comparison with the 10% IGIV samples at equivalent immunoglobulin concentrations. The percentage of the US donor population with past WNV infection was calculated from the number of neuroinvasive cases reported per year and the estimated ratio of neuroinvasive cases to total cases of WNV infection.

US plasma-derived IGIV lots released during 2008 showed variable WNV neutralization titers ranging from 2.8 to 69.8; mean ± SEM titer was 21 ± 1 (n = 256) (Figure 2). Compared with titers shown to be protective in an animal model of WNV infection (equivalent to >21 by the current assay) (*2*), ≈40% of the 2008 IGIV lots had higher titers.

Plasma obtained from persons with NAT-confirmed WNV infection had even higher titers; mean ± SEM titer was 208 ± 40 for 30 persons available for testing. When results were corrected for the immunoglobulin (Ig) G concentration in plasma (≈1%), compared with the 10% IGIV preparations, the mean neutralization titer of the plasma samples was ≈100× higher than that of the IGIV lots tested (2,080 vs. 21).

Conclusions

The most comprehensive collation of information about the incidence of WNV infection in the United States is available from ArboNET. When that information is combined with information obtained from the nationwide screening of the blood supply for WNV RNA by NAT (*1,4,5*), the current prevalence of past WNV in the US population is estimated to be ≈1%.

Busch et al. has noted that large-scale, community-based serologic surveys are hardly feasible because of their expense and because WNV ELISA assays are possibly biased by cross-reactions with other flaviviruses (*1*). Nevertheless, 7 seroepidemiologic studies have been performed (*6–12*). Cumulatively, 5,503 persons were tested for WNV infection by ELISA, and the results have shown highly divergent seroprevalence rates ranging between 1.9% (*6*) and 14.0% (*10*).

The use of IGIV lots, each representing the serostatus of several thousand donors in 1 sample, makes seroepidemiology practical (*13*) because it allows a large donor population to be surveyed by analyzing comparably few samples. The use of a more complex yet functional virus neutralization assay minimizes concerns about cross-reactivity with flaviviruses of other serocomplexes (e.g., dengue virus) that occasionally circulate in the US population. Also, epidemiologic considerations render interference by St. Louis encephalitis virus, a flavivirus within the same serocomplex, highly unlikely (*2*). The specificity of the neutralization assay was confirmed by testing IGIV lots manufactured from European-derived plasma against tick-borne encephalitis virus, a flavivirus closely related to WNV and circulating in Europe. Although these lots contained high neutralization titers against tick-borne encephalitis virus, only 1 of 20 had a detectable neutralization titer of 5 against WNV (unpub. data).

In this study, we determined that the mean titer of samples obtained during 2003–2008 from persons with a confirmed diagnosis of WNV infection was 100× higher than the mean titers of IGIV lots produced in 2008. This determination provides an independent experimental measure of the frequency of past WNV infection in the general US population, as reflected by the plasma/blood donor community, and the results correlate well with results of previously published theoretical extrapolations (*1*), which estimated that ≈1% of the population has already been infected with WNV.

The increasing levels of WNV neutralizing antibodies in IGIV lots from US plasma and the particularly high

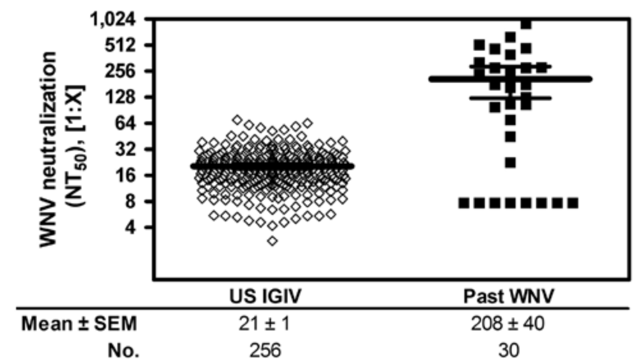

Figure 2. West Nile virus (WNV) neutralization by US plasma-derived immune globulin intravenous (human) (IGIV) released in 2008 and plasma from donors with past WNV infection (past WNV), confirmed by nucleic acid testing. WNV neutralization titers are shown as the mean ± SEM (limit of detection <0.8 for undiluted IGIVs and <7.7 for prediluted sera). NT_{50}, 50% neutralization titer.

titers in donors who have had a WNV infection suggest the possibility of preparing IGIV products with sufficiently high titers to be useful for WNV prophylaxis or treatment. Several ongoing or imminent WNV vaccine clinical trials stress the practical value of an independent confirmation of extrapolations that estimate the percentage of the US population with past WNV infection. Knowing the percentage of preexisting WNV seroprevalence as well as estimates of the mostly asymptomatic incidence rates (*14*) can be of vital importance in designing vaccine trials.

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Ms Planitzer is writing her PhD thesis on virus antibodies in immune globulins at the Global Pathogen Safety Group of Baxter BioScience in Vienna, Austria, in collaboration with the Medical University of Vienna, Austria. Her research focuses on determining functional antiviral properties of immunoglobulin preparations.

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Tick-borne Encephalitis from Eating Goat Cheese in a Mountain Region of Austria

Heidemarie Holzmann, Stephan W. Aberle, Karin Stiasny, Philipp Werner, Andreas Mischak, Bernhard Zainer, Markus Netzer, Stefan Koppi, Elmar Bechter, and Franz X. Heinz

We report transmission of tick-borne encephalitis virus (TBEV) in July 2008 through nonpasteurized goat milk to 6 humans and 4 domestic pigs in an alpine pasture 1,500 m above sea level. This outbreak indicates the emergence of ticks and TBEV at increasing altitudes in central Europe and the efficiency of oral transmission of TBEV.

Tick-borne encephalitis virus (TBEV) is a human pathogenic flavivirus that is endemic to many European countries and to parts of central and eastern Asia (*1*). Even though vaccination can effectively prevent TBE (*2*), >10,000 cases are reported annually for hospitalized persons in areas of Europe and Asia to which TBE is endemic. TBEV occurs in natural foci characterized by ecologic habitats favorable for ticks, especially in wooded areas within the 7°C isotherm (*3*). The major route of virus transmission is tick bites, but TBEV also can be transmitted during consumption of nonpasteurized milk and milk products from infected animals, primarily goats (*3*). Outbreaks resulting from oral virus transmission are rare in central Europe but more common in eastern Europe and the Baltic states (*3*). Our investigation of TBEV transmitted by milk from a goat in an alpine pasture in a mountainous region provides evidence for a changing TBEV epidemiology in central Europe and the expansion of ticks and TBEV to higher regions.

The Study

We investigated a TBE outbreak, comprising 6 cases, in a mountain region in western Austria in July 2008. The index case occurred in a 43-year-old shepherd who had stayed for 24 days at his alpine pasture (1,564 m above sea level) before he was hospitalized for nonbacterial urethritis and nonspecific influenza-like symptoms (including pain in the lower abdomen and legs), followed by clinical signs of meningitis. TBEV infection was confirmed serologically by ELISA demonstration of specific immunoglobulin (Ig) M and IgG in serum and cerebrospinal fluid. The patient did not remember a tick bite but had eaten self-made cheese prepared from a mixture of nonpasteurized goat milk and cow milk 8–11 days before illness onset; further investigation found 6 additional persons who had eaten the same cheese (Figure). For 5 of them, recent TBEV infection was serologically proven (Table). For 3 of these persons (2 men, 44 and 65 years of age; and 1 woman, 60 years of age), similar to the index patient, a typical biphasic course and symptoms of TBE (nonspecific flu-like symptoms followed by fever, cephalea, meningism, and ataxia after 4–10 days) developed and they were hospitalized. The 2 other persons who had eaten the cheese (female, 37 and 7 years of age) were clinically asymptomatic. The noninfected person had vomited shortly after eating the cheese because of a gastric banding. None of the infected persons had been vaccinated against TBEV.

The cheese was prepared from a mixture of fresh milk from 1 goat and 3 cows and was eaten shortly after production. Detection of TBEV-specific hemagglutination inhibiting (HI) and neutralizing antibodies in the goat's serum proved infection in the goat; the 3 cows were seronegative for TBEV. At the time of this investigation (1 month after cheese production), TBEV was already undetectable by PCR in serum and milk of the goat. Cheese from the 3 batches produced after the contaminated batch was TBEV negative by PCR. The original cheese was no longer available for testing.

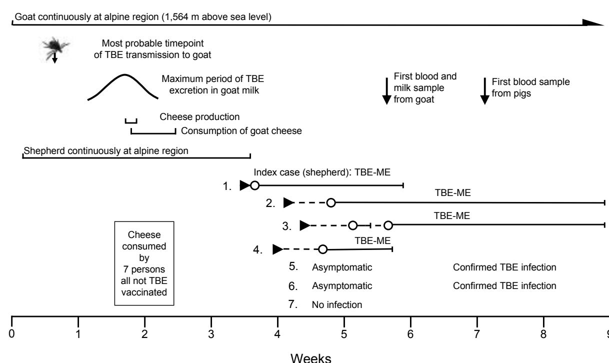

Figure. Time course and series of events of a tick-borne encephalitis (TBE) outbreak from cheese made with goat milk. Week 0, transport of goat to high altitude; ►, onset of disease; **O**—I, hospitalization period; TBEV, tick-borne encephalitis virus; ME, meningoencephalitis.

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Table. Infection parameters of 7 persons exposed to TBEV by eating nonpasteurized goat cheese, Austria, 2008*

Sex/ age, y	Incubation, d	Symptoms/signs	Diagnosis	Hospitalized, d	Virologic parameters				TBEV infection confirmed
					Material	TBEV ELISA		TBEV NT	
						IgM	IgG		
M/43	11	Fever, cephalea, meningism, aseptic urethritis; CSF: pleocytosis	ME	18	Serum	Pos	Pos	Pos	Yes
					CSF	Bor	Pos		
M/65	10	Fever, cephalea, meningism, vertigo, cerebellar ataxia; CSF: pleocytosis	ME	30	Serum	Pos	Pos	Pos	Yes
					CSF	Bor	Bor		
F/60	14	Fever, cephalea, meningism, vertigo, cerebellar ataxia; CSF: pleocytosis	ME	25	Serum	Pos	Pos	Pos	Yes
					CSF	Pos	Pos		
M/44	9	Fever, cephalea, meningism, vertigo, cerebellar ataxia; CSF: pleocytosis	ME	9	Serum	Pos	Pos	Pos	Yes
					CSF	Pos	Bor		
F/37	NA	None	NA	0	Serum	Pos	Pos	Pos	Yes
F/7	NA	None	NA	0	Serum	Pos	Pos	Pos	Yes
F/45	NA	None	NA	0	Serum	Neg	Neg	Neg	No

*TBEV, tick-borne encephalitis virus; NT, neutralization test; CSF, cerebrospinal fluid; Ig, immunoglobulin; ME, meningioencephalitis; pos, positive; bor, borderline; NA, not applicable; neg, negative.

The 4 domestic pigs kept at the alpine pasture and fed with the whey and goat milk, however, were seropositive (TBEV HI- and neutralizing antibodies detected), which indicated TBEV infection, but no clinical signs were observed. Infection with TBEV has been reported in wild boars (*4*,*5*). Serum samples from 105 goats from pastures in the neighborhood also were investigated for TBEV-specific antibodies; all goats were seronegative.

Conclusions

Our analyses showed that the 6 humans and the 4 pigs were infected through the milk of 1 goat, which had been transported by car from a TBE–nonendemic valley to the alp 12 days before production of the TBEV-contaminated cheese. Experiments have demonstrated that infected domestic animals (i.e., goats, sheep, and cows) can excrete TBEV into milk for ≈3–7 days, beginning as early as the second or third day postinfection (*6*–*9*). In addition, although cheese was produced once or twice each week, only this ≈1-kg batch of cheese transmitted TBEV. Therefore, all the evidence indicates that the goat was infected at the alpine pasture at an altitude of 1,564 m. Indeed, some ticks were collected from cows that had stayed at this altitude during the entire summer. Analyses of these ticks for TBEV by PCR, however, yielded only negative results.

Our findings provide further evidence for the expansion of TBEV-endemic regions to higher altitudes in central Europe. For example, longitudinal studies in the Czech Republic, a country with similar climatic and ecologic conditions to those of Austria, showed a shift in *Ixodes ricinus* ticks and TBEV, from 700 m in 1981–1983 to 1,100 m altitude in 2001–2005 (*10*,*11*). Likewise, Zeman and Beneš demonstrated that the maximum altitude at which TBEV is found in the Czech Republic gradually moved upward during 1970–2000, corresponding to the rise in temperature during the same period (*12*). In Scandinavia, a northward extension of the geographic range of *I. ricinus* ticks and TBEV since the mid-1980s has also been recognized (*1*,*13*–*15*). Climatic changes most likely are the major driving forces for the geographic changes in the distribution of TBEV and its main vector, *I. ricinus*, in Europe.

This report also emphasizes the efficiency of oral transmission of TBEV to humans and to pigs. Six of the 7 persons who ate the cheese and all 4 pigs fed residual milk or whey from the same cheese became infected. Given the excellent effectiveness of the TBE vaccine (*2*), vaccination probably could have prevented all 6 human cases.

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Dr Holzmann is a virologist at the Clinical Institute of Virology, Medical University of Vienna, Austria. Her research interests focus on flaviviruses, hepatitis C virus, and antiviral vaccines.

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Surveillance System for Infectious Diseases of Pets, Santiago, Chile

Javier López, Katia Abarca, Jaime Cerda, Berta Valenzuela, Lilia Lorca, Andrea Olea, and Ximena Aguilera

Pet diseases may pose risks to human health but are rarely included in surveillance systems. A pilot surveillance system of pet infectious diseases in Santiago, Chile, found that 4 canine and 3 feline diseases accounted for 90.1% and 98.4% of notifications, respectively. Data also suggested association between poverty and pet diseases.

Communicable diseases challenge health systems and require coordinated efforts for their control. Surveillance systems for human communicable diseases have been implemented since the 19th century. Surveillance of animal infections started later and focused on livestock production. More recently, in response to emerging zoonoses such as avian influenza and West Nile virus infection, novel surveillance systems for wild animals have been implemented (*1*). Although pet-borne infections have become increasingly relevant to human health, systematic notification of these infections is not currently conducted, except for rabies.

Pets (domesticated dogs and cats that live in close proximity to humans) may pose several risks to their owners' health and create occupational hazards for professionals such as veterinarians. They can also serve as sentinels for several diseases by alerting persons to the presence of infectious agents in a community (*2*). These features emphasize the need for surveillance systems of pet infectious diseases, especially those that can be transmitted to humans (*3*).

In Chile, as in other countries, pet infectious diseases, except for rabies, have not been included in any surveillance system; for this reason, information about their epidemiology is scarce. Thus, a pilot surveillance system for infectious disease of pet dogs and cats was implemented for a 2-year period in Santiago, Chile.

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The Study

During October 2004–September 2006, the sentinel surveillance system was implemented in 61 veterinary clinics (30 during the first year and another 31 during the second year) located in 34 districts of Santiago (population 5.4 million). Pet population estimates (1,117,192 dogs; 518,613 cats) were derived from a study conducted previously in Santiago (*4*) and corresponded to a rate of ≈1 sentinel centers per 27,000 pets. Sentinel centers were asked to participate on a voluntary basis and were grouped similarly to the human health services, following geographic criteria.

Of the 12 notifiable infectious diseases in the surveillance system, 5 were nonzoonotic (distemper, canine infectious tracheobronchitis, feline respiratory complex disease, feline leukemia, and hemorrhagic gastroenteritis), and 7 were zoonotic (giardiasis, brucellosis, leptospirosis, rabies, ehrlichiosis, scabies, and tinea infection). Definitions were established for suspected and confirmed cases of each disease. Laboratory confirmation was required for diagnosis of giardiasis, brucellosis, leptospirosis, and rabies. Personnel from each sentinel center recorded their data on a website. They were trained in operative definitions and procedures, which included submitting a weekly report of the total number of cases seen. Participation in the study was voluntary; no funding or incentives were offered.

During the 2-year period, 8,167 cases were reported: 6,974 (85.4%) in dogs and 1,193 (14.6%) in cats. Of these dogs and cats, 4,415 (63.3%) and 730 (61.2%), respectively, were males. Also, 4,524 (64.9%) dogs and 503 (42.2%) cats were <1 year of age. Data submitted during the first year of surveillance accounted for 67.5% of canine and 66.7% of feline diseases notifications.

A negative correlation was found between the average number of notifications per sentinel center (ANC) and time (8 trimesters) for dogs (ρ –0.95, $p<0.01$) and cats (ρ –0.93, $p<0.01$). A positive correlation, although not statistically significant, was found between the average poverty rate of the districts located in each health service (*5*) and the ANC for dogs (ρ +0.77, $p = 0.07$) and cats (ρ +0.43, $p = 0.40$) (Table 1).

During the 2-year surveillance period, 4 canine diseases (hemorrhagic gastroenteritis, distemper, scabies, and infectious tracheobronchitis) accounted for 90.1% of notifications, and 3 feline diseases (respiratory disease complex, feline leukemia, and tinea) accounted for 98.4% of notifications (Table 2). For each disease, ANC during the first year of surveillance was calculated for centers located in South–East Health Service (SEHS), which had the highest poverty rate of its districts, and East Health Service (EHS), which had the lowest. For canine diseases, the ratios of ANC for SEHS/ANC for EHS were 3.5 (scabies), 2.5 (distemper), 2.2 (hemorrhagic gastroenteritis), and 1.8 (infectious tracheobronchitis); for feline diseases, these ratios were 1.2

Table 1. Average number of notifications per sentinel center, according to health service, Santiago, Chile, October 2004–September 2005*

Health Service	Poverty (%)†	Average no. notifications	
		Dogs	Cats
South–East	13.7	160.1	20.1
West	13.2	349.7	52.3
South	11.7	189.6	36.4
North	9.5	143.8	22.6
Central	8.0	62.0	22.3
East	6.6	74.5	18.5

*First year of pilot surveillance system
†Average poverty rate of the districts belonging to each health service.

(respiratory disease complex), 1.1 (feline leukemia), and 0.7 (tinea).

For each of the 7 diseases, we calculated the following ratio: total no. notifications for the whole surveillance period for all sentinel centers for pets <1 year of age/total no. notifications for the whole surveillance period for all sentinel centers for pets ≥1 year of age. Diseases most commonly occurring in pets <1 year of age were hemorrhagic gastroenteritis (10.9) and distemper (3.1); on the contrary, diseases whose ratio favors pets >1 year of age were feline leukemia (0.17) and infectious tracheobronchitis (0.43). Scabies in dogs, feline respiratory disease complex, and tinea in cats had ratios of ≈1.00 (1.07, 0.96, and 0.92, respectively).

Conclusions

This pilot surveillance system indicated that overall notifications predominated for pets with 3 characteristics: canine, male, and age <1 year. These characteristics partially reflect the species and sex distribution of pets in the city, as shown by a household survey of 2,100 homes in 7 districts of Santiago during 2001, which showed that 55.7% of household pets were dogs (62.3% males, 16.0% <1 year of age), and 23.5% were cats (57.9% males, 15.0% <1 year of age) (M.A. Daza, unpub. data). However, according to our data, the predominance of notifications for animals <1 year of age seems to represent a higher risk associated with being <1 year of age.

Table 2. Diseases reported for dogs and cats, Santiago, Chile, October 2004–September 2006*

Disease	No. (%) cases
Dogs	
Hemorrhagic gastroenteritis	2,315 (33.2)
Distemper	1,461 (20.9)
Scabies	1,307 (18.7)
Infectious tracheobronchitis	1,207 (17.3)
Tinea	447 (6.4)
Ehrlichiosis	185 (2.7)
Giardiasis	38 (0.5)
Brucellosis	9 (0.1)
Leptospirosis	5 (0.1)
Cats	
Respiratory complex disease	826 (69.2)
Feline leukemia	222 (18.6)
Tinea	127 (10.6)
Scabies	14 (1.2)
Giardiasis	4 (0.3)
Total	8,167 (100.0)

*All health services, first and second year of surveillance.

The finding that 4 canine and 3 feline diseases were most frequently reported may be useful in many settings, such as disease control prioritization and identification of topics of interest for investigation. From a human health perspective, 1 canine disease (scabies) was zoonotic and 2 others (hemorrhagic gastroenteritis and infectious tracheobronchitis) included zoonotic agents in their list of possible etiologies; thus, the information provided by the surveillance system is useful for human physicians and policy makers. This finding is especially relevant because certain pet diseases may occur on a socioeconomic gradient, affecting a greater proportion of persons in the lowest socioeconomic districts. This socioeconomic gradient could have been underestimated in our study because pet owners in Chile must pay for the healthcare of their pets, and the likelihood of diagnostic tests being performed for diseases requiring laboratory confirmation is low, especially in the poorest areas of the city. We also did not account for the overall number of veterinary clinics that exist in each district, making estimation of disease notification rates among districts or health services, impossible. The finding that the most prevalent diseases were preventable by vaccination (e.g., distemper) raises questions about the coverage and quality of vaccinations among pets in Santiago.

The validity of this pilot surveillance system is limited because the overall ANC showed a declining trend during the 2 years of surveillance. This trend probably does not represent reduced incidence of infectious diseases among pets in Santiago; on the contrary, it may illustrate the difficulty of maintaining a private surveillance system based on professional motivation, a key element for ensuring the sustainability of such a system over time.

This pilot surveillance system may motivate other investigations regarding zoonotic infections of pets in Chile. The resulting information would provide the data needed to calculate disease incidence rates and establish unbiased comparisons, which can be used to further the goal of improved pet and human health.

Dr López is a clinical veterinarian and member of the Chilean Society of Veterinary Infectious Diseases. His research interests include canine and feline arthropod-transmitted diseases such as anaplasmosis, ehrlichiosis, and rickettsiosis and other parasitic infections of pets.

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Independent Lineage of Lymphocytic Choriomeningitis Virus in Wood Mice (*Apodemus sylvaticus*), Spain

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To clarify the presence of lymphocytic choriomeningitis virus (LCMV) in Spain, we examined blood and tissue specimens from 866 small mammals. LCMV RNA was detected in 3 of 694 wood mice (*Apodemus sylvaticus*). Phylogenetic analyses suggest that the strains constitute a new evolutionary lineage. LCMV antibodies were detected in 4 of 10 rodent species tested.

Lymphocytic choriomeningitis virus (LCMV) is a ubiquitous rodent-borne virus belonging to the family *Arenaviridae*, whose genome consists of 2 single strands of RNA, named small (S) and large (L), respectively. The S segment encodes the nucleocapsid protein (NP) and the glycoprotein precursor (GPC). The L segment encodes a viral RNA-dependent RNA polymerase and a zinc-binding protein. The common house mouse (*Mus musculus)* is the principal reservoir for LCMV. Infected mice can shed the virus in large quantities throughout their lives. Some epidemiologic studies show that ≈9% of wild mice are infected with LCMV (*1*,*2*), and other species of rodents have been reported to be possible reservoirs of LCMV (*2–4*).

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Humans become infected with LCMV by inhaling contaminated feces or urine, through bite wounds, by vertical route, or after organ transplants (*5*). LCMV is responsible for aseptic meningitis and encephalitis (*6*) and may cause congenital malformations or abortion (*7*). In Spain, 1 case of encephalitis caused by LCMV has been reported (*8*), and recently, LCMV infection has been detected in 4 patients with aseptic meningitis (*9*). LCMV infection in rodents and the general population has also been demonstrated by serologic tests (*2*). The aim of this study was to improve our knowledge of LCMV in rodents in Spain.

The Study

A total of 866 small mammals were trapped from July 2003 through June 2006 in 19 Spanish provinces. Of those captured, 833 were rodents from 10 species: 694 wood mice (*Apodemus sylvaticus*), 17 yellow-necked mice (*A. flavicollis*), 27 house mice (*M. musculus*), 6 Algerian mice (*Mus spretus*), 21 Norway rate (*Rattus norvegicus*), 50 bank voles (*Myodes* [*Clethrionomys*] *glareolus*), 9 snow voles (*Chionomys* [*Microtus*] *nivalis*), 3 Orkney voles (*Microtus arvalis*), 3 Mediterranean pine voles (*Microtus* [*Pitymys*] *duodecimcostatus)*, and 3 garden dormice (*Eliomys quercinus*). Thirty-three were insectivores (18 shrews [*Sorex* spp.] and 15 white-toothed shrews [*Crocidura russula*]). Tissue samples (lungs, kidneys, spleens) were obtained in all cases and stored at –20°C in RNAlater solution (Ambion Inc., Austin, TX, USA) to preserve the RNA and inactivate the virus. Serum samples were only available from 665 specimens.

Serum samples were assayed against LCMV, diluted 1:16 as previously described (*9*), but using immunoglobulins against mice or rats as secondary antibodies. Western blot assays confirmed 25 of the 35 positive serum specimens detected by the immunofluorescence antibody (IFA) assay. The overall prevalence of antibodies against LCMV was 3.76%. Antibodies were detected in 4 species: *A. sylvaticus* (21/536, 3.92%), *M. musculus* (2/24, 8.33%), *M. spretus* (1/6, 16.67%), and *R. norvegicus* (1/21, 4.76%). Titers ranged from 16 to 2,048 by IFA assay.

LCMV-related genome was detected in 3 of 866 specimens corresponding to *A. sylvaticus* mice trapped in Sierra Nevada (SN05), Cabra (CABN), and Grazalema (GR01), 3 well-preserved natural areas in the southern Spain. Only serum specimens from 2 of these rodents were available, and LCMV antibodies were detected in only 1 sample.

Briefly, pools were prepared by mixing 3- to 4-mm pieces of lung, kidney, and spleen from each trapped animal; the mixture was homogenized and their nucleic acid extracted by using RNeasy Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The extracted RNA was analyzed by reverse transcription and nested PCR. The first round was performed with primers

AREN1+ (5′-$_{2367}$CWATRTANGGCCAICCITCICC$_{2388}$-3′) and AREN1– (5′-$_{2789}$TNRWYAAYCARTTYGGIWCIRTKCC$_{2813}$-3′) and primers AREN2+ (5′-$_{2396}$CANANYTTRTANARNAIRTTYTCRTAIGG$_{2424}$-3′) and AREN2– (5′-$_{2567}$AGYYTNKNNGCNGCIGTIAARGC$_{2589}$-3′) for nested PCR. The symbols + and – correspond to sense and antisense sequences, respectively. Indicated positions correspond to those of LCMV-Armstrong 53b (GenBank accession no. M20869). Primers were designed on conserved motifs of the NP gene and were able to detect arenaviruses from the Old World and from the New World. Amplification products of the expected size (194 bp) were purified and sequenced. Positive results were also obtained when each tissue from these 3 animals was analyzed separately. Viral isolation was not attempted because samples were inactivated with RNA later.

The complete S segment sequence of every detected virus was obtained from lung lysates by using primers designed based on LCMV conserved sequences of the S segments available in GenBank that enable amplification of overlapping complementary DNAs (sequences of the primers are available upon request). The lengths of the S-segments were 3,357, 3,364, and 3,366 nt for samples GR01, SN05, and CABN, respectively (GenBank acces-

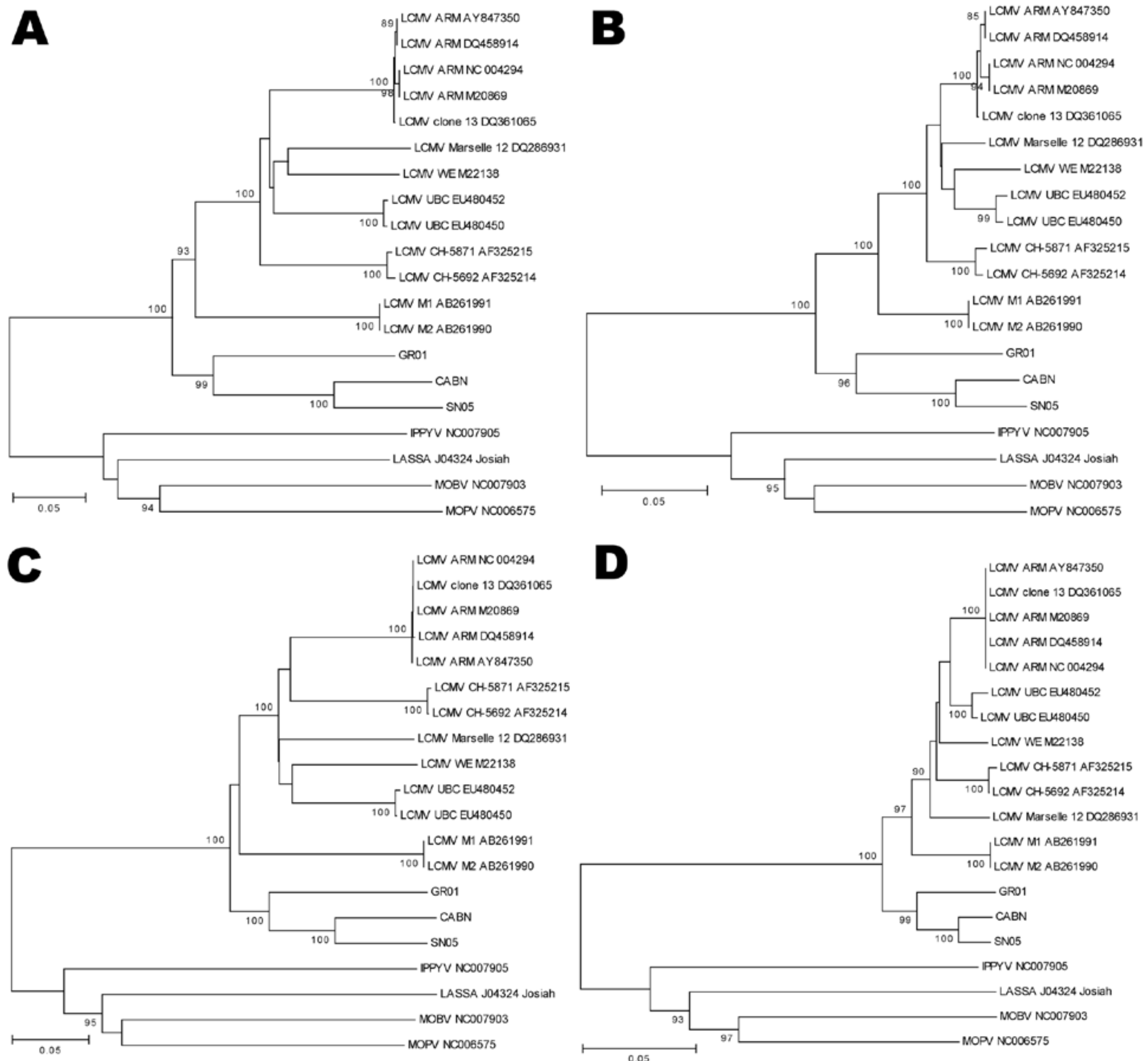

Figure. Phylogeny of lymphocytic choriomeningitis virus (LCMV) strains and the viruses detected in this study based on the analysis of complete sequences of amino acids (aa) and nucleotides (nt) of glycoprotein (GPC) and nucleocapsid protein (NP) genes. A) GPC nt; B) GPC aa; C) NP nt; D) NP aa. Each sequence used shows the name of LCMV strain followed by GenBank accession number. Numbers indicate >80% bootstrap values. Scale bars indicate nucleotide substitutions per site. IPPIV, Ippy virus; LASV, Lassa virus; MOBV, Mobala virus; MOPV; Mopeia virus.

sion nos. FJ895882–FJ895884, respectively). As expected for LCMV, the sequences defined 2 nonoverlapping genes (genes GPC and NP, with 498 and 558 aa, respectively) arranged in ambisense direction, separated by an intergenic noncoding region, and flanked by 5′ and 3′ ends. Sequence comparison with the complete S segment from other LCMV strains showed deletions and insertions of nucleotides in the noncoding regions (information available on request).

Nucleotide and amino acid sequence distances were calculated by the pairwise distance algorithm (p distance) with MEGA version 3.1 (*10*). Phylograms were reconstructed using the neighbor-joining algorithm and tested with the bootstrap method and 1,000 replicates. GPC gene sequences detected in *A. sylvaticus* mice showed 15.9%–19.7% amino acid differences and 23.4%–27.7% nucleotide differences with the rest of the LCMV sequences (online Appendix Table 1, available from www.cdc.gov/EID/content/15/10/1677-appT1.htm). Moreover, *A. sylvaticus*-LCMV sequences of the NP gene differed 8.3%–10.6% at the amino acid level and 19.8%–22.0% at the nucleotide level in comparison with the rest of the LCMV sequences (online Appendix Table 2, available from www.cdc.gov/EID/content/15/10/1677-appT2.htm). Phylogenetic analyses based on the entire amino acid and nucleotide sequences of NP and GPC genes showed that new sequences were grouped with other LCMV strains but in an isolated cluster with a high bootstrap value (Figure).

Conclusions

The LCMV seroprevalence detected in this study was similar to that found in other European countries ranging from 3.6% to 16.3% (*3*,*11*,*12*). Specific LCMV antibodies were detected in 4 of 10 rodent species tested; all belonged to the subfamily Murinae and were trapped throughout the country. These results suggest LCMV infection is widespread in Spain.

Phylogenetic analyses showed the close relationship between the new sequences detected in *A. sylvaticus* mice and the previously known LCMV strains, although they formed a separate cluster with a high bootstrap (Figure). The differences found in NP and GPC genes suggest that the new viruses detected in *A. sylvaticus* mice may constitute a new lineage of LCMV. In Lassa virus, similar differences in NP gene sequences served to group different strains into 4 lineages (*13*). Furthermore, comparison of noncoding regions showed that, in spite of the genetic variability in LCMV strains, CABN, GR01 and SN05 had specific deletions and insertions. In conclusion, our data suggest that the described genetic differences of the new sequences contribute to the definition of a new LCMV lineage.

A. sylvaticus has previously been related to LCMV (*4*) and its role as a reservoir for this virus has also been suggested (*14*). LCMV genome has recently been detected in this species, but the phylogenetic study grouped the sequence within LCMV strains isolated from *M. musculus* (*15*). By contrast, our analysis showed that CABN, GR01, and SN05 strains define a different branch from the previously known LCMVs, suggesting that *A. sylvaticus* might have been responsible for consolidating genetic changes in these new strains during their evolution, and that *A. sylvaticus* could be their natural reservoir. Further research should be conducted on LCMV in Spain to isolate autochthonous strains and establish their serologic and genomic characterization as well as their potential pathogenicity for humans.

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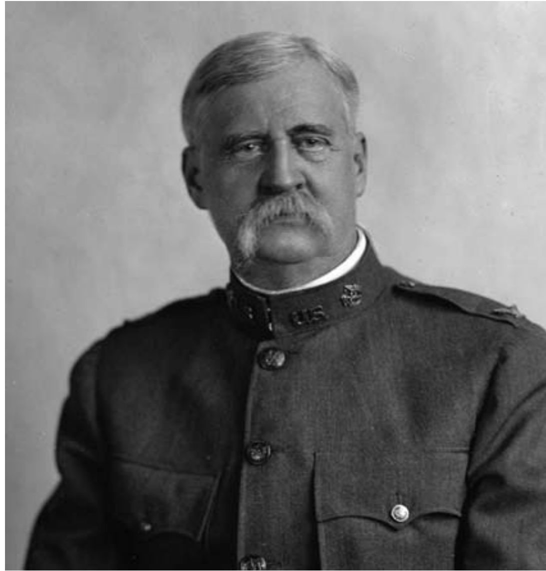

Here is a clue: he discovered the extrinsic incubation period of yellow fever.

Who is he?

A) Henry Rose Carter

B) Carlos J. Finlay

C) William Crawford Gorgas

D) Jesse William Lazear

E) Walter Reed

Decide first. Then turn the page.

Henry Rose Carter

Myron G. Schultz

This is a photograph of Henry Rose Carter (1852–1925). It shows him in his Public Health Service uniform sometime after 1915, when he attained the rank of Assistant Surgeon General. Carter was a quarantine officer and a field epidemiologist, best known for his discovery in 1898 of the extrinsic incubation period of yellow fever. His discovery led directly to the historic finding by Reed, Carroll, Agramonte, and Lazear in 1900 that mosquitoes are the intermediary hosts of the infecting agent that causes yellow fever.

A member of a distinguished Virginia family, Henry Rose Carter was born in Caroline County, Virginia, on August 25, 1852. He graduated as a civil engineer at the University of Virginia; took special courses in mathematics and applied chemistry at the same institution; and studied medicine at the University of Maryland, graduating in 1879. That same year, Carter joined the Marine Hospital Service (MHS), later the United States Public Health Service. Carter's initial MHS assignments placed him at the center of the yellow fever maelstrom. In 1879, he was detailed to Memphis and other southern cities, then in the throes of a second year of devastating epidemics. There began his lifelong interest in the epidemiology and control of yellow fever.

The Deep South of the United States, where Carter conducted most of his work on yellow fever, was affected by periodic outbreaks of the disease. This highly fatal disease had long been a scourge in the United States, especially in port cities. Lack of scientific understanding fueled these outbreaks. In 1793, for example, when a major outbreak of yellow fever struck Philadelphia, Benjamin Rush, the leading resident doctor, thought the fever came from a batch of spoiled coffee from a ship in the harbor. Not until 1900 did Walter Reed and his colleagues solve the mystery by proving that yellow fever was spread solely by mosquitoes. Until then only a few people, including Carter, were pursuing the cause of yellow fever in a methodical, scientific way.

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In 1888, Carter was assigned to the Gulf Coast Maritime Quarantine Station at Ship Island, Mississippi. Here, and at subsequent quarantine station postings along the US Gulf Coast, he thoroughly reviewed the rationale for quarantine policies with a view toward establishing uniform regulations and more thorough disinfection of vessels and minimizing interference with naval commerce. Crucial to the success of these activities was Carter's attention to the incubation period of yellow fever. Carter ably directed the MHS epidemiologic control efforts in numerous threatened regions throughout the South. Contemporaries described Carter as a man of great energy who would work in swamps 10–15 hours a day, often forgetting to shed his clothes when he went to sleep at night. He was completely absorbed in the study of mosquitoes and, from the standpoint of control and sanitation, probably knew more about them than anyone else at that time. He resembled his contemporary, Teddy Roosevelt. Like Roosevelt, he was of less than medium height and solid of physique, wore eyeglasses over a strong nose, and sported a handlebar moustache in a red face.

In conjunction with the sanitary work for the 1898 season, Carter made detailed notes on the development of yellow fever in the rural communities of Orwood and Taylor, Mississippi. The circumstances under which Carter worked were favorable for recording the time between the onset of infection among persons in isolated farmhouses and the occurrence of secondary cases among others in these same houses. Carter observed that when a case of yellow fever occurred in an isolated farmhouse, persons who visited the house at the time did not acquire the disease, but those who arrived 2 weeks later were susceptible to infection. Wade Hampton Frost, a fellow commissioned officer and later a distinguished professor of epidemiology, wrote that one needs to study all of Carter's papers to appreciate his patient persistence in collecting material, his scrupulous care in excluding every observation that might be subject to any question, his skill in analyzing the complex and puzzling data, and his clear logic in establishing a conclusion so remarkable as the existence of a definite period of "incubation in the environment." According to Carter:

> The period from the first (infecting) case to the first group of cases infected at these houses is generally from two to three weeks. The houses having become infected, susceptible individuals who visited them for a few hours fell sick with the disease in the usual period of incubation—one to seven days.

These observations pointed to the presence of an intermediate host, such as the mosquito, which having taken an infecting agent into its stomach soon after entrance of the patient into a noninfected house, was able after a certain interval to retransmit the infecting agent to other persons. Carter called this interval between the primary and secondary cases "the period of extrinsic incubation," and defined its usual range as 10–17 days.

In 1899, before he was able to publish his conclusions, Carter was assigned to Cuba. He served there as the Chief Quarantine Officer for the MHS in the aftermath of the Spanish–American War. This assignment was fortuitous because there he met Jesse Lazear, 1 of the 4 members of the US Army Yellow Fever Commission headed by Walter Reed. Carter had finally arranged for his paper's publication that year in the New Orleans Medical and Surgical Journal and gave a draft to Lazear. "If these dates are correct," Carter later recalled Lazear saying, "it spells a living host." The theory that mosquitoes are the vectors of yellow fever was first advanced in the United States by Dr J.C. Nott of Mobile, Alabama, who in 1848 wrote a paper titled "On the Cause of Yellow Fever" in which he stated his belief that insects play a role as carriers of yellow fever. In the late 19th century, the renowned Cuban physician and scientist Carlos J. Finlay devoted 2 decades to attempting to prove that mosquitoes are the vectors of yellow fever. Periodic epidemics of yellow fever ravaged the population of Finlay's native Cuba, particularly affecting the citizens of Havana, where he had set up a medical practice in 1864. During 1881–1900, Finlay carried out 102 experimental inoculations of human volunteers to prove his hypothesis. He believed he had produced some cases of yellow fever by mosquito inoculation, but the public health community remained skeptical.

One criticism of Finlay's work was that participants were never sufficiently isolated from the general population to eliminate the possibility of contracting yellow fever from sources other than Finlay's experimental mosquitoes. This, and the inconsistency with which fevers developed in the volunteers, kept the mosquito theory on the margins of acceptability. Most important, Finlay's experiments missed 2 essential parts of the development of the agent of yellow fever in mosquitoes: Finlay did not consistently apply mosquitoes to yellow fever patients during the first 3 days of their illnesses, i.e., the period of viremia, and he applied mosquitoes by feeding them on his volunteers too soon after they were presumably infected by feeding on yellow fever patients. He never considered the possibility of an extrinsic incubation period that would require time for the agent of yellow fever to incubate within mosquitoes.

The importance of Carter's observations in determining the direction of Reed's experimental work is contained in Reed, Carroll, Agramonte, and Lazear's first publication on the etiology of yellow fever in October 1900. The authors stated that 3 considerations made them turn their attention to the theory of transmission by mosquitoes:

1. Certain general facts in the epidemiology of the disease (chiefly its sharp seasonal and geographic limitations) that had led Finlay to formulate his theory of mosquito transmission.
2. The work of Ross and the Italian researchers Grassi, Bastianelli, and Bignami in demonstrating the conveyance of malaria by the mosquito (development of parasite in the mosquito; limitation to 1 genus of mosquitoes).
3. Carter's observation (1898) on the extrinsic incubation period.

Reed and his colleagues had the good fortune to begin their investigations with a correct hypothesis that was based on the three considerations cited above. By 1900, the Reed Commission had established the following facts relating to yellow fever: 1) yellow fever is transmitted by a mosquito, now known as *Aedes aegypti*; 2) to become infected, the mosquito must feed on the yellow fever patient during the first 3 days of the disease; 3) the mosquito does not become infective until 10–16 days after it takes blood from a yellow fever patient; and 4) the incubation period in humans, i.e., the time between the moment a person is bitten by an infective mosquito and the time a person's symptoms appear, does not exceed 6 days.

The third point had been established chiefly through the investigations by Carter. He called this 10–16-day interval that occurs in the mosquito the "period of extrinsic incubation." Carter's discovery of the extrinsic incubation period of yellow fever places him with Reed and Gorgas in the distinguished group of scientists and sanitarians who have made the most significant contributions to our knowledge of this disease and the methods of combating it. Walter Reed saluted Carter when he said, "I know of no one more competent to pass judgment on all that pertains to the subject of yellow fever. You must not forget that your own work in Mississippi did more to impress me with the

importance of an intermediate host in yellow fever than everything else put together."

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Suggested Reading

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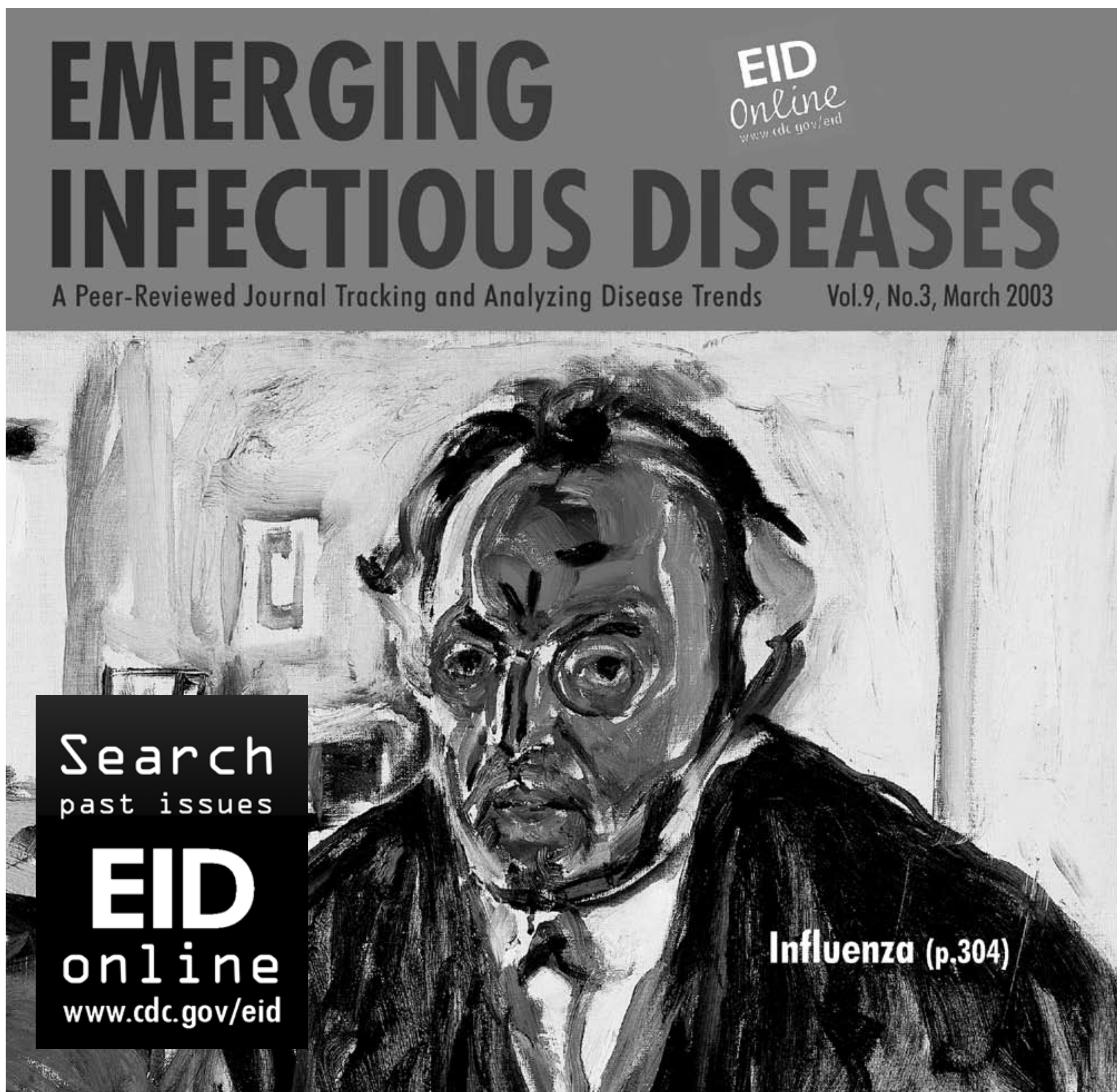

Influenza (H1N1) 2009 Outbreak and School Closure, Osaka Prefecture, Japan

To the Editor: The Osaka Prefectural Government, the third largest local authority in Japan and comprising 43 cities (total population 8.8 million), was informed of a novel influenza outbreak on May 16, 2009. A high school submitted an urgent report that ≈100 students had influenza symptoms; an independent report indicated that a primary school child also showed similar symptoms.

The Infection Control Law in Japan requires that all novel influenza cases diagnosed by physicians and confirmed by laboratory test results be reported to public health centers. Influenza A pandemic (H1N1) 2009 infection was first detected in 2 students in the same high school on May 11, 2009, followed by an outbreak in a high school in city A in northern Osaka Prefecture (online Appendix Table, available from http://www.cdc.gov/EID/content/15/10/1685-appT.htm). Two days later on May 13 in city B in middle Osaka Prefecture, a primary school student and a junior high school student were found to be infected. Infections were also detected among school children in 6 other cities on May 14; six parents of students from the first outbreak school were also infected.

We obtained anecdotal information that influenza seemed to be transmitted from infected students in the first high school outbreak to students in other schools either because students had siblings who attended other schools or students were part of the same extracurricular clubs and cram schools (lessons after school to supplement schoolwork managed by a private company). Therefore, we concluded that the influenza (H1N1) 2009 virus was spreading widely to other schools and communities and that school closures would be necessary (*1,2*).

The governor of Osaka decided to close all 270 high schools and 526 junior high schools in Osaka Prefecture from Monday, May 18, to Sunday, May 24, following the weekend days of May 16 and 17 observed at most schools. Students were ordered to stay at home (*3*). Most nurseries, primary schools, colleges, and universities in the 9 cities with influenza cases voluntarily followed the governor's decision. Antiviral drugs were prescribed by local physicians to almost all students with confirmed infection; families were given these drugs as a prophylactic measure. Family members of an infected student were also strongly encouraged to stay home at least 7 days after the student's symptoms had disappeared. Most newspapers and radio and television stations began reporting the outbreak on May 16, and a national campaign emerged in which facemask use was recommended to the public along with good hygiene practices such as hand washing and gargling.

After the school closures, the number of newly reported cases declined rapidly from 30 cases on May 17 to none by May 25. During that time, 13 schools reported only 1 new case each. After May 25, although no new cases were found among students, some sporadic cases were identified among adults by the end of May. From June 1 through June 22, twenty-five sporadic cases (of which 19 had become infected overseas) were reported, but no further outbreaks were reported in the schools.

Since June 23, smaller school outbreaks have occurred in cities in southern Osaka Prefecture. The government decided not to conduct the prefecture-wide school closure for these outbreaks. Instead, the decision regarding school closure was left to each school's administrator. The prefecture-wide school closure strategy may have had an effect on not only the reduction of virus transmission and elimination of successive large outbreaks but also greater public awareness about the need for preventive measures.

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Maximizing the Value of Drug Stockpiles for Pandemic Influenza

To the Editor: Tamiflu (oseltamivir; Roche, Indianapolis, IN, USA) is destined to be one of the few branded drugs to develop instant street recognition because of its status as 1 of only 2 licensed drugs shown to be active against the influenza A pandemic (H1N1) 2009 virus. Tamiflu is the major drug stockpiled by governments around the world in preparedness against an influenza pandemic. More than 70 governments have placed orders for Tamiflu, and at least 220 million treatment courses have been stockpiled since 2003 at a cost of $6.9 billion (*1*). Roche is producing 110 million courses for the 5 months from May to fall 2009 and will produce up to 36 million courses per month by year's end if necessary. Given the estimated world population of 6.8 billion, it is clear that, on a global basis, stockpiles are woefully inadequate. For the United Kingdom, official estimates indicate sufficient stocks currently exist for half of the population (*2*).

Given the high cost of these stockpiles, every effort should be made to maximize usage of the drug. Most of us are aware of shelf-life assignment to foods, a concept first applied to drugs well before its adoption by food manufacturers. Shelf-life extension could potentially yield significant cost savings in the event stockpiled drugs are not required for use within the typical 5-year shelf life. We use Tamiflu as a case example to suggest how this could be done through careful evidence-based risk assessment.

The chemical integrity of any medicine, including Tamiflu, is important because decomposition may lead to loss of activity or formation of toxic products. For formulated products, decomposition may lead to impaired bioavailability. As it became obvious that there were inadequate drug stockpiles even in affluent countries, one of our authors (A.L.W.P., who had served as a member of the UK Committee on Safety of Medicines [*3,4*]) wrote to his local member of parliament (coauthor N.P.) to suggest that the government institute a program to extend the shelf life of drug stockpiles. A.L.W.P. argued that the relatively minor development work necessary to implement a shelf-life extension program would be highly cost-effective. The UK Department of Health then initiated a collaboration with Roche to extend the shelf life of Tamiflu. On May 8, 2009, the European Medicines Agency independently advised that new batches of Tamiflu would have a shelf life of 7 years instead of only 5 years (*5*).

Oseltamivir is a prodrug that needs metabolic activation (Figure) (*6*). Prodrugs are used typically to reduce toxicity caused by functional groups such as the carboxylate ion, to alter release properties (e.g., prolonging action of antipsychotic agents), or to improve absorption (bioavailability) by making the drug more lipophilic.

Oseltamivir carboxylic acid has poor bioavailability; <5% orally compared to 80% for oseltamivir, the parent drug (*7*). The carboxylate is the only major metabolite and the principal degradation product (*7,8*). Therefore, with poor storage the major risk is reduced activity through reduced absorption rather than formation of toxic by-products.

Health agencies also stockpile other drugs (*9*), most notably antimicrobial drugs and vaccines. Shelf-life extension would need to be assessed on a product-by-product basis. For example, antimicrobial drugs are often quite unstable and the toxicologic implications are less clear; some evidence suggests that allergenic polymers could be formed while the drugs are in storage. On the positive side, antimicrobial drugs are considerably less expensive than neuramidase inhibitors such as Tamiflu, making antimicrobial stockpiles less costly to replenish.

Products with more complicated delivery systems, such as zanamivir in inhalers, would require more validation. For biological products in complex formulations such as vaccines, stability validation may not be cost-effective.

Given the high costs involved in maintaining adequate drug stockpiles, attempts should be made to optimize the value of drugs; shelf-life extension is one of the easiest and most cost-effective ways of doing this. We suggest that governments undertake a systematic program for iterative shelf-life extension, ideally cooperatively. The considerable financial savings could mitigate drug shortages of expensive antiviral drugs. The chemical profile of oseltamivir and its degradation pathway suggest that extending the shelf life of Tamiflu to >20 years should be feasible. Storage in dry airtight containers should be able to maintain the integrity of the product for >7 years.

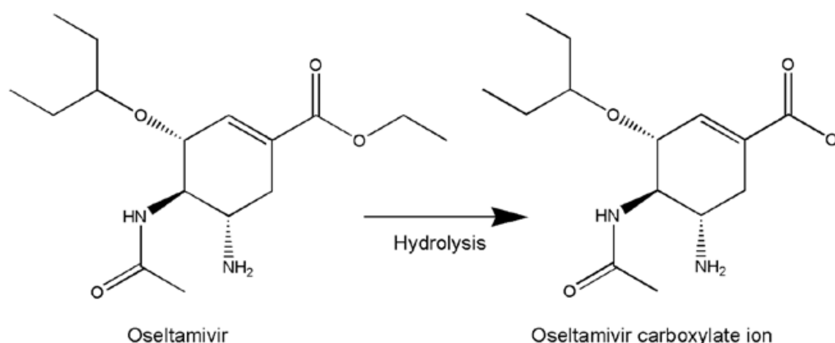

Figure. Metabolic activation of oseltamivir to carboxylic acid.

During a pandemic, when supplies are unavailable, the balance of benefit to harm would favor using the expired product.

The 1918 influenza pandemic is estimated to have killed 50 million persons worldwide (*10*), many in developing countries. By better safeguarding available drug stockpiles, more drugs could be made available to poorer countries that have few drugs stockpiled.

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Intrafamilial Transmission of Methicillin-Resistant *Staphylococcus aureus*[1]

To the Editor: Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infection was first described in our region over 15 years ago (*1*). More recently, CA-MRSA has become a global concern and is now a common cause of skin and soft tissue infections in the United States (*2*). An association between severe CA-MRSA infection (e.g., necrotizing fasciitis and pneumonia) and the synergohymenotrophic exotoxin Panton-Valentine leukocidin (PVL) has been made (*3,4*). Reports have documented CA-MRSA transmission among household members; however, most cases have been mild or moderate infections or asymptomatic colonization (*5–7*). We describe intrafamilial transmission of a PVL-containing CA-MRSA clone common in Australia (ST30-MRSA-IV) between a nurse who experienced recurrent abscesses and her husband, who died of severe pneumonia.

In July 2006, a 61-year-old previously healthy nurse (Mrs A) sought treatment for an infected seborrheic cyst of the scalp. Culture of pus yielded MRSA that was susceptible to clindamycin. She was treated with oral clindamycin. After resolution of the infection, topical MRSA decolonization therapy with 3% hexachlorophane body wash (daily), 20% cetrimide shampoo (3×/wk), and 2% mupirocin nasal ointment (3×/d) was administered for 10 days, as per our institutional protocol for MRSA-colonized healthcare workers. Subsequently, MRSA surveillance swabs from the nose, throat, and scalp obtained weekly for 10 weeks and cultured on selective MRSA chromogenic agar and in selective broth enrichment media were negative. Household members were not screened for MRSA colonization.

Six months later, in January 2007, the patient's husband (Mr A), a 60-year-old smoker who was her only household contact, was admitted with a 1-day history of dyspnea, pleuritic chest pain, cough with sputum, fever, vomiting, and diarrhea. On admission, he was unwell, with tachycardia (pulse rate 132 bpm), hypotension (95/60 mm Hg), tachypnea (40 breaths/min), and hypoxia (oxygen saturation 93% on 15 L O_2/min). A chest radiograph showed bilateral infiltrates and a right pleural effusion. He was diagnosed with community-acquired pneumonia and treated with intravenous ceftriaxone and azithromycin as per local protocol. However, within 12 hours, his condition deteriorated, necessitating admission to the intensive care unit for ventilation and inotropic support.

[1]Results presented in part at the Australasian Society for Infectious Diseases Annual Scientific Meeting, Sunshine Coast, Queensland, Australia, 2008 April 2–5.

Broncho-alveolar lavage (BAL) fluid demonstrated gram-positive cocci in tetrads, and intravenous vancomycin and dicloxacillin were added to therapy. Despite aggressive supportive measures, Mr A's condition continued to deteriorate, and he died 28 hours after admission. MRSA was subsequently cultured from blood, sputum, and BAL fluid; an autopsy was not performed.

In June 2007, Mrs A sought treatment for an abscess with cellulitis on the left thigh. The abscess was surgically drained, and cultures again yielded MRSA. She was treated with intravenous and oral clindamycin for 10 days and subsequently underwent repeat MRSA decolonization therapy; again, swabs taken 1×/wk for 10 weeks postdecolonization were negative.

Molecular typing of the MRSA isolates obtained from Mrs A at the time of her initial skin infection, Mr A's blood culture, and Mrs A's second skin infection was performed by using contour-clamped homogenous electric field electrophoresis (CHEF) according to a previously described method (*8*) (Figure). The CHEF patterns were indistinguishable and were identical to the known CHEF pattern for ST30-MRSA-IV (*9*). All 3 isolates contained the *lukF-PV/lukS-PV* genes that encode PVL and had the same antibiogram (i.e., isolates were resistant only to β-lactam antimicrobial agents).

We describe intrafamilial MRSA transmission (defined as ≥2 family members who live at the same postal address and who are colonized or infected with a MRSA strain having the same CHEF pattern) that resulted in a fatal outcome. The MRSA strain responsible (ST30-MRSA-IV, or Western Samoan phage pattern/Oceania strain MRSA) is a common cause of CA-MRSA infection in Australia.

Recurrent MRSA infection developed in Mrs A several months after completion of apparently successful MRSA decolonization therapy. We could not determine whether this recurrence was because of persistent MRSA colonization not detected by surveillance (e.g., perineal or gastrointestinal colonization) or whether Mrs A was successfully decolonized but Mr A's colonization/infection resulted in recolonization and subsequent infection. Whatever the explanation, this case highlights a potential weakness in MRSA surveillance programs that rely on short-term, limited-site surveillance.

A comprehensive MRSA search-and-destroy policy in place for over 25 years has prevented MRSA from becoming endemic in our institution (*10*). However, the rapidly changing epidemiology of MRSA in becoming a predominantly community pathogen represents a significant challenge to the ongoing success of this policy. In response to this challenge, the Western Australian Department of Health has implemented a community-based MRSA search-and-destroy program for patients with MRSA infection caused by exotic PVL-positive clones (e.g., ST30-MRSA-IV, ST93-MRSA-IV, ST80-MRSA-IV, and ST8-MRSA-IV/USA300). This program includes treatment/decolonization therapy for the index case, screening of household members for MRSA infection/colonization, and simultaneous treatment/decolonization if MRSA is identified. Although a similar approach has proved successful in Denmark (*6*), whether this success can be sustainable on a larger scale remains to be seen.

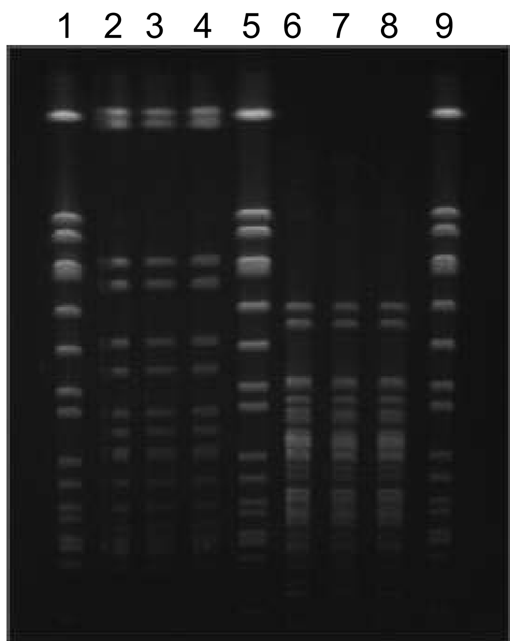

Figure. Contour-clamped homogenous electric field electrophoresis of *Staphylococcus aureus* isolates. Lanes 2, 3, and 4 (*Sma*1 restriction): methicillin-resistant *S. aureus* (MRSA) isolated from Mrs A's first infection, Mr A's blood culture, and Mrs A's second infection, respectively. Lanes 6, 7, and 8 (*Apa*1 restriction): MRSA isolated from Mrs A's first infection, Mr A's blood culture, and Mrs A's second infection, respectively. Lanes 1, 5, and 9: *S. aureus* NCTC8325.

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We thank our patient for allowing us to discuss her case in this report.

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Rhombencephalitis and Coxsackievirus A16

To the Editor: Hand, foot, and mouth disease (HFMD) is a common illness in children and is mainly caused by coxsackievirus A16 (CA16) and enterovirus 71 (EV71). Although its clinical course is usually uneventful and most patients experience a full recovery, serious neurologic complications, including encephalitis, can occur secondarily to HFMD caused by EV71. Such neurological complications occurred during an epidemic in Taiwan in 1998 (*1*). Encephalitis caused by EV71 is characterized by rhombencephalitis, which is a combination of brainstem encephalitis and cerebellitis. Signs and symptoms of rhombencephalitis are irritability, myoclonus, ataxia, and cranial nerve involvement (*1*). In contrast to EV71, HFMD caused by CA16 is associated with few neurologic complications with the exception of infrequent aseptic meningitis (*2*). We report a case of rhombencephalitis that developed in an infant as a complication of HFMD caused by CA16.

HFMD was diagnosed in a 23-month-old girl on the basis of high fever (>40°C, 3 d duration), stomatitis, and multiple papules on her palms, soles, and buttocks. Her illness occurred in the summer of 2007, when sentinel surveillance in the region indicated an epidemic of HFMD caused by both CA16 and EV71. She was admitted to our hospital in Fukoka, Japan, on day 4 of illness because of abnormal eye movement, irritability, and inability to stand. She had intermittent to-and-fro, horizontal oscillations of the eyes (ocular flutter). She also had truncal and limb ataxia and myoclonus in her head and limbs. Brain magnetic resonance imaging (MRI) showed T1-low and T2-high bulbopontine and cerebellar lesions around the fourth ventricle (Figure). Peripheral blood showed a mild leukocytosis (13.13×10^9/L) and a C-reactive protein level within reference range (0.9 mg/L). Blood chemistry results were unremarkable. Cerebrospinal fluid (CSF) examination showed mononuclear pleocytosis (74/µL) with normal protein and glucose levels. CA16 was isolated from her stool specimen on day 4 of illness. Based on reverse transcription–PCR, CSF was negative for enterovirus RNA.

Without specific treatment, our patient's fever resolved on day 5 of illness. The myoclonus, ocular flutter, and irritability subsided by day 16, when MRI findings returned to normal. Ataxia disappeared gradually ≈1 month after onset, and no neurologic sequelae occurred. Neutralizing antibody titers against CA16 and EV71 on day 21 of illness were 32 and <8, respectively. Based on the sequence analysis of the partial VP1 region (876 bp), we classified the patient's CA16 strain phylogenetically as genetic lineage C (*3*). This lineage was identical to lineage 2 (*4*), which became the dominant circulating strain in Asia, including Japan, after the late 1990s (98.2% identical to the 1018T/VNM/05 strain isolated in Vietnam

in 2005 [GenBank accession no. AM292441]) (*4,5*).

The patient's symptoms of irritability, ataxia, myoclonus, and ocular flutter 3 days after the onset of typical HFMD manifestations, along with CSF mononuclear pleocytosis and the lesions around the fourth ventricle shown on MRI, led to the diagnosis of rhombencephalitis associated with HFMD. Virologic examination, including virus isolation and antibody assay, suggested that HFMD was caused by CA16 but not by EV71, although the possibility that CA16 infection was coincidental to the rhombencephalitis could not be excluded.

Although rhombencephalitis can be related to various infectious agents (*6*), HFMD complicated by this condition has been exclusively caused by EV71 (*1,7,8*). In Japan, CA16 and EV71 are consistently the 2 major causative agents of HFMD (*9*). EV71 infection is much more frequently associated with serious neurologic complications and fatalities than is CA16 (*2*). Since 1997, several HFMD outbreaks with multiple cases of severe neurologic pathologies have occurred in the Asia-Pacific region including Malaysia, Taiwan, and Western Australia (*1,7,8*). These complications were associated exclusively with EV71.

Why rhombencephalitis developed in our patient with CA16-related HFMD is unclear. One possibility is that the CA16 strain might have acquired neurovirulence by genetic recombination with EV71; phylogenetic evidence supports the possible occurrence of intertypic recombination involving EV71 and CA16 (*10*). Through phylogenetic analysis of the VP1 sequences, we classified the CA16 strain isolated from the patient's stool phylogenetically as genetic lineage C (*3*), a lineage which was identical to lineage 2 (*4*). Genetic recombinations among enteroviruses occur mainly in noncapsid regions (*10*). We did not conduct phylogenetic analysis of the noncapsid regions of the patient's CA16 strain because sequence data on the regions were very limited. Besides the viral factors, host factors, such as immune status and environmental factors, could confer susceptibility to neurologic complications of enteroviral infections.

Rhomobencephalitis associated with HFMD developed in this patient and was caused by CA16. Therefore, neurologic complications, including rhombencephalitis, should be considered even when CA16 is the prevalent virus causing HFMD.

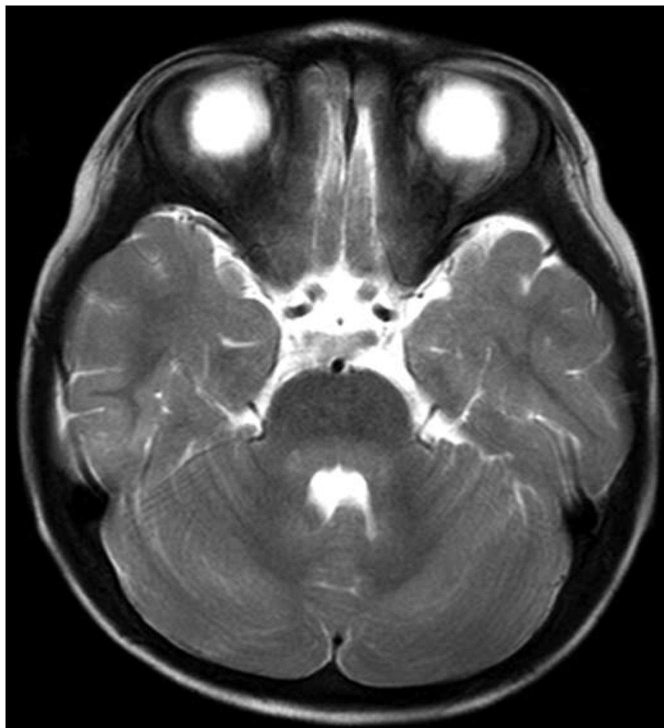

Figure. Axial T2-weighted slice of brain by magnetic resonance imaging, showing hyperintensity lesions in the pons and cerebellum around the fourth ventricle.

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Japanese Encephalitis in Hill and Mountain Districts, Nepal

To the Editor: Nepal, a landlocked country in Southeast Asia with an estimated population of 27 million, is divided administratively into 5 regions; 75 districts comprise 3 ecological zones that run from east to west. Altitude increases from south to north: the 20-district Terai plains in the south, the hill region in the center with 39 districts, and the 16-district mountain regions in the north. Japanese encephalitis (JE) is seasonally endemic to the Terai region, which borders the northern India states of Uttar Pradesh and Bihar. The first outbreak of JE in Nepal was reported in 1978 from the Terai district of Rupendehi (*1*). Since then, JE infection has been reported in animal reservoirs and in humans throughout the Terai region (*1–5*). Although few publications describe the presence of JE outside the Terai, an outbreak of JE in Kathmandu valley in the hill region was confirmed in 1997 (*6*), and a 2006 study reported JE endemicity in Kathmandu Valley (*7*). In recent years, the Ministry of Health and Population in Nepal has introduced public health interventions, including mass immunization campaigns, for JE prevention in these known JE-endemic areas.

JE cases are captured through acute encephalitis syndrome (AES) surveillance conducted by the government of Nepal, with support from the World Health Organization (WHO), through a national sentinel surveillance network. From 2004 through 2006, a total of 108 laboratory-confirmed JE cases were reported from hill or mountain districts (excluding Kathmandu Valley). However, travel histories for case-patients were not available for these years to determine the origin of JE infection. We conducted a study to provide evidence of JE endemicity in hill and mountain districts of Nepal (excluding Kathmandu Valley).

Laboratory-confirmed JE case-patients identified in 2007 who reported residence in 1 of the 52 hill or mountain districts, excluding the 3 hill districts of the Kathmandu Valley, were followed up by surveillance medical officers. All patients (or next of kin if the patient was deceased or unavailable) were visited at home or contacted by telephone to confirm their residence and travel history during the 30 days before the onset of symptoms. Data and sample collection procedures and laboratory methods used for JE diagnosis were as previously reported by Partridge et al. (*7*). Patients were identified by the AES surveillance system if patients' symptoms met the case definition for AES adopted from WHO guidelines (www.who.int/vaccines-documents/DocsPDF06/843.pdf), i.e., acute onset of fever and a change in mental status (e.g., confusion, disorientation, coma, or inability to talk); or if the patient experienced a new onset of seizures (excluding simple febrile seizures) or was identified as having AES, JE, or viral encephalitis. The study population included any person of any age who reported being a resident of 1 of the 52 hill or mountain districts (excluding Kathmandu Valley), who had been seen at any AES reporting site from January 1 through December 31, 2007, and who had been confirmed to have JE antibody by immunoglobulin M capture ELISA on a serum or cerebrospinal fluid (CSF) specimen.

In 2007, a total of 360 AES cases were reported from 40 hill or mountain districts. Of the 344 reported AES cases for which diagnostic samples were obtained, 90 (26%) were laboratory confirmed as JE from 21 hill and 3 mountain districts. Among laboratory confirmed JE cases, CSF samples were collected from 13 (14%) patients and serum samples from 77 (86%) patients (Table). The largest number of AES and laboratory-confirmed JE cas-

Table. Characteristics of Japanese encephalitis case-patients, hill and mountain districts (excluding Kathmandu Valley), Nepal, 2007

Characteristic	No. (%) case-patients
Age, y*	
<1	6 (7)
1–14	67 (74)
≥15	71 (19)
Sex	
M	59 (66)
F	31 (34)
Death associated with Japanese encephalitis	5 (6)
Specimen type collected	
Serum	77 (86)
Cerebrospinal fluid	13 (14)
History of travel outside district of residence†	
No	84 (94)
Yes	3 (3)
Unknown	3 (3)

*Range 4 mo–79 y.
†During the 30 days before onset of symptoms.

es were reported during the monsoon months of August (23 cases) and September (33 cases). The higher proportions of cases in male patients and in those <15 years of age are consistent with the literature (*3*,*8*,*9*).

Although most JE cases in Nepal occur in the lowland plains Terai region bordering India, our findings provide evidence that JE virus is also a cause of acute encephalitis in hill and mountain districts. This finding is consistent with the report of JE endemicity in the Kathmandu Valley, which is located in the hill region (*7*). Although the presence of both JE vectors and amplifying hosts has been confirmed in the hill region (*10*), additional studies are needed in high-altitude mountain districts to confirm the presence of environmental and ecological conditions that promote JE virus transmission. The national JE prevention and control program has focused on the Terai region and, more recently, on the Kathmandu Valley. However, results from our study suggest that Nepal may also have to consider the existence of JE in hill and mountain districts when developing future JE prevention and control programs. In addition, immunization against JE should be recommended for tourists to Nepal, even for those only planning to trek in the hills and mountains.

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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.

Lessons from a Special Service for Public Health, Brazil

To the Editor: Many thanks for your interesting and informative special section on infectious diseases in the Amazon Region (*1*). Your readers should also be interested in a little known, but extremely successful, sustainable health program that had its start in the Amazon.

In 1942, the governments of Brazil and the United States agreed to establish a special service for public health (called the Serviço Especial de Saúde Pública). The purpose of this program was to improve health conditions in key areas in the Amazon, expedite the collection and export of native rubber, and counteract the growing influence of Nazi Germany in Latin America (*2*). The program spread to the Vale do Rio Doce, where there were resources of iron ore, mica, and optical quartz, which were important for the war effort. Although the program eventually moved to all states of Brazil, the Amazon program remained an important activity for ≈50 years before it was integrated into the Brazilian Ministry of Health (*3*).

The program in the Amazon focused primarily on infectious disease. It comprised programs of immunization, provision of small sustainable water systems, development of privy programs (sewer systems in the larger centers of population), malaria control, improvement of residences and living conditions for Chagas disease control, epidemiologic intelligence, and extensive training for auxiliary and professional personnel.

The effects of this program are shown by the increase in life expectancy for all age groups, with an increase of >10 years for those childhood age groups for whom infectious disease control would have the greatest effect from 1939–1941 to 1950–1951 (*4*). This program contains many lessons for the planners of health and disease control projects in tropical, low-income countries.

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Ceftazidime-Resistant *Salmonella enterica*, Morocco

To the Editor: Nontyphoidal salmonellosis (NTS) is a major foodborne illness worldwide. Extended-spectrum cephalosporins (ESCs) are currently preferred drugs for treatment of children with NTS. However, resistance to ESCs has emerged worldwide and has become a serious public health problem. This resistance is caused by production of various class A extended-spectrum β-lactamases (ESBLs) and class C cephalosporinases in *Salmonella enterica* (*1*).

National surveillance systems, ideally based on integration of data for animals, food, and humans, are needed to develop strategies for containing antimicrobial drug resistance. Such systems are primarily based on a network of public or private clinical laboratories that refer *Salmonella* isolates to public health laboratories for identification. However, this laboratory-based surveillance system in developing countries is hampered by cost constraints and poor access to quality health facilities, resulting in a low rate of isolation of bacterial pathogens from patients having mild infections. These constraints account for the lack of data and underestimation of the number of NTS cases in many countries, including Morocco.

According to the World Health Organization Global Salm Surv database (www.who.int/salmsurv/activities/en), the Moroccan National Institute of Hygiene reported only 210 human non-Typhi isolates and 999 animal non-Gallinarum isolates during 1999–2003. Antimicrobial drug resistance data are extremely rare. We report the presence of nontyphoidal *Salmonella* isolates resistant to ESCs during an outbreak of food poisoning and in food products in Morocco.

In March 2008, an *S. enterica* serotype Typhimurium strain was isolated from stool samples of 45 persons who had attended a wedding ceremony in Errachidia. Clinical symptoms were diarrhea, vomiting, and stomach cramps, beginning 24–72 hours after these persons had eaten a tagine prepared with poorly cooked broiler chickens. Five patients were hospitalized for 3 days, but no deaths were recorded. *S. enterica* serotype Typhimurium was isolated from leftovers of a broiler carcass stored in a refrigerator.

PulseNet (http://pulsenetinternational.org/pulsenet/pulsenet.asp)

standard pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested chromosomal DNA showed that human and poultry isolates had identical profiles. Antimicrobial drug susceptibility was determined by the disk diffusion method and E-tests, as described (*2*). Isolates were resistant to penicillins and ceftazidime but were susceptible to other antimicrobial drug classes tested. A positive double-disk synergy test result suggested that these strains produced an ESBL. Isolates showed higher levels of resistance to ceftazidime (MIC 128 mg/L) than to ceftriaxone (MIC 8 mg/L).

For identification of the ESBL gene, we conducted PCR amplifications of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} group genes, as described (*3*). Only the SHV amplicon was obtained, and DNA sequencing showed this amplicon to be 100% identical to bla_{SHV-12}. Resistance to ESCs and the bla_{SHV-12} gene were transferred into *Escherichia coli* by conjugation. An ≈60-kb plasmid was isolated from *E. coli* transconjugants and the parental strain. PCR-based replicon typing analysis identified replicon IncI1 (*4*). Although the broilers had been reared locally, no environmental investigation was conducted.

In November 2007, an *S. enterica* serotype Newport strain was isolated from a pastry made with locally produced eggs during a food survey conducted in southern Morocco. The isolate was resistant to penicillins, cefoxitin (MIC 128 mg/L), ceftriaxone (MIC 64 mg/L), ceftazidime (MIC 128 mg/L), streptomycin, sulfonamides, chloramphenicol, and tetracycline. We identified the bla_{CMY-2} gene carried by a 210-kb nonconjugative plasmid of replicon IncA/C. These CMY-2–producing isolates are also known as *Salmonella* Newport multidrug-resistant (MDR)–AmpC. *Xba*I-PFGE showed a profile similar to the New8a profile described in 2003 in France during a small outbreak linked to consumption of imported horse meat (*2*).

ESC-resistant *Salmonella* isolates have been reported in Morocco (*5*). This report described a serotype Typhimurium clone that produced TEM-3 that was isolated from 10 children hospitalized in Casablanca in 1994. Few studies have been conducted on ESC-resistant *S. enterica* in northern Africa, and most have reported hospital-acquired infections (*1*). Our study identified ESC-resistant *Salmonella* strains in the human food chain and in poultry for human consumption in Morocco. *Salmonella* isolates resistant to ESCs were not identified in food during 2002–2005 at the Institut Pasteur de Casablanca (104 *Salmonella* isolates from 11,516 food samples) (*6*). Emergence in poultry and humans of an MDR serotype Keurmassar strain that produced SHV-12 was reported in Senegal in 2001 (*7*).

Although CMY-2 was originally identified in a serotype Senftenberg isolate from a child in Algeria (*8*), we report the *Salmonella* Newport MDR-AmpC strain in Africa. *Salmonella* Newport MDR-AmpC isolates were reported in 1998 in the United States (*9*), where they quickly spread to cattle and humans. Recent potential spread of this strain into poultry in the United States was suggested by Varma et al. (*10*). Because of the risk for spreading, an efficient national antimicrobial drug resistance monitoring system for foodborne pathogens in Morocco is required to prevent dissemination of bacterial strains resistant to first-line antimicrobial drugs in humans.

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Group B Streptococcus Meningitis in Child with Cochlear Implant

To the Editor: *Streptococcus agalactiae*, designated group B streptococcus (GBS), is a major cause of infections in neonates and young infants (*1*). Invasive GBS disease in children beyond infancy is uncommon, occurring mainly as bacteremia without a focus; meningitis caused by GBS is rarely reported (*2*). Cochlear implant recipients have been documented as having a higher rate of postimplantation bacterial meningitis than a cohort of the same age in the general US population (*3*). However, no cochlear implant recipient described has been reported to be infected with GBS. We report a case of GBS meningitis in a 6-year-old boy with a cochlear implant.

The patient was hospitalized in 2007 with a 1-day history of fever, headache, and vomiting. His medical history indicated congenital bilateral deafness diagnosed at 1 month of age and consistent with Patterson syndrome (i.e., unusual facies, deafness, bronzed hyperpigmentation of the skin, cutis laxa, mental retardation, and bony deformities) (*4*). At 4 years of age, he received a right-ear cochlear implant with good functional result. Preoperative high-resolution computed tomography of the temporal bones showed bilateral inner ear malformations of both the cochlear and vestibular labyrinth, conditions consistent with bilateral Mondini deformity (*5*). Mastoids and middle ears were well aerated. No evidence of cerebrospinal fluid leak appeared during physical examination or imaging. He received a dose of 23-valent pneumococcal polysaccharide vaccine.

At the time of hospital admission, he was somnolent but could be aroused and was cooperative. He had nuchal rigidity, dysmorphic facies, and oligodactyly. Fundi, skin, and ears were unremarkable on examination. Lumbar puncture showed a total protein level of 204 mg/dL, a glucose level of 1.6 mmol/L (blood glucose 3 mmol/L), and 4,800 leukocytes/mm^3 with 88% neutrophils; no bacteria were seen on the Gram stain. Blood count was remarkable for leukocytosis of 30,000/mm^3 and neutrophil predominance.

The patient received treatment with dexamethasone, vancomycin, and ceftriaxone; after treatment, his condition improved rapidly. Blood culture was sterile, but GBS grew in the cerebrospinal fluid culture (the isolate being resistant only to tetracycline). Therapy was continued with ampicillin for 3 weeks. Repeated testing of his hearing and speech perception with the cochlear implant showed no deterioration.

GBS plays a major role in early- and late-onset infections in neonates and young infants (*1*). Infections in older children and adults have been described, especially in elderly patients or those suffering from chronic conditions such as diabetes mellitus, malignancy, or HIV infection (*6*). A review of medical records of patients with GBS infections over a 7-year period at a children's hospital in Memphis, Tennessee, USA, showed that, among 18 patients >3 months of age (13% of all GBS infections in the study), bacteremia was most commonly reported; 3 cases of ventriculo-peritoneal shunt infections were recorded, but no cases of meningitis without foreign devices were found (*2*). GBS meningitis in children beyond infancy is rare; only a few cases have been reported (*7*).

Cochlear implantation is the standard treatment for children and adults affected by severe and severe-to-profound sensorineural hearing loss. The implant is a neural stimulator with an electrode array surgically placed near the auditory nerve fibers in the scala tympani of the cochlea. Pediatric cochlear implant recipients were found to be at higher risk for developing bacterial meningitis than children in the general US population (*3*). Increased risk was evident in the perioperative period but extended to >2 years postimplantation (*8*). Most meningitis cases were associated with an implant with a positioner, a silastic wedge inserted next to the implanted electrode in the cochlea to position the electrode closer to the cochlear nerve endings and thus facilitate electrical signal transmission. Most of those infections were caused by *Streptococcus pneumoniae*, and none by GBS (*3*,*8*). In our patient, the implant did not include a positioner. The timing of meningitis was consistent with the timing indicated in previous reports, but the infecting organism was unique.

Inner ear malformations themselves are associated with increased risk for meningitis (*9*). The patient reported here had bilateral inner ear malformations; therefore, estimating the relative role of the deformity compared with the cochlear implant's role in the pathogenesis of meningitis in

his case is difficult. Meningitis in patients with inner ear malformations is associated with bacteria (e.g., *S. pneumoniae* and *Haemophilus influenzae*) that colonize the upper airways. The prevalence of oropharyngeal colonization with GBS is low (≈5%), explaining the rarity of GBS meningitis (*10*). Unlike for pneumococcal meningitis, which can be prevented at least partially by vaccination, no vaccine is available for GBS.

Our report adds another example to the growing spectrum of invasive GBS disease beyond infancy. GBS is uniformly susceptible to penicillin; therefore, treatment directed at common causes of bacterial meningitis is also appropriate for GBS (*1*,*10*). Cochlear implant recipients with symptoms of fever, otitis media, or headache should be carefully assessed; if meningitis is diagnosed, GBS should be considered as a possible causative organism.

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Severe Necrotizing Pneumonia in Children, Houston, Texas, USA

To the Editor: Routine vaccination of children with the 7-valent pneumococcal conjugate vaccine (PCV-7; Wyeth Pharmaceuticals, Collegeville, PA, USA), initiated in the United States in 2000, was followed within 2 years by an extensive and rapid decline in invasive pneumococcal disease (IPD) (*1*). During the past few years, increasing frequency of invasive disease including necrotizing pneumonia caused by serotypes not included in the vaccine has been reported (*2*). We show an expanded pattern of the changing spectrum of the disease associated with nonvaccine serotypes through this report of 4 cases of necrotizing pneumonia in children, caused by *Streptococcus pneumoniae* serotype 19A.

Over a 6-month period ending in March 2008, 4 children (median age 3.6 years, 1 with asthma) (Table) were brought to our hospital with signs of respiratory distress and a 4- to 7-day history of fever and cough. All had decreased breath sounds or crackles, and radiologic studies showed evidence of complicated pneumonia, which led to hospital admission (3 to an intensive care unit [ICU]). *S. pneumoniae* 19A was isolated from normally sterile sites with each child. All received intravenous antimicrobial drugs followed by an oral antimicrobial drug regimen and were discharged in good health. By reviewing immunization records, we confirmed that all had completed the PCV-7 series before becoming ill.

During the same period, complicated pneumonia was identified in 7 other inpatients by using the International Classification of Diseases, 9th revision, codes for necrotizing pneumonia and empyema and Current Procedural Terminology codes

Table. Characteristics of patients in study of severe necrotizing pneumonia in children, Houston, Texas, USA, 2007–2008*

Characteristic	Patient			
	1	2	3	4
Age, y	4.1	2.8	3.4	3.7
Race	Black	White	Black	Black
Sex	M	M	F	F
Completed PCV-7 series	Yes	Yes	Yes	Yes
Co-illnesses	Asthma	None	None	None
Clinical signs				
Date	2007 Nov	2007 Nov	2007 Dec	2008 Feb
Temperature, °C	37.6	**38.9**	37.9	**38.7**
Pulse, beats/min	**154**	**160**	**162**	**188**
Respiratory rate, breaths/min	**70**	**60**	**40**	**42**
Blood pressure, mm Hg	95/53	**122/84**	**110/78**	106/69
Oxygen saturation on room air, %	**85**	**90**	100	**86**
Symptoms/signs (hospital)	Fever, cough, dyspnea, headache, abdominal pain, decreased activity	Fever, cough, dyspnea, congestion	Fever, cough	Fever, cough, decreased appetite
Hospital course				
Site pathogen isolated	Blood and pleural fluid	Blood and pleural fluid	Pleural fluid	Blood
IV antimicrobial drugs, d	21	9	14	33
IV antimicrobial drugs (MIC, μg/mL)	Cefotaxime (0.06)	Vancomycin (0.75); ceftriaxone (0.19)	Vancomycin (1); resistant to cephalosporin	Vancomycin (0.50); ceftriaxone (0.064)
VATS† (duration, d)	Yes (2)	Yes (2)	Yes (2)	No
ICU stay	Yes	Yes	No	Yes
Intubated (duration, d)	Yes (5)	Yes (5)	No	Yes (22)
Duration of hospitalization, d	22	11	15	28
Oral antimicrobial drugs after discharge (duration, d)	Cefdinir (7)	Cefdinir (14)	Linezolid (14)	Cefdinir (7)

*PCV, pneumococcal conjugate vaccine; IV, intravenous; VATS, video-assisted thoracoscopic surgery; ICU, intensive care unit. **Boldface** indicates clinically significant differences.
†Hospitalization day that VATS was performed.

for thoracoscopic surgery (median age 4.3 years); 2 had asthma, 1 had congenital diaphragmatic hernia with resultant left lung hypoplasia. No causative organism was identified for any of these cases.

As illustrated by our 4 cases, serotype 19A is emerging as an increasing cause of severe disease such as complicated pneumonia. Although the incidence of IPD in general has decreased since the introduction of PCV-7, the emergence of nonvaccine serotypes as a cause of severe disease is becoming more prevalent. Among 8 geographic areas in the United States, the incidence of IPD caused by nonvaccine serotypes in children <5 years of age increased from 16.3 cases/100,000 population to 19.9 cases/100,000 population, respectively, from prevaccine years 1998–1999 to 2004 (*3*).

Because organisms were not isolated from the 7 other patients with necrotizing pneumonia during the same period, we are unable to comment on whether *S. pneumoniae* 19A was the predominant cause of necrotizing pneumonia in our study. However, in comparing our patients with these 7 patients, those with *S. pneumoniae* 19A appear to have had a more complicated course of illness, longer hospital stays (mean 19 days vs. 13 days), and a longer course of intravenous antimicrobial drugs (mean 19.2 days vs. 17 days). Although these 7 patients required more video-assisted thoracoscopic surgery than did our 4 patients (100% vs. 75%), those with *S. pneumoniae* 19A necrotizing pneumonia had a more severe clinical course of illness resulting in more ICU admissions (75% vs. 29%) and intubations (75% vs. 14%).

All 4 of our patients had completed the PCV-7 series before becoming ill. The emergence of nonvaccine serotypes as a cause of severe disease may be caused by serotype replacement and increased nasopharyngeal carriage of nonvaccine serotypes after receiving PCV-7 (*4*). Our report supports the theory of serotype replacement. The increasing incidence of invasive disease caused by nonvaccine serotypes has prompted development of an expanded pneumococcal vaccine to include serotypes 1, 3, 5, 6A, 7F, and 19A in addition to those covered by PCV-7 (*5*). The need for this expanded vaccine is becoming more evident as the number of children with severe pneumococcal disease due to current nonvaccine serotypes increases.

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Human Bocavirus 2 in Children, South Korea

To the Editor: In 2009, Kapoor et al. and Arthur et al. published reports on the prevalence of the newly identified parvovirus, human bocavirus 2 (HBoV-2), in fecal samples (*1,2*). HBoV-1 had been discovered in 2005 (*3*), and reports indicate its possible role in respiratory diseases such as upper respiratory tract infections, lower respiratory tract infections (LRTIs), and in exacerbation of asthma (*4*); in these diseases, the virus co-infects with other respiratory viruses (*5*). Systemic infection with HBoV-1 and possible association of this virus with other diseases such as gastroenteritis, Kawasaki disease, and hepatitis have been reported (*6–8*). We looked for HBoV-2 in clinical samples from children with various diseases, including acute LRTIs, Kawasaki disease, Henoch-Schönlein purpura, and hepatitis.

During September 2008–January 2009, a total of 212 nasopharyngeal aspirates were collected from 212 children (median age 8 months, range 1–59 months) hospitalized with acute LRTIs at Sanggyepaik Hospital in Seoul, South Korea. Previously, during January 2002–June 2006, a total of 173 serum samples had been obtained from children (age range 1 month–15 years) with hepatitis (hepatitis B, 20 samples; hepatitis C, 11 samples; unknown hepatitis, 31 samples), Kawasaki disease (12 samples), and Henoch-Schönlein purpura (18 samples) and from healthy children (same age range, 81 samples) (*9*). The study was approved by the internal review board of Sanggyepaik Hospital.

DNA was extracted from serum samples, and RNA and DNA were extracted from nasopharyngeal aspirates by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and a QIAamp DNA Blood Mini Kit (QIAGEN GmbH), respectively. All nasopharyngeal aspirates were tested by PCR for common respiratory viruses such as respiratory syncytial virus, influenza viruses A and B, parainfluenza virus, and adenovirus, as described previously (*10*). PCRs to detect HBoV-1 were performed by using primers for the nonstructural (NS) 1 and nucleocapsid protein (NP) 1 genes, as described previously (*10*). Additional PCRs for rhinovirus, human metapneumovirus, human coronavirus (hCoV)-NL63, hCoV-OC43, hCoV-229E, hCoV HKU-1, WU polyomavirus, and KU polyomavirus were performed, as described, for HBoV-2–positive samples (*10*).

HBoV-2 was detected by performing first-round PCR with primers based on the *NS* gene, HBoV2-sf1, and HBoV2-sr1. Second-round PCR was performed by using primers HBoV2-sf2 and HBoVsr2, as described previously (*1*). The PCR products were sequenced by using an ABI 3730 XL autoanalyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were aligned by using BioEdit 7.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html) and presented in a topology tree, prepared by using MEGA 4.1 (www.megasoftware.net).

Of the 212 samples tested, the following viruses were detected: human respiratory syncytial virus (in 124 [58.4%] samples), human rhinovirus (24 [11.3%]), influenza virus A (18 [8.4%]), adenovirus (10 [4.7%]), and parainfluenza virus (8 [3.7%]). HBoV-1 was not detected in the study population. HBoV-2 DNA was found in 5 (2.3%) of the 212 samples collected; all positive samples had been obtained in October 2008. The age range of the children with HBoV-2–positive samples was 4–34 months (median 24 months), and all were male. The diagnoses were bronchiolitis for 3 children and bronchopneumonia for 2. The most frequently codetected virus was human respiratory syncytial virus, found in 4 (80%) of 5 samples. One

sample that was negative for respiratory syncytial virus and positive for HBoV-2 was negative for all other respiratory viruses.

Nucleotide sequences were determined for the NS-1 gene, and phylogenetic analyses, which included HBoV-3, a new lineage designated by Arthur et al. (*2*), showed that the NS-1 gene was relatively well conserved and that there were 2 major groups of the virus, the UK strain and the Pakistan strain. HBoV-2 strains isolated from South Korea belonged to the HBoV-PK2255 (FJ170279) cluster (Figure).

Recent studies have detected HBoV-1 in serum samples of children with Kawasaki disease and of an immunocompromised child with hepatitis (*7,8*). However, neither HBoV-1 nor HBoV-2 was detected in the 172 serum samples from 61 patients with hepatitis, 12 with Kawasaki disease, 18 with Henoch-Schönlein purpura, and 81 healthy children.

The absence of HBoV-1 in the samples examined was unexpected because HBoV-1 was detected in >10% of 558 respiratory samples collected from a demographically similar study population during the winter 2 years earlier (*10*). Future studies, with larger populations and over longer periods, are needed to delineate seasonal variations between HBoV-1 and HBoV-2.

We demonstrated HBoV-2 DNA in the respiratory tract secretions of children with acute LRTIs. In most positive samples, the virus was found in addition to other respiratory viruses. A limitation is that the study did not consider health control measures and other clinical disease such as gastroenteritis and was conducted for a short time. The role of HBoV-2 in LRTIs remains unclear; further studies are needed to clarify whether this virus is only shed from the respiratory tract or whether it replicates in the gastrointestinal tract.

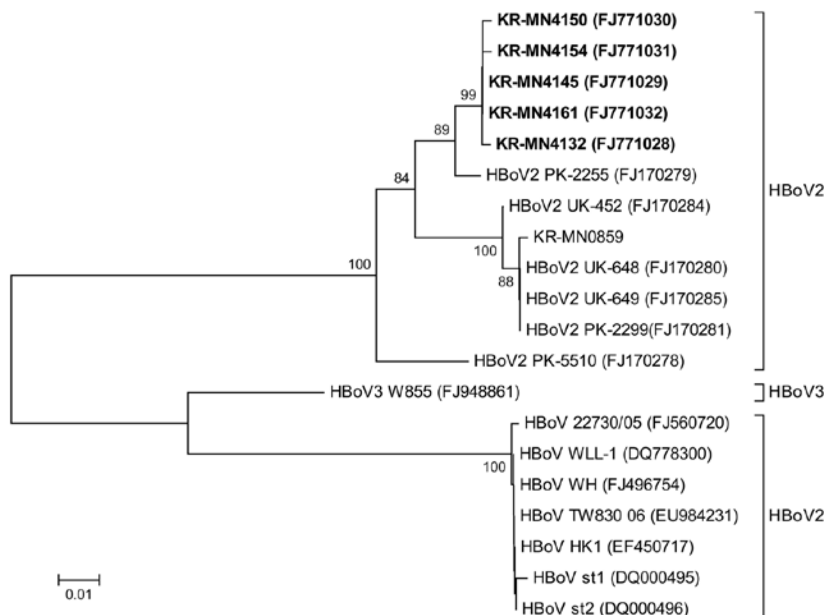

Figure. Phylogenetic analysis of nonstructural (NS) 1 gene sequences from human bocavirus 2 strains from Korea (KR), United Kingdom (UK), and Pakistan (PK), presented on a topology tree prepared by using MEGA 3.1 (www.megasoftware.net). Nucleotide alignment of a 417-bp portion of the NS-1 gene was prepared by using BioEdit 7.0 (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The nucleotide distance matrix was generated by using the Kimura 2-parameter estimation. Nodal confidence values indicate the results of bootstrap resampling (n = 1,000). Five strains from South Korea (FJ771028–32) are in **boldface.** Scale bar indicates estimated number of substitutions per 10 bases.

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Nontuberculous Mycobacterium Infection and Tumor Necrosis Factor-α Antagonists

To the Editor: *Mycobacterium haemophilum* is an aerobic, slow-growing microorganism with optimal growth at 30°C to 32°C. It has a unique requirement for ferric iron–containing compounds (*1*), from which it acquired its name (i.e., *haemophilum*). Infections with *M. haemophilum* are rare, but cervicofacial lymphadenitis caused by *M. haemophilum* has been described in children (*2*). Besides cervicofacial lymphadenitis, extrapulmonary signs of *M. haemophilum* disease include subcutaneous noduli, arthritis, and osteomyelitis, which generally affect immunocompromised patients (*3*). Recently, 2 cases of cutaneous *M. haemophilum* infections after alemtuzumab treatment were reported (*4*). A small number of pulmonary *M. haemophilum* infections associated with AIDS or solid organ or bone marrow transplantation have been described (*1*). We report pulmonary *M. haemophilum* infection in a woman who had been immunosuppressed by tumor necrosis factor-α antagonist (TNF-αA) (adalimumab) treatment for rheumatoid arthritis.

A 72-year-old woman with a history of rheumatoid arthritis and obstructive sleep apnea syndrome had signs and symptoms of fatigue, mild fever episodes, and a nonproductive cough 9 months after treatment for rheumatoid arthritis had begun with methotrexate (MTX) and TNF-αA. Physical examination was unremarkable except for a body temperature of 38.9°C. Laboratory testing showed an increased erythrocyte sedimentation rate (ESR) (77 mm/h), an increased C-reactive protein (CRP) level (60 mg/L), a normal leukocyte count (8,500 cells/μL), and relative monocytosis (12%). HIV serologic testing results were negative. Chest radiograph showed an infiltrate in the right upper lobe. Chest computed tomography confirmed this finding and showed lymphadenopathy in the right hilus and mediastinum.

Notably, the tuberculin skin test result was negative at screening before she began the TNF-αA treatment, but was now positive (20 mm), suggesting mycobacterial infection. Auramine and Ziehl-Neelsen staining of sputum and bronchoalveolar liquids showed no acid-fast bacilli, and *M. tuberculosis* infection was not confirmed by PCR or culture. Eventually, a mediastinal lymph node biopsy was taken by endoscopic ultrasound guidance. Granulomatous inflammation and acid-fast bacilli were seen by microscopy. Corresponding cultures yielded a strain identified as *M. haemophilum* at the Netherlands National Institute for Public Health and the Environment (RIVM) by using the Inno-LiPA Mycobacteria v2 reverse line blot assay (Innogenetics, Ghent, Belgium). Strain identity was confirmed by sequencing of the complete 16S rDNA gene, which was identical to that of *M. haemophilum* available in the GenBank sequence database (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov; accession no. X88923).

The RIVM performed drug susceptibility testing by using a modified agar dilution method (*5*). Middlebrook 7H10 media were enriched with 10% sheep blood hemolyzed by 1:1 dilution with water and subsequent freezing–thawing. Historic drug susceptibility data was reviewed (Table). Initially, adalimumab was discontinued, and our patient was treated with isoniazid, ethambutol, rifampin, and pyrazinimide because *M. tuberculosis* infection was suspected. After identification of *M. haemophilum*, our patient was treated with rifampin and azithromycin. A total treatment duration of 10 months resulted in complete resolution of the

Table. Antimicrobial drug susceptibility test results for *Mycobacterium haemophilum* isolate from rheumatoid arthritis patient and other *M. haemophilum* isolates*

Antimicrobial drug	Case report		RIVM historic data (n = 49)	
	Classification	MIC, mg/L	% Susceptible	% Resistant
Isoniazid	Resistant	10	0	100
Rifampin	Susceptible	0.2	4	96
Ethambutol	Resistant	20	0	100
Streptomycin	Susceptible	<1.0	35	65
Cycloserine	Susceptible	50	78	22
Prothionamide	Susceptible	<1.0	61	39
Amikacin	Resistant	10	29	71
Ciprofloxacin	Resistant	4.0	88	12
Clofazimine	Susceptible	<0.5	92	8
Clarithromycin	Susceptible	<2.0	94	6
Rifabutin	Susceptible	<0.2	96	4

*All isolates submitted to the Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment (RIVM), the Netherlands, January 2000–January 2007. Before January 2004, strains tested were identified by 16S rDNA gene sequencing; after January 2004, strains were identified by the Inno-LiPA assay (Innogenetics, Ghent, Belgium).

pulmonary infiltrate and normalization of ESR and CRP concentration. During the follow- up period of >12 months, the patient remained asymptomatic. Her rheumatoid arthritis was treated with MTX monotherapy.

This case illustrates the risk for infectious diseases during TNF-αA treatment. Rheumatoid arthritis can be treated effectively with MTX and TNF-αA (*6*). Side effects of concern are infectious diseases, which prompt the need for screening for latent mycobacterial infection before commencing treatment (*7*). Despite screening, mycobacterial infections have been diagnosed after prolonged treatment in various patients (*8*). This case shows that not only *M. tuberculosis* but also nontuberculous mycobacteria (NTM) should be considered as possible pathogens. This possibility is of clinical importance because of the diagnostic challenges. Diagnosing NTM infections may require specific culture media and molecular assays. Under optimal conditions, cultures show growth of most NTM species (including *M. haemophilum*) within 2–3 weeks. NTM are less susceptible to antimicrobial drugs than *M. tuberculosis*. *M. haemophilum* is generally resistant in vitro to isoniazid, ethambutol, and rifampicin (Table), but no standardized susceptibility methods for *M. haemophilum* exist. Therefore, following current guidelines from the American Thoracic Society (ATS) is advisable for NTM infections (*9*).

No specific recommendations exist for pulmonary *M. haemophilum* infections, but for disseminated *M. haemophilum* infections, the ATS recommends a multidrug regimen combining clarithromycin, rifampicin/rifabutin, and ciprofloxacin. Although no studies on treatment duration for *M. haemophilum* infections have been conducted, the ATS guidelines recommend treatment until cultures taken during therapy are negative for 1 year (*9*). Whether TNF-αA treatment can be continued during antimycobacterial treatment is a matter of debate in the absence of sufficient safety data (*8*). In active tuberculosis infection, treatment with TNF-αA is contraindicated before patients complete a standard regimen of antituberculosis therapy; no information is available for NTM disease (*10*).

This case is presented especially to demonstrate the diagnostic challenges of NTM infections. For such cases, clinicians are advised to consult experts in the field of NTM infections.

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Transmission of Varicella Vaccine Virus, Japan

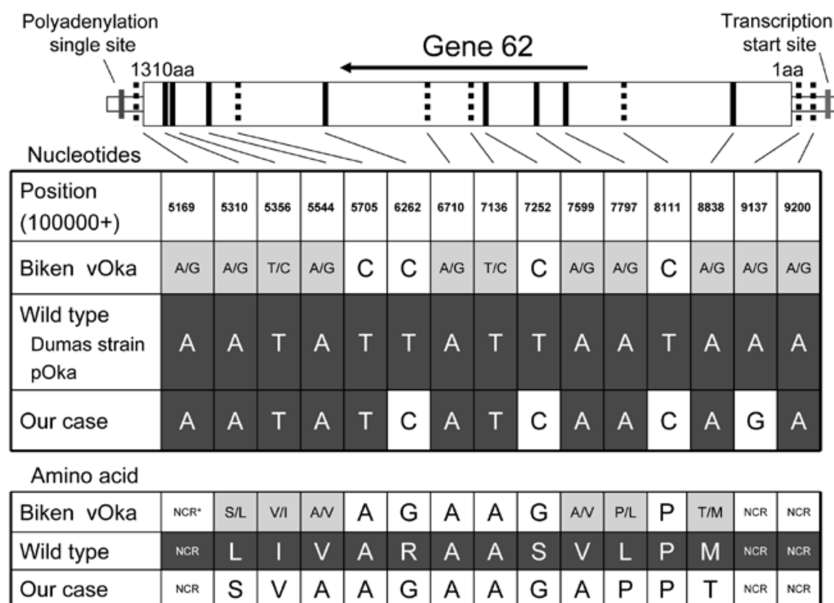
To the Editor: Varicella-zoster virus (VZV), a human herpesvirus, is the causative agent of varicella (chickenpox) and herpes zoster (shingles). Worldwide, children are routinely vaccinated with a live attenuated varicella vaccine containing the Oka vaccine (vOka) strain of VZV, originally developed in Japan (*1–3*). Although the risk for secondary transmission of the vOka strain from immunocompromised vaccinees to susceptible persons is relatively high, the risk for transmission from immunocompetent vaccinees is low (*1*). We report secondary transmission of the vOka strain from an immunocompetent girl with a history of varicella vaccination to her healthy susceptible brother.

Herpes zoster developed in a healthy 3-year-old girl 2 years after she had received the varicella vaccine (lot VZ040; Biken, Osaka, Japan). She received oral acyclovir treatment and fully recovered by day 19 after herpes zoster onset. On the same day that the girl recovered, her immunocompetent 2-year-old brother was found to have fever and a rash consisting of 10–20 papulovesicles; mild varicella was diagnosed. The boy had no known history of contact with persons infected with varicella or with persons who administered the varicella vaccine. After receiving oral acyclovir treatment, the boy recovered without systemic complications.

On day 19 after the girl's onset of herpes zoster, an enzyme immunoassay (Denka Seiken, Tokyo, Japan) confirmed the presence of VZV-specific immunoglobulin (Ig) G (titer 48.9, well above the detection limit of 2.0) but not IgM. The boy showed seroconversion of VZV-specific IgG from a titer of <2.0 on day 3 after his disease onset to 19.3 on day 30. Although vesicular fluid or crust specimens were obtained from both children, only the specimens from the boy contained detectable amounts of VZV DNA.

To determine whether vOka or a wild-type VZV strain caused the varicella in this boy, we performed PCR to amplify the entire region of gene 62 and determine its sequence, as described previously (*4*). The DNA sequence of the PCR product matched that of the vOka sequence with the exception of a single wild-type nucleotide substitution at position 105705 (Figure, GenBank accession no. AB497598). Restriction fragment length polymorphism (RFLP) analysis of the PCR products of the open reading frame (ORF) 38 and ORF54 loci using *Pst*I and *BglI* (*5*) demonstrated that the strain had a vOka-like pattern, i.e., *Pst*I-*BglI*+. Furthermore, the vOka-specific sequences at positions 5,745 and 94,167 were conserved in the strain. Taken together, these results indicate that the strain in the boy likely was derived from the vaccine but was not a recombinant between the Biken vOka strain and a wild-type virus.

Commercial varicella vaccines produced by major manufacturers such as Biken, Merck (Rahway, NJ, USA), and GlaxoSmithKline Biologicals (Rixensart, Belgium) possess similar immunogenicity and safety characteristics (*2*,*3*,*6*). Adverse events involving the vOka products from Merck (e.g., rash, varicella, herpes zoster, neurologic complications, and secondary transmission) have been reported at an overall rate of 3.4–5.3 events/10,000 doses given in the United States (*2*,*6*). Six cases of secondary transmission from 5 immunocompetent persons who had received vOka made by Merck have been documented (*2*,*7*,*8*). Unfortunately, the association of vOka from Merck with some of those cases was defined



Position (100000+)	5169	5310	5356	5544	5705	6262	6710	7136	7252	7599	7797	8111	8838	9137	9200
Biken vOka	A/G	A/G	T/C	A/G	C	C	A/G	T/C	C	A/G	A/G	C	A/G	A/G	A/G
Wild type Dumas strain pOka	A	A	T	A	T	T	A	T	T	A	A	T	A	A	A
Our case	A	A	T	A	T	C	A	T	C	A	A	C	A	G	A

Amino acid															
Biken vOka	NCR*	S/L	V/I	A/V	A	G	A	A	G	A/V	P/L	P	T/M	NCR	NCR
Wild type	NCR	L	I	V	A	R	A	A	S	V	L	P	M	NCR	NCR
Our case	NCR	S	V	A	A	G	A	A	G	A	P	P	T	NCR	NCR

Figure. Sequence of gene 62 from patient with varicella from secondary transmission of Oka vaccine strain (vOka). The diagram at the top shows the structure of gene 62. Amino acid residues are numbered 1–1310 from the amino terminus to the carboxyl terminus. Vertical lines indicate the positions of 15-nt base differences between vOka (GenBank accession no. AB097932) and parental (pOka, accession no. AB097933) strains. The 15 **boldface** and broken lines show substitutions with and without amino acid (aa) alterations, respectively. The charts show a comparison of the gene 62 sequences among vOka, pOka, Dumas (accession no. X04370), and the strain isolated from the secondary case-patient (accession no. AB497598). The black, white, and gray boxes denote pOka-type substitutions, vOka-type substitutions, and mixed-type substitutions (mixture of pOka and vOka nucleotides), respectively. NCR, noncoding region.

by RFLP analyses of only 1 or 2 loci (*2*,*7*). Although 5 of the 6 cases of secondary transmission were linked with vOka-associated cases of varicella, 1 was transmitted from a vaccinee with herpes zoster (*7*). The fact that the sibling reported in that case was already vaccinated before varicella developed confounds the case.

Postmarketing surveillance conducted in Japan by Biken and the governmental Relief Systems for Adverse Reactions have identified no cases of secondary transmission since Biken's vOka was licensed in 1985 (Y.G. and N.I., unpub. data). Thus, the case reported here is considered to be rare in that vOka was transmitted to a healthy susceptible person through close contact with a vaccinee with herpes zoster.

vOka is composed of a mixture of genotypically distinct virus strains that have 15 base substitutions in gene 62 compared with the parental Oka strain. Gomi et al. have suggested that the amino acid alterations in the gene 62 products of vOka are associated with the characteristics of vOka, i.e., slower growth and less efficient cell-to-cell spreading in vitro compared with parental Oka (*4*). Previous studies have suggested that some alleles (positions 107797, 105169, 105356, and 108838) in gene 62 were implicated in the formation of vaccine-associated rash (*9*,*10*). The virus in the case-patient reported here contained 1 synonymous nucleotide substitution from Biken's vOka to a wild-type at position 105705 in gene 62. Because no such alteration was detected in the final Biken vOka products, information about the in vivo process of natural selection for the particular genetic profile is needed.

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Aichi Virus Strains in Children with Gastroenteritis, China

To the Editor: Aichi virus, a member of the *Kobuvirus* genus of the *Picornaviridae* family (*1*,*2*), is a positive-sense, single-stranded RNA virus with a genome of 8,280 nt and a poly(A) tail. The single, large open-reading frame (ORF) encodes a polyprotein of 2,432 aa that is cleaved into the typical picornavirus structural proteins (VP0, VP3, VP1) and nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) (*2*,*3*). Based on the phylogenetic analysis of 519-bp sequences at the 3C–3D (3 CD) junction, Aichi viruses can be divided into 2 genotypes,

A and B, with ≈90% sequence homology (*4*).

Little is known about the epidemiology of Aichi virus. Its presence in fecal specimens of children having diarrhea has been demonstrated in several Asian countries (*5*,*6*), Brazil and Germany (*7*), France (*8*), and Tunisia (*9*). Some reports showed a high level of seroprevalence in adults (*7*,*10*), which suggests widespread exposure to Aichi virus during childhood.

In the present study, 445 fecal samples were collected during April 2008–March 2009 from children 0 to 6 years of age who were hospitalized with acute diarrhea in Shanghai Children's Hospital, People's Republic of China. Ninety-two 2–5-year-old children from 3 childcare centers in Shanghai City were included as healthy control subjects. Viral nucleic acid was extracted from 10% stool suspensions in phosphate-buffered saline (pH 7.5) by QIAamp Viral RNA kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Screening for Aichi viruses was done by reverse transcription–PCR (RT-PCR) with the primers described by Yamashita et al. (*4*), by using a Takara OneStep RT-PCR kit (TaKaRa, Dalian City, Japan). RT-PCR–amplified DNA fragments of the expected sizes, as determined by agarose gel electrophoresis, were excised from the gel, extracted, purified, and sequenced in a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment and phylogenetic analysis were performed by using the ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX) and MEGA4 software (www.megasoftware.net), respectively. Aichi virus RNA was detected in 8 samples (1.8% incidence). The PCR-amplified products of 8 strains were sequenced, and the resulting sequences were submitted to GenBank with the strain names Chshc1–8 and accession nos. FJ890516–FJ890523. Sequence analysis, based on the 529-bp sequences, showed that the isolates shared 98.2%–99.6% identities with each other, which suggests that they can be considered a unique strain. When compared with all Aichi virus strains available in GenBank, the 8 sequences shared 91.3%–96.9% sequence identities, except for a strain from France, DQ145759, which had only 87.2% sequence homology with the other strains in this study. Phylogenetic analysis of those Aichi virus strains, based on the 519-bp sequence, showed that the 8 strains belonged

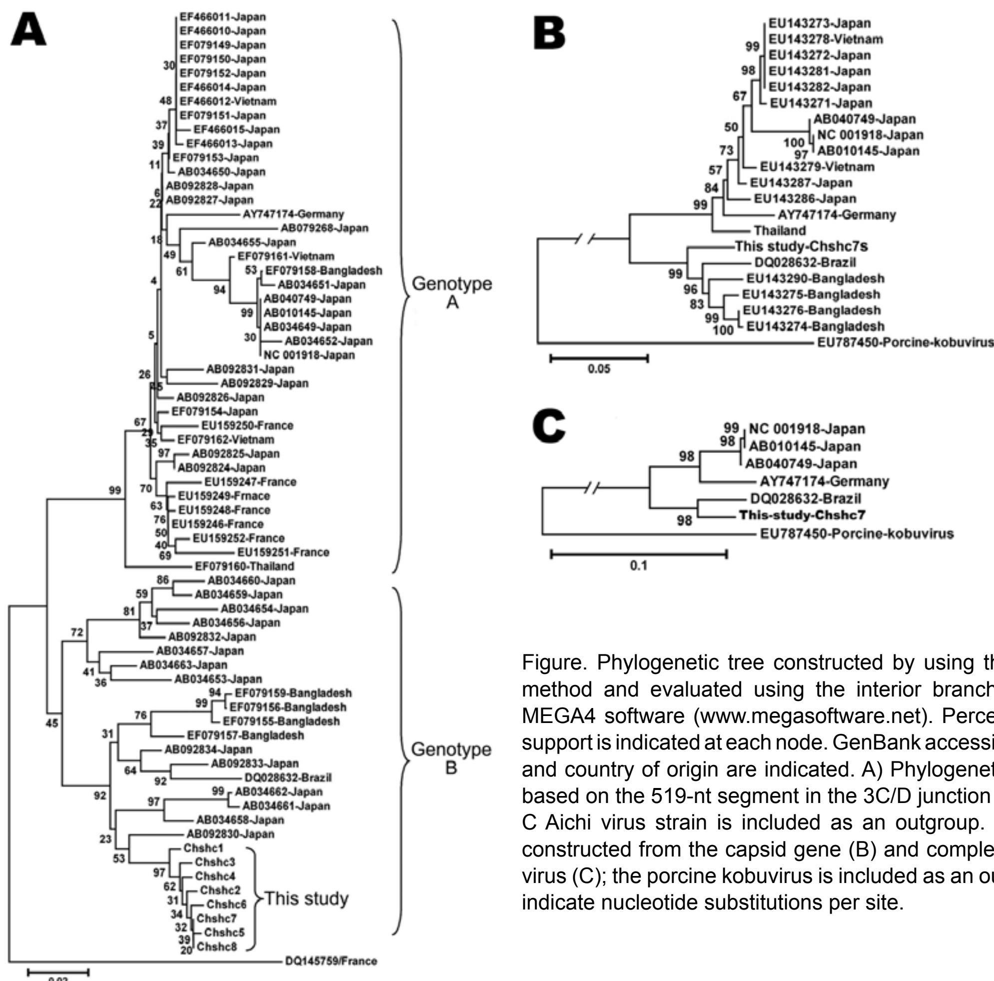

Figure. Phylogenetic tree constructed by using the neighbor-joining method and evaluated using the interior branch test method with MEGA4 software (www.megasoftware.net). Percentage of bootstrap support is indicated at each node. GenBank accession number, source, and country of origin are indicated. A) Phylogenetic tree constructed based on the 519-nt segment in the 3C/D junction region, a genotype C Aichi virus strain is included as an outgroup. Phylogenetic trees constructed from the capsid gene (B) and complete genome of Aichi virus (C); the porcine kobuvirus is included as an outgroup. Scale bars indicate nucleotide substitutions per site.

to genotype B (Figure, panel A) and closely clustered with a Japanese strain AB092830, sharing 96.9% sequence identity with it. The 8 Aichi virus–positive samples were further investigated for norovirus, sapovirus, rotavirus, astrovirus, and adenovirus types 40 and 41 by RT-PCR with the primers described (*9*). Results indicated that one of the samples was also positive for astrovirus, of which the 348-bp–specific fragment was sequenced and deposited in GenBank (accession no. GQ292771). No Aichi virus was detected in samples from the 92 healthy control subjects.

The complete genomic sequence of strain Chshc7 was then determined by using 13 sets of specific oligonucleotide primers designed on the complete genome of 4 Aichi virus strains (NC_001918, AB010145, DQ028632, AY747174). Results showed that the full genome of this virus strain was 8,244 nt and contained a ORF frame with a length of 7,299 nt, encoding a putative polyprotein precursor of 2,433 aa. This ORF is preceded by a 5′ untranslated region (UTR) at least 712 nt in length. The 3′ UTR measure 237 nt, excluding the poly(A) tract. Base compositions of the strain were found to be A, 19.8%, C, 37.8%, G, 20.9%, and U, 21.5%. The polyprotein precursors of this Aichi strain comprise a predicted leader protein of 170 aa and putative VP0, VP3, and VP1 proteins with lengths of 370 aa, 224 aa, and 278 aa, respectively. Regarding the nonstructural proteins, lengths of 111 aa, 165 aa, and 335 aa are predicted for 2A, 2B, and 2C, and of 93 aa (29 aa, 190 aa, 468 aa) for 3A (3B, 3C, 3D).

Phylogenetic trees were constructed on the basis of the capsid protein gene (Figure, panel B), complete genome sequences of the strain Chshc7 (Figure, panel C), and those sequences available in GenBank. Both phylogenetic trees indicated that the strain Chsh7 closely clustered with the Brazilian strain DQ028632, which confirmed that Chsh7 belonged to genotype B. Sequence alignment showed that Chsh7 and the Brazilian strain (DQ028632) shared 95.3% and 98.1% sequence identities with each other over the complete genome and putative amino acid sequences, respectively, which suggested that the 2 strains might come from a common ancestor. These results will provide useful information for further epidemiologic study of Aichi virus in China.

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Appropriate Screening for Leishmaniasis before Immunosuppressive Treatments

To the Editor: We read with great interest the article by Xynos et al. reporting 2 cases of leishmaniasis in patients treated with biologic drugs (*1*). Although we agree with most of the article, we are not totally convinced that serologic analysis alone could be used to screen for leishmaniasis before initiation of biologic or immunosuppressive treatments. Evidence indicates that serologic analysis can identify only symptomatic or asymptomatic cases with recent and still active infection (*2*,*3*).

Leishmania spp. are pathogens that infect hematopoietic cells, where they establish chronic intracellular parasitism and survive for an infected person's lifetime. In leishmaniasis-endemic countries, asymptomatic visceral leishmaniasis (VL) infections occur more frequently than clinically apparent VL cases. An estimated 10%–20% of persons with asymptomatic infections develop clinically overt VL (*4*). The leishmanin skin test (LST) (Montenegro test), an intradermal injection of a suspension of killed promastigotes, measures delayed hypersensitivity reactions and appears to be the only screening test capable of detecting asymptomatic leishmania infections.

A positive LST result is thought to indicate durable cell-mediated immunity after asymptomatic infection or clinical cure of VL. LST positivity may appear months to years postinfection, but once positivity appears, it persists in immunocompetent patients. A survey of VL in Ethiopia showed LST positivity in 32.2% of the population, but leishmania antibodies were found in only 4.1% (*5*).

Because different antigen preparations may affect test sensitivity, LST should use promastigotes of the *Leishmania* spp. present in an area. We believe that ideal screening for leishmaniasis should include LST along with serologic analysis. Unfortunately, little data exist on the use of antileishmania therapies for LST-positive or serologically positive patients. VL with unusual signs and symptoms may develop in immunocompromised patients with previous LST positivity after immunosuppressive treatments. Information about LST positivity is useful for calling attention to this potential risk for VL that may have unusual manifestations in these persons.

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In Response: In their letter responding to our recently published article (*1*) Cascio and Iaria spark an important discussion on the usefulness of screening for *Leishmania* infection before administering biologic agents or other immunosuppressive treatments to patients having autoimmune rheumatic diseases and living in areas where *Leishmania* parasites are endemic (*2*). Although we agree in principle that early detection of asymptomatic *Leishmania* infection will decrease the incidence of the disease in immunosuppressed patients, current diagnostic tools may have a limited (or restricted) role in detecting *Leishmania* infection in this vulnerable patient population. Screening for leishmaniasis has been hampered by the lack of a standard test. Currently available serologic methods have variable sensitivities, specificities, and cross-reactivities, depending on the species being tested and the region where tests are performed. Many experts believe that serologic tests may complement other existing diagnostic tools, raising cost-efficiency concerns, especially in financially deprived countries (*3*).

A positive leishmanin skin test (LST) result indicates exposure to *Leishmania* spp. and is generally thought to reflect a durable cell-mediated immune response. No cross-reaction occurs in patients with Chagas disease, but some cross-reactions are found in patients with glandular tuberculosis or lepromatous leprosy (*4*). Sustained positive responses have been documented for up to 20 years after exposure to the *Leishmania* parasite. Nevertheless, LST has

limitations. In a longitudinal study of visceral leishmaniasis in Bangladesh, Bern et al. reported loss of LST sensitivity attributed to antigen-production issues, such as standardization and documentation of sensitivity, potency, and stability of leishmanin antigens (*5*). Also, prior treatment with immunosuppressive agents, which influence cell-mediated immunity, may decrease LST prognostic potency similarly to changes observed for the tuberculin skin test in similar settings (*6*).

Variations in specificities and sensitivities limit the diagnostic potential of available diagnostic tools. The context of immunosuppression further contributes to the diagnostic complications and increases the need for additional research in leishmaniasis diagnostics.

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ANOTHER DIMENSION

Red Snappers

Erin E. McConnell

for a moment pretend
you are not
the infallible house staff,
but the latest admission—
hacking putrid sputum
from your soulful depths
or your festering chest,
depending on your mood.

slapped with a mask,
you are secured in secluded rooms;
a paucity of guests,
but for the parade of absurd birds—
plastered in Haz-mat
lemon-yellow gowns,
and peach-colored beaks.
your meager dried-up sleep
is aborted by
bloodhungry fowl
covetous of mucus
you no longer produce.

your meals grow cold
waiting for you
in the anteroom of
your negative pressure purgatory.

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DOI: 10.3201/eid1510.090221

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Edgar Degas (1834–1917) Absinthe (c. 1876) Oil on canvas (92 cm × 68 cm) Photo: Hervé Lewandowski. Réunion des Musées Nationaux/Art Resource, New York, NY, USA Musée d'Orsay, Paris, France

Alone Together Then and Now

Polyxeni Potter

"Sickly, neurotic, and so myopic that he is afraid of losing his sight; but for this very reason an eminently receptive creature and sensitive to the character of things," wrote French writer and art critic Edmond de Goncourt about Edgar Degas. The artist knew his own difficult nature. "[I have] one terrible, irreconcilable enemy," he once admitted to Pierre-Auguste Renoir, "myself, of course." From those who associated with him, Degas exacted an emotional toll. "There will be a dish cooked without butter for me. No flowers on the table, very little light …. You'll shut up the cat, I know, and no one will bring a dog. And if there are women there, ask them not to put smells on themselves …. Scent, when there are things that smell so good! Such as toast, for example. And we shall sit down to table at exactly half-past seven."

"All his friends had to leave him," Renoir reported, "I was one of the last to go, but even I couldn't stay till the end." Considered a misogynist by some, Degas counted among his friends Mary Cassatt, Berthe Morisot, and leading opera divas and ballerinas of his day. Accused of being a recluse, he denied it. "I am not a misanthrope, far from it, but it is sad to live surrounded by scoundrels." Despite his uncompromising persona, he was respected by his peers, who were afraid of him, and was popular with art critics and buyers. "I was, or appeared to be, hard with everyone, owing to a sort of tendency towards roughness that originated in my doubts and my bad temper."

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Born in Paris during the same decade as Édouard Manet, Paul Cézanne, and Claude Monet, Degas had many opportunities. His early years were privileged, though tinged by the melancholy that followed him all his life. "I was sulky with the whole world and with myself." Under pressure he agreed to study law but soon abandoned the effort and pursued art with a fervor that convinced his father to support him, first at the Atelier Lamothe and École des Beaux-Arts and then independently in Italy, "the most extraordinary period of my life."

Degas' early works were historical paintings in the classical tradition. Early in his training, he absorbed the methods of Auguste-Dominique Ingres, Eugène Delacroix, and Gustave Courbet and aspired to paint like Michelangelo and Raphael. But by the 1860s, he abandoned history for scenes of everyday life. While copying a Velásquez at the Louvre, he met Manet, who became his friend and brought him into the circle of impressionist painters. Though Degas exhibited with them often, he never identified himself with the movement. "What I do is the result of reflection and study of the great masters. Of inspiration, spontane-

ity, temperament I know nothing." He was not interested in the transient effects of light on landscape. He preferred painting people and abhorred painting *en plein air*. "The gendarmes should shoot down all those easels cluttering up the countryside."

"Draw lines, young man, a great many lines," Ingress once advised Degas, who took the comment to heart. "I always tried to urge my colleagues along the path of draftsmanship, which I consider a more fruitful field than that of color." Always seeking perfection, he reworked every picture, even after it was sold, studying and repeating details until he had mastered and memorized them. Owners were known to chain his works to the wall. He experimented with many media, among them pastels, which he softened over steam into a paste and used over gouache and monotype prints. He disliked the shine of oil paints, so he removed the oil and applied with turpentine, often on paper rather than canvas.

Absinthe, on this month's cover, appears to be a genre scene. But it is a portrait of Degas' friend Marcellin Desboutin, writer, artist, printmaker, and a regular at the Café de la Nouvelle-Athènes, a meeting place for the impressionists and others in the avant garde. "I did not go to either Oxford or Cambridge," Irish art critic George Moore said about his education, "but I went to the Nouvelle-Athènes." Against all convention, the focal point of the portrait is a woman seated at Desboutin's side. She is Ellen Andrée, a model who posed often for Degas and Renoir and aspired to be a serious actress, "like Sarah Bernhardt … in Phèdre."

This painting of an unloving couple was called "the perfection of ugliness" by one critic and caused a stir when exhibited in London. "It is not a painting at all," other critics said, "It is a novelette—a treatise against drink." Desboutin, his elbow a wall between him and his companion, is detached, lost in thought. He is not even entirely in the picture—pipe, arm, and one leg cropped, eyes glaring off somewhere. She is precariously in center stage, her social status exposed. Pushed off one table, not quite at the next one, she sits in-between, as awkwardly positioned as her carafe.

The painting's architecture drives the story, framing it in fresh and innovative ways. The marble tables, zigzagging across the picture, create perspective by drawing the eyes to the figures barricaded behind them, whose reflections in the mirror suggest the presence of other patrons without actually showing any. Newspapers form a bridge between the tables. The artist's signature seems a seal of approval.

This nearly monochromatic snapshot lays bare human isolation in the midst of gaiety. Desputin's drooping companion, propped up behind a glass of absinthe, represents women, many of them in Degas' very neighborhood, caught in ill-fitting bohemianism, absinthe not withstanding, for as Oscar Wilde put it, "After the first glass, you see things as you wish they were. After the second, you see things as they are not. Finally, you see things as they really are, and that is the most horrible thing in the world."

Degas' women were often at a disadvantage, whether they sought solace in cafés, danced *en pointe*, or washed and ironed clothes in substandard conditions for meager pay. They got sick and died young. But through their drab lives, fluffy costumes, or trappings of gaiety in busy venues, the artist's penetrating eye captured for all to see not emotional isolation alone, which had marred his own life, but its many causes: poverty, social stigma, and underlying illnesses, not the least of them tuberculosis, rampant in his day.

The costumes have changed and absinthe is no longer the drug of choice, but emotional isolation lives on as do its many causes. Therapies have curtailed tuberculosis in some parts of the world, despite the emergence of multidrug resistance, but pulmonary infections caused by nontuberculous mycobacteria are on the rise, prompting investigations and gathering of data to explore and identify what Ellen Andrée was not able to find in the bottom of the absinthe glass.

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Article Title

Community-Associated Methicillin-Resistant *Staphylococcus aureus*, Iowa, USA

CME Questions

1. A 25-year-old man is admitted to the intensive care unit with head trauma after a motor vehicle accident, receives a urinary catheter, and is ventilated. He receives prophylactic antibiotics for multiple skin wounds. Which of the following is least likely to be a risk factor for healthcare-associated (HA) verus community-associated (CA) methicillin-resistant *Staphylococcus aureus* (MRSA) in this patient?

A. Young age
B. Hospitalization
C. Catheter use
D. Exposure to antimicrobials

2. Which of the following best describes the definition of multiresistant isolates of MRSA?

A. Resistant to more than 4 of 8 antimicrobial classes
B. Resistant to more than 2 of 7 antimicrobial classes
C. Resistant to more than 3 of 8 antimicrobial classes
D. Resistant to more than 3 of 7 antimicrobial classes

3. Which of the following most accurately describes the trend in CA-MRSA USA300/400 infection in 1 US state from 1999 to 2006?

A. Most USA300/400 isolates were from the skin
B. Distribution of MRSA strains varied according to body site
C. The rate of nosocomial infections increased significantly
D. USA300/400 isolates did not demonstrate multidrug resistance

4. A 64-year-old man in a long-term care facility is admitted to the hospital in the summer for acute stroke. Which of the following is most likely to be a significant positive predictor of CA-MRSA compared with HA-MRSA in this patient?

A. Admission in summer versus other season
B. Age younger than 69 years
C. Hospitalization
D. Concurrent urinary tract infection

5. Which of the following best describes recent patterns of CA-MRSA infection in the United States?

A. Increase in incidence of USA300 strain of MRSA
B. Over 50% of CA-MRSA infections are invasive
C. USA300 is associated with 60% of MRSA bloodstream infections
D. Decrease in urban areas and increase in rural areas

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
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1	2	3	4	5
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Strongly Disagree				Strongly Agree
1	2	3	4	5

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief 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 be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief 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 a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles 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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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.

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.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.