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On the Cover

Henri de Toulouse-Lautrec (1864–1901)
At the Moulin Rouge: The Dance (1890)
Oil on canvas (115.6 cm x 149.9 cm)
Philadelphia Museum of Art: The Henry P. McIlhenny Collection in memory of Frances P. McIlhenny, 1986

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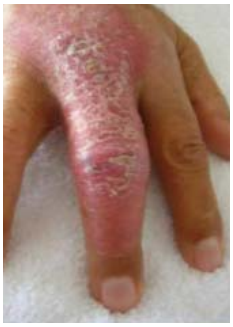

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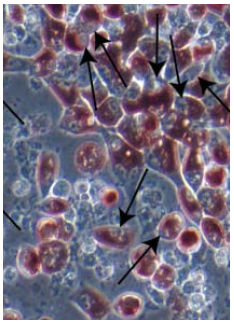

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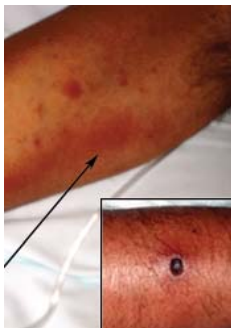

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Rescinding Community Mitigation Strategies in an Influenza Pandemic

Victoria J. Davey*† and Robert J. Glass‡

Using a networked, agent-based computational model of a stylized community, we evaluated thresholds for rescinding 2 community mitigation strategies after an influenza pandemic. We ended child sequestering or all-community sequestering when illness incidence waned to thresholds of 0, 1, 2, or 3 cases in 7 days in 2 levels of pandemic severity. An unmitigated epidemic or strategy continuation for the epidemic duration served as control scenarios. The 0-case per 7-day rescinding threshold was comparable to the continuation strategy on infection and illness rates but reduced the number of days strategies would be needed by 6% to 32% in mild or severe pandemics. If cases recurred, strategies were resumed at a predefined 10-case trigger, and epidemic recurrence was thwarted. Strategies were most effective when used with high compliance and when combined with stringent rescinding thresholds. The need for strategies implemented for control of an influenza pandemic was reduced, without increasing illness rates.

Community goals during an influenza pandemic include protecting people from illness and maintaining critical societal functions by limiting time away from usual occupations. Vaccine and antiviral medications are standards of influenza prevention, postexposure prophylaxis, and treatment (*1*). However, vaccine for a new influenza subtype may not begin to be available for at least 20 weeks after the onset of a pandemic and would be supplied over many months. Antiviral drugs may be in greater supply, but their effectiveness and rapid availability are uncertain (*2*,*3*). The US government has proposed community mitigation strategies for limiting the harm or managing the pace of an influenza pandemic until vaccine becomes available. These behavioral- and pharmaceutical-based strategies rely on reducing viral transmission and include dismissing schools and public gatherings, voluntary sequestering in the home, staggering work shifts, keeping symptomatic persons isolated, and treating ill persons rapidly with antiviral drugs and providing antiviral prophylaxis for their household contacts. These community mitigation strategies would be applied according to a pandemic severity index (PSI) scaled as categories 1–5. Category 5 would be a 1918-like event (case-fatality rate >2.0%) and category 1 (case-fatality rate ≤0.1%) would be akin to a bad seasonal influenza year (*3*). Modeling studies have estimated the effectiveness of mitigation strategies with and without vaccine and antiviral drugs (*4–8*). However, an independent review of pandemic influenza modeling studies raised the question of whether and when community containment strategies might safely be rescinded without reinitiating an epidemic (*9*).

An earlier study of this computational model demonstrated that closing schools and curtailing contacts of children and teenagers for the duration of a mild 1957-like epidemic in a stylized community reduced the number of infected persons by >90% (*10*). The model was constructed with assumptions that children and teenagers are responsible for influenza transmission in a community because of the frequency and nature of their person-to-person contacts (*11*). However, sensitivity analyses showed that permutations of mitigation strategies that included adults were effective at reducing infections in the model population, even for more highly infective 1918-like viral strains or with removal of enhanced children/teenagers' role in transmission (*10*). Several studies have shown that combining strategies such as social distancing of adult groups in and outside the workplace and removing symptomatic persons from community contact substantially reduced infections except in epidemics caused by the most infectious viral strains (*4–10*).

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The US government's community mitigation guidance recommends rapid initiation of strategies, then up to 4 weeks of school closure for a PSI 2–3 pandemic and 12 weeks for a PSI 4–5 pandemic. However, this guidance fails to address the gap between 12 weeks of mitigation strategies and estimated vaccine availability beginning at 20 weeks, especially if antiviral drugs were of limited effectiveness or availability (*3*). A pandemic could recur in the intervening period, and nonpharmaceutical community mitigation strategies with rules for their use would be valuable tools. Although nonpharmaceutical community mitigation measures have been used with apparent success in past pandemics (*12*,*13*), there are concerns about unintended consequences such as economic losses, interruption of education, and restrictions of personal freedom (*9*). The potential impact of community mitigation strategies warrants further study and consideration.

We evaluated effects of rescinding 2 community mitigation strategies for influenza pandemics, seeking a balance of the effect of illness, risk for epidemic recurrence, and minimization of the duration of mitigation strategies. The 2 strategies bracket mitigation measures that might be logically used in a situation in which effective vaccine and antiviral drugs are not available. The strategies are child sequestering, which is included in the US community mitigation guidance of February 2007, and a most conservative measure of all-community sequestering. We instituted strategies early, after 10 cases of mild (1957-like, PSI 1–2) or severe (1918-like, PSI 4–5) pandemic influenza occurred in a stylized community; these strategies were rescinded according to incident cases within a specific period.

Methods

The model used in this study has been described (*10*). Briefly, it is a networked, agent-based computational model, Loki-Infect, developed at the National Infrastructure Simulation and Analysis Center, a collaborative center of Sandia National Laboratories and Los Alamos National Laboratories. Our model application simulates an influenza epidemic in a community of 10,000 populated according to the age breakdown of the 2000 US Census for a small US community (*14*). The community consists of 17.7% children 0–11 years of age, 11.3% teenagers 12–18 years of age, 58.5% adults 19–64 years of age, and 12.5% older adults ≥65 years of age.

The social contact network within the model determines how persons are linked so that transmission of influenza and its consequences may occur. Persons are placed in multiple groups that reflect their roles and functions within the larger social network. Groups include household settings (older adults, adults, teenagers, and children), school settings (teenagers, children), work settings (adults), and community settings (older adults, adults, teenagers, and children). Persons are further assigned to within-age group interactions to reflect routinely occurring social gatherings such as clubs and meetings. Within groups, persons are linked through an average number of person-to-person contact(s) per day on the basis of observations of behavior and activities of group members (*10*). Random links and contacts are built into the model to reflect unscheduled events such as chance face-to-face encounters.

Within the social contact network, each person in the community occupies 1 of 7 positions in the natural history of influenza (uninfected, latent infection, infectious presymptomatic, infectious symptomatic [20% circulating in the community, 80% diagnosed and staying home], infectious asymptomatic, immune, or dead). We also include an eighth position, a noninfectious recovery period (mean 7 days) for diagnosed persons to reflect expected illness caused by a pandemic strain. Opportunities for transmission within the network are selected stochastically and depend on multiple parameters, including infectivity of the virus, position of the person in the natural history of influenza, susceptibility of the person being infected, and infectiousness of the transmitting persons (*10*). Probabilities of progression through the natural history of influenza and susceptibilities follow current understanding of influenza infection, and reflect recent work of Ferguson et al. (*4*,*5*). When diagnosed and staying home, a person reduces contact frequency with all nonhousehold groups by a compliance level.

The 2 community mitigation strategies modeled were school closings with home sequestering of children and teenagers ≤18 years of age (hereafter called child sequestering) and home sequestering of all community members (hereafter called community sequestering). Child sequestering reduces contact frequencies for children and teenagers in school and all nonhousehold settings by a compliance level and doubles within-household contacts of children and teenagers. During child sequestering, 1 household adult stays home in households with children <11 years of age where they similarly adjust their group contact frequencies. Community sequestering reduces all nonhousehold contacts of all community members by a level of compliance and doubles within-household contacts. We considered compliance levels from 50% to 90% in increments of 10%. Adult days at home are a measure of the effect of the mitigation strategy and are counted as number of days adults are either sick at home, taking care of children, or sequestered themselves. Adult days at home in community sequestering are adjusted by compliance to reflect the percentage of time spent outside the home on a daily basis while the strategy is imposed.

Scenarios begin by infecting 10 adults chosen at random. Strategies are imposed after 10 persons within the community receive a diagnosis of influenza and end when

the epidemic slows to the point that 0, 1, 2, or 3 newly diagnosed cases occur in 7 days (≈2 generation times of influenza [*5*]). Strategies are reimplemented if 10 new cases occur and are rescinded at the designated rescinding threshold. Control scenarios are an unmitigated base case (epidemics without mitigation strategies implemented) or continuation of strategies for the epidemic duration (child sequestering or community sequestering implemented at the 10–diagnosed case trigger and ended when the last incident case is recovered or dead).

We designed epidemic severity as a function of case-fatality rate and viral infectivity. We assumed a case-fatality rate of 2% of those with clinical illness. We applied 2 viral infectivities (I_D) to yield 50% and 70% infection rates in the stylized community. Infection is defined as persons with viral infection with or without clinical illness. (Clinical illness rates are 50% of infection rates.) The 2 I_Ds result in reproduction numbers (R_0, or the number of secondary cases produced by a source case in a susceptible population [*15*]) of ≈1.6 and 2.0, respectively. Thus, when the case-fatality rate is factored on 2 I_Ds, simulations yielded epidemics of 2 severities. The mild scenario resulted in a 1957-like epidemic classified by the US PSI as category 1–2. The severe scenario yielded a 1918-like epidemic of PSI 4–5 (*3*,*4*,*10*,*16*).

To capture the real-world heterogeneity of communities experiencing an epidemic, each scenario was simulated in 100 statistically identical networks of the community; each epidemic then propagated through the community stochastically. We include data only from simulations that resulted in epidemics (defined as >100 infected persons or 1% of the population). We report infection rates to emphasize that asymptomatic persons play a role in influenza transmission. We also report peak illness rates to quantify the effect of illness on the community. The Table lists study outcome measures and community epidemic management targets from the literature and our estimations of tolerable epidemic impact to the community (*17*).

Results

We provide scenario outcomes for mild (online Appendix Table 1, available from www.cdc.gov/EID/content/14/3/365-appT1.htm) and severe (online Appendix Table 2, available from www.cdc.gov/EID/content/14/3/365-appT2.htm) epidemics that compare the unmitigated base case, continuation strategies, and rescinding thresholds of 0, 1, 2, or 3 cases in 7 days (hereafter rescinding thresholds assume cases/7 days). Results are displayed for 50%–90% compliance in 10% increments. We also provide epidemic curves for mild (Figure 1) and severe (Figure 2) epidemics beginning with unmitigated epidemics, then with rescinding thresholds under varying conditions, and ending with continuation strategy plots. Both mitigation strategies (child sequestering and community sequestering) are plotted on each graph.

Unmitigated Base Case

In the mild, unmitigated base case epidemic, an average of 8.4% (840 cases) of the population was ill at peak. The combination of a mild epidemic and lack of strategy imposition meant that the average number of days adults spent at home either sick or tending sick children was 2 (range 0.4–2.4) over the course of the pandemic. In the severe epidemic, an average of 17.0% (1,700 cases) of the population was ill at peak, and adults spent an average of 3 (range 2.9–3.4) days at home (online Appendix Table 2). Unmitigated base case epidemics showed a rapid onset, early peak (≈day 23 for mild or day 27 for severe) and resolution (most by day 100 for mild and day 80 for severe) (Figures 1, 2).

Continuation of Strategies for the Epidemic Duration

Addition of mitigation strategies reduced peak illness rates (approximately an order of magnitude; Figures 1, 2). When child sequestering or community sequestering was continued until the last case was recovered or dead (continuation strategies), each equally controlled mild epidemics. For severe epidemics, infection and peak illness rates were higher for child sequestering than for community sequestering (peak illness rates were 1.4% [140 cases] for child sequestering and 0.7% [70 cases] for community sequestering at 90% compliance) (online Appendix Tables 1, 2). Although each strategy reduced the peak illness rate by >90% from the unmitigated base cases, in severe epidemics, child sequestering did not meet epidemic control targets for infection rate and peak clinical illness attack rate; community sequestering was required to meet these targets (Table, online Appendix Tables 1, 2). In contrast to

Table. Epidemic management targets

Outcome	Target
No. epidemics*/1,070 simulations	Fewer than unmitigated base cases
Infection rate	<0.1 of population (n = 1,000)
Peak illness rate	<0.01 of population (n = 100)
Average no. cycles required	<2.0
Average days home per adult	Fewer than continuation strategies
Average no. days strategies imposed	Fewer than continuation strategies

*An epidemic is defined in the model as a simulation with a clinical illness rate >1% of the population.

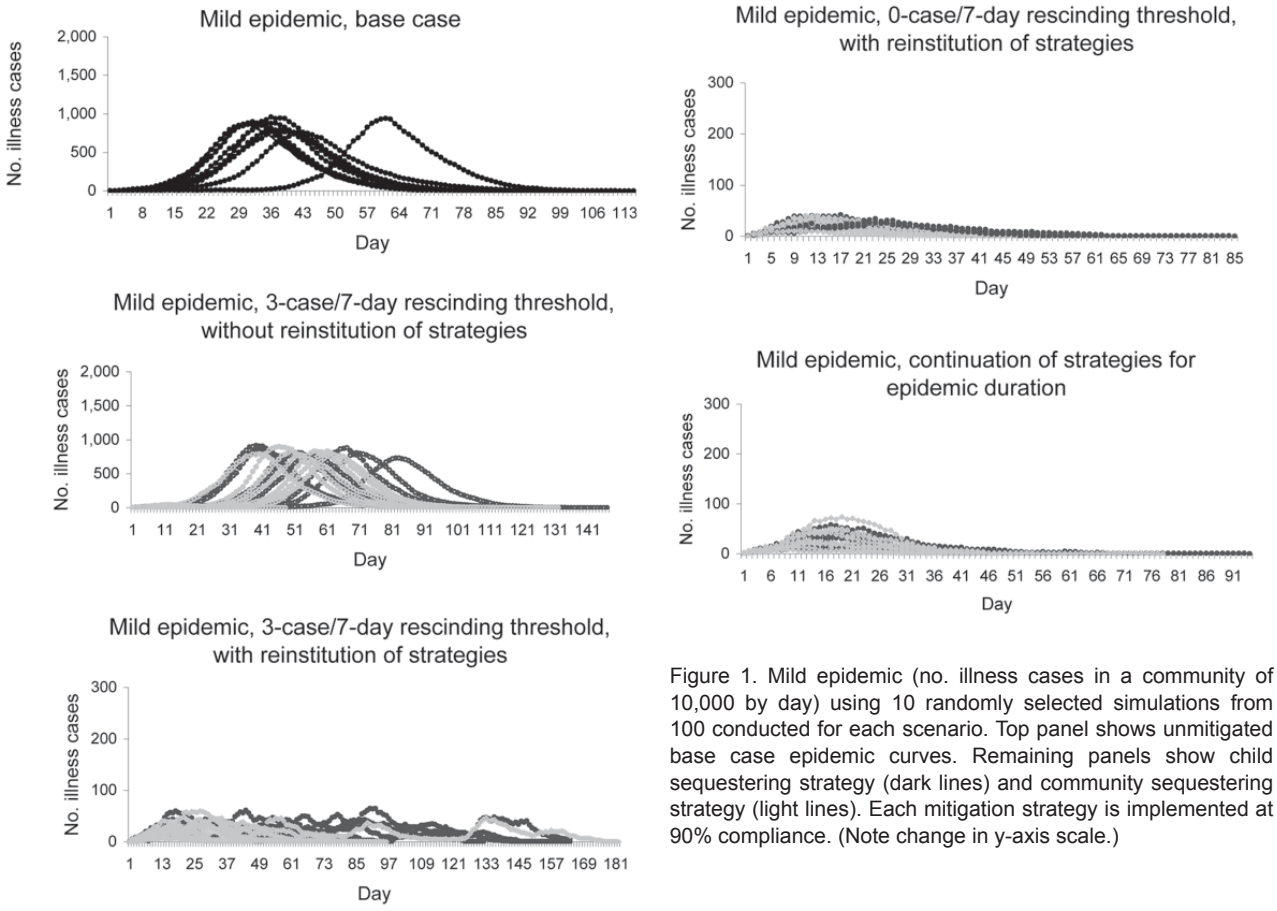

Figure 1. Mild epidemic (no. illness cases in a community of 10,000 by day) using 10 randomly selected simulations from 100 conducted for each scenario. Top panel shows unmitigated base case epidemic curves. Remaining panels show child sequestering strategy (dark lines) and community sequestering strategy (light lines). Each mitigation strategy is implemented at 90% compliance. (Note change in y-axis scale.)

the unmitigated base cases, adults were at home for longer periods with continuation strategies (12 days [mild] and 28 days [severe] for child sequestering; 43 days [mild] and 63 days [severe] for community sequestering; at 90% compliance) (online Appendix Tables 1, 2).

Rescinding Thresholds

Adding rescinding thresholds as a component of mitigation strategies resulted in several beneficial effects, influenced by whether a conservative or lax threshold was used and whether strategies were reinstituted if cases recurred. We assume 90% compliance with strategies; effects of lower compliance are considered in the next section.

Use of a 3-case threshold but not reinstituting strategies at the 10 new case trigger (Figures 1, 2) delayed the full epidemic by interjecting a preliminary period and peak where the epidemic is controlled by the mitigation strategy. After rescinding, a second 10-fold higher peak occurred (especially if child sequestering was used) and total illness attack rates approached those of the base case (Figures 1, 2). In comparison, by reinstituting cycles at the 10-case trigger even with the lax 3-case threshold, peak illness rates were dramatically flattened (to <100 cases for mild and generally <200 cases for severe epidemics), and the epidemic duration was lengthened because of the on-off cycling of mitigation strategies (Figures 1, 2). The 3-case threshold required an average of 3.2 (mild; community sequestering) to 5.8 cycles (mild; child sequestering) (Figures 1, 2, online Appendix Tables 1, 2).

The 0-case threshold was comparable to continuation strategies in controlling infection and peak illness rates (online Appendix Tables 1, 2, Figures 1, 2); <1.1 strategy cycles were needed to achieve control for both mild and severe epidemics. Infection and peak illness rates increased as the rescinding threshold was relaxed to 1, 2, or 3 cases (online Appendix Tables 1, 2). The 0-case threshold decreased duration of epidemics when compared with the 3-case threshold. For example, in a severe epidemic, the maximum epidemic represented by these sample plots lasted 286 days with a 3-case threshold versus 169 days with a 0-case threshold (Figures 1, 2). Adult days at home in a mild epidemic were decreased from the continuation strategy by 25% (12 days to 9 days) for child sequestering and by 33% (43 days to 29 days) for community sequestering, both with the 0-case threshold. For a severe epidemic, adult days at home for child sequestering decreased less (28 to

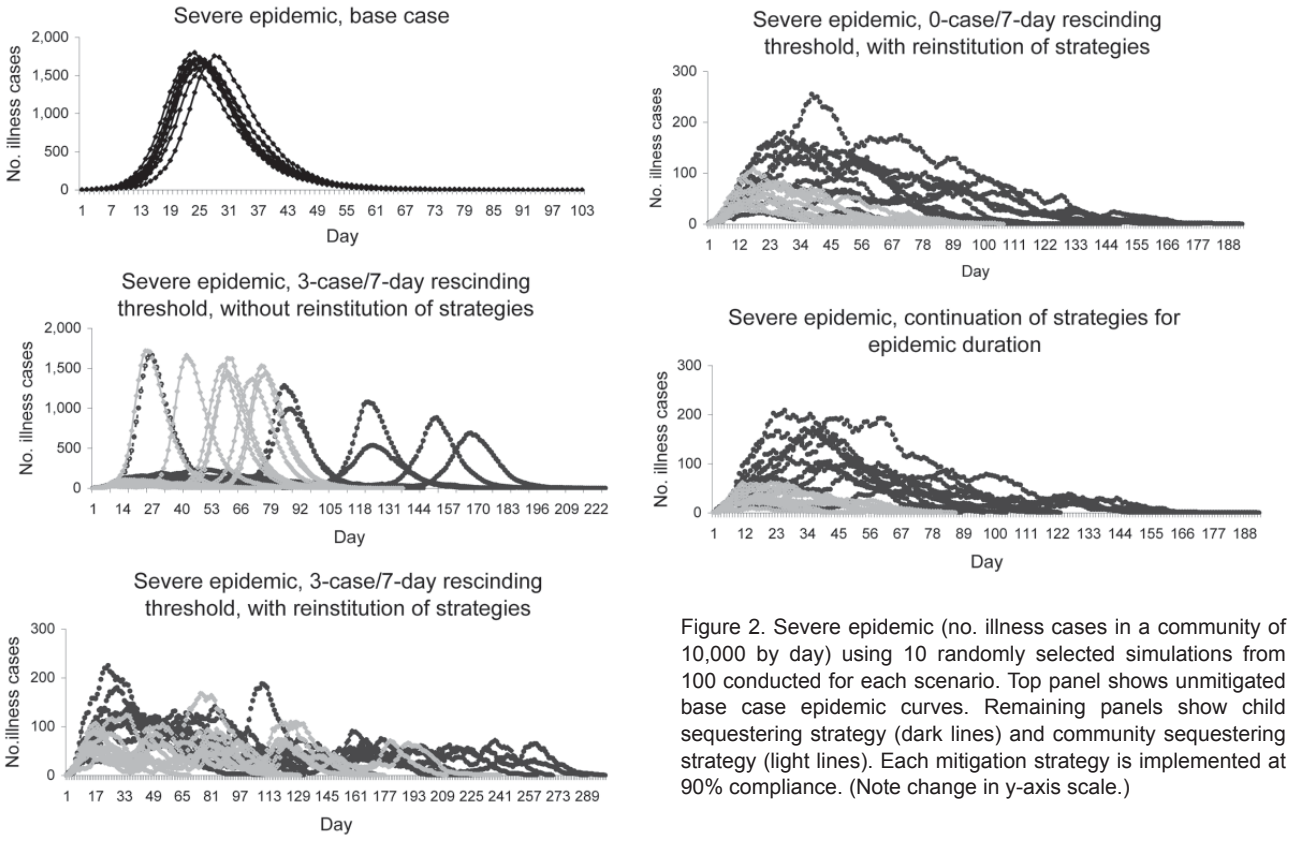

Figure 2. Severe epidemic (no. illness cases in a community of 10,000 by day) using 10 randomly selected simulations from 100 conducted for each scenario. Top panel shows unmitigated base case epidemic curves. Remaining panels show child sequestering strategy (dark lines) and community sequestering strategy (light lines). Each mitigation strategy is implemented at 90% compliance. (Note change in y-axis scale.)

27 days) with the continuation strategy than with community sequestering (63 to 49 days). Relaxing the rescinding threshold generally increased the number of adult days at home often above the continuation strategy (online Appendix Tables 1, 2). Recurrence of epidemics was a function of rescinding threshold; an average of only 1/10 epidemics recurred for the 0-case threshold versus 10/10 for the 3-case threshold (for both mild and severe epidemics).

Role of Compliance

Compliance with imposed child sequestering or community sequestering could be <90% because of persons who provide essential community services, other needs for persons to circulate in the community, or fatigue with the strategy. Figures 3 and 4 compare infection rates and strategy time across the rescinding thresholds and levels of compliance.

For the control continuation strategies, reducing compliance with child sequestering or community sequestering eroded effectiveness in preventing epidemics and also affected infection and peak illness rates. For severe epidemics, high compliance was critical to epidemic control. When compliance decreased from 90% to 50%, infection rates tripled for child sequestering (from 17% to 56%) and increased 10-fold for community sequestering (5% to 50%) (online Appendix Tables 1, 2). In contrast, because low compliance enabled the epidemic to pass through the community more quickly (infecting susceptible persons rapidly), adult days at home were fewer than at higher compliance (e.g., adult days at home in community sequestering were 51 days at 50% compliance and 63 days at 90% compliance (online Appendix Tables 1, 2).

When rescinded at the 0-case threshold with 80%–90% strategy compliance, fewest days of strategies were needed (online Appendix Tables 1, 2, Figures 3, 4). However, for severe epidemics, lower compliance (50%–70%) and the 0-case threshold used more strategy time than less restrictive ending case thresholds. Infection and peak illness rates showed little variation. In a severe epidemic with community sequestering implemented at 50% compliance, the infection rate was 50% with the 0-case threshold with 86 days of the strategy compared with an infection rate of 51% with the 3-case threshold with 71 days of strategy (online Appendix Tables 1, 2). The model shows this result because once half the population is infected (and therefore immune), the epidemic R_0 of 2.0 has effectively been halved. With fewer persons to infect at the end of the epidemic, use of the more conservative rescinding threshold has no effect on ultimate infection rates, requires additional days of strategy, and thus increases adult days at home.

Discussion

We examined whether it was possible to safely rescind child sequestering and community sequestering in a waning mild (PSI 1–2) or severe (PSI 4–5) epidemic by using 0, 1, 2, or 3 new cases in 7 days as a threshold. Defined epidemic management targets reflected community goals aimed at 1) minimizing infection and peak illness rates (to reduce illness cases, transmission opportunities, and to limit healthcare surge); 2) minimizing days adults were kept from their usual occupations (to enable community functions to continue); and 3) minimizing local epidemic duration (to enable all community members to return to usual activities). Community mitigation strategies such as child sequestering and community sequestering may help achieve these goals. Ideally, strategies should be used for the minimum time necessary.

When modeled with the highest compliance and reinstitution of strategies in the event of epidemic recurrence, a rescinding threshold of 0 cases applied as a component of community sequestering contained PSI 1–2 or 4–5 epidemics with fewest days of mitigation strategy needed. Peak illness rates did not exceed 1% of the population and infection rates were <10%. The 0-case threshold applied as a component of child sequestering in severe epidemics did not control as well, but still substantially reduced infection and illness rates and shortened the number of days adults were required to be home and days strategies were used compared with continuing the strategies for the epidemic duration.

When less conservative rescinding thresholds of 1, 2, or 3 cases were used (strategies applied with high compliance), epidemic recurrences required multiple reinstitution of cycles, ultimately yielding marked increases in cases, number of adult days at home, and epidemic duration. Reducing compliance increased infection rates, peak illness rates, and adult days at home, and required duration of strategies. For severe epidemics and low compliance, epidemics were only marginally suppressed. Thus, relaxing ending thresholds to 3 cases did not add to the effect of illness and ultimately cost fewer days of strategies. These findings indicate that low compliance cannot be compensated for by a restrictive rescinding threshold. High compliance is the necessary enabler of successful mitigation strategies (not the rescinding threshold).

Reinstitution of strategies in the event of epidemic recurrence was necessary to prevent near base case levels of illness. Such reinstitution will be a critical action in an epidemic resurgence or in the event of a multiwave pandemic. The 1918 pandemic consisted of an apparent springtime herald wave followed by severe fall-winter waves, and a series of secondary waves lasting into 1920. Historical studies of the 1918 pandemic demonstrated that earlier initiation and longer duration of nonpharmaceutical measures were associated with lower peak and total death rates in US cities (*12*,*13*,*18*). However, pandemic-affected cities that instituted, then abandoned, mitigation strategies had more illness cases in subsequent pandemic waves (because more susceptible persons remained in the population), which points to the need to plan for and follow stringent rules for reinstitution of measures.

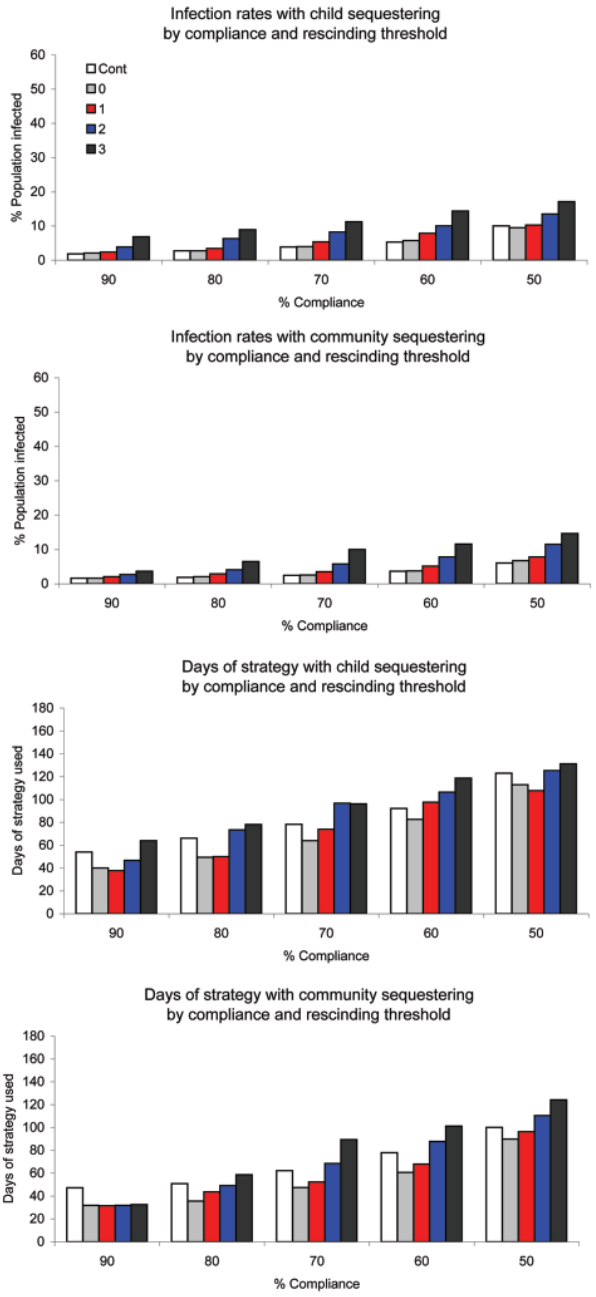

Figure 3. Infection rates and duration of strategy in mild epidemics by level of compliance for child sequestering and community sequestering, and rescinding threshold (Cont, 0, 1, 2, or 3). Cont, strategy continuation for the duration of the epidemic.

The social contact network used for this study was constructed to represent a small community in which complexity of person-to-person transmission can be represented in detail. Once within a community, influenza transmission takes place in the subnetworks simulated by this model, such as workplace, school groups, households, and neighborhoods. A larger population, such as a city, could be best modeled as a set of communities that are in contact through interactions in the work environment or through random interactions in shops or other settings. Mathematically, our model and results apply to such a situation where subnetworks in each community are similar, the epidemic is equivalently initiated in each, and identical mitigation strategies are applied with equivalent thresholds and compliances. Results then apply equally to single and large composite communities; rescinding thresholds should most conservatively be applied at the scale of the composite community.

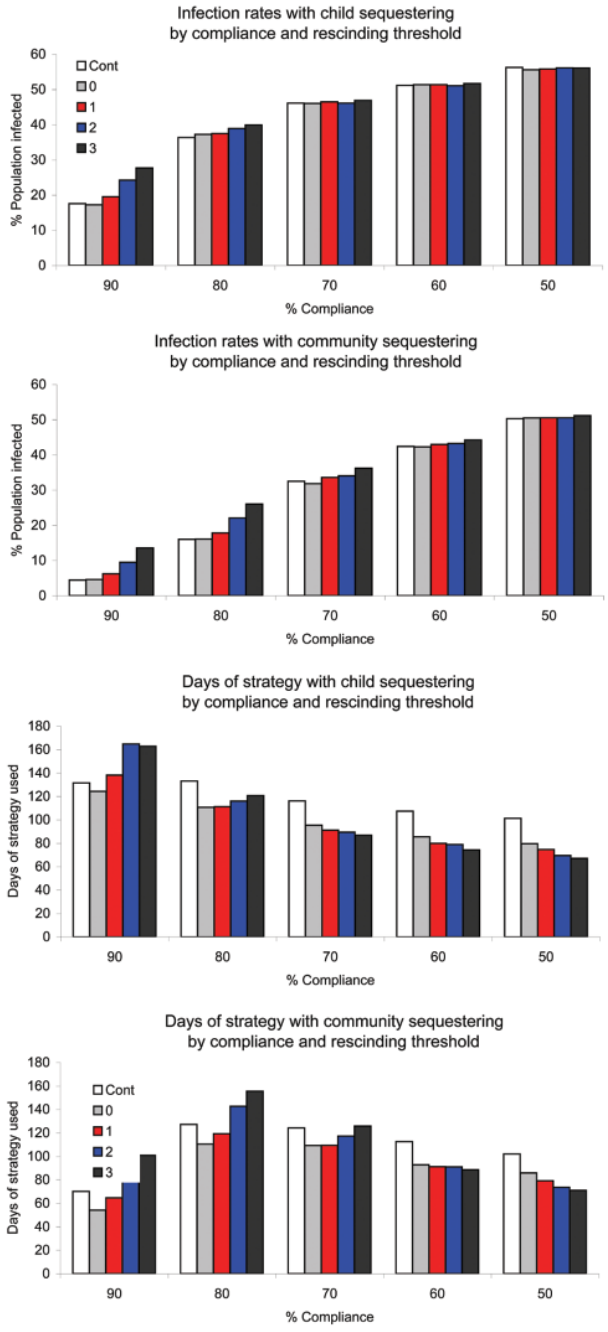

Figure 4. Infection rates and duration of strategy in severe epidemics by level of compliance for child sequestering and community sequestering, and rescinding threshold (Cont, 0, 1, 2, or 3). Cont, strategy continuation for the duration of the epidemic.

The need to formulate regionally based policies for school closings has been suggested (*19*). For comparison, we simulated epidemics in which neighboring communities were not practicing the same community mitigation strategies. In these unmitigated regional simulations, workplace contacts were replaced by random contacts from a fully mixed reservoir of adults from neighboring communities doing nothing to abate the epidemic. When compared with mitigated regional epidemic scenarios, in mild or severe epidemics with child sequestering implemented, peak illness rates doubled and tripled, respectively. However, child sequestering still decreased illness attack rates by ≥75% from base cases. Community sequestering was a more effective strategy in unmitigated regional scenarios for mild and severe epidemics, keeping peak symptomatic cases equivalent to the mitigated regional scenarios, but it lost effectiveness with decreasing compliance. Thus, without regional practice of strategies, effectiveness was less, but the direction of effects was the same.

A recent US Institute of Medicine report properly describes modeling as a simplification of reality (*9*). The stylized community we modeled exemplifies that description. However, modeling is a useful way to explore ramifications of policy. This study outlines potential approaches for reopening schools and workplaces in a waning epidemic, but also points to additional resources required to apply them. Clear case definitions, diagnostic criteria, and availability of diagnostic tests would clarify strategy initiation triggers. Accurate community surveillance would be necessary to count cases and determine when a rescinding threshold had been reached and when measures might need to be reinstituted. Initiation and rescinding of community mitigation strategies could seem frustratingly complex to an unprepared public, pointing to the need for clear messaging and information dissemination about rationale and potential effectiveness of all mitigation measures.

Epidemic modelers and public health experts propose that community mitigation measures might help communities limit pandemic effects. Nonpharmaceutical, behavior-based measures could be critically important in the interval between availability of vaccine and arrival of antiviral medications. Our study builds on previous studies that

examined timing and effectiveness of initiation of nonpharmaceutical pandemic mitigation measures by examining thresholds for rescinding them. We found that measures might be strategically applied and rescinded without the effect of additional illness and with savings of societal costs in terms of restriction of usual activities.

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Mycobacterium ulcerans Disease, Peru

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Eight adult patients (ages 18–58, 5 women) with Buruli ulcer (BU) confirmed by at least 2 diagnostic methods were seen in a 10-year period. Attempts to culture *Mycobacterium ulcerans* failed. Five patients came from jungle areas, and 3 from the swampy northern coast of Peru. The patients had 1–5 lesions, most of which were on the lower extremities. One patient had 5 clustered gluteal lesions; another patient had 2 lesions on a finger. Three patients were lost to follow-up. All 5 remaining patients had moderate disease. Diverse treatments (antituberculous drugs, World Health Organization [WHO] recommended antimicrobial drug treatment for BU, and for 3 patients, excision surgery) were successful. Only 1 patient (patient 7) received the specific drug treatment recommended by WHO. BU is endemic in Peru, although apparently infrequent. Education of populations and training of health workers are first needed to evaluate and understand the full extent of BU in Peru.

Buruli ulcer (BU), a chronic ulcerative disease, has been observed in many tropical areas, but patients have usually come from Africa and Australia (*1*–*6*). Cases were also described in the Americas, mostly in French Guiana (*3*,*6*). A few cases from Surinam have also been recorded in French Guiana, and 8 cases have been reported in Mexico since 1953 (*3*,*6*).

In 1969, the first 2 cases from Peru were reported (*7*). A new case was reported in 1988, along with a redescription of the first 2 cases (*8*). From 1996 through 2005, 8 additional cases, which we describe here, were found in Peru (Figure 1).

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Material and Methods

We conducted a descriptive, retrospective survey of patients seen by members of the Instituto de Medicina Tropical Alexander von Humboldt in Lima and Iquitos. We requested referrals of new patients from other areas. The total of 8 cases occurred from 1996 through 2005.

Patients

We used a collective data sheet proposed by the World Health Organization (WHO) (*9*) to assess the magnitude and severity of the disease and to collect data from patients. We also obtained information from medical records. Patients who had a history of chronic ulcer and who had at least 2 different positive laboratory tests were included in this study (*10*). All the patients gave consent verbally. The publication was approved by the Human Protections Administration Office for Human Research Protection of the Universidad Peruana Cayetano Heredia (SIDISI code 52467).

Figure 1. Map of Peru showing the locations where 8 Buruli ulcer patients were probably infected. Red, Peruvian River basin; gold, coastal area.

Smears and Tissue Collection

Smears obtained by scalpel or swab were prepared with material from the necrotic base and undermined edges of the lesions and were stained with Ziehl-Neelsen. Skin biopsy samples were excised and cut into ≥2 portions. At least 1 portion was fixed in 10% formalin and processed for histologic examination at the pathology laboratory of the Hospital Nacional Cayetano Heredia in Lima. The other portions, minced further, were inoculated after decontamination onto Löwenstein-Jensen medium in Lima as described previously (*10*); the rest was placed in a semisolid transport medium (*11*) and sent to the Institute of Tropical Medicine, Antwerp, for culture and PCR testing (*10*).

Results

We found 8 cases of BU from 1996 to 2006. Case characteristics are indicated in Table 1. Five patients came from the Peruvian rainforest, the likely place of infection. Two patients reported close contact with water in the Marañón (patient 1) and Huallaga (patient 2) River basins. Three patients (patients 3, 4, and 6) lived close to Iquitos, a city on the Amazon River. One patient (patient 5) had briefly visited a swampy area in the north coast of Peru (Tumbes), and 2 other patients (patients 7 and 8) lived in the same area. All of the areas described are warm and humid. The age range at diagnosis was 18–58 years, with a male to female ratio of 3:5. No case patients had a medical or family history of tuberculosis or leprosy. The time between onset of illness and being seen by a physician was 1–8 months. Four patients noticed a nodular lesion before ulceration occurred. All patients had ulcers with typical undermined edges. The site of involvement in our patients was on the extremities, but 1 patient had gluteal lesions. The median number of lesions per patient was 2. Three patients (patients 1, 2, and 5) were lost to follow-up at an early stage, soon after diagnosis, and their disease course was unknown.

Patient 3 had lesions on both knees; he liked gardening and often knelt on soil and organic mulch that contained wood shavings. He first received rifampin and ethambutol for 5 weeks. Drug therapy was stopped because of hepatotoxicity. Trimethoprim-sulfamethoxazole and ciprofloxacin were then administered for 15 days. After completing oral therapy, he treated his lesions with a rifampin spray. Eight months after the start of drug therapy, the lesions were almost closed. A small ulcer remained on the right knee. The patient showed complete remission of lesions without any surgical intervention on his last control visits, 3 and 5 years after diagnosis.

Patient 4 had a medical history suggestive of leishmaniasis, and a smear from an ulcerated lesion was reported as positive for *Leishmania*. She was treated with intramuscular and intralesional sodium stibogluconate, including multiple inoculation sites. One month after receiving a course of 29 intramuscular injections, she showed more lesions in the left gluteal region and was treated with herbal medicine. BU was diagnosed from purulent material removed with a syringe and needle from a closed lesion. After drainage and biopsy her lesions showed improvement, and therefore no other treatment was instituted. She continued herbal treatment until total cure. The lesions have remained inactive for 5 years.

Patient 6 was treated by excision surgery and later received antituberculosis treatment (regimen 1) for 6 months. Patient 7 was pregnant when first seen. After tissue specimens were taken and BU was diagnosed, she was treated conservatively with topical disinfectants until delivery. She then received the WHO-recommended rifampin and streptomycin treatment (*12*) for 31 days and had excision surgery of the largest lesions. Later, all lesions were excised. Patient 8 had lesions on the right middle finger (Figure 2, panel A) that ulcerated after treatment for 1 month with ciprofloxacin, clindamycin, and dexametasone (Figure 2,

Table 1. Case descriptions of Buruli ulcer, Peru*

Patient no.	Geographic origin	Age, y/sex	Patient delay, mo	Localized pain	No. of lesions	Sites	Size of main lesion, cm	Treatment
1	Marañón	18/M	8	Positive	2	Left knee	7 × 6	Lost to follow-up, no treatment
2	Huallaga	22/F	1	Positive	1	Left thigh	2 × 3	Lost to follow-up, no treatment
3	Iquitos	54/M	2	Positive	2 (right knee earlier, larger)	Both knees	6 × 7	Antituberculous drugs and herbal medicines
4	Iquitos	58/F	8	Positive	5 (in a single group)	Left gluteal region	12 × 16 (sum of all 5)	Anti-*Leishmania* and herbal medicines
5	Tumbes	46/F	8	Positive	2 (1 lesion was a scar)	Left foot	5 × 6	Lost to follow-up, no treatment
6	Iquitos	21/F	3	Positive	1	Right thigh	5 × 5	Antituberculous drugs (regimen 1) and surgery
7	Tumbes	34/F	2	Positive	4	Right thigh and leg	6 × 8	WHO BU antibiotics and surgery
8	Tumbes	45/M	1	Positive	2	Right middle finger	8 × 2	Antituberculous drugs (regimen 1) and surgery

*WHO, World Health Organization; BU, Buruli ulcer.

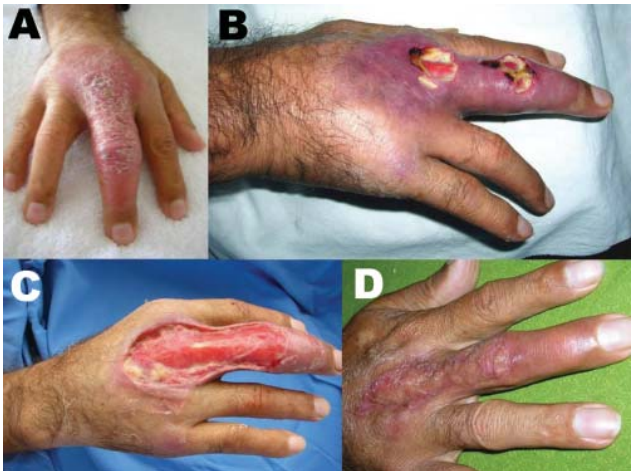

Figure 2. Patient 8. A) Nonulcerative edematous lesion on the right middle finger as first seen; B) ulcerated lesions on the right middle finger ≈4 weeks later; C) extensive debridement, 5.5 weeks after first seen; D) cured lesion 5 months after first seen, 1 month after autologous skin graft.

panel B). Diagnosis was made on the basis of material obtained at the first extensive debridement (Figure 2, panel C). He received 5 weeks of regimen 1 treatment for tuberculosis, to which streptomycin was added for the last 3 weeks before surgical debridement and autologous skin graft. Antituberculous regimen 1 was continued for 2 more weeks. Figure 2, panel D shows the lesion 1 month after surgery. The patient then received 4 months of treatment with minocycline, ciprofloxacin, and trimethoprim-sulfamethoxazole and undertook rehabilitation including exercises. He recovered very good use of his right hand.

Patients 6, 7, and 8 were cured with antimicrobial agents and surgery. They had no recurrence after 2 years of follow-up.

As indicated in Table 2, all patients' cases were confirmed by at least 2 positive laboratory tests. Seven patients showed acid-fast bacilli (AFB) on the initial smear, and 1 was negative (patient 5). The biopsy specimens from all the patients had AFB in histopathologic sections and typical histologic lesions, i.e., necrosis of fat and an abundance of extracellular clumps of AFB. Most biopsy specimens showed little or no inflammatory infiltrate. A granulomatous infiltrate was seen in the biopsy specimen from patient 5. Cultures remained negative, and IS*2404* PCR was positive for all 7 patients tested.

Discussion

From 1969 until 2007, only 11 cases of BU have been reported in Peru, but no countrywide survey has been conducted to evaluate its true prevalence in Peru. BU is probably both infrequent and underreported in Peru and may often be misdiagnosed as leishmaniasis, which is more prevalent and better known. Three separate surveys suggest the rarity of BU. In the first, Saldaña-Patiño reviewed 1,620 ulcers biopsied from 1969 to 1981 and found no other BU patients apart from the 3 he reported (*8*). Second, a preliminary epidemiologic survey was conducted in the general area of the Huallaga Basin close to Tarapoto; several leishmaniasis and vascular lesions were found in 4 communities of ≈4,000 inhabitants, but no BU cases were seen (N. Donaires, MD thesis). Finally, physicians performing populationwide cysticercosis research in Tumbes (on the north coast) included a questionnaire and physical examination of all skin ulcers at the time, using as a guide a booklet in Spanish that was provided to familiarize them with BU (*13*). No skin ulcers were seen in the population surveyed.

The scarcity of BU cases in Peru and Mexico may be due to a lower virulence of the mycobacteria and a better immune response of patients when they become infected by *M. ulcerans*. It is clearly not related to infrequent contact with contaminated water. As in Africa, populations living in the Amazon River Basin have frequent contacts with water for domestic activities. Similarly, the low incidence of BU in Peru does not seem to be related to the absence of *M. ulcerans* in the environment, since the IS*2404* PCR positivity of the environmental specimens from Peru (collected in Tarapoto, in the Huallaga River Basin) and Benin have given comparable results (14% positivity in Peru vs. 10%–20% positivity in Benin) (F. Portaels et al., unpub. data).

Table 2. Diagnostic laboratory studies on patients with Buruli ulcer, Peru*

Patient no.	ITM no.	AFB in smear (ZN)	Histopathologic changes	AFB in histologic sections	IS*2404* PCR	Culture
1	None	Positive	Necrosis of fat	Positive	ND	ND
2	96–0729	Positive	Necrosis of fat	Positive	Positive	Contaminated
3	01–0720	Positive	Necrosis of fat	Positive	Positive	Negative
4	02–1536	Positive	Necrosis of fat	Positive	Positive	Contaminated
5	02–1877	Negative (only 1 AFB seen)	Necrosis of fat + granulomatous infiltrate	Positive	Positive	Negative
6	04–0872	Positive	Necrosis of fat	Positive	Positive	Negative
7	05–2249 05–2411	Positive	Necrosis of fat + inflammation	Positive	Positive	Negative
8	None	Positive	Necrosis of fat + inflammation	Positive	Positive	Negative

*ITM, Institute of Tropical Medicine; AFB, acid-fast bacilli; ZN, Ziehl-Neelsen staining; ND, not done.

The distribution of BU in Peru and elsewhere is strongly associated with wetlands, especially those with slow-flowing or stagnant water (e.g., ponds, backwaters, and swamps) (*4*,*5*). All of our patients had contacts with swampy areas in the Amazon River Basin (5 patients) or on the northern coast (3 patients). The 3 previously reported patients (*8*) had been in contact with water bodies related to tributaries of the Amazon River. In Peru, therefore, BU is present in the Peruvian jungle (*14*) and other swampy regions of the north coast.

In our study, the age of patients when they were first seen with BU ranged from 18 to 58 years. *M. ulcerans* is seen mainly in children and young adults in other BU-endemic regions but may affect any age group (*4*,*5*,*15*). All patients except patient 8 had lesions on the lower limb (Table 1). A similar pattern has been reported in other countries (*4*,*5*,*15*).

Patients sought medical assistance ≈1 month after the first lesion appeared. The longest interval to final diagnosis was 8 months, which led to a very large lesion in patient 4, who was originally being treated for leishmaniasis.

In Africa, the stigma associated with BU appears to be important, and its mysterious nature is often attributed to witchcraft and curses (*16*–*18*). Such concerns were not voiced by any of our patients, as they all had actively sought medical help.

Beside patients lost to follow-up, the clinical outcome of all patients from Peru was favorable. One patient (patient 4) was cured with herbal medicine only. Several authors report that while some topical treatments may heal BU lesions (*4*,*19*), other lesions may heal spontaneously (*20*). Patient 4 may have healed spontaneously or because of herbal medicine. Surgical treatment alone, which was until recently the mainstay of clinical management of BU in BU-endemic areas (*4*) is not practiced in Peru. Surgery is always associated with antimycobacterial drug therapy. Several centers in Africa have started to treat patients with streptomycin and rifampin according to WHO guidelines (*12*), and a recent study indicates that after 8 weeks of drug therapy ulcers may heal without surgery (*21*). The patients with reported infections in Peru up to 1988 had a favorable response to antituberculous therapy, although their lesions were large (*7*). In our study, patient 3 was also successfully treated with drug therapy without surgery. The success of antimycobacterial therapy in some areas may be correlated with a lower virulence of the *M. ulcerans* strains and in particular with lower production of mycolactones. African strains, which produce the greatest number and quantity of mycolactones, are associated with more severe disease forms (*22*), which may explain the difficulty treating some patients with only antimycobacterial drugs.

All patients in our study were PCR positive, but we were unable to cultivate *M. ulcerans* from the clinical specimens in Lima or Antwerp. This is surprising since the procedures used to cultivate *M. ulcerans* in primary culture were identical to those used for thousands of specimens from patients from other parts of the world (which yielded 45% of positive primary cultures) (*11*). Peruvian *M. ulcerans* strains may have different growth requirements or may be more sensitive to the antimicrobial agents in semisolid transport medium (PANTA: polymixin B, amphothericin B, nalidixic acid, trimethoprim, and azlocillin) than those from other geographic locations.

In conclusion, our study confirms that, although infrequently diagnosed, BU is an endemic disease in tropical swampy areas of Peru. Proper diagnosis and treatment require inclusion of simple clinical and laboratory guidelines in tuberculosis, leprosy, and leishmaniasis control programs, which reach health workers at all levels. Known BU-endemic areas should receive special emphasis. Education of populations and training of health workers are first needed to evaluate and understand the full extent of this disease in Peru.

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etymologia

Mycobacterium

[mi′-ko-bak-tēr-eəm], from the Greek—myces (fungus) and baktērion (little rod)

The only genus of bacteria in the family *Mycobacteriaceae*. In 1882, German scientist Robert Koch reported the discovery of a bacillus from the lung tubercles that caused tuberculosis. Earlier, Norwegian researcher G.H.A. Hansen had identified a similar microbe which caused leprosy. In 1896, the genus name *Mycobacterium*, from the Middle Latin noun meaning fungus rodlet, was proposed to include these new pathogens, *M. tuberculosis* and *M. leprae*. The name does not mean that mycobacteria are fungi; rather, the tubercle bacilli grow on the surface of liquid media as moldlike pellicles when cultured. The nomotile, acid-fast, aerobic organisms in this genus cause numerous human and animal diseases.

Source: Sources: Savin JA, Wilkinson DS. Mycobacterial infections including tuberculosis. In: Rook A, Wilkinson DS, Ebling FJG, Champion RH, Burton JL, editors. Textbook of dermatology. Vol.1, 4th ed. Boston: Blackwell Scientific Publications; 1986. p. 791–822. Goodfellow M, Magee JG. Taxonomy of mycobacteria. In: Mycobacteria: basic aspects. Gangadharam PRJ, Jenkins PA, editors. Boca Raton (FL): Chapman & Hall; 1998. p.1. Wayne LG. The "atypical" mycobacteria: recognition and disease association. CRC Crit Rev Microbiol. 1985;12:185–222.

Multicenter Cross-Sectional Study of Nontuberculous Mycobacterial Infections among Cystic Fibrosis Patients, Israel

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This 2-year cross-sectional evaluation of nontuberculous mycobacterial (NTM) infections involved all Israeli medical centers that treat cystic fibrosis patients. The study comprised 186 patients whose sputum was analyzed for NTM. The prevalence of NTM isolates was 22.6%, and 6.5% and 10.8% of the patients fulfilled the 1997 and 2007 American Thoracic Society criteria for NTM lung disease, respectively. *Mycobacterium simiae* (40.5%), *M. abscessus* (31.0%), and *M. avium* complex (14.3%) were the most prevalent. Presence of *Aspergillus* spp. in sputum and the number of sputum specimens processed for mycobacteria were the most significant predictors for isolation of NTM (odds ratio [OR] = 5.14, 95% confidence interval [CI] 1.87–14.11 and OR = 1.47, 95% CI 1.17–1.85, respectively). The incidence of NTM pulmonary infections is increasing among cystic fibrosis patients, reflecting the increase in longevity of such patients as well as environmental exposure to various species of mycobacteria.

The dramatic improvement in the survival of patients with cystic fibrosis (CF) has been complicated by the development of highly resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*; the appearance of new virulent pathogens, such as *Burkholderia cepacia*; and the emergence of organisms of undetermined clinical importance, such as *Alcaligenes xylosoxidans*, *Stenotrophomonas maltophilia,* and the nontuberculous mycobacteria (NTM) (*1–4*). Since 1990, an increasing number of studies have reported the recovery of NTM from the respiratory tract of patients with CF at a prevalence of 2%–28% (*2–13*); higher prevelances have been reported in the United States (*14*) than in Europe (*4*,*11*). Whether such findings indicate infection or simply colonization of the airways by an environmental organism is not clear. The 1997 American Thoracic Society (ATS) guidelines for the diagnosis of NTM lung disease include compatible clinical and radiographic findings as well as bacteriologic findings of 3 positive cultures or, alternatively, 2 positive cultures and a positive smear for acid-fast bacilli (AFB) (*15*). The 2007 ATS microbiologic criteria, however, require the following: 1) 2 positive sputum cultures or 1 positive culture if it was obtained through bronchial wash, lavage, or lung biopsy; or 2) ≥1 sputum or bronchial washings that are culture positive for NTM if mycobacterial histopathologic features were evident (*16*). In a recent multicenter study of CF patients in the United States, Olivier et al. (*14*) reported an overall prevalence of NTM in sputum of 13%. Most isolates in their study were of the *Mycobacterium avium* complex (MAC), although a high prevalence of *M. abscessus* was also noted. Only 20% of the samples met the 1997 ATS microbiologic criteria for disease. It was suggested that patients with CF and multiple positive NTM cultures, charac-

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teristic high-resolution computerized tomographic (HRCT) findings, and progression of HRCT changes should be monitored closely and considered for antimycobacterial drug therapy (*17*).

In Israel, 468 patients with CF are currently treated in 7 medical centers. Although all 7 report that they screen for NTM pulmonary secretions on a regular basis and during most CF exacerbations, we wanted to investigate the various approaches they used to diagnose NTM pulmonary disease. We also wanted to determine the prevalence of NTM infection, the different species involved, and the associated risk factors for the development of NTM pulmonary infections in Israeli patients whose sputum was processed for NTM.

Methods

Definition and Ascertainment of Cases

This retrospective observational study was conducted at all Israeli medical centers that treat patients with CF. The medical records of all CF patients from July 2001 through July 2003 were screened. The number of patients ranged from 15 to 134 per center, with a total of 468 patients. Most patients routinely visited the centers in intervals of at least 3 months. The study population included CF patients ≥5 years of age who had not undergone lung transplantation and in whom sputum specimens were processed for mycobacteria.

We defined NTM infection as a patient having had at least 1 positive isolate over time. NTM disease was defined as the condition in which a patient had a positive NTM isolate and met ATS disease criteria. CF patients who were evaluated at least once without evidence of NTM constituted the control group. We analyzed the data according to the 1997 and 2007 ATS criteria. The study was approved by the respective institutional review boards.

Data Collection and Study Design

The study design was cross-sectional. Demographic, clinical, and laboratory data for all eligible patients were collected from medical records, which included: age, gender, CF genotype, sweat chloride level, body mass index, forced expiratory volume in 1 s (FEV_1); average during the study period), pancreatic function, presence of hemoptysis, sputum cultures during the study period, length of hospitalization (total time throughout the study period), antimicrobial agents administered (yes or no during the study period), and other treatment modalities.

Laboratory Methods

Respiratory tract specimens were assessed in the local microbiology laboratories of each center. The methods were not standardized, but the laboratories operated according to recommendations by international expert groups (*18*). When NTM did grow, however, isolates were forwarded to the National Mycobacterium Reference Laboratory of Israel for further identification. Specimens were processed by standard methods and inoculated onto MB/BacT bottle (BacT/Alert System, bioMérieux, Marcy l'Etoile, France), a Lowenstein-Jensen slant, and a Middlebrook 7H11 selective agar plate (*19–21*). All inoculated media and broths were incubated at 36°C until growth was observed or up to 7 weeks. Direct smears and smears from colonies were stained with Ziehl-Neelsen stain. Species identification was performed by conventional biochemical methods and by determining antimicrobial drug susceptibility patterns using the resistance ratio method and Etest (Biodisk, Solna, Sweden) (*21*,*22*). MAC isolates were confirmed by using commercial RNA/DNA probes (Accuprobe, Gen-Probe, Inc., San Diego, CA, USA).

Data Analysis

Prevalence of NTM was calculated as the ratio between the number of CF patients with at least 1 positive culture and the total study population. Univariate analysis for the comparison of cases and controls was performed by using Student *t* test for continuous variables and the χ^2 test for categorical variables. Multivariate logistic regression analysis was performed to evaluate the effect of predicting variables for NTM-positive cases. Only variables that were significant in the univariate analyses ($p<0.05$) were included in the model (age, number of sputum specimens that were processed for mycobacteria, number of hospitalization days, number of days receiving antimicrobial agents, FEV_1, presence of hemoptysis, growth of *Pseudomonas* or *Aspergillus* spp. in sputum, presence of allergic bronchopulmonary aspergillosis, and treatment with azithromycin or ibuprofen). Data were analyzed by using SAS Software, version 9.0 (Cary, NC, USA).

Results

Study Population

A total of 282 of the 468 eligible CF patients were excluded from the study: 203 did not have any sputum processed for mycobacteria, 59 were <5 years of age, 8 underwent lung transplantation, 2 had received immunosuppressive treatments, and follow-up was not available for the remaining 10 (Table 1). Sputum specimens were processed for mycobacteria for 265 (57%) patients. Four large centers (A, C, D, E, Table 1) evaluated 60%–80% of their CF patients for NTM, and 2 smaller centers (F, G, Table 1) evaluated 45% of their CF patients. One center (B, Table 1) did not evaluate its CF patients for NTM, and those patients were excluded from the study.

For the patients whose sputum was processed for mycobacteria, the average number of sputum samples per pa-

Table 1. Number of patients in all medical centers and their reasons for exclusion from study

Center	Total no. patients	Absence of mycobacterial culture (%)	Age <5 y	Other causes*	No. patients included
A	134	36 (27)	5	11	82
B	82	74 (90)	7	1	0
C	75	28 (37)	13	3	31
D	71	14 (20)	14	1	42
E	60	26 (43)	14	2	18
F	31	17 (55)	4	2	8
G	15	8 (54)	2	0	5
Total	468	203 (72)	59 (21%)	20 (7%)	186

*Other causes; lung transplantation, immunosuppressive therapy, dropped from follow-up.

tient during the study period was 3.1 ± 3.03. Patients whose sputum was evaluated for NTM (n = 265) were older and had markers of the severe form of disease compared to those whose sputum was not evaluated for mycobacteria (n = 203) (Table 2). A total of 186 study participants were eventually enrolled in the study; 42 had NTM infection or disease and 144 were controls. Twelve of the patients with NTM infection had NTM disease according to the 1997 ATS criteria; 20 patients had NTM disease according to the 2007 ATS criteria (p = 0.07).

Prevalence of NTM

The prevalence of NTM isolation among CF patients was 22.6% (42/186) (95% confidence interval [CI] 16.2–27.9). The prevalence of NTM varied by geographic location: no NTM were isolated from patients residing in northern Israel (center E, Table 1), whereas the prevalence was 24%–29% in hospitals located in central and southern Israel (centers A, C, D, F, Table 1), (Figure).

According to 1997 ATS criteria, 12 patients (6.5%) had NTM disease; 7 (58.3%) of these had AFB on smear. According to the 2007 ATS criteria, 20 patients (10.8%) had NTM disease, of whom 8 (40.0%) had AFB on smear. The proportion of patients with NTM disease in centers A, C, and D was 7.3%, 12.9%, and 4.8%, respectively, according to the 1997 ATS criteria, and 14.6%, 13.0% and 9.5%, respectively, according to the 2007 ATS criteria.

NTM Species

The most common mycobacterial species were *M. simiae* (17 patients, 40.5%), *M. abscessus* (13 patients, 31.0%), and MAC (6 patients, 14.3%). *M. fortuitum* was isolated in 4 patients (2 patients had both *M. fortuitum* and *M. simiae,* and 1 patient had both *M. fortuitum* and *M. abscessus*). The species of NTM could not be determined for 5 other patients.

Species distribution differed according to geographic location (Figure). *M. simiae* was the most prevalent in center A, whereas *M. abscessus* was the most prevalent species in centers C and D. No mycobacteria were isolated in 2 centers. Seven of the 12 patients with NTM disease according to the 1997 ATS criteria were infected by *M. simiae* and 4 by *M. abscessus.* The species could not be determined for 1 patient. Nine of the 20 patients with NTM disease according to the 2007 ATS criteria were infected by *M. simiae*, 7 by *M. abscessus,* and 1 by MAC. The species could not be determined in 3 patients.

Case-Control Study

Patients with NTM were significantly older (by 4.8 years) than culture-negative study participants, had more sputum specimens processed for mycobacteria, had more episodes of hemoptysis (23.8% vs. 8.3%), a lower FEV_1 (14.5 L/s), a longer hospital stay (14.8 days), and more exposure to intravenous antimicrobial treatment (35.2 days) (Table 3). Patients with NTM were treated with azithromycin and

Table 2. Comparison of patients included in the current study, patients tested for NTM, and patients not tested for NTM*

Parameter	Patients included in the current study (n = 186)	Patients tested for NTM (n = 265)	Patients not tested for NTM (n = 203)	p value
Age (mean ± SD)	20.51 ± 10.40	20.22 ± 10.53	13.99 ± 10.70	0.0001
Sex, F/M	74/112	105/160	86/117	0.54
Hemoptysis	22	23	4	0.002
FEV_1, L/s (mean ± SD)	67.90 ± 22.09	65.18 ± 21.69	82.94 ± 16.20	0.0001
Pancreatic insufficiency	135	151	131	0.09
Hospitalization, d (mean ± SD)	21.77 ± 28.54	23.80 ± 32.44	8.44 ± 14.03	0.0001
Administration of antimicrobial agents, d (mean ± SD)	22.66 ± 47.51	19.98 ± 45.52	2.30 ± 10.28	0.0001
Azithromycin	104	114	51	0.001
Ibuprofen	7	15	6	0.16
Insulin	17	20	9	0.16
Systemic steroids	13	37	19	0.12
Inhaled steroids	96	106	105	0.01

*NTM, nontuberculous mycobacteria; SD, standard deviation; FEV_1, forced expiratory volume in 1 s.

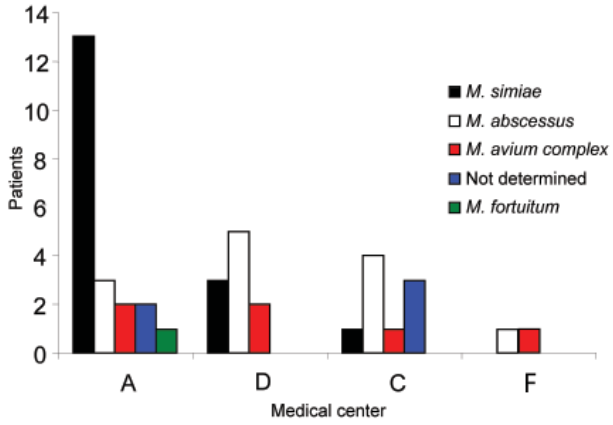

Figure. Different species of nontuberculous mycobacteria isolated from patients with cystic fibrosis (unique patient isolate) in 4 medical centers. *M*., *Mycobacterium*.

ibuprofen more frequently. They had higher rates of *P. aeruginosa* (95.2% vs. 65.3% of controls) and *Aspergillus* spp. (66.7% vs. 21.5% of controls) in sputum samples. Both study and control groups were similar with respect to sex, sweat chloride level, pancreatic insufficiency, and requirement for insulin and steroids. Culture-negative and culture-positive groups had similar frequencies of mild and severe genotypes and also had the same rates of *S. aureus* and *S. maltophilia* growth. A multivariate analysis demonstrated that the presence of *Aspergillus* spp. in sputum and the number of sputum specimens processed for mycobacteria remained the only statistically significant predictors for developing NTM infection (odds ratio [OR] 5.14, 95% CI 1.87–14.11 and OR 1.47, 95% CI 1.17–1.85, respectively).

According to the 1997 ATS criteria, patients with NTM disease had more sputum specimens processed for mycobacteria, longer hospital stays, more courses of ibuprofen, higher isolation rate of *Aspergillus* spp., higher frequency of allergic bronchopulmonary aspergillosis, and more positive sputum smears than patients with NTM infection (Table 4). Analysis of the data according to the 2007 ATS criteria disclosed that patients with NTM disease had more sputum specimens processed for mycobacteria, used more inhaled steroids, and had more positive sputum smears. Patients with *M. abscessus* growth in sputum were younger than patients with the growth of other NTM (18.46 ± 6.42 vs. 26.79 ± 11.63 years, $p<0.05$), tended to have positive smears (53.8% vs. 13.8%, $p<0.001$), and had frequent growth of *S. maltophilia* in sputum (15.4% vs. 0, $p<0.05$). Patients with *M. simiae* growth in sputum had worse sweat test results than those with the growth of other NTM (92.18 ± 45.42 vs. 60.08 ± 49.88, $p<0.05$); they had more episodes of hemoptysis (52.9% vs. 12.0%, $p<0.05$), and they were treated more often with inhaled steroids (82.3% vs. 48.0%, $p<0.05$) and systemic steroids (29% vs. 0, $p<0.01$).

Discussion

This multicenter study included 40% of the registered CF patients in Israel during a 2-year period and is the most representative study on NTM pulmonary infection among

Table 3. Comparison between patients and controls in univariate and multivariate analyses*

Parameter	Patients (n = 42)	Controls (n = 144)	p value	Adjusted OR	95% CI
Age, y (mean ± SD)	24.2 ± 10.9	19.4 ± 10.0	0.014	1.03	0.99–1.09
Sex, F/M	18/24	56/88	0.64		
No. sputum specimens (mean ± SD)	5.7 ± 4.8	2.4 ± 1.7	<0.0001	1.47	1.17–1.85
Hemoptysis	10	12	0.006	1.08	0.29–4.05
FEV_1, L/s (mean ± SD)	56.7 ± 19.6	71.2 ± 21.8	0.0001	0.97	0.94–0.99
Pancreatic insufficiency	35	100	0.07		
Sweat chloride, Meq/ L (mean ± SD)	73.1 ± 50.2	66.2 ± 48.3	0.43		
Hospitalization, d (mean ± SD)	33.2 ± 37.5	18.4 ± 24.5	0.019	0.99	0.97–1.01
Administration of antimicrobial drugs, d (mean ± SD)	49.9 ± 78.7	14.7 ± 29.4	0.007	0.99	0.98–1.00
Azithromycin treatment	30	74	0.02	1.00	0.99–1.00
Azithromycin treatment, d (mean ± SD)	367.6 ± 302.7	221.9 ± 288.4	0.007	1.00	0.99–1.00
Ibuprofen treatment	5	2	0.001	4.72	0.60–36.85
Insulin treatment	4	13	0.92		
Systemic steroids treatment	3	10	0.96		
Inhaled steroids treatment	26	70	0.13		
Pseudomonas aeruginosa	40	94	0.0005	0.76	0.32–1.79
Staphylococcus aureus	18	58	0.95		
Aspergillus	28	31	<0.0001	5.14	1.87–14.11
Allergic bronchopulmonary aspergillosis	3	3	0.10		
Haemophillus influenza	3	24	0.13		
Alcaligenes xylosoxidans	0	5	0.22		
Klebsiella pneumoniae	3	4	0.17		
Stenotrophomonas maltophilia	2	3	0.34		

*OR, odds ratio; CI, confidence interval; SD, standard deviation; FEV_1, forced expiratory volume in 1 s.

Table 4. Comparison of patients with NTM infection and NTM disease according to 1997 and 2007 ATS criteria*

Parameter	1997 criteria			2007 criteria		
	NTM disease (n = 12)	NTM infection (n = 30)	p value	NTM disease (n = 20)	NTM infection (n = 22)	p value
Age, y (mean ± SD)	19.8 ± 9.1	26.0 ± 11.2	0.17	24.2 ± 11.7	24.3 ± 10.4	0.97
Sex, F/M	7/5	11/19	0.20	9/11	9/13	0.79
No. sputum specimens (mean ± SD)	9.0 ± 5.1	4.4 ± 4.0	0.01	8.0 ± 5.2	3.6 ± 3.2	0.003
BMI, kg/m^2 (mean ± SD)	18.9 ± 1.4	20.4 ± 4.0	0.19	19.7 ± 2.6	20.2 ± 4.1	0.63
Hemoptysis	4	6	0.36	6	4	0.37
FEV_1, L/s (mean ± SD)	55.0 ± 23.0	57.4 ± 18.4	0.75	50.7 ± 20.3	62.1 ± 17.6	0.06
Pancreatic insufficiency	9	26	0.36	17	18	0.78
Sweat chloride (Meq/L) (mean ± SD)	66.5 ± 53.3	75.7 ± 49.6	0.61	81.9 ± 45.6	65.0 ± 53.7	0.28
Hospitalization, d (mean ± SD)	50.8 ± 52.5	26.2 ± 27.7	0.05	43.9 ± 44.1	23.6 ± 28.0	0.09
Administration of antimicrobial drug therapy, d (mean ± SD)	87.8 ± 110.9	34.7 ± 57.1	0.14	70.1 ± 90.6	31.5 ± 62.7	0.12
Azithromycin	10	20	0.55	15	15	0.63
Azithromycin treatment, d (mean ± SD)	379.5 ± 258.6	362.9 ± 322.7	0.87	360.7 ± 297.5	374.0 ± 314.2	0.89
Ibuprofen	4	1	0.01	4	1	0.12
Insulin	1	3	0.87	2	2	0.92
Systemic steroids	2	1	0.13	2	1	0.49
Inhaled steroids	10	16	0.07	16	10	0.02
AFB in sputum	7	4	0.01	8	3	0.05
Pseudomonas aeruginosa	12	28	0.36	20	20	0.17
Staphylococcus aureus	7	11	0.2	8	10	0.72
Aspergillus spp.	11	17	0.03	16	12	0.12
Allergic bronchopulmonary aspergillosis	3	0	0.004	3	0	0.06
Hemophillus influenza	2	1	0.14	2	1	0.52
Klebsiella pneumoniae	1	2	0.85	2	1	0.55
Stenotrophomonas maltophilia	2	0	0.02	2	0	0.14

*NTM, nontuberculous mycobacteria; ATS, American Thoracic Society; SD, standard deviation; BMI, body mass index; FEV_1, forced expiratory volume in 1 s; AFB, acid-fast bacilli.

CF patients thus far. The only other comparable published report was a multicenter study from the United States by Olivier et al. (*14*), in which only 10% of the CF population was sampled. The overall prevalence of NTM in sputum in the current study was 22.6%, higher than the 13% reported from the United States (*14*). Twenty-six percent of the culture-positive study participants had positive smears, and one third to half of patients met the 1997 and 2007 ATS microbiologic criteria, respectively, for NTM disease. Half of the case-patients in our study had at least 2 positive cultures in contrast to 29% among the US CF population. The high prevalence in this study reflects the different mycobacterial ecology that exists in Israel. The findings also may be related to the 2-year cross-sectional design of this study compared to point prevalence studies. Furthermore, our study included patients for whom their physician had a reason to obtain a mycobacterial culture. This may have preselected a population with a higher likelihood of having mycobacteria. The study by Olivier et al. (*14*), which reported a lower prevalence, enrolled patients and prospectively obtained 3 sputum specimens over the course of 1 year.

There was some variability in the frequency of these bacteria between different centers. This finding may be due to differences in the quality of microbiology laboratories, differences in antimicrobial drug treatment, or endemic occurrence of bacteria in certain centers.

In the present study, *M. simiae* was the most common species isolated (40.5%), followed by *M. abscessus* (31.0%) and MAC (14.3%). *M. simiae* was also isolated among half of the patients with NTM disease. In the US study, MAC and *M. abscessus* were isolated in 72% and 16% of the cases, respectively. *M. simiae* is an infrequently found environmental organism that has rarely been associated with human disease. It has been reported as a cause of disseminated disease in AIDS patients (*23–25*) and the source of pulmonary disease in patients with underlying bronchiectatic lungs (*26*,*27*). To our knowledge, this is the first report on *M. simiae* pulmonary disease in CF patients. *M. simiae* was also associated with an outbreak due to a contaminated hospital water supply, which was distributed from an aquifer (*28*). Interestingly, the medical center with the highest prevalence of *M. simiae* did have an aquifer as the primary water source. Repeated water surveys, however, did not find contamination of facility water reservoirs as the source of the high prevalence of this species. *M. simiae*

seems to have a limited geographic distribution; most clinical isolates have come from Arizona, New Mexico, Texas, Cuba, and Israel (*29*–*31*). In Israel, *M. simiae* is the most common NTM isolated from clinical specimens; the species usually colonizes damaged lungs (*30*,*31*). The distribution of *M. simiae* varied among treatment sites, with the highest incidence in central Israel (center A). In previous studies in Israel, ≈99% of *M. simiae* isolates were obtained from patients who resided on the coastal plain, mainly the Tel Aviv area (*30*).

As had been noted earlier by others (*14*), CF patients with NTM infection were older than those without NTM. In contrast to other studies, however, our CF patients with NTM infection had markers of severe disease, including lower FEV_1, hemoptysis, higher frequency of *P. aeruginosa* and *Aspergillus* spp. growth in sputum, longer hospital stay, and higher exposure to intravenous antimicrobial agents, azithromycin, and ibuprofen. Mycobacterial colonization may be secondary to severe disease or that these mycobacteria cause the disease to be more severe. The association between NTM and markers of severe disease may be related to longer duration of disease or to the mycobacterial species involved. Severe disease could also promote altered mucociliary clearance, colonization, and infection with *M. simiae* and *M. abscessus*, while infection with MAC causes less progressive disease, mainly in older patients with mild disease (*14*).

In Olivier et al.'s study (*14*), *P. aeruginosa* was inversely associated with NTM, while the presence of *S. aureus* was positively associated with NTM. In our current study, patients with NTM tended to have less *S. aureus* and more *P. aeruginosa* in their lower airways. The strong association between infection with NTM and *Aspergillus* spp. probably reflects the severity of the disease. The presence of *Aspergillus* spp. or NTM may create favorable conditions for the colonization and infections of each other.

Azithromycin has a potential immunomodulatory effect in the treatment of CF, mainly for chronic *P. aeruginosa* respiratory tract infection (*32*). In our population, azithromycin was administered chiefly for its immunomodulatory properties and not to treat NTM pulmonary disease. Our study patients with NTM were treated more often with azithromycin (71.5% of case-patients vs. 51.4% of controls). Because macrolides are the treatment of choice for infections caused by MAC and *M. abscessus*, subtherapeutic doses of macrolides can induce selection of macrolide-resistant mycobacteria. The effect of long-term treatment with azithromycin on the antibimicrobial selection of NTM in CF patients remains undefined.

High doses of ibuprofen inhibit the inflammatory response to chronic infection, which contributes to lung destruction in patients with cystic fibrosis (*33*), and our patients with NTM were treated more frequently with ibuprofen. Furthermore, prostaglandin E inhibitors up-regulate the Th1 function with increasing levels of tumor necrosis factor, γ-interferon, and interleukin-2, which are necessary for the control of mycobacterial infections (*34*). The effect of prostaglandin inhibitors on mycobacterial infection has not been assessed in depth.

We did not find any correlation between the gene mutation profile and NTM infection. By contrast, others have demonstrated that 60.7% of patients with emerging bacteria were homozygous for the Delta F508 mutation in comparison to only 23.8% of the isolates from the control group (*4*).

Given the possibility that NTM may merely represent environmental contamination or simple colonization of the airways, we compared patients who were diagnosed as having NTM pulmonary disease with those with NTM infection, and found that, according to both 1997 and 2007 ATS criteria, those with NTM disease had more sputum specimens processed for mycobacteria and higher rates of positive smears. According to the 1997 criteria, patients with NTM disease had more severe pulmonary disease, and *P. aeruginosa* and *Aspergillus* spp. grew in their sputum more frequently.

The current high level of interest in NTM disease is the result of the recognition that NTM disease is encountered with increasing frequency in non-AIDS populations and in unrecognized settings with new manifestations. Furthermore, advances in mycobacteriology laboratories facilitated the publication of new diagnostic and therapeutic guidelines (*16*). The percentage of patients in our study who meet the current criteria is higher than those who met the previous criteria. It should be noted, however, that these guidelines apply to patients with lung disease due to MAC, *M. kansasii*, and *M. abscessus*, and it is not certain that these diagnostic criteria are universally applicable for all NTM respiratory pathogens.

This study has several limitations. First, retrospective studies can be limited by ascertainment bias, despite our best efforts to review all available paper and electronic records. Second, although 6 of the 7 centers report that they screen for NTM pulmonary secretions on a regular basis and during most exacerbations, only 45%–80% of patients in these centers were actually evaluated for the presence of NTM. Because testing for NTM was not routine in all centers, and since testing may have been performed preferentially on patients who showed clinical deterioration and in whom NTM-related disease was suspected, our data may not precisely reflect the overall prevalence of these bacteria in the population. Furthermore, an average of 6 sputum specimens were analyzed during the study period for each study patient while only 2.4 specimens were analyzed for each control. Nevertheless, this survey did provide some interesting insights about how often CF physicians look for NTM in sputum and gives an overview of the Israeli experience.

As the life expectancy of patients with CF increases and surveillance and microbiologic methods of detection improve, the prevalence of mycobacterial infection among the CF population appears to be increasing. The implication of this has not yet been conclusively established, and distinguishing between colonization and active disease remains difficult.

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Mycobacterium xenopi Clinical Relevance and Determinants, the Netherlands

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In the Netherlands, isolation of *Mycobacterium xenopi* is infrequent, and its clinical relevance is often uncertain. To determine clinical relevance and determinants, we retrospectively reviewed medical files of all patients in the Netherlands in whom *M. xenopi* was isolated from January 1999 through March 2005 by using diagnostic criteria for nontuberculous mycobacterial infection published by the American Thoracic Society. We found 49 patients, mostly white men, with an average age of 60 years and pre-existing pulmonary disease; of these patients, 25 (51%) met the diagnostic criteria. Mycobacterial genotype, based on 16S rRNA gene sequencing, was associated with true infection. Most infections were pulmonary, but pleural and spinal infections (spinal in HIV-infected patients) were also noted. Treatment regimens varied in content and duration; some patients were overtreated and some were undertreated.

Mycobacterium xenopi was first described by Schwabacher in 1959; it was isolated from skin lesions in a clawed frog and named after the official species designation of the frog, *Xenopus laevis* (*1*). Thereafter, these slow-growing mycobacteria have been recovered from heated water systems in many countries and more recently from natural waters in Finland (*2*). Transmission to humans is believed to originate from the environment, through aerosol inhalation or ingestion. Human-to-human transmission and transmission from animal reservoirs remain controversial because these routes have not been proven by molecular typing (*3*,*4*).

Pulmonary *M. xenopi* infections are most common, but extrapulmonary and disseminated infections have also been recorded (*5*,*6*). A predisposing factor is impaired immunity, either local (e.g., pre-existing pulmonary disease) or systemic (e.g., hematologic malignancy, immunosuppressive medication, or HIV/AIDS) (*5*,*7*).

Its survival in flowing water systems and resistance to common disinfectants enables *M. xenopi* to contaminate laboratory samples and medical devices such as bronchoscopes, thus causing healthcare-acquired (pseudo) infections and laboratory cross-contaminations (*3*,*6*,*8*,*9*). Differentiating true infection from pseudoinfection is of paramount importance because treatment of *M. xenopi* infections is time-consuming and often complicated. The British Thoracic Society (BTS) trial in 2001 established that treatment for pulmonary infections should consist of a 2-year course of rifampin and ethambutol; regimens including macrolides or fluoroquinolones are still being investigated (*10*). The American Thoracic Society (ATS) established general criteria for the diagnosis and treatment of nontuberculous mycobacterial, not specifically *M. xenopi*, infections. The treatment guidelines are similar to those by the BTS, although the ATS guidelines advocate macrolide-containing regimens (*5*).

To assess frequency and clinical relevance of *M. xenopi* isolation and its determinants in the Netherlands, we performed a retrospective case study. We used the ATS diagnostic criteria available during the study period to differentiate true infection from pseudoinfection.

Methods

To determine clinical relevance, we examined medical records of all patients in the Netherlands from whom *M. xenopi* had been isolated from January 1999 through March 2005. The following variables were extracted from the records: sex, age, predisposing factors, symptoms, chest imaging results, treatment and outcome, and drug susceptibility and status according to the ATS diagnostic criteria (*5*).

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Laboratory diagnosis of the isolates was made by the Dutch National Institute of Public Health and the Environment (RIVM) or by a local hospital laboratory. RIVM acts as the national reference laboratory that provides identification, drug-susceptibility testing, and genotyping of mycobacterial isolates for all hospitals and other healthcare institutions in the Netherlands. To identify a mycobacterial isolate, Hain GenoType MTBC line-blot (Hain Lifescience, Nehren, Germany) was used after PCR-based amplification to determine whether an isolate was a member of the *M. tuberculosis* complex. If the reaction was negative, an INNO-LiPA MYCOBACTERIA (Innogenetics, Gent, Belgium) reverse hybridization multiple DNA probe assay was used to differentiate between the more common species of nontuberculous mycobacteria, including *M. xenopi* (*11*). Before 2004, 16S rRNA gene sequence analysis was performed, after ruling out membership in the *M. tuberculosis* complex, by using the AccuProbe MTB DNA probe kit (GenProbe, San Diego, CA, USA). The result was compared with the RIVM and BLAST (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) 16S rRNA gene sequence databases (*12*).

On the basis of the results at position 90 in the 151-bp hypervariable region of the 16S gene, 2 *M. xenopi* genotypes were discerned; a C at position 90 distinguished *M. xenopi* I and a T distinguished *M. xenopi* II. Retrospectively, the 16S rRNA genes of all *M. xenopi* isolates at RIVM were sequenced and assigned to their respective genotypes.

Susceptibility testing was performed by using an agar dilution method (*13*). Drugs in the susceptibility testing panel were isoniazid, rifampin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin, and rifabutin. The Pearson χ^2 test was used for statistical correlations. The local ethics committee approved the study.

Results

We found 49 patients with nontuberculous mycobacterial infection (Table 1); of these, 25 (51%) met the ATS diagnostic criteria. Isolates from 46 patients were identified to the genotype level, I or II. Sequencing failed for 2, and 1 was unavailable at RIVM. *M. xenopi* I was found for 28 patients, *M. xenopi* II for 13, and mixed (types I and II) for 5. Isolation of type II was significantly associated with fulfillment of the ATS criteria compared with isolation of type I only (77% vs 39%; odds ratio [OR] 5.1, 95% confidence interval [CI] 1.2–23.0, p = 0.025). When we defined *M. xenopi* II cultures as "involving *M. xenopi* II," and thus included the mixed cultures, the correlation increased in significance (OR 5.4, 95% CI 1.4–20.8, p = 0.011).

Clinical signs and symptoms varied widely and were not associated with fulfillment of the ATS diagnostic criteria (Table 1). Chest radiographs were taken for all patients, except for 2 who had spinal infection (Table 1). Cavitation was the only radiographic finding significantly associated with fulfillment of the ATS diagnostic criteria (OR 14.3, 95% CI 2.7–75.6, p = 0.001). Results of additional computed tomography scanning, performed for 27 patients, were not associated with fulfillment of the ATS diagnostic criteria (data not shown).

We found 4 cases of extrapulmonary disease, 2 cases of pleural *M. xenopi* infection, and 2 cases of spondylodiscitis (in HIV–co-infected patients). The pleural infections were diagnosed by biopsy of pleural tissue for 1 patient and repeated culture of pleural fluid for the other, after chest radiograph demonstrated pleural thickening and fluid collection. The spinal infections were diagnosed by bone biopsy. In the pleural and bone biopsy specimens, granulomatous lesions with central necrosis were observed.

For most patients, *M. xenopi* was first isolated from sputum (51%), bronchoalveolar lavage fluid (35%), or lung biopsy sample (4%). Remaining isolates were from bone biopsy samples (4%), pleural fluid (2%), pleural biopsy samples (2%), and stool samples (2%). Acid-fast bacilli were detected with direct microscopy of primary samples for 39% of patients. An acid-fast bacilli–positive primary sample, regardless of its nature, was significantly associated with fulfillment of the ATS diagnostic criteria (OR 8.2, 95% CI 2.1–31.6, p<0.001).

Treatment was started for 25 of 49 patients, of whom 19 met the ATS diagnostic criteria. Therapy consisted of medication for 21 patients, surgery for 2, or both for 2. Surgery consisted of lobectomy, pulmonary wedge resection, Clagett pleurostomy, and vertebral surgery with psoas muscle abscess drainage. Medication regimens varied widely but generally included rifampin, isoniazid, ethambutol, clarithromycin, ciprofloxacin, and pyrazinamide in various 3- to 4-drug combinations. Duration of therapy varied between 5 days and 2.5 years, with a mean duration of 9 months. Macrolides were included in regimens for 58% and quinolones for 37% of the patients who met the ATS diagnostic criteria and received drug treatment.

Antimycobacterial treatment cured 11 (58%) patients who met the ATS diagnostic criteria: 7 with *M. xenopi* II, 2 with *M. xenopi* I, and 2 with *M. xenopi* I and II. We defined cure as resolution of symptoms and negative cultures after finishing treatment, until the end of our study period (range 0–60 months, median 25 months). Treatment failure, defined as protracted culture positivity for *M. xenopi* during and after adequate treatment, was noted for 4 (21%). Four other patients died. Treatment failure or death was not associated with genotype, susceptibility pattern, predisposing conditions, or radiographic imaging results.

Although they fulfilled the ATS diagnostic criteria, 4 patients did not receive treatment. Of these, 1 recovered spontaneously; 2 remained positive for acid-fast bacilli,

Table 1. Baseline population characteristics of 49 patients with nontuberculous mycobacterial infection, the Netherlands, January 1999 through March 2005*

Characteristic	ATS+ (n = 25)	ATS– (n = 24)	Total (%)
Demographics			
Male sex	19	18	37 (76)
Mean age, y	60	60	60
Dutch origin	24	20	44 (90)
Concurrent and predisposing conditions			
Pre-existing pulmonary disease	21	18	39 (80)
Chronic obstructive pulmonary disease	17	14	31 (63)
Lung cancer	1	3	4 (8)
Prior tuberculosis	0	2	2 (4)
Recurrent pulmonary infection†	5	2	7 (14)
Bronchiectasis	2	4	6 (12)
Smoker, current/ past	15/ 6	11/ 3	35 (71)
Alcohol abuse	2	3	5 (10)
High-dose steroid use‡	3	5	8 (16)
HIV infection	2	5	7 (14)
Mean CD4 count in HIV-infected patients, cells/mL	226	126	159
Hematologic malignancy	0	1	1 (2)
Otherwise impaired immunity§	2	1	3 (6)
Signs and symptoms			
Productive cough	21	20	41 (84)
Hemoptysis	5	4	9 (18)
Dyspnea	14	9	23 (47)
Fever	11	6	17 (35)
Weight loss	12	7	19 (39)
Malaise	16	10	26 (53)
Chest radiographic abnormalities			
Infiltrate	15	12	27 (55)
Cavity	12¶	3	15 (31)
Pleural thickening	3	4	7 (14)
Emphysema	9	9	18 (37)
Space-occupying lesion	1	3	4 (8)

*ATS+, American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection met; ATS–, ATS diagnostic criteria for nontuberculous mycobacterial infection not met.
†>3 requiring treatment in 6 months before primary *Mycobacteria xenopi* culture.
‡>15 mg prednisone/day for >3 months before primary *M. xenopi* culture.
§Diabetes mellitus, cisplatinum chemotherapy, anorexia nervosa (all n = 1).
¶Significant association (odds ratio 14.3, 95% confidence interval 2.7–75.6, p = 0.001).

culture, or both throughout the study period; and 1 died. These outcomes were not associated with specific genotypes or patient factors.

Susceptibility testing was performed for 47 isolates from 42 patients. For 5 patients, cultures failed to grow for testing; for 2 others, cultures were not available. Results for isoniazid, rifampin, and ethambutol are shown in Table 2. Isolates were susceptible to all other compounds tested. Susceptibility testing of follow-up cultures was performed for 5 patients. *M. xenopi* bacteria in 2 patients treated with rifampin became resistant to rifampin and to ethambutol in 1 patient. For 9 patients, susceptibility testing results influenced the treatment regimens, mostly by inclusion or exclusion of rifampin and ethambutol or by adding a quinolone or macrolide agent.

For 6 patients with cavitation visible on chest radiograph, of whom 4 met the ATS diagnostic criteria, fungi were cultured simultaneously (*Aspergillus fumigatus* from 4, *A. flavus* from 1, and *Scedosporium apiospermum* from 1). Antifungal treatment was initiated for 4 patients, which meant true nontuberculous mycobacteria infection was left untreated for 2.

Four patients who had received antimycobacterial treatment for *M. xenopi* infection before (mean duration 8 months) had relapses; the mean interval between discontinuation of drug treatment and relapse was 28 months (range 12–39). We found no evidence of geographic clustering, which suggests nosocomial transmission or a pseudo-outbreak. The number of new isolates per year remained steady at ≈8 per year. At least 5 patients were treated in preventive isolation for 2–15 days until *M. tuberculosis* complex infection was excluded by PCR.

Discussion

Clinical relevance of *M. xenopi* isolation, defined by fulfillment of the ATS diagnostic criteria, was likely in 51% of

Table 2. Baseline in vitro susceptibility of 47 primary isolates from 42 patients with nontuberculous mycobacterial infection, the Netherlands, January 1999 through March 2005

Susceptibility	Drug, no. (%), MIC		
	Isoniazid	Rifampin	Ethambutol
Susceptible	9 (21), MIC 0.2 mg/L	29 (69), MIC ≤1mg/L	5 (12), MIC 5 mg/L
Intermediate	32 (76), MIC 0.5–1.0 mg/L		11 (26), MIC 10 mg/L
Resistant	1 (2), MIC >1 mg/L	13 (31), MIC >1 mg/L	26 (62), MIC >10 mg/L

patients; mycobacterial genotype II was a major determinant. To our knowledge, this phenomenon and its causal mechanisms have not been described. If further evidence emerges, 16S rRNA gene sequencing may become a relevant addition to the diagnostic algorithm of *M. xenopi* infection.

The ATS diagnostic criteria are designed for *M. avium*, *M. kansasii,* and *M. abscessus* infections, although the authors state "there is no reason to believe these criteria would not be applicable to other species" (*5*). Because the BTS statement focuses on management rather than specific diagnostic criteria (*14*), the ATS diagnostic criteria are recommended for the clinical setting. Of the main ATS diagnostic components, 2 were each significantly associated with true infection in our study (cavitary lesions on chest radiograph and acid-fast bacilli on primary samples), thereby supporting the ATS criteria.

The ATS diagnostic criteria, however, have 1 limitation. Patients with pre-existing cavitary lesions are likely to have respiratory symptoms; they meet the radiologic criteria and are more likely to harbor mycobacteria in the cavity, which are not necessarily responsible for their symptoms and cavity formation. Cavity characteristics cannot reliably predict the cavity's origin or pathogenesis (*15*). The uncertainty is compounded when fungi are cultured simultaneously, which suggests matching requirements for in vivo success of these microorganisms or selective impaired immunity. Determining which organism causes disease in the patient is difficult.

The undertreatment and overtreatment that we noted indicates a relative lack of knowledge in physicians, mainly those specializing in pulmonary conditions, concerning nontuberculous mycobacteria infections. Unnecessary drug treatment could harm the patient in terms of adverse effects and costs (*16*), and undertreatment of patients who fulfill the diagnostic criteria is potentially harmful to the patients' health.

The baseline characteristics of our study group are similar to those in studies of nontuberculous mycobacteria patients in societies with low HIV prevalence (*5*,*10*). The North American series included more HIV-infected patients, which lowered the mean age of patients in these studies (*17*,*18*). Pre-existing pulmonary diseases are major predisposing factors and may be causally associated with isolation of *M. xenopi*. However, *M. xenopi* may have been isolated more often because physicians were more focused on mycobacterial cultures for this category of patients.

Minor predisposing factors were HIV infection or other causes of impaired immunity, which, in addition to the causal relationship, probably reflect the low prevalence of HIV infection compared with chronic pulmonary disease in the Netherlands. Also, because most HIV-infected patients receive highly active antiretroviral therapy, fewer cases of severe HIV immunosuppression and its co-infections are seen. HIV infection predisposes patients to extrapulmonary *M. xenopi* infection, especially spinal infection (*6*,*19*). Those with HIV-associated spinal *M. xenopi* infection had rising CD4 counts after starting or changing highly active antiretroviral therapy regimens (Table 1). Possibly, their *M. xenopi* infection was an expression of immune reconstitution inflammatory syndrome (*20*). We found no previous reports of pleural infections.

Although the treatment regimens recorded in our study were not in accordance with the current standards of the BTS (*14*) and ATS (*5*), cure rates were high. Although partly the result of the restricted definition of "cure" resulting from our research methods, this finding does bring the validity of the ATS diagnostic criteria into question. Despite meeting these criteria, some patients might have cleared *M. xenopi* infection without treatment. This possibility is endorsed by the spontaneous recovery of a minority of patients who met the ATS criteria but were not treated. Alternatively, the regimen of 24 months of rifampin and ethambutol advised by the BTS may not be better than similar regimens of shorter duration. The addition of macrolides and quinolones to therapy regimens might also account for the high cure rates after relatively short treatment durations.

Susceptibility testing results were similar to those published previously (*21*), but their value in clinical practice is uncertain. Interpretation of the laboratory results is difficult because of discrepancies between in vitro susceptibility and in vivo response to treatment (*5*,*21*). In our study, results rarely influenced treatment regimens; when they did, choice of regimen was controversial and not in accordance with ATS and BTS guidelines (*5*,*14*). Increasing use of PCR to rule out *M. tuberculosis* infection can be valuable for preventing or shortening patient isolation.

In conclusion, clinical isolation of *M. xenopi* was relevant for 51% of the patients; mycobacterial genotype was a major determinant. Currently, the ATS diagnostic criteria are the best tool for determining clinical relevance. We strongly recommend increased awareness of these diagnostic criteria and management guidelines by ATS and BTS.

Dr van Ingen is a physician at the Department of Pulmonary Diseases, Radboud University Nijmegen Medical Center, Nijmegen, and at the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. His research interests are the clinical relevance of isolation of a group of less common nontuberculous mycobacteria (*M. xenopi*, *M. malmoense*, *M. szulgai*, *M. chelonae*, *M. abscessus*, *M. simiae,* and "*M. noviomagense* sp. nov.") in clinical samples, their molecular epidemiology, and the possibility of human-to-human transmission of these mycobacteria and implications for disease control.

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Epidemiology of Nontuberculous Mycobacteria in Patients without HIV Infection, New York City

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We reviewed medical records of patients without known HIV and with positive cultures for nontuberculous mycobacteria (NTM) isolated during 2000–2003 from 1 large hospital in New York, New York. Overall, 505 patients had positive NTM cultures; 119 (24%) met the criteria for NTM disease. The difference between demographic characteristics of case-patients in our study (66% female, 61% white, and 59% >60 years of age) and those of the base population as determined by regional census data was statistically significant. Estimated incidences for positive cultures, all disease, and respiratory tract disease were 17.7, 2.7, and 2.0 per 100,000 persons, respectively. More patients with rapidly growing mycobacteria (61%), *Mycobacterium kansasii* (70%), or *M. marinum* (100%) met criteria for disease than did patients with *M. avium* complex (MAC) (27%, (p<0.01). NTM disease in patients without HIV is increasing. Laboratory-based surveillance may be useful for detecting non-MAC and non–respiratory tract disease.

Although the pathogenic potential of nontuberculous mycobacteria (NTM) was reported throughout the 20th century, widespread appreciation of the clinical syndromes caused by NTM began during the 1980s in association with the AIDS pandemic and the consequent dramatic increase in disseminated *Mycobacterium avium* complex (MAC) infections (*1*,*2*). However, the epidemiology of NTM disease in patients without HIV infection remains somewhat difficult to determine. NTM disease is relatively uncommon (*3*); it is not a reportable health event, and environmental exposure varies greatly by geographic region (*1*,*4*). Further, clinically insignificant colonization or contamination can be difficult to distinguish from true disease, which can render laboratory-based surveillance potentially inaccurate (*5*), and the risk factors for disease have not yet been fully defined.

To expand our understanding of the epidemiology of NTM, we reviewed the demographic and clinical characteristics of patients without known HIV infection who had positive cultures for NTM from 2000–2003. We sought to determine the incidence of NTM disease and colonization, the risk factors for NTM disease, and the species of mycobacteria associated with different clinical syndromes at our urban medical center.

Methods

Study Design and Site

We conducted a retrospective study of patients without known HIV infection and with positive cultures for NTM obtained during 2000–2003 at Columbia University Medical Center (CUMC), New York-Presbyterian Hospital, the only medical center in northern Manhattan. The study was approved by the Institutional Review Board of Columbia University.

Study Patients

Study patients had positive cultures for NTM and no laboratory evidence of HIV infection. Our mycobacteriology laboratory compiled the medical record numbers of patients with positive NTM cultures from 2000 through 2003. To maintain privacy regarding HIV status, the list was electronically purged of the names of patients with positive HIV serologic test results, patients with HIV viral load, and patients who had had genotyping studies performed. Patients identified in clinical notes as HIV infected were excluded.

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Data Collection

Demographic characteristics, coexisting medical illnesses, and results of computed tomography (CT) studies of the chest and mycobacteriologic studies were collected from electronic medical records. These records were generally complete for demographic characteristics and clinical microbiology laboratory, surgical, and radiographic reports but sometimes lacked progress notes or treatment records, which were often written by hand in bedside charts. Electronic medical records were considered adequate to assess risk factors if the clinical notes (progress notes, consultation notes, discharge summaries) documented the medical history, coexisting illnesses, and medication regimens, including use of antimycobacterial agents.

Case Definitions of NTM

Patients with blood cultures or tissue biopsy specimens positive for NTM were considered to have NTM disease. Patients with positive respiratory tract cultures were considered to have pulmonary disease if they met the following American Thoracic Society (ATS) guidelines (*6*): chest CT scan performed within 6 months of an NTM-positive culture demonstrating infiltrates, nodules, cavities, bronchiectasis, or tree-in-bud formations and ≥3 NTM-positive respiratory cultures; 2 positive cultures with ≥1 positive acid-fast smear; or 1 positive culture with moderate, many, or heavy acid-fast bacilli noted on smear. Patients were considered not to have disease if NTM had been isolated from stool or urine or if a nonpathogenic NTM species (e.g., *M. gordonae* or *M. gastri*) had been isolated but symptoms attributed to another etiology.

Estimated Incidence of NTM Disease

We estimated the annual incidence of NTM disease by using previously described methods (*7*). Since the total population at risk was unknown, we calculated a rough incidence estimate by studying only those patients with positive NTM cultures who resided in the geographic area, which was closer to CUMC than to any other New York City hospital. Residents in this area, which encompassed 5 ZIP codes, were assumed to have the highest probability of receiving medical care at CUMC, and in fact, these ZIP codes were the most commonly noted among patients at CUMC. The number of cases of NTM disease diagnosed in this geographic subset per year was then divided by the 2000 US Census population for the same area to calculate the incidence estimate (*8*). The population was adjusted downward by 1.5% based on current estimates of the HIV/AIDS prevalence in New York City.

Demographic and Geographic Analysis

The demographic characteristics of the study patients were compared with those of the New York Public Health Department (NYPH) catchment population using 2000 US Census data (*8*). We calculated the distribution of sex and race in the catchment population from weighted census tract data. To evaluate the potential role of environmental exposure, we compared the distribution of the residence ZIP codes of patients with NTM isolates to the ZIP codes of all CUMC patients.

Statistical Analysis

Associations and confidence intervals (CIs) were calculated with SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and EpiInfo 3.3.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Single-proportion CIs were derived from the binomial distribution with continuity correction. We calculated CIs for the incidence estimate by using the formula provided by the National Center for Health Statistics (*9*). Median ages were compared by using the Mann-Whitney-Wilcoxon test. Univariate and multivariate associations between clinical and mycobacteriology data used Fisher exact test and logistic regression, respectively. Reported CIs and 2-tailed p values were for the 95% confidence level; p values are given without correction for multiple comparisons.

Results

During the 4-year study period, the clinical microbiology laboratory identified 769 patients with at least 1 positive NTM culture. Of these, 264 were excluded from further analysis by electronic purge of HIV-infected patients as previously described (Figure 1). The remaining 505 study patients had 820 positive NTM cultures; 282 (56%) were hospitalized when their first positive NTM culture was obtained. MAC and the rapidly growing mycobacteria (RGM) species were most common, isolated from 84% (n = 422) and 9% (n = 45) of patients, respectively (Table 1).

Proportion of Patients with NTM Disease

Of the 505 study patients with NTM-positive cultures, 375 (74%) had adequate clinical data to determine disease status. In all, 119 (32%) of 375 were considered to have NTM disease (Table 1, Figure 1). A significantly higher proportion of patients with RGM (61%), *M. kansasii* (70%), or *M. marinum* (100%) isolates were categorized with disease compared with those with MAC (27%) isolates ($p<0.01$).

Body Site and NTM Species

Respiratory Tract

Although only 24% (81/344) of patients with NTM-positive cultures from the respiratory tract met ATS criteria for NTM disease, 68% of cases of disease occurred in the respiratory tract (Table 2). However, MAC, RGM,

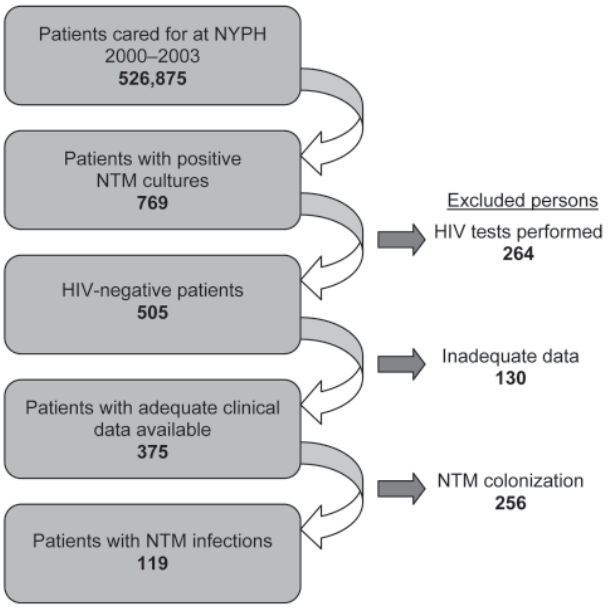

Figure 1. Flowchart of patient selection for cases of nontuberculous mycobacteria (NTM) colonization and NTM disease among patients without HIV infection, New York–Presbyterian Hospital (NYPH), Columbia University Medical Center, 2000–2003.

M. xenopi, and *M. kansasii* caused 80%, 9%, 6%, and 5% of these cases, respectively. No NTM species predicted disease; although a higher proportion of patients with *M. kansasii* (57%) and *M. xenopi* (44%) isolates were categorized with disease compared with MAC (25%); these differences were not statistically significant (p = 0.08 and 0.24, respectively). No patient with *M. gordonae, M. flavescens*, or *M. scrofulaceum* isolates met ATS criteria for disease.

Skin, Soft Tissue, and Surgical Wounds

Skin and soft tissue sites were the second most common sites of disease and occurred in 21 (18%) patients. RGM caused 4 (33%) of 12 nonsurgical skin and soft tissue infections and 7 (78%) of 9 surgical wound infections. Seven of the latter were associated with cosmetic procedures; 4 had been performed in the Dominican Republic, 2 in Ecuador, and 1 in the United States. All 4 cases of *M. marinum* infection occurred in the upper extremities.

Bloodstream Infections and Disseminated Disease

Seven patients had bloodstream infections (5 with RGM and 2 with MAC). Two additional subjects had positive blood cultures (both with MAC) and other infected body sites and, thus, were categorized with disseminated disease.

Gastrointestinal (GI) Tract Isolates

Of the 11 patients with MAC cultured from the GI tract, 7 had adequate clinical information to assess disease status. Two (29%) of 7 had disseminated disease as described above, and 5 had no clinical signs or symptoms of infection.

Urine Isolates

No patients had NTM disease of the urinary tract. One patient had 4 urine cultures positive for MAC but was not categorized as having NTM disease because no symptoms of urinary tract infection and no treatment with antimycobacterial agents had been documented.

Estimated Incidence of NTM Disease

Data from the 2000 US Census showed that 276,032 people resided within 5 ZIP codes that are closer to our medical center than any other hospital. During the study period, 37% of the 536,875 patients cared for at CUMC listed their home addresses within these 5 ZIP codes. Adjusted for the HIV prevalence rate of ≈1.5% in New York City, the base HIV-negative population was 271,892. Overall, 192 (38%) of 505 patients with positive cultures for NTM and 29 (24%) of 119 patients with NTM disease

Table 1. HIV-negative patients with positive nontuberculous mycobacteria cultures and disease, New York–Presbyterian Hospital, Columbia University Medical Center, New York, New York, 2000–2003

NTM species*	No. positive cultures	Adequate data to assess case status†	No. patients with disease (%)‡
All species	505	375	119 (32)
Mycobacterium avium complex (MAC)	422	297	79 (27)
Rapidly growing mycobacteria‡	45	41	25 (61)
M. abscessus	14	13	11 (85)
M. chelonae	15	13	4 (31)
M. fortuitum	16	15	10 (67)
M. gordonae	25	6	0
M. kansasii ‡	12	10	7 (70)
M. marinum ‡	4	4	4 (100)
M. scrofulaceum	5	4	0
M. xenopi	13	9	5 (56)

**M. flavescens*, *M. gastri*, *M. haemophilum*, and *M. neoaurum* were isolated once each.
†Patients with adequate clinical, radiographic, and mycobacteriologic data to assess case status.
‡Greater proportion of rapidly growing mycobacteria, *M. kansasii,* and *M. marinum* caused nontuberculous mycobacteria disease when compared with MAC (p<0.01).

Table 2. Site of disease and species of nontuberculous mycobacteria, New York–Presbyterian Hospital, Columbia University Medical Center, New York, New York, 2000–2003*

Site of disease	No. patients with MAC infection	No. patients with RGM infection	No. patients with other species infections	Total no. patients (%)
Respiratory tract	65	7	9	81 (68.1)
Skin and soft tissue, nonsurgical	2	4	6	12 (10.1)
Surgical sites	0	7	2	9 (7.6)
Bloodstream	2	4	1	7 (5.9)
Lymph node	5	1	0	6 (5.0)
Disseminated	2	0	0	2 (1.7)
Central nervous system	0	1	0	1 (0.8)
Gastrointestinal tract	0	0	1	1 (0.8)
All body sites	76	24	19	119 (100)

*MAC, *Mycobacterium avium* complex; RGM, rapidly growing mycobacteria.

resided in this same area. Thus, the estimated annual incidences of patients with positive NTM cultures in the area defined by these 5 ZIP codes, NTM disease (inclusive of the respiratory tract), and NTM disease specifically of the respiratory tract were 17.7 (95% CI 15.2–20.2), 2.7 (95% CI 1.8–3.8), and 2.0 (95% CI 1.3–3.1) cases per 100,000 persons, respectively.

Demographic Characteristics

Sex

By adjusting 2000 US Census data for age, the expected proportion of women in the base population was 57% (Figure 2). The same proportion was observed in patients with NTM-positive cultures (57.0%, 95% CI = 52.6%–61.4%). In contrast, patients with NTM disease were significantly more likely to be female than were those in the base population (66.4%, p = 0.04). Among those with disease of the respiratory tract caused by MAC, the distribution of patients by sex was similar to that of the base population (60.0% female, p = 0.71).

Race and Ethnicity

The overall distribution of race and ethnicity was significantly different for patients with positive NTM cultures (p<0.01) or disease (p<0.001) when compared with the age-adjusted base population (Figure 3). A greater proportion of patients with NTM disease were white and fewer were Hispanic. Similarly, patients with NTM disease were more likely to be white than patients with a positive culture (61% vs. 48%, p = 0.008).

Age

The median age of the study patients with positive NTM cultures was 66 years. Most (59%, n = 70) patients with disease were >60 years of age; only 8% (n = 9) were children <15 years of age. Patients with MAC disease were older than those with RGM disease (68 vs. 53 years of age, respectively, p<0.01). The median ages of patients with disease of the respiratory tract caused by different species were similar (71 years vs. 69 years of age for MAC and RGM, respectively); patients with nonpulmonary disease caused by MAC were substantially younger than those with nonpulmonary disease caused by RGM (11 vs. 41 years of age, respectively).

Geographic Distribution of Patients

The ZIP codes of patients with positive NTM cultures were compared with those of all patients registered at CUMC. Patients with positive cultures were less likely to live in northern Manhattan within 3 miles of the medical center than were the hospital's overall patient population (OR 0.72, p<0.001). In contrast, substantially more patients with positive cultures resided in the northwestern area of the Bronx (OR 2.17, p<0.001) or in Staten Island (OR 2.25, p<0.001).

Coexisting Illness and Concomitant Medications

At least 1 coexisting illness or concomitant medication considered to be a potential risk factor for NTM disease was noted for 73% of patients who fulfilled the study case definitions for disease. Ninety-four (79%) of 119 patients with NTM disease had adequate data to assess their medical

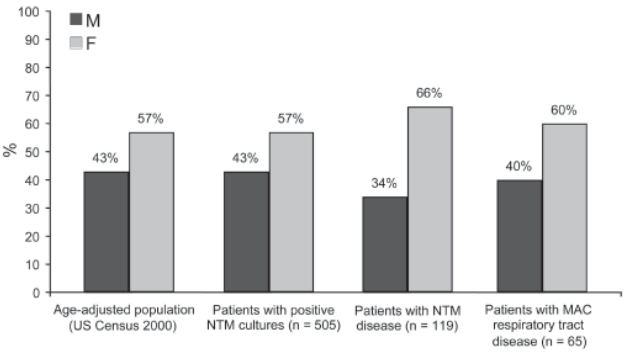

Figure 2. Distribution by sex of patients with positive nontuberculous mycobacteria (NTM) cultures, NTM disease, and disease of the respiratory tract caused by *Mycobacterium avium* complex (MAC), New York–Presbyterian Hospital, Columbia University Medical Center, 2000–2003, compared with age-adjusted base population from 2000 US Census data.

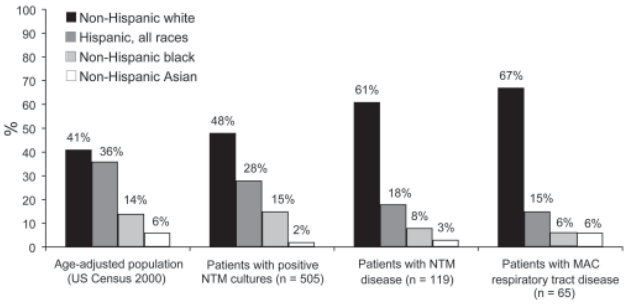

Figure 3. Distribution by race of patients with positive nontuberculous mycobacteria (NTM) cultures, NTM disease, and disease of the respiratory tract caused by *Mycobacterium avium* complex (MAC), New York–Presbyterian Hospital, Columbia University Medical Center, 2000–2003, compared with age-adjusted base population from 2000 US Census data.

histories, and 66% (62/94) had ≥1 coexisting illness (Table 3). Preexisting lung disease was most common (44%), and patients with respiratory tract disease with NTM were more likely to have preexisting lung disease than patients with disease of other body sites (OR18, 95% CI 4.9–64, p<0.001).

Eighteen percent of patients had ≥1 immunosuppressive condition (other than transplantation), including diabetes mellitus (14%), chronic renal failure (4%), or rheumatologic disease (5%), and 17% had solid organ or hematologic malignancy. When site of disease (respiratory tract vs. nonrespiratory tract) was adjusted for, patients with MAC disease were more likely to be transplant recipients than were patients with disease caused by other NTM species (OR 7.2, p = 0.01).

For 79 (66%) of 119 patients with NTM disease, data were adequate to assess concomitant medications. Steroids or other immunosuppressive medications were prescribed for 25% and 15% of patients, respectively, within the 6 months before the first positive NTM culture. Although the use of steroids did not predict the site of NTM disease, the use of other immunosuppressive medications was less common in those with disease of the respiratory tract compared with those with disease of nonrespiratory sites (OR 0.30, 95% CI 0.10–0.89, p<0.05). However, when body site was adjusted for, patients with MAC were more likely to have received steroids than were those infected with other species (OR 5.2, 95% CI 1.2–24, p = 0.03). Also, more patients with bloodstream infections received cancer chemotherapeutics than did patients with disease of other body sites (OR 28, 95% CI 3.6–220, p<0.01).

Discussion

This study is one of the largest recent studies of NTM and reflects the current epidemiology and risk factors for disease and colonization with these microorganisms as assessed in our medical center in northern Manhattan. The rate of NTM disease observed in patients without HIV infection appears to be increasing, but it is difficult to compare studies because different epidemiologic methodols have been used. In a laboratory survey from 1993 to 1996 performed by the Centers for Disease Control and Prevention, the rate of positive NTM cultures was 7.5–8.2 cases per 100,000 persons, compared with our positive culture rate of 17.7 per 100,000 (*5*). The rate of NTM disease derived from several studies conducted through the mid-1990s was estimated to be 2 per 100,000 (*10*). Surveys conducted in Europe estimated the rate of respiratory tract disease with MAC to be 0.2 cases per 100,000, and investigators in the United Kingdom estimated the rate of disease of any body site to be 0.8–3.1 per 100,000 (*11*,*12*). We presented higher estimates of the incidence of NTM respiratory tract disease (2.0 per 100,000) and disease of any body

Table 3. Comparison of coexisting conditions and concomitant medications with body site of nontuberculous mycobacteria (NTM) disease, New York–Presbyterian Hospital, Columbia University Medical Center, New York, New York, 2000–2003

	% Patients with coexisting condition (n = 94)				
Site of NTM disease	Lung disease	Transplant recipient	Immunocompromised*	Cancer	None
Blood (n = 7)	0	0	33	67	0
Respiratory tract (n = 81)	63	9	16	13	28
Skin and soft tissue, surgical sites (n = 21)	6	19	25	6	62
All†	44	11	18	17	34
	% Patients receiving concomitant medications (n = 79)				
Site of NTM disease	Systemic steroids	Immunosuppressants	Chemotherapeutics	Immunomodulators	None
Blood (n = 7)	50	33	50	17	17
Respiratory tract (n = 81)	26	13	2	4	70
Skin and soft tissue, surgical sites (n = 21)	17	17	8	8	75
All†	25	15	6	5	66

*Defined as diabetes, chronic renal failure and/or rheumatologic disease.
†Includes 2 patients with disseminated disease following bone marrow transplantation, 1 patient with central nervous system disease receiving steroids, 1 patient with gastrointestinal disease, and 2 patients with lymph node disease/cancer.

site (2.7 per 100,000), potentially attributable, in part, to improved detection methods. However, our incidence calculation may actually have underestimated the rate of NTM disease if persons who resided in the 5 ZIP codes of interest received care for NTM at another medical facility. Alternatively, had we used a larger geographic region to calculate incidence, we may have compounded the underestimate because additional persons most likely would have sought care at medical facilities other than CUMC. Nevertheless, in the absence of mandatory statewide or nationwide reporting, large institution-based studies can produce the best incidence data.

Variation in the rates of NTM disease and colonization among different populations may also reflect differences in the risk for exposure to environmental mycobacteria. Our data demonstrate geographic variations in the incidence of NTM disease within New York City. Although neighborhood demographics may act as confounding variables, these findings suggest that environmental factors deserve further study. For example, patients residing in the northwestern Bronx had higher rates of disease with NTM; this area receives water from the smaller Croton Reservoir as opposed to the Catskills-Delaware Reservoir that supplies most of New York City (*13*). To test this hypothesis, results of environmental sampling would need to be correlated with cases of human disease (*14*,*15*).

Our study provided an opportunity to study risk factors in a population without referral center bias that can occur in centers specializing in NTM care. The predominance of women among persons with NTM disease is consistent with previous reports (*12*,*16*). For pulmonary disease caused by MAC, the greater proportion of affected women appeared to reflect the higher proportion of women in the older age strata of the base population of NYPH. The correlation of NTM disease with gender did not appear to be attributable to a higher prevalence of concurrent medical conditions or concomitant medication use among women. For example, chronic obstructive pulmonary disease is the most common risk factor for pulmonary disease with NTM, but it is more prevalent among men than women in the United States (89 vs. 61 per 1,000 persons) (*17*,*18*). We speculated that cosmetic surgery could explain, in part, the higher risk of nonrespiratory tract disease in women, although the number of these procedures performed during the study period was unavailable, and cosmetic surgery accounted for only a small number of cases (*19*–*21*). We were also able to uniquely examine the effect of race. As in previous studies, most cases of NTM disease occurred in white persons (*3*), but our base population was unique in having a lower proportion of whites. Furthermore, the higher prevalence of NTM disease noted in whites in our study could not be attributed to cystic fibrosis because only 4 subjects had this medical condition (*22*–*26*).

Only one-third of patients with positive cultures for NTM were categorized with disease. A significantly higher proportion of patients with positive cultures for RGM, *M. kansasii*, or *M. marinum* were considered to have NTM disease than patients with MAC (*27*). Although respiratory tract isolates were most common (*28*,*29*), most reflected colonization or contamination. Thus, laboratory-based surveillance may reasonably estimate the incidence of nonrespiratory tract disease caused by RGM, *M. kansasii,* and *M. marinum* but provide less accurate estimates of the incidence of MAC disease and of respiratory tract disease.

Among the expected risk factors for NTM disease, we found that preexisting pulmonary conditions were most common. However, many cases of NTM disease occurred in patients with concurrent illnesses or medications that were immunosuppressive. Our finding that MAC was the most common pathogen causing posttransplant NTM disease was consistent with results of prior studies (*30*,*31*). Notably, one fourth of patients with NTM disease did not have a known risk factor, which suggests the possibility of a unique genetic susceptibility or environmental exposure.

Our study did have limitations. We used a convenience sample of patients receiving care at our medical center, which introduced potential bias if our sample was not representative of the general population. Our findings may not be applicable to other geographic regions, particularly given the different rates of disease we noted among different areas in New York City. The rare nature of NTM disease makes an accurate measure of the incidence in the population exceptionally difficult. Our incidence rate calculation was a gross estimate and likely an underestimate. Patients residing in the selected base population may have sought care elsewhere; patients with positive cultures and presumptive colonization may have progressed to active disease; and our case-patients were often hospitalized at the time of diagnosis, which suggests limited detection of outpatient cases. In addition, the high proportion of hospitalized case-patients could overestimate coexisting illnesses and concomitant medications. Potential cases of respiratory tract disease could have been missed due to incomplete data, usually a lack of chest CT results. Cultures or radiographic imaging may have been performed at other medical facilities, which could have resulted in misclassification of disease status. Racial differences could reflect, in part, differential access to healthcare. Furthermore, although CUMC is not a referral center for NTM, it is a referral center for other conditions, including lung transplantation.

In conclusion, we found an increased incidence of NTM-positive cultures and disease compared with results in previous reports. Our results suggest that laboratory-based surveillance may produce reasonable estimates of the incidence of nonrespiratory tract disease and of disease caused by RGM, *M. kansasii,* and *M. marinum.* However,

such surveillance is relatively inaccurate for estimating the incidence of pulmonary disease and disease caused by MAC. Larger, multicenter regional studies or mandatory reporting will be required to better understand the changing epidemiology of NTM in patients without HIV infection.

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Exposure to Novel Parainfluenza Virus and Clinical Relevance in 2 Bottlenose Dolphin (*Tursiops truncatus*) Populations

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Parainfluenza virus (PIV) is a leading cause of respiratory infections in humans. A novel virus closely related to human and bovine parainfluenza viruses types 3 (HPIV-3 and BPIV-3), named *Tursiops truncatus* parainfluenza virus type 1 (TtPIV-1), was isolated from a dolphin with respiratory disease. We developed a dolphin-specific ELISA to measure acute- and convalescent-phase PIV antibodies in dolphins during 1999–2006 with hemograms similar to that of the positive control. PIV seroconversion occurred concurrently with an abnormal hemogram in 22 animals, of which 7 (31.8%) had respiratory signs. Seroprevalence surveys were conducted on 114 healthy bottlenose dolphins in Florida and California. When the most conservative interpretation of positive was used, 11.4% of healthy dolphins were antibody positive, 29.8% were negative, and 58.8% were inconclusive. PIV appears to be a common marine mammal virus that may be of human health interest because of the similarity of TtPIV-1 to BPIV-3 and HPIV-3.

Parainfluenza viruses (PIVs) are often associated with respiratory illness in terrestrial mammals, including croup in humans (*1*), kennel cough in dogs (*2*), and bovine respiratory disease in cattle (*3*). A novel PIV tentatively named *Tursiops truncatus* parainfluenza virus type 1 (TtPIV-1) was cultured from lung tissue in an Atlantic bottlenose dolphin (*T. truncatus*) (*4*). This animal had respiratory disease including laryngitis, tracheitis, and bronchointerstitial pneumonia with mild to moderate growth of *Candida glabrata.*

Phylogenetic analyses of 2 genomic fragments of TtPIV-1 showed that the virus strain was monophyletic with, but genetically distinct from, bovine parainfluenza virus 3 (BPIV-3) strains and human parainfluenza type 3 (HPIV-3) (*4*). BPIV-3 is an effective antigenic stimulator in humans and is used in human vaccines that protect against HPIV-3 (*5–7*). TtPIV-1 may provide similar protection in humans. Dolphins have been recognized as useful marine ecosystem sentinels (*8*), and changes in marine PIV may reflect changes in terrestrial PIV.

ELISAs have been characterized as the most sensitive diagnostic tool to identify rising titers due to PIV-associated respiratory illness in humans (*9*). Although ELISA is the ideal tool for identifying infections caused by PIV, high levels of antigenic cross-reactivity among various PIV subfamilies and other closely related viruses hinder the ability for ELISA to determine which type of PIV has infected an animal (*10*,*11*). In wild marine mammal populations, ELISA-based serosurveys for suspected viral or bacterial pathogens are common (*12–14*). Limitations of these studies include unknown health status of animals or lack of paired samples that can differentiate exposures from active infections.

The US Navy Marine Mammal Program (MMP) manages a population of bottlenose dolphins that live in San Diego Bay, California. These animals are provided high-quality medical and preventive care throughout their lifetime. Standardized health data and voluntary blood samples are

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collected routinely, uniquely enabling MMP to amass routine physiologic information on dolphins living in a marine environment at all age stages. Since 1988, health assessments have been conducted by the Chicago Zoological Society and collaborators on a free-ranging, resident coastal population of bottlenose dolphins in Sarasota Bay, Florida, 2,500 miles away, as part of the world's longest running study of wild dolphins. Serum samples from this presumably healthy population are archived for use in retrospective health assessments.

An indirect, dolphin-specific PIV-antibody ELISA was developed and applied to archived serum samples collected from MMP dolphins in San Diego (1999–2006) and healthy, free-ranging dolphins living near Sarasota (2004–2005). We used this ELISA to assess the clinical relevance of PIV exposure and seroconversion in bottlenose dolphins living along US coasts.

Methods

The MMP is routinely reviewed by an Institutional Animal Care and Use Committee (IACUC) and Navy Bureau of Medicine; the MMP is accredited by the Assessment and Accreditation of Laboratory Care International. All sample collection protocols for the Sarasota wild dolphin population were approved by the University of Florida IACUC (IACUC no. C233).

San Diego, California

Blood samples from MMP dolphins were initially collected by venipuncture from animals trained either to present their tail for sampling in the water or to rest on a foam mat during a routine physical examination out of the water. Samples were collected from the caudal peduncle vein by using a 20- or 21-gauge, 1.5-inch Vacutainer needle (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) or from a fluke vein by using a 21-gauge, 1-inch butterfly needle. Blood was collected into a Vacutainer serum separator tube or a Vacutainer EDTA (K_3) tube for serum chemistries and complete blood counts, respectively.

Samples for chemistry analysis were centrifuged within 2 h of collection. Centrifugation was performed at 3,000 rpm at 21°C for 10 min. Fibrin clots were removed, and serum was transferred to a 5-mL plastic submission tube. Whole blood was collected in EDTA Vacutainer tubes. All samples were sent on wet ice by courier to Quest Diagnostic Laboratories in San Diego.

Automated hematologic analyses were conducted by Quest Diagnostic Laboratories with the Coulter LH 1500 Series (Beckman Coulter, Inc., Fullerton, CA, USA). The Fisherbrand Dispette 2 (Fisher Scientific, Pittsburgh, PA, USA), correlating with the Westergren method, was used in house to determine 60-min erythrocyte sedimentation rates (ESRs) from 1 mL EDTA whole blood. Remaining serum from these samples was archived at –80°C at the MMP facility at the time of initial blood collection.

Upon completed development of the PIV-antibody ELISA, the archived, frozen serum samples were shipped frozen overnight to the laboratory for PIV-antibody analysis. Total leukocyte count, absolute neutrophils, absolute lymphocytes, absolute monocytes, absolute eosinophils, and ESR results were incorporated into the retrospective PIV seroprevalence study and linked to animal age, sex, clinical signs, and PIV-antibody ELISA results.

Sarasota Bay, Florida

Blood samples from free-ranging dolphins were obtained as part of a long-term health assessment conducted near Sarasota, involving a multigenerational resident population of ≈150 dolphins (*8*). Most of these dolphins are recognizable; are of known age, gender, and maternal lineage; and medical histories have been recorded for many years. Small groups of dolphins were encircled with a 5,000-m long × 5-m deep seine net in shallow water (<1.8 m). Each dolphin was shaded, kept cool and wet, and carefully monitored for signs of discomfort by 1 to 3 veterinarians experienced with cetaceans. The veterinary staff monitored the dolphin's respiration rate and quality, responsiveness to external stimuli, mental alertness, skin temperature, and heart rate to evaluate the animal's comfort level and immediate health status. Blood samples were drawn from the ventral fluke vasculature by a 19-gauge × ¾-inch butterfly catheter with a multisample adaptor (Becton Dickinson), which allowed the blood collection tubes to be filled directly from the venipuncture set.

ELISA Development

Antigen Production

Archived TtPIV-1 was propagated in BSC40 cells as previously described (*15*). Uninfected BSC40 cells were cultured as negative controls. When >90% of the infected monolayers showed cytopathic effect (CPE), all infected and uninfected cultures were harvested and centrifuged for 15 min at 1,500 rpm, and the supernatant media was removed. The remaining cell pellets were freeze-thawed 3× and centrifuged for 15 min at 3,000 rpm. The cell pellet was discarded, and the supernatant cell lysates were pooled for use as antigen in the ELISA. The protein concentration of the infected and uninfected cell lysates was determined by using a modified Bradford assay for ELISA, after which the cell lysates were diluted in phosphate-buffered saline (PBS) to the desired coating concentration.

Optimization of ELISA Parameters

A serum sample collected postmortem from the case dolphin was used as the positive reference serum. A nega-

tive reference serum sample was collected from an immunologically naive neonate bottlenose dolphin that had not yet nursed. The positive and negative reference serum samples were used to optimize the ELISA conditions. All assay parameters were varied (working volume 50–100 μL; coating concentration 1–20 μg/mL; serum dilution 1:50–1:400; developing time 15–60 min), and the assay conditions with the highest ratio of the optical density at 405 nm (OD_{405}) of positive reference serum sample to the OD_{405} of negative reference serum sample were selected. The conditions of the optimized ELISA protocol were as follows. Wells of a high protein-binding microplate (Nunc Maxisorp, Fisher Scientific) were coated with 50 μL of infected or uninfected cell lysates at 5 μg/mL in PBS and were left to adsorb overnight at 4°C. After this and each subsequent step, all wells were washed 3× with PBS with 0.05% Tween by using an automated EL 404 microplate washer (Biotek Instruments, Winooski, VT, USA). After washing, all wells were blocked with 300 μL of Superblock blocking buffer (Pierce, Rockford, IL, USA) in PBS with sodium azide (PBS/Az), after which the dolphin serum samples were applied (1:500 in 1% bovine serum albumin [BSA] in PBS/Az). All sera were applied in triplicate to wells that were coated with either infected or uninfected cell lysate antigen. The positive and negative control sera were included on each plate. A biotinylated monoclonal antibody specific for bottlenose dolphin IgG (*15*) was used at a concentration of 5 μg/mL (in 1% BSA in PBS/Az) as the secondary reagent for the detection of bound antibodies. Each step of the ELISA was left to incubate with gentle agitation (Nutator; Adams, Fisher Scientific) for 1 h at ≈22°C. Finally, 1.0 mg/mL p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) substrate was added. The OD_{405} was recorded 60 min after addition of the substrate by using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). For analysis, the mean OD_{405} of the triplicate readings on the uninfected antigen was subtracted from the mean OD_{405} of the triplicate readings by using the infected antigen. All results were presented as OD_{405} ratios, defined as the OD_{405} reading of the unknown samples divided by the OD_{405} reading of the positive control sample for that plate.

Cut-off Values, Definitions, and Data Analysis

All data were analyzed with SAS software (Release 8e; SAS Institute, Inc., Cary, NC, USA). p values ≤0.01 were defined as significant.

PIV Seroconversion

Archived hematologic and clinical observation data (1999–2006) were mined to identify dolphins with hemograms similar to that of the positive control animal, including a neutrophilic, monocytic leukocytosis or a high ESR, as defined by MMP reference ranges (*16*). Archived serum samples collected within 60 days before, during, and ≤90 days after an inflammatory hemogram or clinical illness from suspected PIV cases were subsequently analyzed for PIV antibodies with the dolphin-specific ELISA. PIV seroconversion was defined as a ≥4-fold increase in PIV antibody OD_{405} level within a 3-month period.

Analyses were conducted to describe frequencies of abnormal clinicopathologic values and clinical signs among PIV seroconversion cases from 60 days before the highest OD_{405} ratio to 30 days after the highest OD_{405} ratio. Median clinicopathologic blood values were calculated among PIV seroconversion cases by using the serum sample with the highest leukocyte count from each identified animal (e.g., the blood sample most likely representing the most severe phase of disease).

Seroprevalence among Healthy Populations

Serum samples collected during July to December 2006 from 58 MMP bottlenose dolphins in San Diego and samples collected during 2003–2005 from 56 free-ranging bottlenose dolphins living near Sarasota were analyzed for PIV antibodies. Because only 1 positive control animal had been identified, a conservative interpretation of ELISA results was adopted. Samples with an OD_{405} ratio >1.0 (seropositive) contained an antibody level at least as high as the positive control's highest antibody level during the time of PIV infection. Samples with an OD_{405} ratio of 0.0 (seronegative) contained an anti-PIV antibody level that was less than or equal to the negative control. OD_{405} ratios >0 and <1 were categorized as inconclusive. Midrange values were used to compare mean OD_{405} ratios by population location, age, and sex. Midrange values were also used to assess changes within 1 animal over time (see Methods, PIV Seroconversion).

Descriptive statistics were used to determine the prevalence of PIV-seropositive, -seronegative, and -inconclusive animals among the 2 study populations. To assess the clinical relevance of PIV exposure among presumably healthy dolphins, mean values of hematologic and serum biochemical inflammatory indicators (leukocyte counts and ESR) were subsequently compared between PIV-seropositive and -seronegative animals in the MMP 2006 population by using analysis of covariance (ANCOVA) with a general linear model to control for varying numbers of samples, age, and sex of animals. Age and sex were controlled covariates because of the previously documented effects of age and sex on healthy, normal reference ranges in dolphins (*16*). A type I sum of squares p value was used to determine significance. Mean comparisons were reported by using least squares means when controlling for covariates.

PIV-antibody levels were compared between healthy MMP dolphins in San Diego and free-ranging dolphins living near Sarasota. Differences in age and sex between the 2

study populations were analyzed by using a general linear model (PROC GLM; CLASS population; MODEL age = population; MEANS population) and a Mantel-Haenszel $\chi 2$ test, respectively. Mean OD_{405} levels were compared by population, age, and sex (controlling for age due to identified differences in ages between the 2 populations) by using ANCOVA with a general linear model to control for varying numbers of samples by animal (PROC GLM Overview, SAS Online Doc, Version 8, SAS Institute, Inc.).

Results

Positive Control Case

PIV serum antibody OD_{405} levels were determined in a dolphin from which TtPIV-1 was successfully isolated from antemortem and postmortem lung samples. Low PIV antibody levels appeared to be present 300 days before illness, and rising antibody levels were detected during the course of TtPIV-1–associated respiratory illness (Figure 1).

PIV Seroconversion

PIV antibody levels were determined in 588 serum samples collected during 1999–2006 from 58 selected MMP bottlenose dolphins before, during, and after an inflammatory hemogram similar to that of the positive control. Within this sample set, 22 dolphins were identified that seroconverted within a 3-month period (examples, Figure 2, panels A–C). Eleven (50%) of the dolphins that seroconverted were female; median age was 22.2 years (range 0.3–43 years).

As expected because of the selection criteria for suspected cases, dolphins with PIV seroconversion had a neutrophilic leukocytosis (18, 81.8%) or high ESR (16, 72.7%). Other clinicopathologic abnormalities that were present in at least half of the case dolphins included hyperglobulinemia, monocytosis, thrombocytosis, and high alanine aminotransferase (ALT) levels (Tables 1, 2).

Of 22 dolphins that seroconverted, 5 (22.7%) maintained normal behavior and appetite, and 17 (77.3%) had at least 1 clinical sign. The most commonly reported behavioral abnormalities were decreased appetite (12, 54.5%) and lethargy (10, 45.5%). Veterinary observations also included respiratory (7, 31.8%) and ocular (5, 22.7%) clinical signs (Table 3). Respiratory signs included ventral left lung consolidation, pleuritis, tachypnea, coughing, and abnormal blowhole fluid. Ocular clinical signs were reported as bilateral or unilateral blepharospasm, intermittent squinting, or corneal opacities. Other clinical signs were diffuse, miliary skin or mucosal lesions (2 dolphins) and orange-yellow liquid feces and excessive flatulence (2 dolphins).

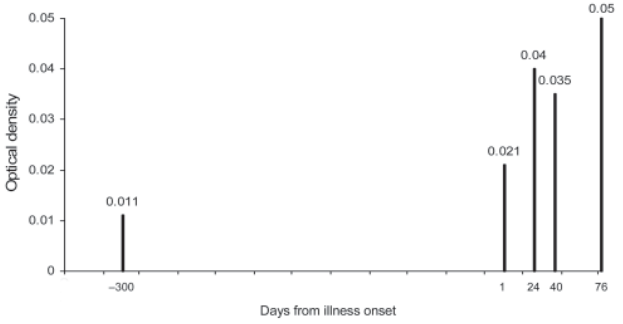

Figure 1. Sample series of parainfluenza virus (PIV) antibody optical density in the positive control bottlenose dolphin (*Tursiops truncatus*) during time of respiratory illness and multiple positive viral isolations from grossly affected lung.

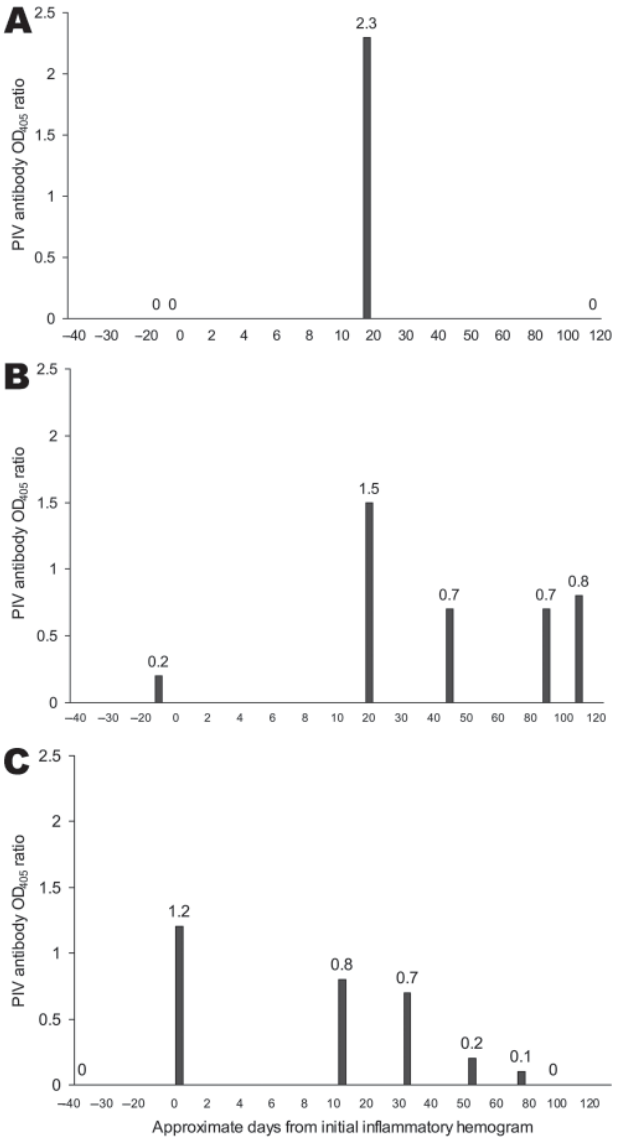

Figure 2. Evidence of active parainfluenza virus (PIV) infection in bottlenose dolphins (*Tursiops truncatus*) with inflammatory hemograms or clinical illness. A) PIV antibody optical density at 405 nm (OD_{405}) ratios in a 3-year-old female bottlenose dolphin; B) PIV antibody OD_{405} ratios in a 26-year-old male bottlenose dolphin; C) PIV antibody OD_{405} ratios in a 22-year-old male bottlenose dolphin.

Table 1. Frequencies of abnormal hematologic and inflammatory indicator values in bottlenose dolphins (*Tursiops truncatus*) within 60 d before and 30 d after PIV antibody seroconversion (n = 22)*

Blood variable	Adult dolphin reference range	Median value (range), cases	No. (%) cases below reference range	No. (%) cases above reference range
Leukocytes, cells/μL	4,275–10,089	12,950 (5,700–38,800)	2 (9.1)	18 (81.8)
HCT, %	38–46	39 (29–50)	11 (50.0)	0
Platelets, cells/μL	55,000–143,000	116,000 (25,000–333,000)	6 (27.3)	11 (50.0)
Neutrophils, cells/μL	2,737–7,570	9,750 (3,760–36,470)	2 (9.1)	18 (81.8)
Lymphocytes, cells/μL	270–1,500	1,425 (290–5,210)	3 (13.6)	7 (31.8)
Monocytes, cells/μL	0–576	300 (0–3, 080)	NA	11 (50.0)
Eosinophils, cells/μL	78–1,792	900 (0–3,000)	8 (36.4)	3 (13.6)
ESR, mL/60 min	0–18	22 (1–131)	NA	16 (72.7)
Iron, μg/dL	92–300	193 (22–1,036)	8 (36.4)	9 (40.9)

*PIV, parainfluenza virus; HCT, hematocrit; NA, not available; ESR, erythrocyte sedimentation rate.

Of 13 dolphins that had clinical signs and recovered, the average duration of clinical illness was 9.8 days (range 1–40 days). Four dolphins had PIV antibody seroconversion within 30 days of death, but the presence of TtPIV-1 was not confirmed on virus culture from any animal tissues upon necropsy except from the positive control dolphin. Of these 4 dolphins, 2 died from bacterial pneumonia confirmed by laboratory culture and histologic examination, and 1 had mild to moderate growth of *Candida glabrata* from lung samples. All 4 animals had a mild to moderate tracheitis or laryngitis not explained by finding intralesional bacterial or fungal infection upon histopathologic examination.

Population Seroprevalence

Comparisons of age and sex distribution among the 2 dolphin populations are provided in Table 4. The median age of dolphins was 15.5 years (range 0.2–49.2). Sex was determined for 110 dolphins; of these, 50% were female.

Of 114 clinically healthy dolphins tested for PIV antibodies, 13 (11.4%) were positive, 34 (29.8%) were negative, and 67 (58.8%) were inconclusive ($0 < OD_{405}$ ratio < 1.0). Mean and median PIV OD_{405} ratios were 0.4 (standard deviation = 0.4) and 0.2 (range 0.0–2.8), respectively. There were no significant differences in OD_{405} ratio by age ($p = 0.2$) or sex (females = 0.33, males = 0.43, $p = 0.3$). When Florida free-ranging healthy dolphins were compared with San Diego managed healthy dolphins, there were no significant differences in mean PIV OD_{405} ratios ($p = 0.6$) or percentages of animals categorized as negative, positive, or inconclusive (Table 4; $p = 0.5$). Among the 2006 MMP healthy dolphin serosurvey population, no significant differences were identified when hematologic

Table 2. Frequencies of abnormal serum biochemical values in bottlenose dolphins (*Tursiops truncatus*) within 60 d before and 30 d after PIV antibody seroconversion (n = 22)*

Blood variable	Adult dolphin reference range	Median value (range), cases	No. cases below reference range (%)	No. cases above reference range (%)
Protein, g/dL	6.2–7.6	7.1 (5.7–9.6)	4 (18.2)	12 (54.5)
Albumin, g/dL	3.9–4.9	4.2 (3.4–5.1)	6 (27.3)	2 (9.1)
Globulin, g/dL	2.1–3.1	3.0 (1.7–5.0)	2 (9.1)	12 (54.5)
Glucose, mg/dL	85–144	110 (70–214)	7 (31.8)	9 (40.9)
Sodium, mEq/L	153–159	156 (127–169)	8 (36.4)	0
Chloride, mEq/L	115–125	118 (109–134)	12 (54.5)	0
Potassium, mEq/L	3.5–4.1	3.8 (3.0–7.5)	5 (22.7)	10 (45.5)
Calcium, mg/dL	8.3–9.7	9.1 (6.6–10.8)	3 (13.6)	9 (40.9)
BUN, mg/dL	36–59	48 (29–76)	8 (36.4)	6 (31.8)
Creatinine, mg/dL	1.2–2.0	1.3 (0.7–2.4)	7 (31.8)	4 (18.2)
Uric acid, mg/dL	0.0–0.7	0.2 (0.0–1.8)	NA	8 (36.4)
LDH, U/L	270–494	399 (247–1672)	0	10 (45.5)
AST, U/L	118–398	228 (111–987)	0	9 (40.9)
ALT, U/L	13–54	33 (15–541)	0	11 (50.0)
GGT, U/L	21–48	39 (18–876)	0	10 (45.5)
Carbon dioxide, mEq/L	17–28	24 (9–34)	3 (13.6)	4 (18.2)
Inorganic phosphate, mg/dL	3.9–5.9	5.1 (3.2–7.5)	6 (27.3)	9 (40.9)
Alkaline phosphatase, U/L	158–556	240 (36–1070)	8 (36.4)	3 (13.6)
Cholesterol, mg/dL	153–262	202 (115–382)	9 (40.9)	5 (22.7)
Triglyceride, mg/dL	11–175	95 (27–667)	0	8 (36.4)
CPK, U/L	51–183	122 (24–596)	5 (22.7)	9 (40.9)

*PIV, parainfluenza virus; BUN, blood urea nitrogen; NA, not available; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; CPK, creatine phosphokinase.

Table 3. Clinical signs noted in selected bottlenose dolphins (*Tursiops truncatus*) within 60 d before and 30 d after PIV antibody seroconversion and an abnormal hemogram (n = 22)*†

Animal	Clinical signs	Decreased appetite	Lethargy	Respiratory signs	Ocular signs	Epidermal signs	GI signs
A							
B	X				X		X
C							
D	X		X				
E	X	X		X	X		
F	X	X					
G	X	X	X				
H	X	X	X		X	X	
I	X	X	X	X			
J	X	X	X	X			
K	X	X			X		X
L							
M							
N	X		X	X	X		
O							
P	X	X	X				
Q	X						
R	X		X				
S	X	X	X	X			
T	X	X		X			
U	X	X	X			X	
V	X	X		X			
Total	17 (77.3%)	12 (54.5%)	10 (45.5%)	7 (31.8%)	5 (22.7%)	2 (9.1%)	2 (9.1%)

*PIV, parainfluenza virus; GI, gastrointestinal.
†No viruses were cultured from clinical samples collected from these animals, with the exception of the positive control dolphin, and 2 dolphins had culture-confirmed bacterial pneumonia concurrent with PIV seroconversion.

indicators of inflammation among healthy PIV antibody–positive dolphins were compared with PIV antibody–negative dolphins (Table 5).

Discussion

Using an indirect dolphin-specific antibody ELISA, we demonstrated an increase in PIV serum antibodies during culture-confirmed TtPIV-1 respiratory illness in an adult bottlenose dolphin. Although ELISA has been recognized as the most sensitive indicator of PIV infections in human populations (*9*), virus isolation and genotyping are needed to confirm which type of PIV is associated with an infection (*10*). As such, antibody ELISA results in our study were interpreted as dolphin immune responses to PIV or a closely related virus (e.g., a mumps-like virus).

We report 21 additional dolphins in which PIV antibody seroconversion occurred within 3 months of an abnormal hemogram similar to that of the positive control animal during 1999–2006. Approximately 23% of these dolphins did not have overt clinical signs, indicating that PIV infections may affect hematologic values without affecting animal behavior. Further, no significant differences in inflammatory indicators were identified when PIV antibody–seropositive and –seronegative animals were compared in our cross-sectional serosurvey of healthy animals. Subclinical BPIV-3 infections are frequent in cattle popu-

Table 4. Comparisons of age, sex, and PIV antibody levels among 2 healthy bottlenose dolphin (*Tursiops truncatus*) populations (n = 114)

Descriptor	Free-ranging dolphins, Sarasota, Florida (n = 56)	Managed dolphins, San Diego, California (n = 58)	p value
Mean age, y	11.5	20.7	<0.0001
Sex, %			
Female	45.6	54.7	0.3
Male	54.4	45.3	
Mean PIV antibody OD_{405} ratio*	0.42	0.36	0.6
PIV result, %			0.5
Negative	33.9	25.9	
Equivocal	58.9	58.6	
Positive	7.1	15.5	

*PIV, parainfluenza virus; OD_{405}, optical density at 405 nm.

Table 5. Comparisons of mean inflammatory indicator values between bottlenose dolphins (*Tursiops truncatus*) seropositive or seronegative for parainfluenza virus antibodies, July–December 2006*

Blood variable, cells/μL	Least squares means, TtPIV1 seropositive (n = 9 animals)	Least squares means, TtPIV1 seronegative (n = 15 animals)	p value
Leukocytes	6,969	7,602	0.23
HCT	41.6	40.3	0.25
Lymphocytes	1,357	1,379	0.93
Monocytes	141	133	0.89
Neutrophils	4,389	5,189	0.05
60-min ESR	10.5	10.2	0.93

*TtPIV1, *T. truncatus* parainfluenza virus type 1; HCT, hematocrit; ESR, erythrocyte sedimentation rate.

lations (*17*). In a case-control study comparing acute- and convalescent-phase serum samples among calves with respiratory disease and calves that were clinically normal, the incidence of BPIV-3 seroconversion was actually higher in clinically normal calves (*18*).

In our survey involving dolphins that seroconverted within 3 months of an abnormal hemogram, clinical signs were most often nonspecific and limited to lethargy and decreased appetite lasting an average of 9–10 days. Of animals that seroconverted, 32% had respiratory clinical signs, and 3 of 4 animals that died within 30 days of seroconversion had intralesional bacterial or fungal pathogens in lung tissue. Further evidence of primary PIV infections in animals that died from bacterial or fungal pneumonia was inflamed laryngeal or tracheal tissue without intracellular bacterial or fungal pathogens. Despite confirmed bacterial or fungal pneumonia in these animals, pathologists' interpreted the tracheitis and laryngitis to be of possible viral origin. In terrestrial mammals, PIV most commonly affects the upper and lower respiratory tract (*1–3*,*9*), and frequent conditions include tracheitis and laryngitis. Further, PIV infections are commonly associated with bacterial or fungal coinfections in terrestrial mammals (*19–21*).

In our study, nonrespiratory signs associated with PIV seroconversion involved the ocular, epidermal, and gastrointestinal systems. Additionally, 50% of dolphins with PIV seroconversion had high ALT levels not associated with medications, indicating potential hepatic involvement. Similarly, nonrespiratory clinical signs reported in a study involving 46 human patients with PIV virus infections included conjunctivitis, exanthema, oral mucosal lesions, diarrhea, and increased levels of transaminases (*22*).

Lacking in all active case dolphinss, except the positive control, was culture of PIV from clinical or postmortem samples. In humans, reported HPIV-3 viral culture success rates from clinical samples can range from 42% to 50% (*23*,*24*), and detecting PIV by looking for CPE is not considered reliable or useful (*9*). The diagnostic laboratory we routinely used to culture viruses from MMP samples relied primarily upon CPE in monkey kidney cell lines over multiple passages. To improve the likelihood of isolating PIV during active infections in bottlenose dolphins, diagnostic workups should include PCRs and indirect immunofluorescent-antibody assays (IFAs), as these tools have proven effective for rapidly identifying PIV cases in other species (*25*,*26*).

In our seroprevalence study involving 114 clinically healthy dolphins, we demonstrated that 11.4% had PIV antibodies at least as high as our positive control, and 70% had PIV antibodies higher than our negative control. No significant differences in PIV antibody levels were found when comparing dolphin location, age, or sex. Similar findings regarding equivalent PIV exposures by age, sex, and geographic location have been reported in humans (*9*) and cattle (*27*). High rates of PIV infection among these populations support incomplete immunization with infections and high rates of reinfection throughout life (*9*). Serologic studies have demonstrated common PIV exposure among wild hooved species in Alaska (67%) (*28*), the central Italian Alps (17%) (*29*), Argentina (43%) (*30*), Alberta (49%) (*31*), South Africa (25%) (*32*), and Quebec (82%–84%) (*33*).

The primary limitation of our study was interpretation of PIV antibody levels based upon 1 positive control. To compensate for the limitation of positive controls among marine mammal samples, we applied conservative definitions for positive and negative ELISA results and tested for significant changes in antibody levels in the same animal over time. Use of PCR and IFA as standard assays on prospective samples will help to increase the number of positive controls for future studies (*22*).

TtPIV-1 is a novel virus most closely related to bovine PIV3 (BPIV-3) (*4*), and attenuated BPIV-3 has been demonstrated as a safe and effective vaccine against human PIV3 (HPIV-3) in human populations (*34*,*35*). Given the genetic similarity of TtPIV-1 to BPIV-3 and HPIV-3, TtPIV-1 may provide therapeutic benefit to human populations.

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Dr Venn-Watson is a veterinary epidemiologist at the US Navy MMP. Her research interests include the epidemiology of diseases in wild and exotic animal species.

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Hantavirus RNA in Saliva from Patients with Hemorrhagic Fever with Renal Syndrome

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Hantaviruses cause 2 zoonotic diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome. Infection is usually initiated after inhalation of virus-contaminated rodent excreta. In addition to the zoonotic infection route, growing evidence suggests person-to-person transmission of Andes virus. For this reason, we studied whether saliva from HFRS patients contained hantavirus. During an outbreak in northern Sweden of nephropathia epidemica (NE), a milder form of hemorrhagic fever with renal syndrome, we collected saliva and plasma from 14 hospitalized NE patients with verified Puumala virus (PUUV) infection. PUUV RNA was detected in saliva from 10 patients (range 1,530–121,323 PUUV RNA copies/mL) by quantitative reverse transcription–PCR. The PUUV S-segment sequences from saliva and plasma of the same patients were identical. Our data show that hantavirus RNA could be detected in human saliva several days after onset of disease symptoms and raise the question whether interhuman transmission of hantavirus may occur through saliva.

Members of the family *Bunyaviridae* cause severe and often fatal human diseases in a large and increasing number of persons worldwide each year (*1*). This family contains 5 genera, and the genus *Hantavirus* causes 2 febrile illnesses: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in North and South America. Hantaviruses are rodent-borne pathogens. In Sweden, Finland, Norway, Russia, and parts of central Europe, Puumala virus (PUUV) is endemic in bank voles (*Myodes glareolus*). PUUV causes nephropathia epidemica (NE), a milder form of HFRS. The most common symptoms of NE are fever, headache, nausea, vomiting, myalgia, abdominal pain, back pain, and visual disturbances (*2*). One third of the patients have hemorrhagic manifestations, 10%–20% have respiratory tract symptoms, and most have signs of renal failure (*2*). There is no effective treatment or available vaccine.

The most common route of hantavirus infection is infectious aerosols originating from saliva, urine, and feces of infected rodents (*1*). Because rodent bites have been demonstrated to cause human hantavirus infections (*3*,*4*), saliva of hantavirus-infected rodents must contain infectious virus. Infectious PUUV has been found in oropharyngeal secretions from *M. glareolus* (*5*) and Andes virus (ANDV) RNA is present in saliva from infected pigmy rice rats (*Oligoryzomys longicaudatus*) (*6*). Furthermore, Sin Nombre virus RNA is present in saliva but not in urine and feces from deer mice (*Peromyscus maniculatus*) (*7*), confirming that hantaviruses are transmitted to humans through rodent saliva. However, person-to-person transmission has recently been documented for the ANDV that causes HCPS (*8*–*13*), and contaminated human saliva suggests a possible route of infection (*13*). For herpesviruses, such as Epstein-Barr virus and herpesviruses-7 and -8, person-to-person transmission can occur from saliva (*14*–*16*), but human salivary secretions could also negatively modulate herpesvirus infectivity as shown for herpesvirus-1 (*17*).

Because the saliva of hantavirus-infected rodents contains infectious virus that is transmitted to humans (*3*,*4*,*7*) and person-to-person transmission is strongly suspected for ANDV (*13*), we studied whether the virus could be detected in saliva of hantavirus-infected patients. For this reason, we collected saliva from patients during an NE outbreak in northern Sweden and analyzed the samples for the presence and levels of PUUV RNA by using a real-time reverse transcription–PCR (RT-PCR) assay.

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Material and Methods

Collection of Human Saliva

Saliva was collected from PUUV-infected patients who were hospitalized from January through May 2007 at the Department of Infectious Diseases at Umeå University Hospital (Umeå, Sweden). Samples were obtained from patients with typical clinical symptoms of acute NE, which were verified by detection of PUUV-specific immunoglobulin (Ig) M in serum, a PUUV-specific real-time RT-PCR, or both. The collection was random, with no consideration of time of the day or food intake. The samples were only collected on 1 occasion during each patient's hospitalization by asking the patient to spit in a single-use plastic mug. The saliva was then removed with a syringe (5 mL; Becton Dickinson, Franklin Lakes, NJ, USA) and put in sterile plastic test tubes (Nunc, Roskilde, Denmark). The samples were immediately stored at –80°C until they were further analyzed. Plastic gloves and protective clothing were used during the procedure. A total of 9 samples were stored without additives, and 5 were diluted 1:3 in virus transport medium (2% HEPES, 10 μg/mL bovine serum albumin, 50 μg/mL sucrose, 0.016 μg/mL fungizone, and 2.5 μg/mL garamycin) before storage. The project was approved by the Research Ethics Committee of Umeå University and all patients gave written and informed consent.

Real-Time RT-PCR

RNA from 140 μL of patient saliva and plasma was extracted by using a QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The real-time RT-PCR was performed as previously described (*18*). Briefly, the RNA was reverse-transcribed followed by a real-time PCR TaqMan assay in triplets with PUUV-specific primers and probe from the S segment (*18*). The real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System 2.0 (Applied Biosystems, Foster City, California, USA). As internal positive control, we used a 72-bp *Drosophila melanogaster* fragment (nt position 1783–1854, GenBank accession no. NM_144343) cloned into a pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The plasmid was linearized with the restriction enzyme *Apa*I, and RNA was transcribed and purified as described (MEGAscript High Yield Transcription Kit, Ambion, Promega, Madison, WI, USA; www.megasoftware.net). The RNA control was diluted, added to patient sample, and used as template for quantitative real-time RT-PCR as described above. The primers and probe used for amplification of control RNA was *D. melanogaster* forward primer 5′-AGGTGCCCGTGTGTATCCAT-3′ (900 nM), reverse primer 5′-GCTCGTCCTCCGCCTCAT-3′ (900 nM) and probe 5′FAM-TACCACGAATCTGCGACATTACCAGGG-TAMRA-3′ (200 nM). Primers and probe were designed by using Primer Express version 2.0 software (Applied Biosystems).

Immunofluorescence Assay

An immunofluoresence assay was performed as previously described (*18*). Briefly, patient serum was added to spot slides covered with fixed PUUV-infected Vero E6 cells, and IgG levels were determined by using fluorescein-conjugated rabbit anti-human IgG (F202, DAKO A/S, Glostrup, Denmark) diluted in phosphate-buffered saline (PBS) with Evans blue. For IgM analysis, patient serum was pretreated with rheumatoid factor absorbent (Virion\Serion GmbH, Würzburg, Germany) to eliminate possible interference of rheumatoid factor and PUUV-specific IgG. Slides were incubated overnight at 37°C, and antibody was detected by using fluorescein-conjugated rabbit F(ab′)2 anti-human IgM antibodies (F0317, DAKO A/S) diluted in PBS with Evans blue.

Sequencing

cDNA from patient saliva and plasma were amplified by using Pfu DNA Polymerase (Fermentas Life Sciences, Helsingbord, Sweden), and the sequencing reactions were performed by using the Big Dye Terminator v 1.1 Cycle Sequencing kit (Applied Biosystems). The PCR and sequencing reactions were performed with primers from the S-segment. For patients 1 and 4, the primers for PCR was Puu1s (5′-CAAGAGGATATAACCCGCCA-3′) and Puu6as (5′-GCCATCCCTGCAACATAGAT-3′) followed by Puu3s (5′-AACTGGGATTGAGCCAGATG-3′) and Puu5as (5′-TGGGCATTCCTTTTCCATAA-3′) for sequencing. For patient 2, the primers for PCR was Puu1s and Puu5as followed by Puu2s (5′-ACCCGCCATGAACAACAACT-3′) and Puu4as (5′-TAGGGCTTTCAAAATAATAGGTAG-3′) for sequencing. Before the sequencing reaction, the PCR fragment was purified by using a QIAquick PCR Purification kit (QIAGEN) and precipitated in ethanol. Assembly, analysis, and alignment of sequences were performed by using Geneious Basic 3.0.6 (www.geneious.com) and BLAST (www.ncbi.nlm.nih.gov/blast).

Results

Hantavirus RNA in Saliva from NE Patients

We collected saliva 2–9 days after onset of symptoms from 14 hospitalized NE patients to determine whether hantaviruses were present in human saliva. All patients were positive for PUUV RNA in plasma by a real-time RT-PCR, and 13 patients had PUUV-specific IgM antibodies in serum (Table). We then analyzed the saliva by real-time RT-PCR for the presence and levels of viral RNA. Saliva samples from 10 of 14 patients were positive for PUUV RNA (range 1,530–121,323 PUUV RNA copies/mL),

Table. Comparison between detection of PUUV RNA in saliva and plasma and antibodies in serum samples from 14 patients with nephropathia epidemica*

Patient no.	Sex	Age, y	Date saliva collected (d of disease)	PUUV RNA† in saliva, copies/mL	PUUV RNA† in plasma,‡ copies/mL	PUUV IgM§ in serum‡	PUUV IgG titer in serum‡
1	M	65	6	121,323	959,294	+	40
2	M	64	2	66,994	117,562	+	80
3	M	33	5	44,898	40,427	+	≥640
4	F	38	6	17,516	1,381,413	+	40
5	F	59	9	9,582	3,724	+	≥640
6	M	54	5	6,372	26,626	–	320
7	M	39	7	3,745	189,233	+	320
8	M	28	5	2,589	36,231	+	≥640
9	F	26	5	2,163	54,315	+	80
10	F	39	5	1,530	3,044	+	80
11	M	38	6	0	1,952	±	≥640
12	M	41	6	0	5,215	+	40
13	F	57	2	0	41,271	+	320
14	F	62	7	0	81,330	+	160

*PUUV, Puumala virus; Ig, immunoglobulin.
†Detected by real-time reverse transcription–PCR.
‡Most plasma and serum samples were collected the same day or within 1 day before or after the saliva sample and antibody was detected by immunofluorescence assay.
§Serum was diluted 1:16 and scored as negative (–), positive (+), or weakly positive (±).

demonstrating that viral RNA could be detected in saliva during disease (Table). Furthermore, we detected no inhibition of real-time RT-PCR in saliva or plasma when we analyzed our patient samples by using an internal positive control (data not shown). All patients were also IgG positive in serum with varying titers.

Sequencing of PUUV RNA in Saliva

To study in more detail whether the RNA we detected in saliva was truly specific, we sequenced regions of RT-PCR products. Sequencing of amplification products from saliva and plasma from 3 NE patients—patient 1 (GenBank accession nos. EU337014 and EU337015), patient 2 (GenBank accession nos. EU177629 and EU177630), and patient 4 (GenBank accession nos. EU337016 and EU337017)—demonstrated a PUUV S-segment sequence that was identical for each saliva/plasma pair (data not shown). When we compared the sequences for patients 1 and 4, we found 24 mismatches in the 292-nt S-segment sequence, which clearly demonstrated that they were derived from different strains (Figure). The observed nucleotide mismatches did not result in different amino acid sequences. The sequence from patient 2 was from another S-segment region. All PUUV sequences from the NE patients were most closely related to PUUV strains from the disease-endemic region in northern Sweden and grouped with the northern branch of PUUV strains (*19*,*20*).

Respiratory Symptoms in NE patients

All 14 NE patients required hospital care and displayed the symptoms of NE. All survived and none had to be treated by dialysis. Seven patients had respiratory symptoms including cough, dyspnea, or both. In 2 of the patients with respiratory symptoms, a chest radiograph revealed infiltrates (data not shown). The 2 patients with the highest levels of viral RNA in saliva had dyspnea (patient 1) and cough and lung infiltrates (patient 2) (Table). All patients with respiratory symptoms, except 1 (patient 12), had PUUV RNA in their saliva.

Discussion

This investigation showed that hantavirus RNA could be detected in saliva from HFRS patients. We found PUUV RNA with varying PUUV genome copy numbers in the saliva of most of the 14 hospitalized NE patients. Previously, we have shown that PUUV RNA in plasma decreases with time in the individual patient (*18*), and although we did not study this finding, PUUV in saliva likely would display similar kinetics. PUUV viremia is thought to persist for 5–7 days, but in our study, 1 patient had PUUV in both saliva and plasma 9 days after onset of symptoms. Furthermore, we recently detected PUUV RNA in plasma 16 days after first appearance of disease (*18*). It would also be interesting to study whether nonhospitalized patients with milder symptoms also had PUUV RNA in their saliva. We do not know if PUUV RNA in saliva could originate from PUUV viremia, but the PUUV RNA detected in saliva was likely produced in salivary glands or through coughing. Recently, ANDV antigen was found in secretory cells of the salivary glands of human patients (*21*). In our study, 7 of the patients had respiratory symptoms, and virus shedding in saliva is known to occur for several respiratory viruses, such as severe acute respiratory syndrome–associated coronavirus, respiratory syncytial virus, and human metapneumovirus (*22*,*23*). Symptoms in the upper respiratory tract are also often present in NE (*2*,*24*), which makes our

Figure. Comparison of nucleotide sequences between reverse transcription–PCR products isolated from nephropathia epidemica patients 1 and 4. Mismatches are underlined.

finding of PUUV in saliva credible. One may argue that NE, the HFRS endemic in northern and central Europe, is a different entity than HCPS, caused by Sin Nombre hantavirus and ANDV, and that the finding of viral RNA in saliva from NE patients is a pure coincidence. However, NE in Scandinavia shows several common characteristics with HCPS in the Americas. For example, pulmonary involvement and respiratory symptoms are common in NE. In addition, radiologic examination–detectable infiltrates, decreased pulmonary function, and a local inflammatory response in the lungs have previously been demonstrated (*25–28*).

Could hantaviruses in human saliva be infectious and initiate infection? Today, the only hantavirus suggested to be transmitted between humans is ANDV (*8–13*). By using sophisticated epidemiologic data, Ferres et al. showed that the risk for HCPS caused by ANDV was higher among sex partners of the index case than among other household contacts (*13*). These investigators suggested that ANDV needs close person-to-person contact, such as sexual relations or deep kissing, to be transmitted between humans (*13*). Our finding of hantavirus in saliva support the conclusion that hantavirus infection could be transmitted between humans through saliva, such as during kissing or coughing. However, we do not know at this stage whether the hantavirus RNA in saliva detected in our study is infectious. When we tried to infect bank voles with human PUUV RNA–positive saliva, no seroconversion was found after 21 days (J. Hardestam et al., unpub. data). Furthermore, we have not been successful in isolating PUUV from saliva specimens by infecting Vero E6 cells. Clinical isolates of hantaviruses do not grow readily on cell lines, and mutations of the noncoding regions were shown to be needed for the PUUV strain Kazan, originally isolated from bank voles, to grow in Vero E6 cells (*29*). The only human PUUV isolate from Sweden, PUUV strain Umeå/hu, adapted to growth in Vero E6 cells, was isolated with phytohemagglutinin-stimulated leukocytes from an NE patient, and PUUV antigen was not detected until 6 months after infection of Vero E6 cells (*30*).

How long after onset of disease viral hantavirus RNA is present in saliva and whether saliva contains neutralizing antibodies need to be studied further, even though ANDV-specific IgA antibodies have been detected in saliva in patients with acute HCPS from 5 to 31 days after onset of symptoms (*31*). Detecting hantavirus-specific antibodies in human saliva may be useful for diagnostics, and detecting viral RNA in saliva could be used as a tool to study hantavirus epidemiology. ANDV RNA was shown to be present in human peripheral blood cells for 5 to 15 days before the onset of symptoms (*13*), and PUUV RNA was detected in plasma from *Cynomolgus* macaques 4 days before symptoms appeared (*32*). Studies are needed to determine whether the same is true for saliva in animal models. If this was the case, the hantavirus in antibody-free saliva could have a rather high infectious potential. On the other hand, saliva has antimicrobial functions (*33*) and has been shown to inhibit certain viruses, such as HIV-1 (*34*), influenza A virus (*35*), and herpes simplex virus type 1 (*17*); however, saliva does not inhibit Epstein-Barr virus (*14*) and adenovirus (*34*). So far, little is known regarding the effect of human saliva on hantavirus infectivity, but results of experiments we have performed indicate that human saliva could reduce at least part of the infectivity in vitro (J. Hardestam et al., unpub. data). Apparently, hantaviruses in rodent saliva are infectious, but whether the composition of rodent and human saliva differs is not known. Further studies are needed to elucidate the mechanisms behind hantavirus transmission between the natural rodent hosts and humans.

After finding PUUV RNA in saliva from NE patients, we searched for evidence of possible person-to-person transmission of PUUV. When the NE patients were followed-up 1–2 months later, we discovered several clusters of household members with NE symptoms. However, none had severe enough disease to require physician care, and no samples were collected for laboratory diagnosis. Furthermore, during the large NE outbreak in early 2007, there was a very high incidence (313/100,000) of NE in the region where our university hospital is situated (*36*). Since all patients lived in this area, we cannot exclude inhalation of rodent excreta as the main or only transmission route. Although it remains to be clearly shown, our results sup-

port the hypothesis that person-to-person transmission may occur by this route.

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Increased Mortality Rate Associated with Chikungunya Epidemic, Ahmedabad, India

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In 2005–2006, Réunion Island in the Indian Ocean reported ≈266,000 cases of chikungunya; 254 were fatal (case-fatality rate 1/1,000). India reported 1.39 million cases of chikungunya fever in 2006 with no attributable deaths; Ahmedabad, India, reported 60,777 suspected chikungunya cases. To assess the effect of this epidemic, mortality rates in 2006 were compared with those in 2002–2005 for Ahmedabad (population 3.8 million). A total of 2,944 excess deaths occurred during the chikungunya epidemic (August–November 2006) when compared with the average number of deaths in the same months during the previous 4 years. These excess deaths may be attributable to this epidemic. However, a hidden or unexplained cause of death is also possible. Public health authorities should thoroughly investigate this increase in deaths associated with this epidemic and implement measures to prevent further epidemics of chikungunya.

Chikungunya virus, an alphavirus of the family *Togaviridae*, is native to tropical Africa and Asia. This virus is transmitted to humans by mosquitoes. *Aedes aegypti* and *Ae*. *albopictus* are the 2 main vectors that transmit this disease (*1*). The first reported chikungunya outbreak occurred in Tanganyika (now Tanzania) in 1952–1953 (*2*). The word chikungunya is derived from the *Makonde* language in southeastern Tanzania and means "bent down or become contorted," which indicates the classic posture the patient adopts because of severe joint pain. Symptoms of chikungunya include sudden onset of fever, severe arthralgia, and maculopapular rash. A specific symptom is severe incapacitating arthralgia, often persistent, which can result in long-lasting disability (*3*).

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A major epidemic of this disease was reported in 2005–2006 in Réunion Island; ≈266,000 residents (34.3% of the population) of this Indian Ocean island were affected by chikungunya fever as of February 19, 2007. This epidemic also spread to France through imported cases from Réunion Island (*4*). Historically, chikungunya was considered self-limiting and nonfatal. However, 254 deaths on Réunion (case-fatality rate 1/1,000) that were attributed directly or indirectly to chikungunya during the epidemic changed this perspective (*1*,*4*).

India reported a massive chikungunya epidemic in 2006. Chikungunya has reemerged in India since 1973, when the attack rate was 37.5%. However, in the 2006 epidemic, the attack rate increased to 45% in some places (*4*). More than 1.39 million cases across 151 districts and 10 states were reported during this period (*5*). However, unlike the epidemic on Réunion Island, no deaths directly attributable to this disease were reported (*6*). The dominant vectors are *Ae*. *albopictus* on Réunion Island and *Ae*. *aegypti* in India (*4*). However, *Ae*. *albopictus* was also implicated in Kerala State, India (*7*).

Studies have indicated that the recent outbreak in the Indian Ocean islands was initiated by a strain related to East African isolates, from which viral variants have evolved with a traceable history of microevolution. This history could provide information for understanding the unusual magnitude and virulence of this chikungunya epidemic (*8*).

The purpose of this study was to analyze the association between the chikungunya epidemic in India and the mortality rate in the city of Ahmedabad. Such findings could show correlations between reported genomic mutations in chikungunya virus and its increased virulence. Such information is valuable for public health systems in

developing countries that frequently underreport or misreport epidemics.

Methods

Collection of Death Data

The registrar of births and deaths (RBD) of Ahmedabad, who is a subordinate officer to the medical officer of health, registers all births and deaths within the city limits under the Registration of Births and Deaths Act. Deaths are registered in 2 ways. Deaths that occur in a hospital are reported by hospital authorities, who provide a medical certificate of death that is sent to the RBD officer in the city ward in which the hospital is located. Deaths that occur at home are reported by the family to the local RBD officer of the ward in which their home is located.

Deaths are compiled and sent from the RBD ward office to the RBD central office and subsequently communicated to the state level registrar of birth and death. Death data used in this study were provided by the medical officer of health of the city. Data include monthly total deaths registered in Ahmedabad during 2002–2006.

Collection of Chikungunya Case Data

During the chikungunya epidemic, the city health department collected, compiled, and reported data on suspected cases of chikungunya from municipal hospitals and health centers. Data include monthly reported cases of chikungunya, blood samples sent for testing, and samples positive for chikungunya virus infection in Ahmedabad starting in April 2006. Few data were reported by private hospitals, dispensaries, and private practitioners in the city, who treat many patients.

Statistical Analysis

Average mortality rate for each month during 2002–2005 (years before the epidemic) was calculated by dividing the average number of deaths for each month by the average population. Average mortality rate for each month in 2006 was calculated by dividing the number of registered deaths for each month by the monthly population. The expected number of monthly deaths for each month in 2006 was calculated by multiplying the average mortality rate for each month (2002–2005) by the monthly population in 2006. Because there were 12 estimates of expected deaths (1 for each month), we used the more conservative simultaneous confidence interval (CI) and the Bonferroni method (*9*) instead of a simple CI for each month separately. Excess deaths for each month in 2006 were the difference between actual observed number of deaths and expected number of deaths. Average monthly mortality rates for 2002–2005 were then compared with the mortality rate for 2006 (epidemic year).

Results

The medical officer of health in Ahmabadad reported 60,777 suspected chikungunya cases in 2006. The peak of the epidemic occurred in August and September 2006 when 55,593 (91.5%) of the cases were reported. A total of 84 (54.5%) of 154 blood samples tested were positive for chikungunya virus. Of these 84 confirmed chikungunya cases, 10 were fatal (case-fatality rate 11.9%).

A monthly distribution of cases of chikungunya, actual and expected number of deaths in 2006, and monthly average mortality rates for 2002–2005 and 2006 per 10,000 persons are shown in the Table. The number of deaths and mortality rates increased substantially from August through November 2006 compared with values for 2002–2005 for the same months. Mortality rates for August, September, and October 2006 increased 22%, 57%, and 33%, respectively, compared with average mortality rates for these months for 2002–2005. The highest numbers of chikungunya cases were also reported during these months. A total of 31,496 deaths were registered in 2006 compared with 28,440 (99% CI 27,500–29,380) expected deaths for the same year based on average number of deaths for the last 4 years. There were ≈3,056 additional deaths registered in Ahmedabad in 2006 compared with the expected number of deaths for 2006. A comparison of the monthly distribution of actual deaths in 2006 with expected deaths showed a rapid increase in deaths registered from August through November 2006. In these 5 months, 2,944 additional deaths (96.34% of total additional deaths for 2006) occurred when compared with the expected number of deaths for the same months for the previous 4 years. Excess number of deaths peaked in September 2006, when 1,448 additional deaths (47.38% of total additional deaths for 2006) occurred when compared with the expected deaths for September (Figure).

The temporal relationship between chikungunya cases and expected mortality rates and actual mortality rates in 2006 is shown in the Figure. The peak in chikungunya cases in August–September coincides with the peak in actual deaths in 2006.

Discussion

Analysis of our data shows that the mortality rate in Ahmedabad increased substantially in 2006 when compared with rates for the previous 4 years. A total of 3,056 excess deaths occurred in 2006 (the epidemic year) when compared with the expected number of deaths for that year. A substantial increase in deaths reported was observed from August through November 2006 (2,944 excess deaths in these months). The number of reported chikungunya cases also showed a peak in August and September 2006, which coincided temporally with the peak in number of deaths in Ahmedabad (Figure).

Table. Monthly chikungunya cases, deaths, and mortality rates, Ahmedabad, India, 2002–2005 and 2006*

Month	Chikungunya cases, 2006	Mortality rate/10,000 (99% CI), 2002–2005	Expected deaths, 2006 (99% CI)	Actual deaths, 2006	Excess deaths, 2006	Mortality rate/10,000, 2006	% Change in mortality rate
Jan	ND	6.19 (6.00–6.41)	2,422 (2,342–2,502)	2,559	137	6.54	+5.66
Feb	ND	5.56 (5.37–5.76)	2,180 (2,105–2,255)	2,227	47	5.68	+2.14
Mar	ND	5.76 (5.56–5.95)	2,264 (2,187–2,341)	2,337	73	5.95	+3.24
Apr	434	5.75 (5.53–5.92)	2,260 (2,183–2,337)	2,150	–110	5.47	–4.89
May	141	6.16 (5.93–6.33)	2,428 (2,349–2,507)	2,510	82	6.37	+3.37
Jun	31	5.80 (5.56–5.95)	2,290 (2,213–2,367)	2,156	–134	5.46	–5.86
Jul	184	5.50 (5.27–5.65)	2,177 (2,102–2,252)	2,270	93	5.73	+4.27
Aug	28,233	6.08 (5.82–6.21)	2,410 (2,331–2,489)	2,942	532	7.42	+22.09
Sep	27,360	6.40 (6.12–6.52)	2,541 (2,460–2,622)	3,989	1,448	10.05	+56.96
Oct	3,555	5.92 (5.64–6.03)	2,355 (2,277–2,433)	3,121	766	7.85	+32.51
Nov	539	6.27 (5.97–6.38)	2,500 (2,420–2,580)	2,698	198	6.77	+7.90
Dec	300	6.54 (6.22–6.63)	2,613 (2,531–2,695)	2,537	–76	6.35	–2.90
Total	60,777		28,440 (27,500–29,380)	31,496	3,056		

*CI, confidence interval; ND, no data available.

The main issues of contention are whether these excess deaths were caused by chikungunya and whether such excess deaths will occur in future years without chikungunya epidemics. No major adverse event or other epidemic occurred in Ahmebabad in August–November 2006 other than the chikungunya epidemic. Our epidemiologic evidence shows that the epidemic is the most plausible explanation for the large increase in deaths in Ahmedabad in August–November 2006. However, other unidentified causes cannot be ruled out. Similar data from other cities and areas affected by the chikungunya epidemic may help establish the link between chikungunya and excess deaths.

There are 2 major problems with reporting of deaths in Ahmedabad. The cause of death is poorly reported, and the RBD does not separate death data for residents and nonresidents. Inclusion of patients from surrounding rural areas who died in city hospitals could have resulted in excess deaths being reported during the epidemic. However, this was a problem in years before the epidemic (2002–2005) as well. A review of deaths registered in rural areas outside the city limits of Ahmedabad showed no major decrease during the epidemic months of 2006 over previous years. Thus, the increase in number of deaths caused by migration of sick patients cannot explain this major increase in deaths in 2006, although this factor may have contributed to it.

An excess in total deaths was also reported for the chikungunya epidemic on Réunion Island during February–April 2006 (*10*). A total of 260 excess deaths were reported on Réunion Island during the epidemic, of which 254 were directly or indirectly attributed to chikungunya (mortality rate attributed to chikungunya 1/1,000) (*4*,*10*). Most of the excess deaths on Réunion Island were persons ≥75 years of age. Of 10 confirmed deaths in Ahmedabad caused by chikungunya, 2 were persons >80 years of age, 4 were persons 60–70 years of age, and 3 were persons <60 years of age.

The genomic sequences of chikungunya virus isolates from India were similar to that of a recent isolate from Réunion Island (*11*). Because of this finding, the mortality rate on Réunion Island can be applied to the epidemic in India to estimate the probable number of deaths that may have occurred. With limited case data reported from India and a mortality rate on Réunion Island of 1 per 1,000 cases, it was previously estimated that India would have ≈1,200–19,000 deaths caused by the chikungunya epidemic (*12*). The excess number of deaths observed during the epidemic in Ahmedabad suggests that estimates of deaths caused by chikungunya in India need to be revised.

Despite the increase in deaths in Ahmedabad and reports of suspected deaths caused by chikungunya in Kerala State, India (*13*), no systematic and comprehensive investigation of deaths in relation to this epidemic has been conducted by government authorities at the national or state level in India. The government of India has declared repeatedly in the parliament that "there are no deaths directly attributable to Chikungunya" in India (*6*). Although 10 deaths caused by chikungunya were reported by the medical officer of health in Ahmebadad, the website of the government of India continues to report "zero deaths" (*5*). Further investigations on the cause of excess deaths are urgently needed to conclusively establish that chikungunya was the cause of excess deaths in Ahmedabad. Given the possible association of deaths with the chikungunya fever epidemic in Ahmedabad, public health authorities should investigate such epidemics in other countries. These investigations will help determine whether the virus has increased in virulence, which may also pose a greater threat outside the Indian Ocean region. Such studies would help detect and control similar epidemics and help governments to provide adequate warnings to travelers to chikungunya-endemic countries.

We report an increase in mortality rates in Ahmedabad during August–November 2006 (when a chikungunya epidemic occurred in this city) compared with previous months in 2006 and the same months in the past 4 years. The highest number of chikungunya cases was also reported in

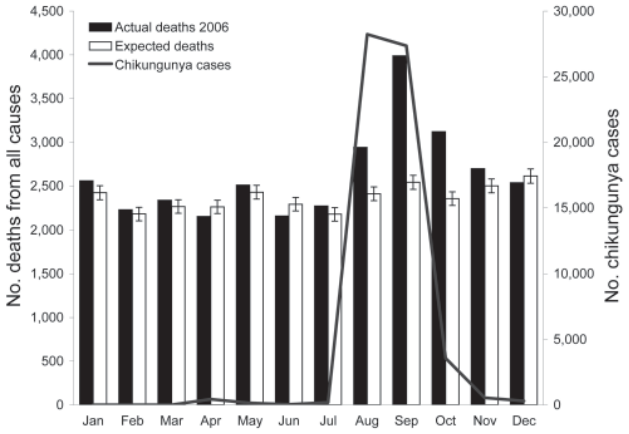

Figure. Monthly chikungunya cases, expected deaths, and reported deaths, Ahmedabad, India, 2006. Error bars show 99% confidence intervals. Jul–Dec, differences were statistically significant.

August and September. The city had ≈2,944 additional deaths during August–November 2006. Epidemiologic evidence shows that the increase in deaths in Ahmedabad was largely attributable to the chikungunya epidemic. Given poor reporting of deaths, an unexplained cause of death cannot be ruled out. Mortality rate data for Ahmedabad are consistent with observations of other researchers that the virus may have mutated and become more dangerous than reported (*8*). Public health authorities must investigate recent epidemics. Otherwise, developing countries may not be able to detect and combat severe future epidemics of other reemerging diseases such as avian influenza and severe acute respiratory syndrome. If our findings are validated by studies in other regions of India and elsewhere, it would assist the international health community to be better prepared in dealing with future epidemics of emerging infectious diseases and reduce associated deaths.

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Chikungunya Fever in Travelers Returning to Europe from the Indian Ocean Region, 2006

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Chikungunya fever has spread through several Indian Ocean islands and India, including popular travel destinations. To compare usefulness of diagnostic tests and to understand reasons for the magnitude and severity of an outbreak, we used 3 diagnostic methods to test 720 samples from 680 patients returning to Europe from the Indian Ocean region in 2006. Chikungunya infection was confirmed for 24.4% patients in the first half of the year and for 9.9% in the second half. Reverse transcription–PCR was positive for all samples taken up to day 4 after symptom onset. Immunofluorescence detected immunoglobulin (Ig) M on day 1 and IgG on day 2 for some patients, and in all patients from day 5 onward. Soon after onset of symptoms, patients had IgG and IgM and high viral loads (some >10^9 copies/mL plasma). These data will help healthcare providers select diagnostic tests for returning travelers.

In 2005 and 2006 an outbreak of chikungunya fever of unprecedented magnitude spread over the western Indian Ocean region, including the Comoros Islands, Mauritius, Réunion Island, Madagascar, and the Seychelles (*1*,*2*). By October 2006 on Réunion Island alone, which has a population of 760,000, at least 266,000 cases had been reported (*3*). The epidemic swept eastward into the Indian subcontinent, where by the end of the year it had caused >1.3 million cases; attack rates were 45% in some regions (*4*,*5*). By the beginning of 2007, the epidemic was on the decline on La Réunion and the Seychelles (*6*), but it seems to be continuing in areas of India. New outbreaks have been reported from early 2007 in Malaysia and mid 2007 in Indonesia (*7*,*8*).

Chikungunya virus (CHIKV) is an arthropod-borne RNA virus of the genus *Alphavirus,* family *Togaviridae*. Its genome is single stranded, of positive polarity, and 11.7 kb long. Based on partial sequences of the envelope protein E1, CHIKV strains can be grouped into 3 distinct genetic lineages, which share a common ancestor in tropical Africa (*9*). The virus is transmitted to humans by numerous *Aedes* mosquito species, including *Ae. aegypti* and *Ae. albopictus* (*2*,*10*–*12*). The latter is thought to be less competent as a vector (*2*).

Strict mosquito-control measures in 2006 ameliorated the outbreaks on the Indian Ocean islands, but the spread of the same strain of CHIKV to India proves that the virus is not easy to contain (*2*,*5*). Tourists visiting these regions have imported the virus back to Europe and the United States, including regions of these countries in which the vector is known to be present (*13*,*14*).

CHIKV infection in humans is characterized by a sudden onset of fever, rash, and severe arthralgia (*15*–*17*). No specific treatment exists and symptoms are generally self-limiting (*16*,*18*). Because in popular tourist destinations in the Indian Ocean region, the disease is endemic, along with malaria and dengue, CHIKV testing is now being conducted in outpatient settings. Antibody assays, virus isolation, and reverse transcription–PCR (RT-PCR) are available (*19*–*21*).

For clinical management of chikungunya, knowing which laboratory assays provide what information at given points of time during disease is helpful; cross-sectional detection rates and kinetics of virologic parameters over time (virus RNA, immunoglobulin [Ig] M, and IgG) are crucial. Unfortunately, these data for CHIKV are minimal because studies on large patient cohorts were completed before relevant laboratory tests, particularly RT-PCR, became avail-

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able. More recent studies have used such methods, but the numbers of studied patients have been limited (*14*,*22*,*23*). To provide support for the selection of diagnostic tests, we collected a cumulative figure of virologic parameters (viral RNA detection and antibody testing results over time) for the largest cohort of returning travelers studied to date. In addition, we sought possible reasons for the magnitude and severity of current outbreaks. Of relevance is a recent finding that Indian Ocean CHIKV strains display genetic characteristics in their structural E1 gene (*22*), especially at amino acid position 226. Similar to the related Semliki Forest virus, in which a homologous mutation causes enhanced membrane fusion capacity in insect cells, the virus could have an advantage in insects or even in humans (*22*,*24*). We determined whether the mutation had an influence on viral loads in the patients in our cohort and how it was distributed geographically and temporally during 2006.

Materials and Methods

Patients and Clinical Samples

From January 1 through December 31, 2006, we tested 720 samples from 680 patients at the Bernhard-Nocht Institute for Tropical Medicine in Hamburg, Germany, for CHIKV infection. All had symptoms compatible with acute or recent CHIKV infection (sudden onset of fever, muscle and joint pain, headaches, rash) upon return to Europe (Germany, n = 515; Belgium, n = 99; Switzerland, n = 42; Denmark, n = 22; Poland, n = 2). For 189 patients, exact travel destinations were reported: Madagascar (n = 9), Mauritius (n = 92), the Seychelles (n = 23), Réunion Island (n = 18), Bali (n = 2), Indonesia (n = 6), Sri Lanka (n = 5), India (n = 28), Malaysia (n = 2), Kenya (n = 1), and Thailand (n = 3). Average ages of travelers to each country did not differ significantly (analysis of variance F-test, $p>0.05$). For 121 patients, exact dates of onset and sampling could be retrieved through voluntary questionnaires completed by telephone or fax after issuance of results. Age and sex distribution and travel histories for these patients are shown in Table 1. The day of onset of symptoms was defined as day 0. All samples with possible CHIKV in 2006 were tested for IgG and IgM by indirect immunofluorescence. During the first half of 2006, all samples were tested by real-time RT-PCR in addition; however, in the second half of the year RT-PCR testing was restricted to samples from patients with acute infection only (on the basis of experiences from the first 6 months as described below). Classification of patients as having laboratory-confirmed cases required either a positive RT-PCR or IgM result or an isolated IgG detection with at least a subsequent 4-fold increase in titer. The Statgraphics Plus 5.1 software package (Manugistics, Dresden, Germany) was used for all statistical analyses.

Table 1. Characteristics of chikungunya fever patients for whom travel and disease histories were known

Location visited	Age, y		Sex	
	Mean	Range	M	F
Mauritius (n = 69)	48.2	16–72	28	41
Seychelles (n = 17)	47.9	24–74	7	10
India (n = 15)	45.6	22–76	5	10
Réunion Island (n = 10)	47.3	17–78	3	7
Madagascar (n = 5)	45.2	29–53	4	1
Sri Lanka (n = 3)	38.7	34–48	3	0
Kenya (n = 1)	47	NA*	1	0
Indonesia (n = 1)	56	NA	0	1
Total (n = 121)	47.4	16–78	51	70

*NA, not applicable.

Indirect Immunofluorescence

CHIKV strain S27 was grown on Vero cells at a multiplicity of infection of 0.5. Cells were spread on slides after 24 h, air dried, and fixed in ice-cold acetone. Serum samples were incubated for 1 h (IgG) or overnight (IgM) on fixed cells. Antibodies were detected by anti-human IgG or IgM labeled with fluorescein isothiocyanate (Sifin, Berlin, Germany). For IgM testing, IgG was absorbed before testing by Biosorb resin (Biomed, Munich, Germany). Specificity of antibodies was confirmed by plaque-reduction neutralization assay on a selection of samples (data not shown).

Nucleic Acid Testing

Two versions of real-time RT-PCR targeting the *nsp1* gene of CHIKV were used: 1 for CHIKV in general and 1 adapted specifically to the current epidemic strain. Reaction conditions and oligonucleotides are listed in Table 2. Quantified in vitro–transcribed RNA was generated by cloning the RT-PCR target region in plasmid pCR2.1 (Invitrogen, Karlsruhe, Germany). Inserts together with a 5′-T7 promotor sequence were amplified with M13 plasmid-specific primers and transcribed into RNA by use of Megascript T7 reagents (Ambion, Austin, TX, USA). After DNase digestion and affinity purification, transcripts were quantified by photometer and used as quantification standards.

Competitive internal controls were generated by overlap-extension PCR as described (*25*,*26*), by using ChikS/ChikAs primers and mutagenic primers 5′-ATCGTTCGTTGAGCGATTAGCAGCAGGAAGTACCACTGCGTTTGCC-3′ and 5′-CTGCTAATCGCTCAACGAACGATGCACTACCAATATCCAGGATGGTTG-3′. Mutated constructs were cloned into pCR2.1 and transcribed into RNA. Both assays did not cross-react with RNA extracted from serum samples with high titers or undiluted cell culture supernatants of the following viruses: Barmah Forest, dengue, Epstein-Barr, hepatitis C, herpes simplex type 1, human immunodeficiency type 1, Japanese encephalitis, poliomyelitis type 1, Ross River, Sindbis, Venezuelan equine encephaltitis, West Nile, and yellow fever. According to series of parallel limiting-dilution experiments and

Table 2. Real-time reverse transcription–PCR (RT-PCR) assay results for chikungunya virus (CHIKV)

Oligonucleotide name	Purpose*	Sequence and label (5′ → 3′)	Position (GenBank accession no.)
ChikSI	Forward primer, general CHIKV assay	TGATCCCGACTCAACCATCCT	241–261 (AF369024)
ChikSII	Forward primer, adapted assay for Indian Ocean strain	CCGACTCAACCATCCTGGAT	246–265 (DQ443544)
ChikAsI	Reverse primer, general CHIKV assay	GGCAAACGCAGTGGTACTTCCT	323–302 (AF369024)
ChikAsII	Reverse primer, adapted assay for Indian Ocean strain	GGCAGACGCAGTGGTACTTCCT	323–302 (DQ443544)
ChikP	Detection probe, CHIKV	FAM-TCCGACATCATCCTCCTTGCTGGC-BHQ1	300–277 (AF369024)
ICP	Detection probe, internal control	DyXL-ATCGTTCGTTGAGCGATTAGCAG-BHQ2	Not applicable

*All oligonucleotides were used in the following assay: 25-µL reaction volume, 3 µL of RNA extract (Viral RNA Mini Kit, QIAGEN, Hilden, Germany), QIAGEN OneStep RT-PCR Kit, 600 nmol/L each primer, 200 nmol/L each probe. Cycling at 50°C for 30 min, 95°C for 15 min, 45 cycles each at 95°C for 15 s and 58°C for 30 s, LightCycler (Roche, Mannheim, Germany).

probit analysis (*25*,*26*), the general CHIKV assay detected 3,844 RNA copies/mL of serum at >95% certainty (95% confidence interval [CI] 2,834–6,832 copies/mL); the assay adapted to the current Indian Ocean strain detected 2,285 copies/mL at 95% certainty (95% CI 1,694–5,326 copies/mL) (online Appendix Figure 1, available from www.cdc.gov/EID/content/14/3/416-appG1.htm). On the whole panel of clinical samples described in this study, quantitative correlation between both assays was close (regression analysis slope = 1, SD 0.04, $p<0.001$; online Appendix Figure 2, available from www.cdc.gov/EID/content/14/3/416-appG2.htm).

Sequence Analysis

A fragment of the CHIKV E1 gene (positions 10265–11158, GenBank accession no. DQ443544) was amplified directly from patient plasma and sequenced. Sequencing was performed with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany) as recommended. Sequences were analyzed with Lasergene software package (DNASTAR, Madison, WI, USA).

Dengue Virus Testing

Serum samples were tested for dengue antibodies by an IgM µ-capture assay (Pan-Bio, Sinnamon Park, Queensland, Australia) and by indirect IgG immunofluorescence assay as described (*27*). All samples that were reportedly taken during the first 12 days of illness were also tested for dengue virus by real-time RT-PCR (*28*).

Results

Cross-sectional Laboratory Results

CHIKV infection was laboratory-confirmed for 152 (22%) symptomatic patients and 188 (26%) samples. Median IgG titer for all 156 IgG-positive samples was 2,560 (range 40–40,960). Median IgM titer for 136 IgM-positive samples was 320 (range 20–10,240). Median viral load for 50 RNA positive samples was 1.7×10^5 RNA copies/mL (range 1×10^3–1.2×10^{10} RNA copies/mL). Table 3 summarizes cross-sectional testing results for individual patients at time of first visit.

In the first half of the year, the rate of confirmed infection for patients with suspected CHIKV was 24.4%; in the second half, 9.9%. Although the numbers of laboratory tests were comparable in the first and second half of the year (396 vs. 326), laboratory confirmation rates in travelers were significantly higher in the first half (χ^2 test, $p<0.001$).

Cumulative Antibody Kinetics

We were able to retrieve 153 samples, exact information on travel destination, onset of symptoms, and date of sampling for 121 patients (Figure 1). CHIKV IgG was found for 79% of patients (125 [81.7%] of 153 samples). The earliest sample with a detectable IgG titer had been drawn on day 2 after onset; the latest, on day 252. An isolated IgG titer, i.e., no concomitant IgM, was detected in 15 samples, 14 of which had been collected late after onset (median 86.5 days, range 39–252 days). The remaining sample, collected on day 2, was positive by RT-PCR.

Demonstrable CHIKV IgM was found in 111 (72.5%) of 153 samples from 88 (72.7%) patients. The earliest IgM-positive result was obtained from a sample drawn on day 1; the latest was obtained on day 170, from 1 sample. The only IgM sample with no concomitant IgG titer was drawn on day 1 after onset of fever; it was concomitantly positive by RT-PCR.

For 11 patients whose first sample yielded antibodies, consecutive samples were available. All paired samples were drawn at least 14 days apart. The first samples were collected after a median of 13 days from onset (range 5–68 days), and all had IgM (median titer 320) and IgG (median titer 2,560). The second samples were collected after a median of 77 days from onset (range 20–207 days). Median IgG titer was 5,120; median IgM titer was 40. For 4 patients, IgM was not detectable in the second serum sample, the earliest of which was drawn 50 days after onset of symptoms.

Table 3. Detection of chikungunya virus infections in patients at initial examination, by laboratory test, 2006*

Period	IgG and IgM		IgG only		IgM only		RT-PCR†		RT-PCR only	
	No. (%)	Total	No. (%)	Total	No. (%)	Total	No. (%)	Total	No. (%)	Total
Jan–Jun	85 (23.1)	368	5 (1.4)	368	2 (0.5)	368	42 (11.8)	355	9 (2.5)	355
Jul–Dec	13 (4.2)	312	9 (2.9)	312	0	312	6 (30.0)	20	0	20
Total	98 (14.4)	680	14 (2.1)	680	2 (0.3)	680	48 (12.8)	375	9 (2.4)	375

*Ig, immunoglobulin; RT-PCR, reverse transcription–PCR.
†Some patients were tested by using both RT-PCR and immunofluorescence; only RT-PCR results are given.

Cumulative Diagnostic Parameters in Early Acute Illness

Of the 121 patients with complete histories, 45 (37.2%) had positive real-time RT-PCR results. To compare the efficiency of diagnostic methods in acute disease, we selected all samples collected during the first 10 days of symptoms from patients in whom CHIKV was confirmed by virus isolation or subsequent seroconversion (n = 63). RT-PCR was 100% positive up to day 4, regardless of antibody results (Figure 2). IgG and IgM were 100% positive from day 5 onward. IgM was detected only 1 day earlier than IgG. Overlapping RT-PCR and antibody results occurred from day1 to day 7. No positive RT-PCR result was found after day 7.

To assess whether CHIKV RNA detection may be influenced by antiviral antibodies, we compared RNA concentrations in seronegative (n = 21) and seropositive (n = 6) samples, all collected within the first 4 days of illness (Figure 2). Median RNA concentration was 9.85×10^7 copies/mL in seronegative samples, which was significantly higher than the 2.35×10^5 copies/mL found as median concentrations in seropositive samples ($p<0.017$, analysis of variance F-test). To characterize the course of viremia in absence of antibodies, we plotted viral loads of all antibody-negative, PCR-positive samples against sampling days. The highest concentrations were found in samples drawn on day 0 with a mean of 1.2×10^9 RNA copies/mL (95% CI 8.2×10^8–1.6×10^9 copies/mL) (Figure 2). Viral loads in later samples continuously decreased.

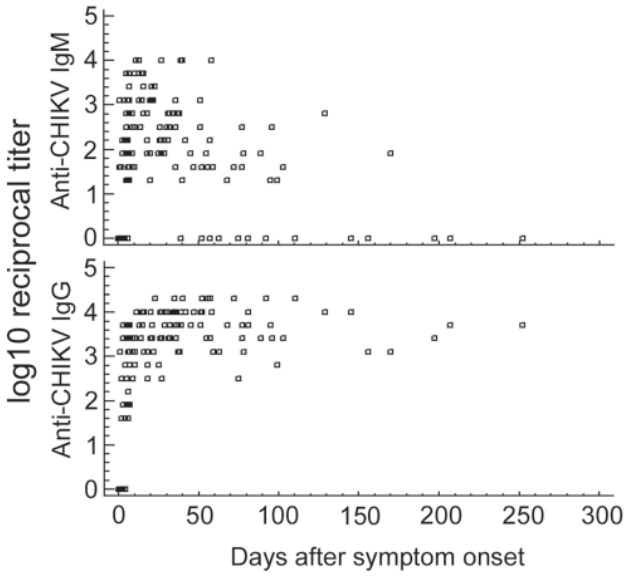

Figure 1. Immunoglobulin (Ig) M and IgG titers in 153 samples from 121 patients. Some patients are represented more than once if multiple specimens were submitted for testing. CHIKV, chikungunya virus.

Efficiency of Virus Isolation

To reassess the diagnostic value of classic virus isolation techniques, we cultured 47 PCR-positive serum samples on Vero cells. All material had been frozen and thawed only 1 time before cell culture. Only 11 (23.4%) of 47 samples yielded a virus isolate. Virus was isolated only in samples containing $>1 \times 10^7$ RNA copies/mL (Figure 2). All culture-positive samples had been taken on or before the second day of fever, after a mean of 0.75 days from onset. Antibodies were not detectable in any of these samples, which suggests that infectivity in culture may be neutralized by antibodies.

Dengue Virus Co-infections

Dengue virus is endemic to parts of the Indian Ocean region and resembles CHIKV infection. All serum samples received in the first half of 2006, including those from patients for whom travel and disease histories were incomplete, were tested for dengue virus. Only 3 of 368 patients had dengue IgG. Dengue IgM antibodies without IgG were found for 2 additional patients, which suggests acute infection. Both patients had traveled to Mauritius. One patient had dengue IgM and CHIKV RNA. This patient had a long period of fever (38°C–40°C for 11 days) and severe arthralgia. Another patient had dengue IgM and anti-CHIKV virus IgM and IgG. This patient had severe arthralgia for 1 month.

E1 Protein Genotypes

We sequenced 40 PCR-positive samples, of which 18 (45%) showed the original E1-226A genotype and 22 (55%) had the E1-226V mutation (2 from Réunion Island, 20 from Mauritius, all sampled in the first half of 2006). Samples from India (n = 2) and from Sri Lanka (n = 2), which were collected in the second half of 2006, displayed the original E1-226A genotype. CHIKV viral loads among genotypes were compared (Figure 3). Median concentrations (1.6×10^7 copies/mL for E1-226A; 1.6×10^5 copies/mL for E1-226V) were not significantly different at the

95% confidence level. To exclude an influence of CHIKV antibodies on this result, we analyzed samples without antibodies separately. Again, the difference was not significant (226A samples (n = 12), 5×10^7 copies/mL; 226V samples (n = 12), 2.5×10^6 RNA copies/mL).

Discussion

Chikungunya fever has become a major differential diagnosis, along with malaria and dengue fever, for ill travelers returning to Europe from the Indian Ocean region. Because chikungunya fever has similar symptoms but requires different management than malaria and dengue, laboratory testing for CHIKV is now part of routine clinical decision making. Our analysis of baseline virologic findings by using a modern repertoire of diagnostic tests comprised the largest cohort of travelers returning from sites of the current epidemic. Tourists are of particular interest because they are not seen in the context of community outbreaks, and thus, their diagnoses need individual laboratory confirmation. The large number of cases in our study enabled us to establish a gapless time course of virologic parameters by cumulating single- and dual-point laboratory determinations from individual infected travelers.

Cross-sectional laboratory data provided a good indication of the extent of this large outbreak of a usually underdiagnosed arbovirus infection in travelers. In the first half of 2006, as much as 24.4% clinically suspected cases of CHIKV infection could be confirmed. This predictive value of clinical diagnosis is impressive compared with that for suspected dengue virus infection in travelers (*29*,*30*). The decrease to 9.9% in the second half of 2006 is probably a reflection of the decreasing CHIKV activity in tourist destinations, along with a shift to the Indian subcontinent where tourism is less concentrated.

In the cumulative time course, anti-CHIKV IgG and IgM antibodies were detected soon after onset of symptoms. They were present in all patients in whom acute infection was confirmed after 5 days. IgG was present so early that additional testing for IgM provided only limited additional sensitivity for detecting acute cases. Most likely this finding was caused not by low sensitivity of our IgM assay but by an unusually early IgG response. One reason for early IgG findings could be lack of specificity of the applied immunofluorescence assay. However, we have shown recently that indirect immunofluorescence is a sensitive and specific method for determining antibodies against CHIKV (N. Litzba et al., unpub. data). Moreover, we have confirmed specificity of our immunofluorescence assay results by retesting a subset of serum samples by plaque-reduction neutralization test (data not shown); all antibody-positive samples subjected to this test had positive results. Finally, because most patients resided in Germany, where alphaviruses are not endemic, preexisting

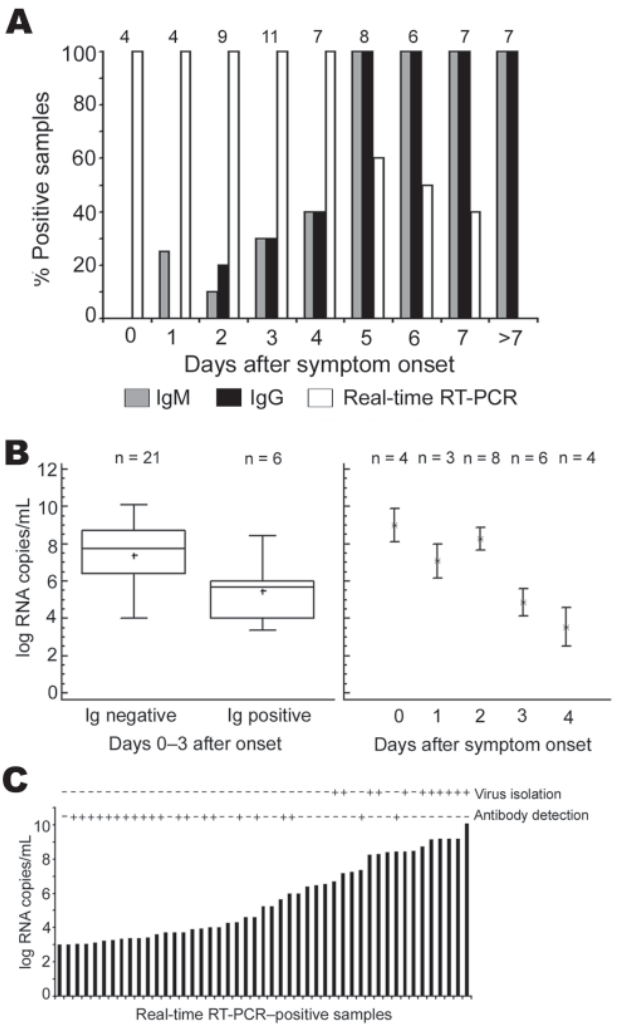

Figure 2. A) Rates of positive results from assays for immunoglobulin (Ig) M, IgG, and virus RNA, first 10 days of symptoms. Numbers above bars are numbers of samples (and patients). B) Left panel, viral loads in serum or plasma in antibody-negative, PCR-positive patients (n = 21, left column) and in antibody-positive, PCR-positive patients (n = 6, right column). All patients were sampled during first 3 days of symptoms. Right panel, viral loads in all antibody-negative, PCR-positive samples. Error bars represent interquartile ranges. C) Viral loads, antibodies, and virus isolation from 47 samples positive by reverse transcription–PCR (RT-PCR). Virus isolation + indicates isolation success as confirmed by cytopathogenic effect and direct immunofluorescence assay. Antibody detection + indicates an IgG or IgM titer >10 by immunofluorescence assay.

antibodies are unlikely. We must therefore assume that the IgG results are technically valid. An explanation for early IgG production would be the presence of unusually high presymptomatic viremia that provided an early antigen stimulus for antibody formation.

Cell culture in our study was not particularly diagnostic. It was rather insensitive (23.4%) compared with RT-PCR, and the presence of antibody seemed to prevent isola-

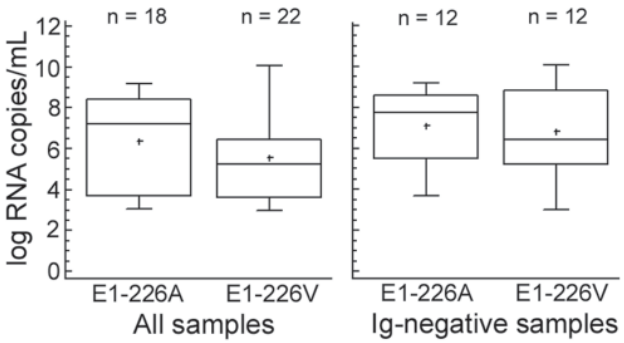

Figure 3. Viral loads for all PCR-positive samples (left panel) and immunoglobulin (Ig)–negative/PCR-positive samples (right panel), depending on types of mutation (alanine or valine at amino acid position 226 of the envelope 1 protein, as shown on the x-axis). Boxes represent the innermost 2 quartiles of data; horizontal line shows the mean; whiskers represent the outermost 2 quartiles.

tion of virus. In successful cultures, cytopathic effects were not observed before 3–4 days of incubation, often requiring at least 1 passage, which made time for culture longer than time to seroconversion. Nevertheless, culture is essential for more specific investigation such as virus neutralization tests, as well as for ecologic and basic research. For clinical virus detection, RT-PCR seemed to be the method of choice. Our study provides 2 alternative real-time RT-PCR protocols that have analytical performance comparable to that of major diagnostic PCR systems (*25*,*26*); each protocol has been clinically evaluated on our whole cohort.

Apart from practical diagnostic implications, our findings identify important questions for future research. As an example, courses of antibodies and viremia were different from those of dengue virus infections (*27*,*31*). Probably the most important difference was an early IgG response to CHIKV, compared with a clear delay of IgG (appearing after IgM) in most acute cases of primary dengue virus infection. For a substantial fraction of patients, dengue IgG is not detected until after 2 weeks of illness (*27*,*31*). One could speculate that the reason for this difference is the different tropism of each virus. Dengue virus infects predominantly monocytes (*31*) and thereby may induce a relevant, short-term immunosuppression (*32*). CHIKV probably does not share this tropism (*33*), although the primary target site of CHIKV infection is unclear. Whether co-infection with dengue and CHIKV would lead to altered symptoms is another question; 2 patients in our study had this co-infection. Their disease courses may be more severe or prolonged than those of most patients (*17*), but detailed and controlled studies on more patients are needed.

Data on viral load determined in this study are crucial for infection control. During their first few days of illness, our patients had tremendous virus concentrations, sometimes >10^9 copies/mL of plasma. Such high viral loads are uncommon with other arboviral diseases (*34*–*36*) and would make CHIKV prone to nosocomial transmission, e.g., by needle-stick injury or mucous membrane exposure. Indeed such an event has already been documented (*23*). Blood and other body fluids of CHIKV patients should be considered infectious during the first week of symptoms. We cannot determine from this study whether the potential infectivity of CHIKV may also become important in the context of transfusion, because we analyzed only symptomatic patients. However, virus concentrations in our patients were already decreasing when symptoms were detected, and therefore a high viremia in presymptomatic patients seems likely. None of our patients had antibodies on day 0, and presymptomatic patients would therefore be highly infectious. The issue of transfusion transmissibility of CHIKV deserves intensive investigation. Targeted interventions like on Réunion Island, where blood donation was suspended during the epidemic and blood was imported from France (*37*), are not an option in India or Southeast Asia.

Why such a large epidemic of CHIKV infections could occur and why courses of disease were so unusually severe are unclear. A previous study recognized an A226V exchange in the E1 envelope glycoprotein for the first time in CHIKV and found it to increase over time in isolates from Réunion Island (*22*). In the related Semliki Forest virus, a corresponding mutation conferred a replication advantage in cholesterol-depleted insect cells (*24*), which for the virus would implicate an advantage in its reservoir. In addition to the above findings of Schuffenecker et al, we could confirm presence of the mutation on Mauritius, where a larger part of our patients had visited. However, viruses isolated in the late phase of our study (September to December 2006) from patients returning from India and Sri Lanka, respectively, did not show the mutation but were otherwise clearly related to the Indian Ocean strain. The significance of the mutation thus remains uncertain, and functional studies are needed. Confirmation of this or other genetic connections would provide another example of minor genetic differences that account for major changes of an arthropod virus. After the past and ongoing experiences with agents such as the coronavirus of severe acute respiratory syndrome and avian influenza viruses, the CHIKV epidemic provides more proof of the importance of research into the ecology of emerging and reemerging diseases.

Acknowledgments

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High Rate of Mobilization for *bla*$_{CTX-M}$s

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We constructed a phylogenetic analysis of class A β-lactamases and found that the *bla*$_{CTX-M}$s have been mobilized to plasmids ≈10 times more frequently than other class A β-lactamases. We also found that the *bla*$_{CTX-M}$s are descended from a common ancestor that was incorporated in ancient times into the chromosome of the ancestor of *Kluyvera* species through horizontal transfer. Considerable sequence divergence has occurred among the descendents of that ancestral gene sequence since that gene was inserted. That divergence has mainly occurred in the presence of purifying selection, which indicates a slow rate of evolution for *bla*$_{CTX-M}$s in the pre–antimicrobial drug era.

Antimicrobial drug–sensitive bacteria become resistant to antimicrobial drugs through a variety of mechanisms, such as chromosomal mutations that up-regulate the expression of antibiotic-resistance genes, DNA uptake through transformation, or the process of conjugation. The ability of plasmids to evolve independently of their hosts has allowed numerous resistance genes from diverse species of bacteria to assemble within single plasmids and spread into a wide variety of organisms (*1*). The mobilization of a chromosomal resistance gene to a plasmid is an important event because the mobilized gene is now capable of spreading widely throughout diverse species of bacteria and because the fitness advantage that a plasmid confers generally increases as it acquires more resistance genes (*1*).

The class A β-lactamases have been the most frequently encountered plasmidic resistance genes. Class A β-lactamases from the TEM group have occurred at a particularly high frequency; in many surveillance studies, they have been identified as the resistance determinants most frequently encountered (*2*–*9*). The first *bla*$_{TEM}$ allele, *bla*$_{TEM-1}$, is a plasmidic allele that was first isolated in 1963 (*10*,*11*). Currently, ≈160 different plasmidic alleles encode unique TEM β-lactamase enzymes (www.lahey.org/Studies), and all are descended from a single plasmidic ancestor, *bla*$_{TEM-1}$ (*12*).

The SHVs are another group of class A β-lactamases that have been frequently observed in surveillance studies. As with the TEMs, numerous alleles encode unique SHV enzymes (≈105), and the SHVs are all descended from a single ancestor (*13*). The first *bla*$_{SHV}$ allele was detected in 1974 (*10*,*14*). Unlike *bla*$_{TEM}$s, the *bla*$_{SHV}$s are present in the chromosome of nearly all *Klebsiella pneumoniae* isolates belonging to the KP1 group. Evidence suggests that *bla*$_{SHV}$s have been chromosomally located since the pre–antimicrobial drug era (*15*), and they may have been mobilized to plasmids up to 4 times, although the sequence divergence among them is insufficient to clearly resolve the independent mobilizations of the *bla*$_{SHV}$s.

The CTX-Ms are another group of class A β-lactamases that are located on plasmids and that have been of particular clinical interest because they are rapidly spreading through clinical populations of bacteria. The first plasmidic *bla*$_{CTX-M}$ observed in human-associated clinical populations was isolated in 1989 (*16*,*17*). Unlike the usual pattern of class A β-lactamase mobilizations in which the plasmidic alleles are all descended from a single common plasmidic ancestor, evidence shows that CTX-Ms have been mobilized numerous times from the chromosomes of *Kluyvera* (*16*,*18*–*21*). Because *Kluyvera* chromosomal genes have been found that exactly match the sequence of plasmidic CTX-Ms (*18*), many of the mobilizations have likely occurred recently. To investigate the mobilization of the *bla*$_{CTX-M}$s to plasmids, we generated a phylogenetic

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analysis of the CTX-Ms that included a representative sampling of other class A β-lactamases.

Methods

BLAST Search

bla$_{CTX-M}$ and *bla*$_{CTX-M}$ homologue DNA sequences were identified with a TBLASTN (www.ncbi.nlm.nih.gov/blast) (*22*,*23*) search of the nonredundant National Center for Biotechnology Information (NCBI) sequence database and the completed microbial genomes database by using characterized *bla*$_{CTX-MS}$ as query sequences (*bla*$_{CTX-M1}$ and *bla*$_{CTX-M2}$). The BLAST search of the completed microbial genomes identified positive matches for organisms that contain *bla*$_{CTX-M}$ homologs. The BLAST search of completed genomes also showed which microbes have no close *bla*$_{CTX-M}$ homologue, and thus enabled horizontal transfer events to be identified.

Alignment

The protein sequences of the *bla*$_{CTX-M}$s and their homologs were aligned with ClustalX 1.8 (*24*) by using the Gonet 250 similarity matrix with a gap-opening penalty of 35 and a gap-extension penalty of 0.75 for the pairwise alignment stage, and a gap-opening penalty of 15 and a gap extension penalty of 0.3 for the multiple alignment stage. The corresponding DNA coding sequences were aligned by introducing triplet gaps between codons corresponding to gaps in the aligned protein sequences with the CodonAlign program. (CodonAlign for Macintosh and for PC [Windows] computers and source code that can be compiled for other platforms are available at no charge from http://sinauer.com/hall.)

Estimation of Positive Selection

Estimation of the nonsynonymous (d_N) and synonymous (d_S) substitution rates is an important means of understanding mechanisms of molecular evolution. A d_N/d_S ratio >1 is taken as evidence of positive selection, whereas a d_N/d_S ratio <1 is taken as evidence of purifying selection (*25*). The Codeml program of the PAML package (available from http://envgen.nox.ac.uk/bioinformatics/docs/codeml.html) (*25*) was used to estimate d_N/d_S ratios in the phylogenetic analysis shown in Figure 1. The values were calculated by using model 1 in the program, and default parameters were used for the execution of the program.

Phylogenetic Reconstruction

Phylogenies were constructed by the Bayesian method, as implemented by the program MrBayes (*26*) (available at no charge from www.mrbayes.net). The evolutionary model used was the General Time Reversible model (*27*). Because evolutionary rates are not homogeneous for every site in a gene, among-site variation in evolutionary rate was estimated separately for first, second, and third positions of sites within codons. Four chains, with a "temperature" of 0.2 for the heated chains, were run for each tree. Trees were sampled every 100 generations. A total of 10 million generations were run with a burn-in of 5,000 trees. The length of each burn-in was set at a value that exceeded twice the number of trees required for convergence upon a stable likelihood value. Because the consensus trees calculated by MrBayes do not include the posterior probabilities of the clades, each entire set of trees was imported into PAUP* (*28*) and the same trees used by MrBayes to calculate a consensus were used to calculate a 50% majority rule consensus in PAUP* (*28*). The resulting tree shows the posterior probabilities of the clades, i.e., the percentage of time that those taxa are included in the clade. The consensus trees calculated by MrBayes were imported into PAUP* for the purposes of displaying and printing the tree.

Results

Ancient Horizontal Transfer of *bla*$_{CTX-M}$ Ancestor

The NCBI genomes database (www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=genome) currently contains the completed genomic sequences of 139 eubacterial organisms. Because *Kluyvera* are members of the *Enterobacteriaceae* group of the gamma subdivision of Proteobacteria, the genomes of other members of the gamma subdivision, and especially the chromosomes of *Enterobacteriaceae*, were of the greatest interest. BLAST searches of these mi-

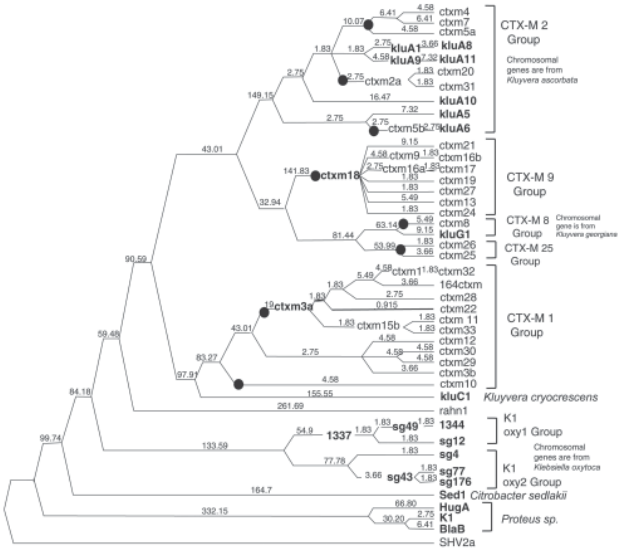

Figure 1. Phylogenetic analysis of *bla*$_{CTX-M}$s. This tree was calculated by Bayesian inference. Number of mutations occurring along each branch are given along the length of the branch. Black dots represent mobilizations. **Boldface** indicates chromosomal genes. CTX-M-14 and 3a exist as both unmobilized chromosomal genes and plasmid-borne CTX-M alleles.

crobial genomes show that among the complete genomic sequences available for 9 species of *Enterobacteriaceae (Escherichia coli*, *Salmonella typhimrium* LT2, *S. enterica*, *Shigella flexneri*, *Photorhabdus luminescens, Buchnera aphidicola, Candidatus Blochmannia floridanus, Wigglesworthia glossinidia,* and *Yersinia pestis*) none contain chromosomal homologs of the bla_{CTX-M}s that are detectable through sequence comparison. BLAST searches similarly show that many non-*Enterobacteriaceae* members of the gamma subdivision of Proteobacteria, *Shewanella oneidensis, Haemophilus ducreyi, H. influenzae, Pseudomonas aeruginosa, P. putida, P. syringae, Vibrio cholerae, V. parahemolyticus, V. vulnificus, Xanthomonas axonopodis, X. campestris,* and *Xylella fastidiosa,* also do not contain chromosomal homologs of the bla_{CTX-M}s. However, BLAST searches of the translated nonredundant nucleotide database revealed that *Kluyvera* species, *Citrobacter sedlakii*, and *Klebsiella oxytoca* contain close chromosomal homologs of the bla_{CTX-M}s.

These results show that the bla_{CTX-M} homologs originally came into the chromosomes of *K. oxytoca, Kluyverra* species, and *C. sedlakii* by horizontal transfer because most species of gamma Proteobacteria do not contain bla_{CTX-M} homologs. If bla_{CTX-M} homologs were vertically transmitted into the species that contain them, numerous deletions would be required to explain absence of those homologs in the majority of gamma Proteobacteria. However, only 3 horizontal insertions are required to explain the presence of bla_{CTX-M} homologs in the chromosomes of *K. oxytoca*, *Citrobacter* species. Because fewer insertions than deletions are required to explain these data, insertion of bla_{CTX-M} homologs into the chromosomes of those bacteria that contain them is the most likely explanation for their current distribution.

Divergence of the bla_{CTX-M}s

The GenBank DNA and protein accession numbers of the sequences included in this analysis are shown in the online Appendix Table (available from www.cdc.gov/EID/content/14/3/423-appT.htm), along with the organism in which the gene exists and whether the gene was located on a plasmid or a chromosome. The results of our phylogenetic analysis are presented in Figure 1. The groupings of bla_{CTX-M}s on our phylogenetic analysis agree with the dendrogram published by Bonnet in a recent review (*16*); for purposes of clarity, we will use the same group names used in that review, as shown in Figure 1.

The phylogenetic analysis shows that the bla_{CTX-M}s represent a fairly divergent group of β-lactamase genes descended from a common ancestor. The genes encoding the CTX-M-1 and CTX-M-2 groups are separated by over 400 mutations, which indicates considerable diversification of the bla_{CTX-M}s. The average distance separating the bla_{CTX-M}s from their most recent common ancestor is 226.2-nt ± 22.8-nt mutations, which indicates that the rates of evolution among the bla_{CTX-M}s have been similar.

Positive selection testing within the phylogenetic analysis shows that positive selection has occurred throughout the evolutionary history of the class A β-lactamases. More positive selection appears to exist at branches deep within the tree than along more recent branches. The branches during which most of the divergence of the bla_{CTX-M}s occurred are characterized by purifying selection. The detection of purifying selection suggests a slow evolutionary rate and that the bla_{CTX-M}s diverged in ancient times. More recent evolution of the bla_{CTX-M}s likely can be characterized by intense positive selection, but the branches at the tips are still too short to obtain reliable d_N/d_S ratios.

Mobilization of bla_{CTX-M}s to Plasmids

The bla_{CTX-M}s have been mobilized from the chromosomes of various *Kluyvera* species to plasmids at least 8 times since they diverged from their most recent common ancestor as indicated in Figure 1. The alleles in the CTX-M-2 group have been mobilized from the chromosome of *Kluyvera ascorbata* at least twice (*29*). The alleles in the CTX-M-9 group have been mobilized once from the chromosome of *Kluyvera georgiana* (*30*). The alleles from the CTX-M-8 group were mobilized once from the chromosome of *K. georgiana* (*20*). The CTX-M-25 group has been mobilized once, although the species from which it originates has not yet been determined. The alleles in the CTX-M-1 group have been mobilized at least 3 times (*17*,*18*,*31*), and one of those mobilizations has been from the chromosome of *K. ascorbata*. When compared with the bla_{TEM}s, which have been mobilized once, and the bla_{SHV}s, which have been reported to have been mobilized 2–4 times (*32*), the number of mobilization events that have occurred among the bla_{CTX-M}s is atypically high.

To compare the number of mobilizations that have occurred in the CTX-M group with those that have occurred in the rest of the class A β-lactamases, we constructed a phylogenetic analysis of class A alleles that spans the breadth of this group and that contains representatives of all groups of class A alleles known to the authors (Figure 2). Among all of the class A β-lactamases, including the bla_{CTX-M}s, only 22 mobilizations to plasmids were found. To quantitatively compare the numbers of times that CTX-Ms have been mobilized to plasmids with the number of times that other class A β-lactamases have been mobilized to plasmids, the total number of mutations that have occurred within the bla_{CTX-M} clade were summed and divided by the number of mobilizations that have occurred in that region of the phylogenetic analysis. Among the bla_{CTX-M}s, the ratio of mobilizations to mutations is 1 mobilization per 191 mutations. Among the remainder of the tree when the

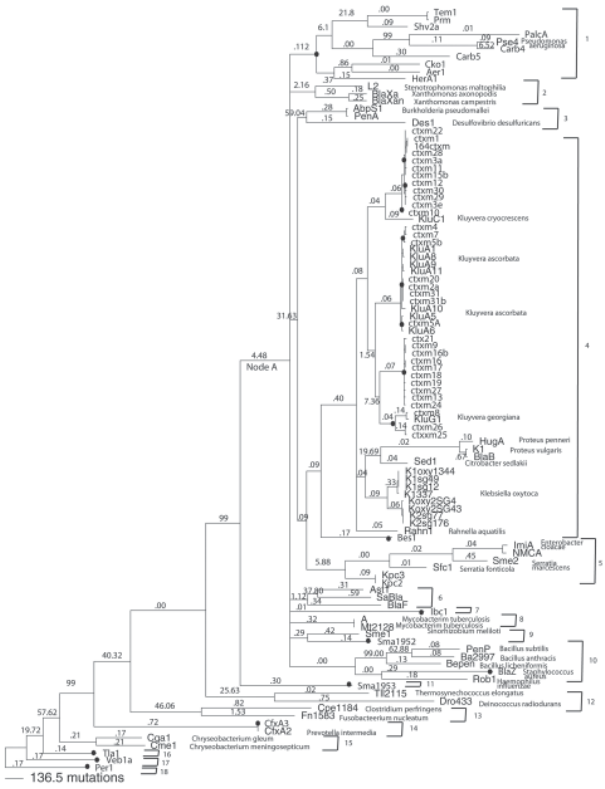

Figure 2. Phylogenetic analysis of class A β-lactamases calculated by Bayesian inference. Number of mutations occurring along each branch are represented visually by the lengths of the branches. d_N/d_S ratios for all branches except the tips are given along the lengths of the branches. **Boldface** indicates plasmidic genes. Black dots indicate mobilizations to plasmids. Numbered brackets indicate monophyletic divisions within the tree. d_N, nonsynonymous substitution rate; d_S, synonoymous substitution rate.

bla$_{CTX-M}$ clade is excluded from the analysis, 14 mobilizations occur with the ratio of mobilizations to mutations being 1 mobilization per 2,471 mutations. When the complete phylogenetic analysis is considered, 1 mobilization occurs per 1,870 mutations. By that comparison, the mobilization of the *bla*$_{CTX-M}$ genes to plasmids has occurred at an unusually high rate. This result is unlikely to be an artifact of sampling bias or clinical interest because other class A β-lactamases have been intently studied for a longer period than the *bla*$_{CTX-M}$s. If any bias exists in the data, it would be the undersampling of *bla*$_{CTX-M}$ mobilizations relative to other class A β-lactamases.

Because nearly one half of the mobilizations that have occurred in the class A phylogenetic analysis have occurred among the *bla*$_{CTX-M}$s, it seemed reasonable to conclude that the circumstances associated with the mobilizations of the *bla*$_{CTX-M}$s may differ from the circumstances associated with the mobilizations of other class A β-lactamases. To rule out any effect that varying intensities of selection or varying evolutionary rates might have on mobilizations to plasmids, we divided the phylogenetic analysis into several monophyletic groups for further analysis. Within the class A β-lactamase phylogenetic analysis (Figure 2) are several monophyletic clades that descended from a single ancestor (node A). Each of the monophyletic clades that descended from node A were considered separately during positive selection testing except for the monophyletic clade that contains the *bla*$_{CTX-M}$s; it was divided into 2 separate clades so that a clade containing only the *bla*$_{CTX-M}$s and their closest relatives could be considered. Monophyletic clades that diverged before the point represented at node A were also examined individually.

The d_N/d_S ratios were computed for each clade (Table), and a correlation coefficient for mobilizations and d_N/d_S ratios of 0.21 ($p = 0.40$) was calculated. The average distance of each clade from the root of the tree was also computed (Table), and the correlation coefficient for mobilizations and average distance from the root is 0.21 ($p = 0.41$). The nonsignificant p values yielded by those results mean that the unusually high number of mobilizations among the *bla*$_{CTX-M}$s are probably not an artifact caused by positive selection or evolutionary rate.

Most of the mobilizations of the *bla*$_{CTX-M}$s have occurred in recent years because genes that are identical (*bla*$_{CTX-M3a}$ [*18*] and *bla*$_{CTX-M-18}$ [*19*]) or nearly identical to the ancestors of plasmidic clades (Figure 1) have been found in the chromosomes of *Kluyvera* species, whereas many of the other plasmidic class A β-lactamases have been mobilized much longer, perhaps even since ancient times. In many cases, no chromosomal ancestor is identified and the plasmidic resistance genes are not closely related to the chromosomal resistance genes of any identified groups of bacteria.

Table. Average distances from root and d_N/d_S ratios of monophyletic clades

Clade	Average distance from root	d_N/d_S ratio*	Mobilizations
1	3140.865	0.0935	5
2	2534.35	0.4535	0
3	2538.9	0.2446	0
4	2819.37634	0.0929	9
5	3139.5	0.0426	0
6	2575.3	0.5024	0
7	2975.7	0.1019	1
8	2443.35	0.2618	0
9	2532.075	0.1969	1
10	3267.81	0.0338	0
11	2429.7	0.0118	1
12	2811.9	0.016	0
13	2218.125	1.1595	0
14	1747.2	0.9331	1
15	873.6	0.2056	0
16	518.7	0.1069	1
17	559.65	0.1595	1
18	778.05	0.2496	0

*d_N, nonsynonymous substitution rate; d_S, synonoymous substitution rate.

Discussion

Although the use of antimicrobial agents generally has enhanced the spread of antimicrobial drug resistance among bacteria by providing the selective pressure needed for the emergence of novel resistance determinants, selective pressure alone does not explain the increasing frequency with which bla_{CTX-M} alleles have been noted in bacterial populations in recent years (*16*,*33*). Although bla_{CTX-M} alleles tend to be located on transmissible plasmids and transposable elements, which clearly facilitate their dissemination, the repeated mobilization of the bla_{CTX-M}s from the chromosomes principally among *Kluyera* species is most intriguing. The mechanistic basis underlying this repeated mobilization to plasmids remains unknown. Whether the chromosomes of *Kluyvera* species have some unique aspect that enhances the mobilization of the bla_{CTX-M} genes remains to be determined. Other factors, such as exposure of the isolates to specific antimicrobial agents or to environmental changes that facilitate the mobilization of bla_{CTX-M}s to plasmids also need to be investigated.

Two insertion elements are known to contribute to the mobilization of bla_{CTX-M}s. The first, which is associated with the CTX-M-2 and CTX-M-9 groups, is IS*CR1* (*34*) and the second, which is associated with the CTX-M-1, CTX-M-8, and CTX-M-25 groups is IS*Ecp1* (*35*). According to our phylogenetic analysis (Figure 1), 4 mobilizations can be attributed to each of these insertion type elements. Thus, both elements seem to promote equal frequencies of mobilization of bla_{CTX-M}s. Notably, IS*CR1* was also reported to be responsible for the mobilization of both bla_{VEB} and bla_{PER} alleles, but neither of these resistance determinants has been reported to have an unusually high rate of mobilizations from chromosomal locations to plasmids.

Another factor that may contribute to the rate of mobilizations of the bla_{CTX-M} resistance determinants is the frequency of plasmids in bacterial populations. As the number of plasmids increases in microbial populations, so does the number of target replicons. A comparison of the percentage of bacterial strains that contained plasmids in the pre–antimicrobial drug era (*36*) with the percentage of contemporary strains that carry plasmids (*37*,*38*) indicates that the frequency of plasmid carriage has increased from 19% to 58%–100%, depending on the species surveyed. Although the collection methods and resistance detection assays varied in the studies used for this comparison (which may have introduced biases toward an increasing frequency of plasmids), few doubt that plasmid carriage is much more common among bacterial strains in the antimicrobial drug era (*39*,*40*). Unfortunately, specific information about plasmid carriage of *Kluyvera* species versus other *Enterobacteriaceae* is not available.

Regardless of the mechanism, the increased number of mobilizations of bla_{CTX-M}s from their chromosomal locations among relatively rare human pathogens to plasmids that circulate widely among several important human and animal pathogens (particularly among *E. coli*) is a serious public health concern. The results of our study indicate the potential for an increase in the rate of mobilization of a variety of other resistance determinants to plasmids. Such an increase could result in more rapid mobilizations of novel resistance determinants and contribute to the accelerated spread of antimicrobial resistance determinants among a large spectrum of bacterial pathogens.

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Dr Barlow is a founding faculty member at the University of California, Merced. Her research focuses on the evolution of plasmidic resistance determinants, with particular emphasis on β-lactamases.

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Integrated Food Chain Surveillance System for *Salmonella* spp. in Mexico[1]

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Few developing countries have foodborne pathogen surveillance systems, and none of these integrates data from humans, food, and animals. We describe the implementation of a 4-state, integrated food chain surveillance system (IFCS) for *Salmonella* spp. in Mexico. Significant findings were 1) high rates of meat contamination (21.3%–36.4%), 2) high rates of ceftriaxone-resistant *S*. Typhimurium in chicken, ill humans, and swine (77.3%, 66.3%, and 40.4% of *S*. Typhimurium isolates, respectively), and 3) the emergence of ciprofloxacin resistance in *S*. Heidelberg (10.4%) and *S*. Typhimurium (1.7%) from swine. A strong association between *Salmonella* spp. contamination in beef and asymptomatic *Salmonella* spp. infection was only observed in the state with the lowest poverty level (Pearson r = 0.91, p<0.001). Pulsed-field gel electrophoresis analysis of 311 *S*. Typhimurium isolates showed 14 clusters with 102 human, retail meat, and food-animal isolates with indistinguishable patterns. An IFCS is technically and economically feasible in developing countries and can effectively identify major public health priorities.

Diarrheal diseases are leading causes of childhood illness and death in developing countries (*1*). Many of these infections are acquired through contaminated food and water, but the fraction attributable to each food category is unknown. In the face of worldwide increase in antimicrobial-resistant foodborne pathogens (FBP) and the growing globalization of travel and food trade, the World Health Organization has recommended the establishment of national networks that conduct surveillance along the entire food chain (*2*). Industrialized nations have implemented FBP surveillance systems that include a combination of passive, active, and outbreak data sources (*3*,*4*). In certain countries, outbreak data constitute a considerable proportion of human surveillance (*4*).

In developing countries, information on food-borne disease is scant. Passive surveillance systems are generally inadequate because 1) patients with diarrhea do not seek medical attention; 2) appropriate samples are not sent for culture; or 3) physicians may not report culture-based cases, including deaths, to a public health reference center. Similarly, outbreak information is frequently unsubstantial, either because health authorities lack the capabilities or resources for detection, or presumably, because diarrheal diseases are highly endemic and outbreaks may be less common or obvious than in industrialized countries. Furthermore, comparability between human, food, and animal data is hindered by the lack of standardized laboratory methods and the absence of joint collaboration between the medical, food, and veterinary sectors.

Integrated food chain surveillance systems (IFCSs) are presently established in only a few industrialized countries. Before this study, national data on FBD in Mexico were based on the syndromatic diagnosis of diarrhea. Beginning in 2002, we established an IFCS for *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* in 4 states from geographically different regions. The system enabled us to identify important human health risks and to establish fu-

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ture research and public health priorities. This article summarizes the data on *Salmonella* spp. obtained from March 2002 through August 2005.

Materials and Methods

Study Setting and Epidemiologic Design

The food safety authorities in Mexico (Sistema Federal Sanitario, Secretaría de Salud) divide the country's 32 states into 5 regions. The 4 states included in this network belong to and are representative of each of the 4 largest regions as follows: Sonora (Region I, Northwest), San Luis Potosi (Region II, Gulf-Central), Michoacan (Region III, Central-Pacific), and Yucatan (Region V, Southeast). In all states, food-animal production is a major economic activity, and most of the circulating retail meat is locally produced. Active surveillance was initiated in 2002 with samples from ill and asymptomatic persons and retail pork, chicken, and beef. Collection of food-animal intestines from chicken, swine, and cattle at slaughterhouses was initiated in mid-2003. The number of samples from food animals and retail meat was designed to reflect regional consumption of each meat product and was limited to domestic production only. In each state, 1 city was sampled per month. Cities with large populations were sampled on repeated occasions; however, different retail outlets and kindergartens were selected for each sampling session. The sampling scheme was designed to follow the food chain in a temporal fashion. Food-animal intestines were collected at each municipal slaughterhouse on day 1, followed by raw retail meat on days 2–4, and fecal samples from asymptomatic kindergarten children on days 7–14. To make the samples in each city as representative as possible, meat was purchased from at least 3 different retail outlets, and only those kindergartens with ≥40 children were selected for the study. Samples from ill children (which included those with severe and moderate diarrhea as well as those with systemic infections) were collected at the major state government hospitals through active surveillance.

Network activities were incorporated into existing programs at each state health department to increase the likelihood of long-term sustainability of the IFCS. Department inspectors conducted the slaughterhouse surveillance, and diarrheal diseases were monitored at the oral rehydration units and pediatric emergency services that participated in national programs for acute enteric diseases.

Study Definitions and Ethical Considerations

Case definitions for diarrhea and asymptomatic children have been described (*5*). Socioeconomic indicators previously used to measure poverty (*6*), such as literacy, household sanitary infrastructure, and income, were obtained from recent national population surveys (*7*,*8*). The internal review boards and ethics committees at all participating institutions approved the protocol, and written informed consent was collected from all participants or their guardians to obtain fecal samples and use the clinical and microbiologic information for scientific studies.

Microbiologic Methods

Participating laboratories used the same standardized methods for isolating and identifying *Salmonella* spp. from human and nonhuman specimens (*9*). Biochemically confirmed isolates were serotyped according to the Kaufmann-White scheme (*10*). All isolates were tested for susceptibility to ampicillin, chloramphenicol, ceftriaxone, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline by disk diffusion according to standard guidelines (*11*). External quality control was performed twice a year by the coordinating center in Yucatan. MICs for ceftriaxone, ciprofloxacin, and nalidixic acid were determined by agar dilution (*12*). For purposes of this study, the terms "resistant" or "resistance" refer to strains with zone diameters below or MICs above the susceptible breakpoint. For ciprofloxacin, resistance was defined as an MIC ≥2 μg/mL. Because isolates with an MIC to ciprofloxacin of 0.12–1.0 μg/mL have been associated with therapeutic failure (*13*), they were included in the analysis and are referred to in this article as "decreased susceptibility." Isolates that were resistant to ceftriaxone (MIC ≥16) were further tested for susceptibility to piperacillin, ticarcillin, aztreonam, cefoxitin, ceftazidime, cefotaxime, ceftiofur, cefepime, and imipenem, and also screened for extended spectrum β-lactamases by disk diffusion using ceftazidime and cefotaxime with and without clavulanic acid (*11*). Data analysis was performed with Whonet software version 5.3 (www.who.int/drugresistance/whonetsoftware/en).

Pulsed-field Gel Electrophoresis

Because of its capacity for virulence and multidrug resistance, *S.* Typhimurium isolates were selected for pulsed-field gel electrophoresis (PFGE) analysis to determine genetic relatedness among human, retail meat, and food-animal isolates from all 4 states. PFGE was performed according to the standard protocol developed by the Centers for Disease Control and Prevention (*14*), which used digestion by *XbaI*. Results were analyzed by using the BioNumerics Software version 3.0 (Applied-Maths, Kortrijk, Belgium), and banding patterns were compared by using Dice coefficients with a 1.5% band position tolerance. A cluster was defined as a group of ≥2 strains that shared a unique PFGE restriction pattern.

Statistical Methods

The χ^2 test was used for comparison of proportions, with an α value of 0.05. Association between type of infection (diarrhea-associated vs. asymptomatic) and the isolation of an extended-spectrum cephalosporin (ESC)–resistant *S.* Typhimurium were analyzed in a 2 × 2 table, and the odds ratio (OR) and its 95% confidence interval (CI) were calculated. Associations between the median rates of *Salmonella* spp. in retail chicken, pork, and beef and the median percentage of asymptomatic *Salmonella* spp. infection in children for each city were calculated with Pearson's correlation coefficient (r) using SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Epidemiologic Surveillance

Figure 1 shows the number of specimens sampled from each source and the percentage that were positive for *Salmonella* spp. The prevalence of *Salmonella* spp. was highest in swine intestines and pork meat (42.1% and 36.4%, respectively), followed by cattle intestines and beef (20.9% and 29.9%, respectively), and chicken intestines and chicken meat (16.9% and 21.3%, respectively). *Salmonella* was isolated from 12.3% of hospital-samples from children with cases of diarrhea and 5.3% of asymptomatic kindergarten children.

Socioeconomic indicators and the rates of meat contamination and human *Salmonella* infections for each state are compared in Table 1. Socioeconomic levels for each state were graded on the basis of the 4 indicators shown in the table; Yucatan was classified as the state with the lowest level, followed by San Luis Potosi, Michoacan, and Sonora (highest level). One hundred forty-one sampling sessions were conducted in 64 cities. Samples from all 3 retail meats and kindergarten children were available for 61 cities in the 4 states for correlation analysis. In 11 cities in the state of Sonora, a strong direct correlation was found between each city's prevalence of beef contamination and its prevalence of asymptomatic *Salmonella* spp. infection (r = 0.91, p<0.001). A moderate correlation was found between pork meat and asymptomatic infection (r = 0.62, p = 0.04). No association was observed for retail meat contamination and human asymptomatic infection in the cities from the other 3 states.

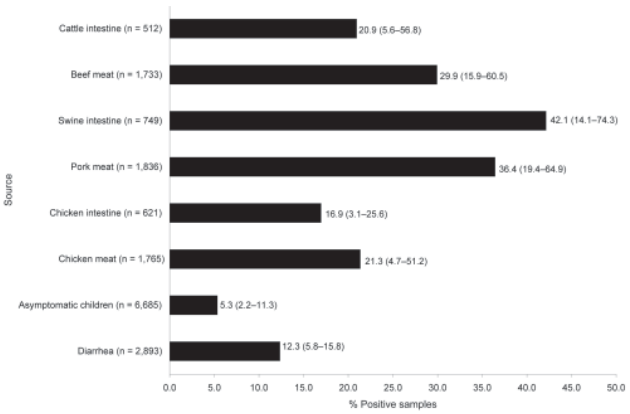


Figure 1. Percentage of human, retail meat, and food-animal samples positive for *Salmonella* spp. detected by an integrated food chain system in Mexico, 2002–2005. Numbers to the right of bars indicate average values, and numbers in parentheses indicate the frequency of positive samples in the states with the lowest and highest prevalence, respectively. The number of specimens examined from each source (n) is shown next to each source heading.

S. Typhimurium and *S.* Enteritidis were the top 2 serovars isolated from ill humans (22.2% and 14.5%, respectively). Among food animals, swine were the most important reservoir of *S.* Typhimurium (10.2% of *Salmonella* spp. isolates), and chickens were the main reservoir of *S.* Enteritidis (11.9% of *Salmonella* spp. isolates) (Table 2). Both humans and animals harbored a considerable diversity of serovars, ranging from 47 to 56 serovars among the different sources. A total of 392 isolates were collected from clinically ill humans. Of these, 26 were isolated from patients with bacteremia and meningitis; 20 (77%) of these isolates were *S.* Typhimurium (8), *S.* Enteritidis (6), and *S.* Typhi (6).

Antimicrobial Drug Resistance

The percentages of isolates from human and food-animal sources that were resistant to antimicrobial agents are given in Table 3. Antimicrobial drug resistance was highest in ill

Table 1. Comparison of socioeconomic indicators and prevalence of retail meat contamination and human *Salmonella* infection, by state, Mexico, 2002–2005

Indicator	Yucatan, %	Sonora, %	San Luis Potosi, %	Michoacan, %
Population >15 y illiterate or with incomplete primary education	40.1	24.0	37.1	44.0
Households with no toilet or latrine	24.6	7.0	14.0	15.4
Households with no sewage system	40.8	20.2	37	24.4
Working population earning <$4 US/d	23.4	6.7	16.4	13.0
Average prevalence of *Salmonella* in retail meat	59.1	14.2	29.7	16.0
Average prevalence of *Salmonella* in diarrheal episodes	15.8	12.6	10.8	5.8
Average prevalence of *Salmonella* in asymptomatic children	11.3	4.4	2.2	1.9

Table 2. *Salmonella* serovars in ill humans and their relative frequency in asymptomatic children and food animals, Mexico, 2002–2005

Serovar	% for each serovar relative to the total no. of *Salmonella* serovars				
	Ill humans* (n = 392)	Asymptomatic children (n = 373)	Chicken† (n = 546)	Swine† (n = 1237)	Cattle† (n = 767)
Typhimurium	22.2	6.7	4.6	10.2	6.8
Enteritidis	14.5	3.2	11.9	0.1	0.1
Agona	6.6	8.3	9.5	9.3	7.3
Muenchen	5.1	3.5	0.6	1.9	2.5
Oranienburg	4.1	4.3	0.9	0.3	0.3
Anatum	3.8	8.0	4.8	13.0	17.7
Newport	3.8	5.4	0.0	0.7	2.6
Meleagridis	3.1	6.4	5.3	11.6	13.0
Other	36.8	54.2	62.4	52.9	49.7

*Ill humans include 366 isolates from enteric infections and 26 isolates from invasive infections.
†Data for food animal intestine and the corresponding retail meat have been combined because serovars were similar.

humans and swine. Resistance to nalidixic acid was highest in *S.* Albany (57%, 65/115) and *S.* Enteritidis (53%, 36/68) from chicken and in *S.* Typhimurium (74%, 133/180) and *S.* Anatum (31%, 92/294) from swine and cattle. Resistance to ciprofloxacin emerged in 2003 in *S.* Heidelberg (10.4%, 5/48) and *S.* Typhimurium (1.7%, 2/127) from swine. Decreased susceptibility to ciprofloxacin was detected in 16.4% of all *Salmonella* spp. isolates (545/3,315) and was most commonly found in chickens (24.9% of all isolates from source), followed by swine (18% of all isolates from source) and ill humans (17.3% of all isolates from source). ESC resistance was first detected in serovar Typhimurium in 2002. From 2002 to 2005, ESC resistance increased from 1.6% to 4.9% and was detected in 6 other serovars. Isolates resistant to ceftriaxone were also resistant to piperacillin, ticarcillin, cefoxitin, ceftazidime, cefotaxime, ceftiofur, and aztreonam and did not show increased susceptibility in the presence of clavulanic acid, which suggests the presence of an AmpC-like β-lactamase. ESC resistance was highest in *S.* Typhimurium (42% of all *S.* Typhimurium isolates, 132/314), followed by *S.* Bredeney (7.1%, 1/14), *S.* Newport (6.3%, 4/64), *S.* Reading (2.4%, 2/85), *S.* Uganda (2.4%, 1/42), *S.* Kentucky (2.2%, 1/46), and *S.* Anatum (0.5%, 2/365).

S. Typhimurium showed particularly high antimicrobial-drug resistance rates in both humans and food animals (Table 4). High resistance rates to ESCs were observed in poultry (77.3%), ill humans (66.3%), and swine (40.4%); multidrug resistance to ≥4 antimicrobial drug classes was found in 86.6% of isolates. *S.* Typhimurium isolated from an ill patient was 6 times more likely to be ESC-resistant than isolates from asymptomatic children (OR 6.3, 95% CI 2.3–17.6; χ^2 14.4, p<0.001).

PFGE

The network collected 314 *S.* Typhimurium isolates, of which 311 were available for PFGE (Figure 2). A total of 126 PFGE patterns were identified. Fourteen clusters (boxes A–N), comprising a total of 102 strains (37 human, 37 retail meat, and 28 food-animal isolates), were common to both humans and food animals. Three patterns (012, 101, 113) were common to humans, and all 3 food animals, and 1 pattern (113) was found in all 4 states. For each state, we found clusters of human isolates that were indistinguishable or closely related to those found in retail meat, food animals, or both.

Discussion

Our experience shows that the establishment of an IFCS is technically and economically feasible in a developing country such as Mexico. The system effectively identified the serovars that caused the greatest effects of disease as well as major animal reservoirs of these serovars in a setting where *Salmonella* spp. infections are highly endemic and passive surveillance is inadequate. The epidemiologic design also corrected constraints present in other monitoring systems, such as the temporal and spatial dissociation of human, food, and animal isolates, as well as the lack of uniform laboratory methods.

Table 3. Antimicrobial drug resistance in *Salmonella* isolates from humans, retail meat, and food animals in Mexico, 2002–2005*

Source	% Resistant†										
	AMP	CHL	CIP	CRO	GEN	KAN	NAL	STR	SU	SXT	TET
Ill humans (n = 392)	25.5	23.4	0.0	14.5	11.7	11.2	24.6	61.1	49.7	24.3	41.2
Asymptomatic children (n = 373)	7.8	8.4	0.0	1.4	2.2	0.5	8.6	45.9	35.4	8.0	26.1
Chicken‡ (n = 546)	7.7	7.4	0.0	3.6	2.6	1.8	30.6	58.9	38.9	11.5	36.8
Swine‡ (n = 1,237)	18.3	22.9	0.6	4.2	8.4	9.0	26.0	73.1	62.1	24.2	55.3
Cattle‡ (n = 767)	11.9	14.1	0.0	1.2	6.6	7.2	20.8	71.6	53.1	19.2	48.8

*AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SU, sulfisoxazole; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.
†Includes resistant and intermediate.
‡Includes isolates from food-animal intestines and the corresponding retail meat.

Table 4. Antimicrobial drug resistance in *Salmonella* Typhimurium isolates from humans, retail meat and food animals in Mexico, 2002–2005*

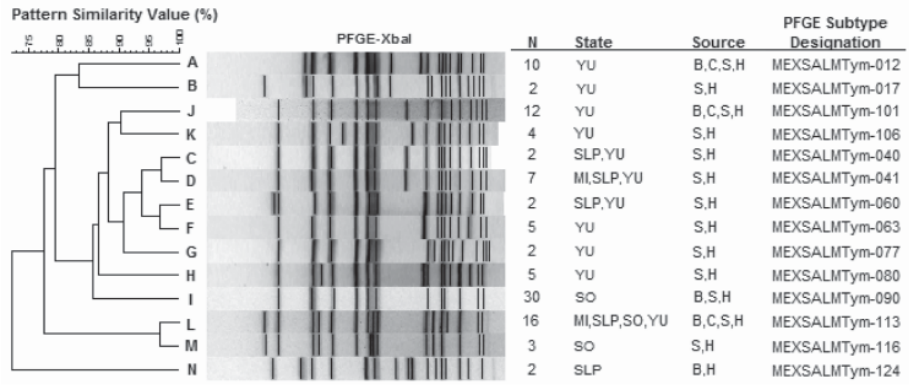
Source	% Resistant†										
	AMP	CHL	CIP	CRO	GEN	KAN	NAL	STR	SU	SXT	TET
Ill humans (n = 87)	79.3	80.5	0.0	66.3	44.8	33.3	55.1	97.3	91.9	71.2	88.5
Asymptomatic children (n = 25)	36.0	36.0	0.0	25.0	4.0	4.0	12.0	73.9	60.0	24.0	44.0
Chicken‡ (n = 22)	100.0	86.4	0.0	77.3	18.2	18.1	27.2	100.0	90.9	54.5	90.9
Swine‡ (n = 127)	61.9	88.9	1.7	40.4	47.6	41.3	72.2	97.2	92.9	63.5	94.4
Cattle‡ (n = 53)	47.2	71.7	0.0	7.5	45.3	35.9	79.2	94.6	90.6	56.6	92.5

*AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SU, sulfisoxazole; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.
†Includes resistant and intermediate.
‡Includes isolates from food-animal intestines and the corresponding retail meat.

Furthermore, the system yielded epidemiologically meaningful data that provided evidence of the magnitude of *Salmonella* spp. as a health risk. Our average prevalence of *Salmonella* spp. contamination in retail meats (21.3%–36.4%) and the high frequency of human *Salmonella* spp. infection, in conjunction with PFGE clusters of geographically and temporally related human and food-animal isolates, led us to conclude that food animals are a major source of salmonellosis in Mexico. A proportion of these clusters may constitute undetected outbreaks; however, this can only be determined once reliable baseline data have been obtained.

Higher rates of meat contamination were observed in those states with higher poverty levels. This finding can likely be explained by the multiple inadequacies in the sanitary infrastructure that lead to increased contamination and dissemination of FBP throughout the environment, in particular, along the food chain. Interestingly, in Sonora, the state with the lowest poverty level that borders the United States, cities with high rates of beef contamination (and to a lesser degree, pork contamination) also had high rates of asymptomatic carriage of *Salmonella* spp. among children. Sonora is one of the major beef- and pork-producing states in the country, and these retail meats are consumed more frequently than chicken by the local population, which might explain the absence of an association with the latter. Conversely, those cities with low rates of meat contamination had lower rates of asymptomatic carriage. Such associations, however, were not found in the other 3 states with higher poverty levels. The conditions in Sonora may more closely resemble the transmission of *Salmonella* spp. infections in industrialized countries, where most infections are acquired through the food chain (*15*), whereas in the other states *Salmonella* spp. infections are probably acquired by other modes of transmission aside from contaminated food, such as from person to person or by contact with animal feces. In settings with greater fecal-oral transmission, asymptomatic infections would not directly reflect contamination rates in the retail meat. Our findings suggest that cattle, swine, and chicken are important, but not unique, reservoirs of *Salmonella* spp. in Mexico. Other animals not included in this study may contribute to an important proportion of these infections, and therefore, other food categories should be included in the surveillance system in the future.

The high rates of ESC resistance and the emerging resistance to fluoroquinolones are other important public health risks detected by the system. Our increasing resistance rates are consistent with a worldwide upsurge of multidrug-resistant nontyphoidal *Salmonella* spp. (*16*,*17*). Multidrug resistance in our *S.* Typhimurium isolates is the most pressing concern. bla_{CMY-2} *S.* Typhimurium is now a major public health problem in Yucatan, where its prevalence increased from 0% in 2000 and 2001 to 75% in 2004 and 2005



N	State	Source	PFGE Subtype Designation
10	YU	B,C,S,H	MEXSALMTym-012
2	YU	S,H	MEXSALMTym-017
12	YU	B,C,S,H	MEXSALMTym-101
4	YU	S,H	MEXSALMTym-106
2	SLP,YU	S,H	MEXSALMTym-040
7	MI,SLP,YU	S,H	MEXSALMTym-041
2	SLP,YU	S,H	MEXSALMTym-060
5	YU	S,H	MEXSALMTym-063
2	YU	S,H	MEXSALMTym-077
5	YU	S,H	MEXSALMTym-080
30	SO	B,S,H	MEXSALMTym-090
16	MI,SLP,SO,YU	B,C,S,H	MEXSALMTym-113
3	SO	S,H	MEXSALMTym-116
2	SLP	B,H	MEXSALMTym-124

Figure 2. Selected pulsed-field gel electrophoresis (PFGE) clusters that represent 102 strains of *Salmonella* Typhimurium and shared indistinguishable PFGE patterns among humans (H), chicken meat and intestine (C), pork meat and swine intestine (P), and beef meat and cattle intestine (B). Several clusters (C,D, E, and L) were present in more than one state. MI, Michoacan; SLP, San Luis Potosi; SO, Sonora; YU, Yucatan. An expanded version of this figure containing the complete set of PFGE patterns is available from www.cdc.gov/EID/content/14/3/429-G2.htm.

and where it has caused fatal infections in young infants (*18*). The higher rate of ESC resistance in ill children with *S.* Typhimurium infection than in asymptomatic children has 2 possible explanations. The first is that these children received antimicrobial drugs shortly before their diarrheal episode, a known risk factor for acquiring multidrug-resistant *Salmonella* spp. infection (*19*,*20*). A second possibility propounded by other investigators is that antimicrobial drug–resistant *Salmonella* spp. is associated with increased virulence (*21*,*22*). We believe that the higher prevalence of ESC resistance in our ill children is of substantial public health importance and that the mechanisms leading to this increased resistance should be further investigated.

Because the network is in its early stages, it does not yet have the capabilities of a mature surveillance system. At present, it is not designed to measure the human health impact of contaminated food consumption or to perform cost-benefit analyses of prevented deaths or reduced days of hospitalization. Furthermore, it does not have a high sensitivity for outbreak detection. All of these capabilities need to be developed in the future. The main objective of the initial phase of the IFCS (2002–2005) was to collect baseline data with good internal validity. Enormous effort was devoted to training staff and achieving good laboratory quality control. During subsequent phases, priority should be given to measuring illness, proportion of deaths, and the economic impact of salmonellosis. This goal could be achieved by increasing the number of sentinel hospitals per state and organizing the systematic collection of clinical and cost-related data. Furthermore, future surveillance should include animal farms and data on local food consumption and antimicrobial usage. Lastly, due to its nature as an early stage study, the system yielded predominantly descriptive information. These data have served to generate several hypotheses that can be tested in future research.

The network described in this study included 4 representative states and was originally established in collaboration with state health authorities. It is currently being transferred to the federal food safety authorities. Based on this study, we believe that a sustainable IFCS could include a 5-state network (one from each representative region). Operating costs (laboratory supplies and staff salaries) for this system, including PFGE analysis for the top 2 serotypes found in ill children, would run US $500,000 per year, which is equivalent to ≈2% of the Federal Sanitary System's yearly budget (*23*).

In conclusion, this project can serve as a model for developing countries to establish an IFCS. We believe that, in the initial stages, efforts should focus on training of staff; laboratory quality control; and achieving good integration of human, food, and animal data. Once this stage has been consolidated and good baseline data have been obtained, the system can become increasingly complex according to needs and available resources. In a period of 3 years and with a modest financial investment, our system was able to identify several important emerging public health threats that should be the focus of future evidence-based interventions. The reduction of *Salmonella* spp. contamination throughout the food chain, as well as the prevention and control of ESC-resistant and fluoroquinolone-resistant *Salmonella* spp. in food animals and retail meats, should receive major priority. The striking differences between the various states can be used by policy makers to design interventions and allocate resources by region to reduce the effects of FBD in those areas with the greatest risk. Finally, control measures instituted throughout the food chain should be linked to other national programs that include the use of oral rehydration therapy, the promotion of breast feeding, and improved nutrition as part of a joint effort to reduce illness and death from diarrheal disease (*24*,*25*).

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Dr Zaidi is currently head of the Microbiology Research Laboratory at the Hospital General O'Horan in Merida, Mexico. Her main interests are in surveillance and control of antimicrobial resistance in community and hospital-acquired pathogens. Dr Zaidi was responsible for study coordination and execution and for writing the report.

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Genetic Variability of West Nile Virus in US Blood Donors, 2002–2005

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West Nile virus (WNV) was detected in the United States in 1999, has reoccurred every summer since, and has become endemic. Transfusion transmission was documented in 2002, and screening of blood donations for WNV began in 2003. We investigated genetic variation of WNV in human isolates obtained from specimens collected from 30 infected blood donors who tested positive for WNV RNA during 2002–2005. Complete genomic sequences of 8 isolates and structural gene sequences from 22 additional isolates were analyzed. We found some genetic diversity in isolates from different geographic regions and genetic divergence from reported sequences from epidemics in 1999–2001. Nucleotide divergence of structural genes showed a small increase from 2002 (0.18%) to 2005 (0.37%), suggesting absence of strong selective pressure and limited genetic evolution of WNV during that period. Nevertheless, WNV has continued to diverge from precursor isolates as geographic distribution of the virus has expanded.

West Nile virus (WNV) is a small, enveloped, positive-strand RNA virus of the genus *Flavivirus* and a member of the Japanese encephalitis serocomplex. The plus-sense RNA genome is ≈11 kb and contains a single open reading frame flanked by 5′ and 3′ untranslated regions (UTRs). The encoded polyprotein is processed into 3 structural and 7 nonstructural (NS) proteins that are essential for viral replication, assembly, and release. WNV is maintained in nature by transmission between mosquitoes and birds but can also infect humans and other mammals (*1*) and reptiles (*2*). Most human infections are asymptomatic (70%–80%); symptomatic cases range from flulike illness to severe neurologic disease (>1% of cases) (*3*–*5*).

The first US outbreak of WNV occurred in 1999 in New York City; 68 human infections, mostly as meningoencephalitis, were confirmed, resulting in 7 deaths. Since 1999, WNV epidemics have reoccurred yearly with 23,975 reported human cases of disease and 962 deaths through 2006 (www.cdc.gov/ncidod/dvbid/westnile).

In 2002, the virus spread westward and the number of reported human cases increased dramatically. The North American epidemics of 2002 and 2003 represent the largest WNV outbreaks ever reported (*6*). Additional modes of WNV transmission were identified in 2002, including human-to-human transmissions from mother to child, organ transplantation, and blood transfusion (*7*–*9*). The quick spread of the virus raised questions regarding viral adaptation and prompted a detailed investigation of the genetic evolution of the virus.

The prototype WNV isolate, NY99-flamingo382–99 (WN-NY99; GenBank accession no. AF196835) was obtained from a flamingo infected during the 1999 outbreak in New York. This isolate belongs to lineage I and is most closely related to the Israel-98 goose isolate AF481864 (IS-98) (*6*,*10*). Phylogenetic comparisons of partial and complete nucleotide sequences from US isolates collected between the 1999 and 2000 epidemics with WN-NY99 isolate showed a high degree of genetic similarity with >99.8% nucleotide homology and >99.9% amino acid homology (*11*–*14*). A study of 22 different WNV isolates from 2001 and 2002 showed genetic variation of 0.35% (mean 0.18%)

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in the premembrane (*preM*) and envelope (*env*) genes compared with WN-NY99 (*15*).

Subsequently, 2 distinct genotypes were detected in strains obtained from Texas in 2002 (*15*). One new genetic variant widely spread over the United States diverged from the original WN-NY99 strain by several conserved nucleotide mutations and 1 aa substitution in the env protein. Recent studies have highlighted the emergence of a new WNV dominant genotype, named WN02, which has been increasingly prevalent in the United States since 2002 (*16–19*). Although 13 nt mutations became fixed in the new dominant genotype compared with the WN-NY99 prototype, the highest nucleotide sequence divergence of WNV strains isolated after 2002 is still in the range of 0.4%–0.5% (*18*,*20*). The reason for displacement of the WN-NY99 genotype by a new dominant genotype in North America is not clear, but could have been caused by differences in transmission efficiency of domestic mosquitoes that may offer a selective advantage for the newly emerged genotype (*20*,*21*).

Several studies on the genetic variation of WNV have been published (*12*,*15*,*17*,*19*,*21*,*22*). However, continuous monitoring of variability is needed because sensitivity of blood donor screening and diagnostic assays may be affected, producing a negative effect on public health. Genetic variability could also affect viral pathogenesis, development of vaccines, and development of efficacious therapeutic agents.

This study reports the genomic variation of WNV observed in clinical isolates obtained in the continental United States during 4 consecutive years (2002–2005). We observed an increase in the number of mutations in the full WNV genome from 0.18% in 2002 to 0.37% in 2005. It should be noted that 80% of the nucleotide changes in structural regions are transitions (T ↔ C) and 75% are silent mutations. Thus, WNV has continued to slowly diverge from precursor isolates as geographic distribution of the virus expanded.

Materials and Methods

Study Samples

This study included 30 plasma specimens (Table 1) obtained from blood donor units positive for WNV by

Table 1. Sequences of 30 West Nile virus isolates from the United States*

Isolate ID	Year	Geographic location	No. passages in Vero cells†	GenBank accession no.
FDA/HU-02	**2002**	**NY**	**3**	**AY646354**
ARC10–02	**2002**	**MI**	**1**	**AY795965**
ARC12–02	2002	OH	1	DQ666453
ARC13–02	2002	MI	1	DQ666454
ARC15–02	2002	MI	1	DQ666455
ARC16–02	2002	IN	1	DQ666456
ARC17–02	2002	GA	1	DQ666457
BSL5–03	**2003**	**UT**	**1**	**DQ005530**
BSL9–03	2003	TX	1	DQ666458
BSL56–03	2003	ND	1	DQ666459
BSL62–03	2003	SD	2	DQ666460
BSL114–03	2003	TX	2	DQ666461
RMS1–03	2003	MN	1	DQ666462
RMS2–03	2003	IN	1	DQ666463
RMS3–03	2003	IN	1	DQ666464
RMS4–03	2003	IA	1	DQ666465
BSL1–04	2004	AZ	1	DQ666466
BSL2–04	2004	AZ	1	DQ666467
BSL4–04	2004	AZ	2	DQ666468
BSL5–04	**2004**	**AZ**	**1**	**DQ666448**
BSL6–04	2004	AZ	1	DQ666469
BSL7–04	2004	AZ	2	DQ666470
BSL8–04	2004	AZ	2	DQ666471
GCTX1	**2005**	**TX**	**1**	**DQ666449**
GCTX2	**2005**	**TX**	**1**	**DQ666450**
BSL2–05	**2005**	**SD**	**1**	**DQ666452**
BSL6–05	2005	AZ	1	DQ666472
BSL9–05	2005	TX	1	DQ666473
BSL10–05	2005	LA	1	DQ666474
BSL13–05	**2005**	**AZ**	**1**	**DQ666451**

*Isolates in **boldface** have been completely sequenced.
†Indicates passage of viral isolate from which RNA extracts were used to obtain the genomic sequence.

nucleic acid tests used to screen blood donations under Food and Drug Administration (FDA)–approved nationwide clinical trials; the specimens were collected in 13 states in the continental United States. All samples were collected under Institutional Review Board–approved informed consent provided by each of the institutions performing donor screening.

Virus Isolation in Vero Cells

Vero cells were plated in T75 flasks and grown to 85% confluence in Eagle minimal essential medium (GIBCO BRL, Gaithersburg, MD, USA) containing 5% fetal bovine serum (Hyclone, Logan, UT, USA) and 10 μg/mL of penicillin/streptomycin (GIBCO-BRL). For viral isolation, medium was removed, and 500 μL of each plasma sample was added to individual flasks and the volume was adjusted to 5 mL with fresh medium. Cultures were incubated for 2 hours with the plasma, either at room temperature with gentle rocking or at 37°C with sporadic mixing. A total of 10 mL of fresh medium was then added and cultures were incubated at 37°C in an atmosphere of 5% CO_2. Cultures were observed daily for cytopathic effect under phase microscopy. Supernatants were harvested when an extensive cytopathic effect was observed, centrifuged to remove cell debris, and frozen at –70°C until further analysis.

RNA Extraction, Reverse Transcription, and PCR

RNA extracts were obtained from 1-mL plasma samples by using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and extracts were resuspended in 20 μL of water. RNA samples from viral passages were isolated from 140 μL of culture supernatants by using the QiaAMP viral RNA extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. RNA extracts were stored at –70°C until further analysis. Reverse transcription reactions were performed in a final volume of 20 μL that contained 10 μL of RNA and specific WNV reverse primers by using SuperScript III (Invitrogen) according to the manufacturer's instructions. Specific primers used for both PCR amplification and sequencing were designed according to available sequence information in GenBank and based on alignments of published WNV sequences. PCR amplification of overlapping fragments to cover the complete viral genome was performed by using 15 pairs of primers (Table 2). Partial sequences for the structural region were obtained by using primer sets 1, 2, and 3. cDNA specimens

Table 2. Primer sets used for PCR analyses of West Nile virus and sizes of overlapping amplicons*

Set/location	Amplicon size, kb	Name	Sequence (5′ → 3′)
1/F1–R1300	1.3	F1	AGTAGTTCGCCTGTGTGAGCTGAC
		R1300	TTGGCGCATGTGTCAATGCT
2/F980–R2000	1.0	F980	CTTGGAATGAGCAACAGAGA
		R2000	GTTAGGTCGTTCAATGAAGC
3/F1690–R2685	1.0	F1690	GAGACGTTAATGGAGTTTGA
		R2670	CTTCACTGCTTCCCACATTTG
4/F2340–R3420	1.1	F2340	TTCGGAGGCATGTCCTGGAT
		R3420	CTGATCTCCATACCATACCAACA
5/F3330–R4120	0.8	F3330	GAGAGCTGCGGACACCGTGGACC
		R4120	CATAGCAGACTTGCTCCTTTCT
6/F4070–R4950	0.9	F4070	CTGTTGATGGTCGGAATAGG
		R4950	CCTGGTTTCGTCTGGACGTT
7/F4810–R5650	0.85	F4810	CGCCTGGACCCATACTGG
		R5650	CCATTCGTATCCAGAGTTCCA
8/F5510–R6430	0.9	F5510	AGCATTGCAGCAAGAGGTTA
		R6430	TAGTGCCTGGTGATCCGAGTACAC
9/F6290–R6770	0.5	F6290	CGACCGGAGGTGGTGCTTTGATGG
		R6770	CCTGGAACTTCAGCCATCCA
10/F6690–R7550	0.85	F6690	CCTCCTCATGCAGCGGAA
		R7550	GAGCTTGCTCCATTCTCCCA
11/F7420–R8260	0.85	F7420	CCACACCCATCATGCAGAA
		R8260	CGTTGGAGCAGCTCCATCTT
12/F8170–R9050	0.9	F8170	CATAGGACGATTCGGGTCCT
		R9050	CTCTTTCCCATCATGTTGTAAATGC
13/F8920–R9810	0.9	F8920	CAGCTTTGGGTGCCATGTT
		R9810	GAACCTGCTGCCAATCATACC
14/F9750–R10630	0.9	F9750	TCCTCAATGCTATGTCAAAGGT
		R10630	GGTCCTCCTTCCGAGACGGT
15/F10550–R11029	0.5	F10550	TGAGTAGACGGTGCTGCCTG
		R11029	GATCCTGTGTTCTCGCACCACCAG

*Internal sequencing primers were also used and their sequences are available upon request.

were amplified by using the Hi-Fidelity PCR system (Invitrogen) according to the manufacturer's instructions.

DNA Sequencing

PCR products were purified by agarose gel electrophoresis by using the MinElute Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. Both strands were subjected to direct sequencing by using amplifying primers (Table 2) and additional internal sequence primers. Sequencing reactions were performed by using the ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and analysis by using the ABI Prism 3100 system (Applied Biosystems).

Sequence Analysis

Sequencing data were assembled and analyzed by using the Vector NTI Advance 10 software package (Invitrogen). Nucleotide and deduced amino acid sequences from each isolate were aligned by using the Align X program in Vector NTI and compared with prototype WN-NY99 and previously published sequences of isolates from different regions of the United States and other countries. Phylogenetic relationship studies were based on several methods of analysis (distance, parsimony, and likelihood algorithms) by using MEGA version 3.1 (www.megasoftware.com). All studied isolates were compared with each other, and phylogenetic trees were constructed by using the Kimura 2-parameter method that included transitions and transversions to show genetic relationships of isolates in this study with other WNV isolates in GenBank.

Results

Because of limited volume of plasma specimens available, viruses were isolated from Vero cell cultures for sequence analysis. We assessed the potential of the viral isolation procedure to cause mutations in the viral genome by comparison of sequences of the structural region of 6 specimens and their respective isolates after 3 passages in Vero cells. There were no changes in the RNA sequences obtained in each original plasma sample and the RNA sequences obtained in each of the passages in culture.

We investigated the genetic variability of 30 WNV isolates collected from plasma specimens from nucleic acid amplification testing–positive blood donors in 13 states during 2002–2005 (Table 1). Isolates were generated by cultivation in Vero cells. The fragment that encompasses the 5′-UTR, all structural genes, and part of *NS1* (bp 1–2,685) from all 30 specimens were subjected to genomic sequencing. Eight of 30 isolates were fully sequenced.

The Appendix Table (online Appendix Table, available from www.cdc.gov/EID/content/14/3/436-appT.htm) compares conserved nucleotide mutations and deduced amino acid substitutions identified in structural regions of all 30 isolates with the WN-NY99 isolate (AF196835). Approximately 80% of the nucleotide changes in structural regions were transitions (T ↔ C) and 75% were silent mutations. All mutations in the preM and membrane (M) regions were silent, and 16 isolates shared the transition 660 C → T. Several WNV strains from Europe and Asia, as well as Kunjin virus isolates, also had T at position 660, but both the prototype WN-NY99 and the IS-98 (AF481864) isolates contain 660 C. Twenty-nine of 30 isolates shared 2 conserved nucleotide mutations in the *env* gene: 1442 T → C (Val 159 → Ala) and 2466 C → T that differentiates the new dominant genotype, WN02, from the preceding genotype WN99.

Construction of a phylogenetic tree by the maximum parsimony method (Figure 1) showed the degree of divergence of isolates from WN-NY99. The average nucleotide divergence for structural genes has increased from 0.18% in 2002 to 0.37% in 2005.

The env protein has several biologic roles, which include viral entry, virion assembly and release, agglutination of erythrocytes, and induction of B- and T-cell responses that are associated with protective immunity. Thus, this protein may be involved in WNV evolution. The phylogenetic tree shown in Figure 2 was constructed by maximum parsimony analysis with *env* gene sequences from US isolates from 1999–2006 in GenBank and sequences of

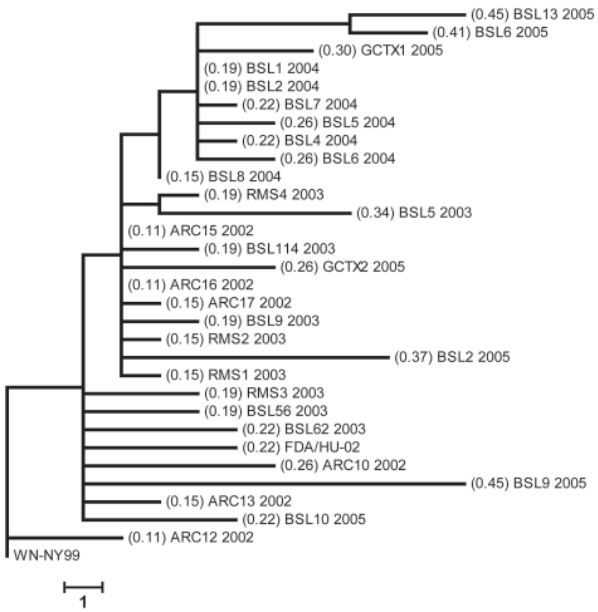

Figure 1. Phylogenetic analyses based on maximum parsimony comparing the 2,685-bp nucleotide sequence, including the complete structural and the 5′-untranslated region of prototype West Nile virus (WNV) strain WN-NY99 with 30 WNV isolates collected during the 2002–2005 epidemics in the United States. Values in parentheses show percentage of nucleotide sequence divergence from WN-NY99. Scale bar represents a 1-nt change.

human isolates in our study. The isolates clustered in 2 clades correlated with the parsimony-revealing mutation sites at positions 1442 and 2466.

Although *preM*, *M*, and *env* sequences show adequate phylogenetic representation, we also analyzed 8 complete genomes of WNV isolates for stronger evidence of evolutionary relationships between isolates and additional mutations that may have implications in phenotypic properties of these isolates. Nucleotide changes and deduced amino acid substitutions of complete genomic sequences from 8 isolates are shown in Tables 3 and 4, respectively. When compared with WN-NY99 sequences, these sequences showed an increased number of nucleotide mutations. FDA/Hu-02 isolated in 2002 showed 20 nt mutations plus 1 insertion at position 10497, and 5 mutations resulted in amino acid substitutions on the basis of deduced sequence of viral polyprotein. ARC10–02 isolated in 2002 had 22 mutations, 3 of which resulted in amino acid substitutions. These 2 isolates showed ≈0.2% nucleotide divergence.

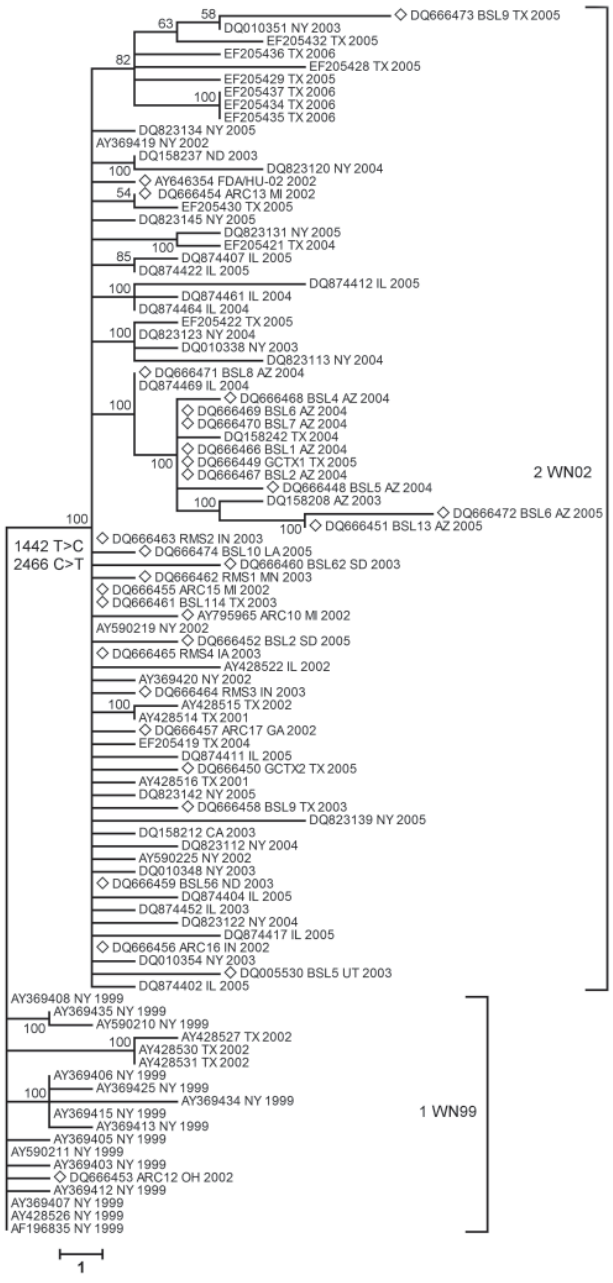

Figure 2. Distance analysis of envelope glycoprotein of West Nile virus isolates collected during 1999–2006 epidemics in the United States. Phylogram is based on maximum parsimony analysis of complete nucleotide sequences of the envelope gene. Diamonds indicate isolates from this study. All isolates from clade 2 (WN02 strain) contained conserved mutations at positions 1442 (T → C) and 2466 (C → T). Values near branches represent percentage support by parsimony bootstrap analysis. Scale bar represents a 1-nt change.

The 2003 isolate BSL5–03 showed 39 mutations (nucleotide divergence 0.35%), 7 of which were associated with predicted amino acid substitutions. These 3 isolates from 2002 and 2003 had 11 common mutations: 2 were in env (1442 T → C resulting in Val 449 → Ala and 2466 C → T, a silent mutation); 8 were in the NS regions (4146 A → G in NS2A; 2 C → T transitions at positions 4803 and 6138 in NS3; 6996C → T and 7015T → C in NS4B; T → C transitions at positions 7938 and 8811 and 9352 C → T at NS5); and 1 in the 3-′UTR (10851 A → G).

The 2004 isolate BSL5–04 had 42 mutations with 7 aa substitutions. The isolates from 2005 were as follows: GCTX1-05 had 56 mutations with 17 aa substitutions; GCTX2-05 had 41 mutations with 3 aa substitutions; BSL2–05 had 44 mutations with 10 aa substitutions plus a deletion of 14 nt (10480 to 10493) in the 3′-UTR; and BSL13–05 had 48 mutations with 8 aa substitutions. Isolates from 2004 and 2005 shared a nucleotide mutation 6721 G → A, which resulted in amino acid substitution Ala2209Thr in the NS4A. Four isolates from 3 consecutive years (BSL5–03 from 2003, BSL5–04 from 2004, GCTX1 and BSL13–05 from 2005), shared an amino acid substitution (Lys2842Arg) in NS5. Three isolates from 2005 (GCTX1–05, BSL2–05 and BSL13–05) plus 1 isolate from 2004 (BSL5–04) also shared a silent mutation at position 8550 C → T.

The overall nucleotide divergence from the WN-NY99 isolates from 2003, 2004, and 2005 showed a steady but small increase (BSL5–03, 035%; BSL5–04, 0.38%; BSL2–05, 0.39%; BSL13–05, 0.43%; GCTX1–05, 0.5%; and GCTX2–05, 0.37%). These findings suggest relative stasis in WNV divergence. The 8 completely sequenced isolates shared conserved nucleotide mutations in the *preM*, *M*, *env*, *NS2A*, *NS3*, *NS4B*, and *NS5* genes and the 3′-UTR (Table 4). The largest number of conserved mutations was in the *NS3* and *NS5* genes. No conserved mutations were observed in the *core*, *M*, *NS1*, and *NS2B* genes or the 5′-UTR.

Table 3. Deduced amino acid substitutions in 8 completely sequenced West Nile virus isolates compared with isolate WN-NY99*

	Residue no.													
	Nucleocapsid			Envelope glycoprotein									NS1	
Isolate	11	21	52	294	298	342	449	472	529	602	663	684	799	895
WN-NY99	Ser	Met	Ala	Leu	Asn	Asn	Val	Glu	Leu	Leu	Ile	Asn	Ile	Leu
FDA/HU-02		Thr	Val				Ala					Ser		
ARC10–02							Ala							
BSL5–03							Ala				Val		Val	
BSL5–04							Ala							
GCTX1–05					Ser		Ala							
GCTX2–05							Ala							Phe
BSL2–05					Ser	Ser	Ala	Gly		Ile				
BSL13–05	Asn			Pro			Ala		Phe					
Protein	NS2A	NS2B				NS3					NS4A			NS4B
Residue no.	1169	1438	1611	1688	1690	1702	1971	1991	2188	2193	2209	2213	2261	2301
WN-NY99	Lys	Ser	Val	Met	Arg	Gly	Pro	Phe	Thr	Phe	Ala	Val	Leu	Ser
FDA/HU-02														Gly
ARC10–02												Ala		
BSL5–03	Arg						Ser							
BSL5–04		Asn							Ser		Thr			
GCTX1–05			Ala	Thr	Lys	Asp				Leu	Thr		Met	
GCTX2–05														
BSL2–05								Leu			Thr			
BSL13–05											Ile			
	NS4B	NS5												
Isolate	2488	2549	2755	2788	2820	2826	2842	3056	3104	3132	3191	3238	3240	
WN-NY99	Ser	Lys	Gly	Asn	Ser	Glu	Lys	Pro	Arg	Arg	Met	Gln	Pro	Total
FDA/HU-02														5
ARC10–02						Gly								3
BSL5–03							Arg							6
BSL5–04			Ser				Arg					Lys	Gln	8
GCTX1–05			Arg	Ser	Leu		Arg		Cys	Ser	Thr			16
GCTX2–05								Ser						3
BSL2–05	Gly	Arg											Gln	10
BSL13–05	Gly						Arg						Gln	8

*NS, nonstructural.

Discussion

Our study describes genetic variability observed among 30 clinical isolates of WNV from 13 states in the United States obtained during 2002–2005. Since the initial recognition of WNV in North America in 1999 (*10*), the virus has spread from the East to the West Coast and has become endemic. Genetic studies have shown that >50% of all WNV isolates in 2002 and >80% of all isolates in 2003 had a new genotype that emerged in the United States in 2001. The new dominant genotype (WN02) was characterized by 2 conserved nucleotide mutations in *env* (1442 T → C and 2466 C → T) and 1 deduced amino acid substitution (Val 159 → Ala) in the env protein (*16–18*). Displacement of the initial genotype (WN99) by WN02 has been attributed to differences in efficiency of transmission of the virus by domestic mosquitoes (*21*). Genetic studies conducted during the early years of WNV activity in the United States identified a small number of mutations and showed no changes suggesting specific adaptations (*6*,*12*,*15*). After the genetic shift in 2001–2002, most nucleotide changes observed were silent transitions (T ↔ C and A ↔ G). Limited variability in structural genes observed by our group and others (*15*,*16*,*18*,*20*,*22*) suggests the absence of strong immune selective pressure, which led to limited evolution of WNV during 2002–2005. Data in this report show that WNV has continued to diverge from precursor isolates as geographic distribution of the virus expanded.

On the basis of nucleotide sequences, 6 aa substitutions were predicted in the core protein of 5 isolates. Two amino acid substitutions, Ser 11 → Asn in isolate BSL13–05 and Met 21 → Thr in isolate FDA-Hu2002, were located within the core hydrophilic region outside α helices. The other amino acid substitutions (Met 34 → Val in isolate BSL06–04, Ala 52 → Val in isolate FDA-Hu2002, Ala 77 → Val in isolate ARC16–02, and Lys 79 → Arg in isolate BSL10–05) were located within α1, α2, α3, and α4 helices, respectively (*23*) and were not expected to affect conformation, function, or antigenic properties of core protein.

Studies of WNV evolution have focused on the env protein because it plays a major role in immune response to

Table 4. Nucleotide mutations conserved in fully sequenced West Nile virus isolates from 2002–2005 epidemics in the United States compared with isolate WN-NY99*

Isolate	Gene or region															
	preM	*Env*		*NS2A*	*NS3*				*NS4A*	*NS4B*		*NS5*				3′-UTR
	Nucleotide no.															
	660	1442	2466	4146	4803	6138	6238	6426	6721	6996	7015	7938	8621	8811	9352	10851
WN-NY99	C	T	C	A	C	C	C	C	G	C	T	T	A	T	C	A
FDA/HU-02		C	T	G	T	T				T	C	C		C	T	G
ARC10 2002		C	T	G	T	T		T		T	C	C		C	T	G
BSL5 2003	T	C	T	G	T	T		T		T	C	C	G	C	T	G
BSL5 2004	T	C	T	G	T	T	T	T	A	T	C	C	G	C	T	G
GCTX1 2005	T	C	T	G	T	T	T	T	A	T	C	C	G	C	T	G
GCTX2 2005	T	C	T	G	T	T	T	T		T	C	C		C	T	G
BSL2 2005	T	C	T	G	T	T	T	T	A	T	C	C		C	T	G
BSL13 2005	T	C	T	G	T	T	T	T	A	T	C	C	G	C	T	G

*preM, premembrane; Env, envelope; NS, nonstructural; UTR, untranslated region.

infection and immunologic pressure could lead to its variation (*12*,*15*–*21*,*24*,*25*). A total of 29 of 30 isolates in this study contained 2 conserved nucleotide mutations in *env* (1442 T → C and 2466 C → T) that differentiate the dominant genotype from other genotypes and place these isolates in the WN02 clade relative to WN-NY99 (Figure 2). The mutation 2466 C → T was a silent mutation. Mutation 1442 T → C leads to the amino acid substitution Val 159 → Ala, located in the variable region close to the glycosylation site. Glycosylation of env protein can influence virus infectivity and has been considered a potential determinant of virulence in a mouse model (*26*–*28*).

All 7 isolates from 2004 were from Arizona and had 2 common silent mutations in *env*: 1320 A → G present in all isolates, and 1974 C → T, present in 6 of 7 isolates. Mutation 1320 A → G is also present in reported attenuated strains of WNV (AY532665, AY688948, M12294) (*29*) and was also reported in human isolate DQ164201 from Arizona in 2004 and in the red-tailed hawk isolate DQ164204 from Colorado in 2003 (*18*). Mutation 1974 C → T, found in isolates from Arizona and Texas, was detected in 2000 in New York (AF404756), which indicated that this mutation occurred at least twice. A Kunjin virus isolate from 2003 (AY274505) and isolate EthAn4766 from Ethiopia (AY603654) also had that mutation.

Phylogenetic analysis of complete genomes of WNV isolates provided stronger evidence of evolutionary relationships between isolates. Eight fully sequenced isolates were compared by phylogenetic analysis with 39 published complete WNV genomes present in the United States during epidemics in 1999–2005 (Figure 3). The phylogenetic tree was constructed by maximum parsimony analyses and rooted by using WN-NY99 and IS-98; it clearly shows that these 2 isolates were the predecessors of other US WNV isolates. Analysis of nucleotide sequence alignment of complete WNV genomes indicated that most mutations that occur each year were not fixed. However, WNV has continued to diverge and the total number of fixed mutations and the overall nucleotide divergence have increased. Some mutations in US isolates may have appeared as a result of native adaptation and genetic drift of parental WNV isolates (*18*).

Within the WN02 genotype, a sequence from NY 2003 (DQ164189) formed 2 outgroups with moderately strong (96%) bootstrap support (Figure 3). Five of 8 isolates in outgroup 2a were from Arizona and had the 6721 G → A mutation resulting in amino acid substitution Ala 209 → Thr. This mutation was already present in the 2003 magpie isolate DQ164203 from Colorado and in the 2004 human isolate DQ164201 from Arizona (*18*). These isolates also shared silent mutation 8550 C → T, which had been reported in the 2004 human isolate DQ164201 from Arizona (*18*).

Local concentration of closely related isolates in Arizona (Figures 2, 3) or in California (outgroup 1; Figure 3) may have been caused by introduction of 1 or few genetically similar viruses in the area with rapid spread to mosquitoes and local birds, which amplified the original genome. Human infections in that area would therefore result from 1 or a few local WNV-colonizing genotypes and reflect stochastic introduction of 1 or a few infected vectors, followed by rapid localized amplification (*19*). In contrast, a larger number of different viruses were introduced in other areas, as observed in Texas, which lead to a broader diversity of variants.

Several studies showed that mutations in conserved structures within the 3′-UTR did not affect WNV translation but could affect RNA replication and interaction between cellular protein eEF1A and WNV 3′-UTR, facilitating viral minus-strand synthesis (*30*–*32*). Isolate BSL2–05 from South Dakota had a 14-nt deletion (10480–10493) and isolate FDA/Hu-02 had 1-nt insertion at position 10497 in the 3′-UTR; the second isolate had larger plaques than WN-NY99. The recently published 2004 human isolate DQ431705 from North Dakota (*19*) had a 5-nt deletion (10434–10439) in the same 3′-UTR variable region.

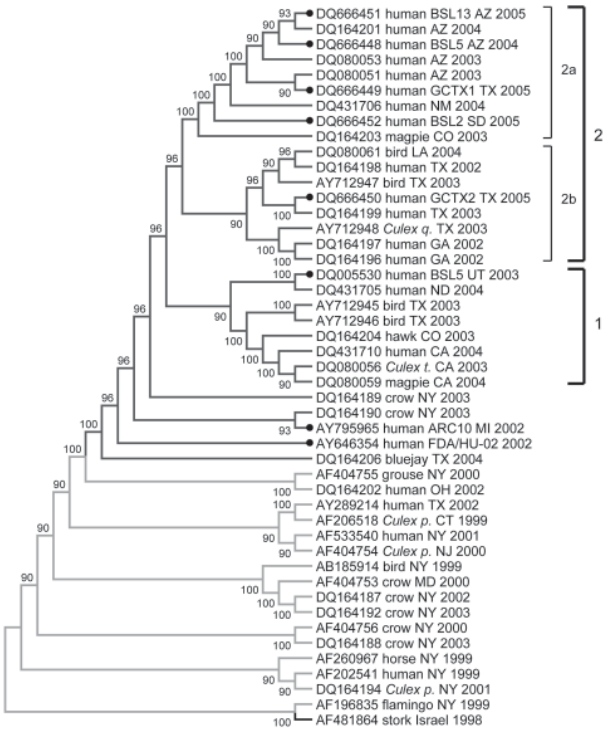

Figure 3. Phylogenetic tree of complete genomes of West Nile virus (WNV) isolates collected during the 1999–2005 epidemics in the United States. Phylogeny reconstruction was estimated by using MEGA version 3.1 (www.megasoftware.com) on the basis of maximum parsimony analysis. Solid circles indicate isolates from this study. Values near branches represent percentage support by parsimony bootstrap analysis. Some parsimony-informative positions (1442, 2446, 4146, 6138, 6721, 8811, 10408, and 10851) play an important role in topologic arrangement of the tree and outgroup configurations. The tree was rooted with prototype WNV isolate WN-NY99 (AF196835) and the most closely related Old World isolate, IS-98 (AF481864). *Culex q.*, *Culex. quinquefasciatus*; *Culex t.*, *Cx. tarsalis*; *Culex p.*, *Cx. pipiens*. WNV genotype is color coded: green, WN99; blue, WN02.

Previous studies had reported low genetic variation among WNV isolates in North America and emphasized a low frequency of nucleotide variants in the 3′-UTR. Subsequent observations showed that nucleotide changes in the 3′-UTR played a role in rapid spread of this virus in the New World because nucleotide changes in the 3′-UTR had co-evolved with amino acid changes and affected interactions of helicase and RNA polymerase with their RNA substrate (*33*). Mutations in 3′-UTR appear to play a role in production of small-plaque temperature-sensitive variants or mouse-attenuated phenotype variants (*34*).

Our study showed limited but continuous genetic variability with amino acid substitutions in WNV strains. We also found insertions and deletions in the 3′-UTR region that should be studied for assessment of their functional role. Phylogenetic studies of WNV isolates are needed to monitor microevolution of WNV that may affect pathogenic properties of the virus and to assess the effectiveness of available commercial donor screening and diagnostic assays for detection of variant strains. Study of genetic variability may also provide insights into the development of vaccines and therapeutic agents.

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Discovering and Differentiating New and Emerging Clonal Populations of *Chlamydia trachomatis* with a Novel Shotgun Cell Culture Harvest Assay

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Chlamydia trachomatis is the leading cause of preventable blindness and bacterial sexually transmitted diseases worldwide. Plaque assays have been used to clonally segregate laboratory-adapted *C. trachomatis* strains from mixed infections, but no assays have been reported to segregate clones from recent clinical samples. We developed a novel shotgun cell culture harvest assay for this purpose because we found that recent clinical samples do not form plaques. Clones were strain-typed by using outer membrane protein A and 16S rRNA sequences. Surprisingly, ocular trachoma reference strain A/SA-1 contained clones of *Chlamydophila abortus*. *C. abortus* primarily infects ruminants and pigs and has never been identified in populations where trachoma is endemic. Three clonal variants of reference strain Ba/Apache-2 were also identified. Our findings reflect the importance of clonal isolation in identifying constituents of mixed infections containing new or emerging strains and of viable clones for research to more fully understand the dynamics of in vivo strain-mixing, evolution, and disease pathogenesis.

Chlamydia trachomatis is a ubiquitous human pathogen that is responsible for the most prevalent bacterial sexually transmitted diseases (STDs) worldwide (*1*). As an obligate intracellular bacterium, it has a distinctive biphasic developmental cycle (*2*). The cycle begins when metabolically inactive elementary bodies (EBs) infect the host cell and reside in a vacuole termed an inclusion body. EBs differentiate into noninfectious, metabolically active reticulate bodies that multiply by binary fission and redifferentiate into EBs after 30–48 hours and then are released from the cell by lysis or exocytosis to initiate a new round of infection (*2*).

The organism comprises 2 biovars, trachoma and lymphogranuloma venereum (LGV) (*3*). These biovars comprise 19 serologic variants (serovars), which are identified by monoclonal antibodies that react to epitopes on the major outer membrane protein (MOMP) (*4*). Variants of *ompA*, the gene that encodes MOMP, differentiate genotypes of these serovars (*5*–*7*). Phylogenetic analyses and statistical modeling have enhanced *ompA* genotyping. For example, serovar B is restricted to the ocular mucosa while Ba is found in the eye and urogenital tract (*8*). The LGV biovar (L_1, L_2, L_2', L_2a, L_2b, L_3) causes invasive STDs (*9*,*10*). The trachoma biovar (A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, Ja, K) is responsible for ocular disease, termed trachoma, and for STDs globally. The former is caused by serovars A to C and Ba and the latter by D through K, Da, Ia, Ja, and rarely Ba and C (*4*,*5*,*11*).

Approximately 8%–57% of clinical STD samples mixed infections (*5*–*7*,*9*,*12*,*13*). Thus, an inherent problem with strain typing is detecting mixed infections. These infections can be identified by using PCR primers that are specific for each strain followed by sequencing (*5*), by cloning PCR products and sequencing >10 clones (*11*,*13*), or by reverse dot-blot hybridization of PCR amplicons to serovar-specific probes (*14*). However, none of these techniques can detect new genetic strains that fail to either anneal with the current selection of primers or hybridize with the available probes. The end product is also a nonviable DNA sequence.

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Increasingly, isolates representing single clones are needed for in vitro and in vivo research, including genomic, murine, and translational studies, to advance our understanding of chlamydial pathogenesis. Although a few studies have described methods for segregating clones of laboratory-adapted *C. trachomatis* clinical and reference strains (*12*,*15*,*16*), none has clonally purified all 19 *C. trachomatis* reference strains nor determined optimal methods to clonally segregate clinically mixed samples. Consequently, we modified the plaque-forming assay of Matsumoto et al. (*16*) to segegrate clones from reference strains and developed a novel cell culture shotgun harvest assay to segregate viable clones from recent clinical samples because typical plaques do not form for most of these samples.

Our culture techniques coupled with outer membrane protein A (*ompA*) and 16S rRNA sequencing identified the constituents of mixed infections that represented new and emerging *Chlamydiaceae* strains and clonal variants in human disease. These results stress the importance of clonal isolation for these types of discoveries. Clonal isolates will also be essential for chlamydial research to ensure reproducibility of experiments among laboratories and to understand the dynamics of in vivo strain-mixing, evolution, and disease pathogenesis.

Materials and Methods

C. trachomatis Reference and Clinical Strains

We studied 19 *C. trachomatis* reference strains (A/SA-1, B/TW-5, Ba/Apache-2, C/TW-3, D/UW-3, Da/TW-448, E/Bour, F/IC-Cal3, G/UW-57, H/UW-4, I/UW-12, Ia/IU-4168, J/UW-36, Ja/UW-92, K/UW-31, L1/440, L2/434, L2a/TW-396, and L3/404) and 5 clinical strains, representing *ompA* genotypes G, F, H, Ja, and K (Table 1). Reference strains were the original isolates. A/Har-13; *Chlamydophila caviae*, strain GPIC; *Chlamydia muridanum*, strain Nigg; *Chlamydophila abortus*, strain S26/3; and another seed stock of A/SA-1 were included for PCR amplification analyses (see Preparation of Genomic DNA and Sequencing of ompA and 16S rRNA for Each Clone). Clinical strains were isolated from acute (Ja and K strains; no prior history of chlamydial STD) and persistent cervical strains (F, G, and H; same-ompA genotypes occurring in the same woman over several years despite antimicrobial drug therapy). Clinical samples were identified by a unique identification number with no link to patient names.

C. trachomatis Culture and Titration of Inclusion-Forming Units

Confluent monolayers of McCoy cells were inoculated with reference and clinical strains by centrifugation at 550 × *g* for 1 h at 35°C. Cultures were maintained at 37°C and 5% CO_2 in chlamydial growth medium (CMGH), which contains minimal essential medium (MEM; Cellgro, Mannassas, VA, USA]; 10% fetal bovine serum (FBS; University of California, San Francisco [UCSF] Cell Culture Facility, San Francisco, CA, USA); 0.45% glucose solution (Cellgro); 20 mmol/L HEPES (UCSF Cell Culture Facility); 0.08% $NaHCO_3$, 10 μg/mL gentamicin (MP Biomedicals, Solon, OH, USA); 25 μg/mL vancomycin (Acros Organics, Morris Plains, NJ, USA); 25 units/mL nystatin (MP Biomedicals), 375 μg/mL amphotericin B (PharmaTek, Huntington, NY, USA); 1 μg/mL cyclohexamide for 48 h and harvested as described (*18*,*19*). Inclusion-forming units (IFUs) were titrated after 30–48 h of growth, depending on the strain, by using chlamydial lipopolysaccharide (LPS)–specific monoclonal antibodies (LPS-MAbs; Virostat, Portland, ME, USA) (*2*,*18*).

Plaque Assay for Reference Strains and Clinical Samples

We modified the plaque assay of Matsumoto et al. (*16*) by using low speed centrifugation at 550 × *g* and 6-well plates for infections, and 1-dram shell vials (Kimble Chase Inc., Vineland, NJ, USA) for propagation. To ensure detection of mixed infections, 1:3 and 1:1 ratios of IFUs for reference strains E/Bour and D/UW-3, and a 1:1 ratio for clinical strains F and G, were created for inoculation and harvest.

Reference and clinical strains were serially diluted in sucrose-phosphate-glutamine (SPG) (219 mmol/L sucrose; 3.82 mmol/L KH_2PO_4; 8.59 mmol/L Na_2HPO_4; 4.26 mmol/L glutamic acid; 10 μg/mL gentamicin; 100 μg/mL vancomycin; 25 U/mL nystatin in distilled water, pH 7.4). Each 6-well plate contained dilutions from 1.25×10^6 IFUs in the 1st well to 1.25 × 10 IFUs on 60%–70% confluent McCoy cell monolayers. Two 6-well plates were prepared identically per strain except that the second plate contained a glass coverslip in each well. After centrifugation, the inocula were removed and replaced with CMGH plus 1 μg/mL cyclohexamide and maintained at 37°C in 5% CO_2.

At 24 h postinfection (p.i.), culture medium was aspirated, and wells were overlaid with initial agarose (IAO: 0.5% SeaKem ME agarose [BMA, Rockland, ME, USA]) in phenol red–free MEM (BioWhittaker, Walkersville, MD, USA); 10% FBS; 1 μg/mL cyclohexamide. Two milliliters of CMGH without cyclohexamide were added to the solidified IAO. Medium was replaced every 4 days to optimize chlamydial growth.

Once small plaques formed by visual inspection at 7–12 days p.i., medium was removed, and final agarose overlay (FAO: 0.5% SeaKem ME agarose in phenol red–free MEM; 10% FBS; 1/100 volume of 3% neutral red [Sigma-Aldrich, St. Louis, MO, USA]) was dispensed onto IAO. CMGH, without cyclohexamide, was added, and the plates were incubated for 12–24 h.

At 48 h, duplicate plates with coverslips were fixed with methanol and stained with a fluorescein isothiocyanate (FITC)–conjugated *C. trachomatis* LPS-MAbs (*18*). Inclusions on each cover slip were counted to determine IFUs per milliliter per well and efficacy of infection given the calculated IFUs inoculated for each well.

Plaques were visualized as a central area of cellular debris surrounded by viable infected cells with red staining of cytoplasm at the cell periphery (Figure 1, panels A and C). Inclusion bodies and nonviable cells remained clear. Any plaque (≈1–2 mm) that was clearly isolated from another plaque, or appeared as a solitary plaque in a well, was se-

Table 1. Results of the modified plaque assay for *Chlamydia* reference strains and shotgun cell culture harvest technique for clinical strains representing acute and persistent infections*

Strain	Days p.i. to plaque formation or harvest	No. plaques or shotgun harvested areas	*ompA* genotype (no.)	Location of nucleotide substitutions in *ompA* (amino acid substitution location)	16S rRNA (no.)
Reference D/UW-3 and E/Bour 1:1†	9	25	D (13)	–	*Chlamydia trachomatis* D (13)
			E (9)	–	*C. trachomatis* E (9)
			D/E (3)	–	*C. trachomatis* D/E (3)
Reference D/UW-3 and E/Bour 3:‡	9	9	D (9)	–	*C. trachomatis* D (9)
			E (0)	–	*C. trachomatis* E (0)
D/UW-3/E/Bour mixed infection§	9	11	D (7)		*C. trachomatis* D (7)
			E (4)		*C. trachomatis* E (4)
Acute clinical Ja	8	11	Ja (11)	–	*C. trachomatis* Ja (11)
Acute clinical K	10	11	K (11)	–	*C. trachomatis* K (11)
Persistent H	7	5	H (5)	–	*C. trachomatis* H (5)
Persistent G	14	7	G (7)	–	*C. trachomatis* G (7)
Persistent F	10	5	F (5)	–	*C. trachomatis* F (5)
Persistent clinical F and G strains 1:1†	10	13	F (1)	–	*C. trachomatis* F (1)
			G (12)	–	*C. trachomatis* G (12)
A/SA-1	10	18	A (14)	–	*C. trachomatis* A (14)
			Chlamydophila abortus (4)	–	*C. abortus* (4)
	10¶	21	A/*C. abortus* (21)		*C. trachomatis* A/*C. abortus* (21)
B/TW-5	9	15	B (15)	–	*C. trachomatis* B (15)
Ba/Apache-2	8	14	Ba (9)	–	*C. trachomatis* Ba (14)
			Ba_1 (1)	**C662T (P221L)**	
			Ba_2 (1)	**C662T (P221L)** **G673A (E225K)**	
			Ba_3 (1)	**C662T (P221L)** A717C (K239N)	
C/TW-3	12	13	C (13)	–	*C. trachomatis* C (13)
D/UW-3	9	11	D (11)	–	*C. trachomatis* D (11)
Da/TW-448	10	12	Da (12)	–	*C. trachomatis* Da (12)
E/Bour	9	9	E (9)	–	*C. trachomatis* E (9)
F/IC-Cal3	9	13	F (10)	–	*C. trachomatis* F (13)
			F-III (3)	G269A (G90**E**)#	
G/UW57/Cx	7	10	G (10)	–	*C. trachomatis* G (10)
H/UW-4	12	12	H (10)	–	*C. trachomatis* H(10)
I/UW-12	11	14	I (14)	–	*C. trachomatis* I (14)
Ia/IU-4168	12	11	Ia (12)	–	*C. trachomatis* Ia (12)
J/UW-36	11	15	J (15)	–	*C. trachomatis* J (15)
Ja/UW-92	12	12	Ja (12)	–	*C. trachomatis* Ja (12)
K/UW-31	11	13	K (13)	–	*C. trachomatis* K (13)
L_1/440	9	11	L_1 (11)	–	*C. trachomatis* L_1 (11)
L_2/434	9	11	L_2 (8)	–	*C. trachomatis* L_2 (10)
			L_2' (3)	C471G, G496A (A166T)¶	*C. trachomatis* L_2a (1)
L_2a/TW-396	9	13	L_2a (13)	–	*C. trachomatis* L_2 (13)
L_3/404	9	14	L_3 (14)	–	*C. trachomatis* L_3 (14)

*p.i.,.postinoculation; ompA, outer membrane protein A; –, no evidence for nucleotide substitution.
†Represents an equal mixture of inclusion-forming units (IFUs) of each strain.
‡Represents a 3:1 mixture of IFUs of reference strains D/UW-3 and E/Bour.
§Represents the plaques from the 1:1 mixture of D/UW-3 and E/Bour when both reference strains were found in 3 plaques.
¶Represents the plaques that were harvested from the wells with 10^{-8} dilution.
#**Boldface** denotes nonconservative amino acid substitution (*17*).

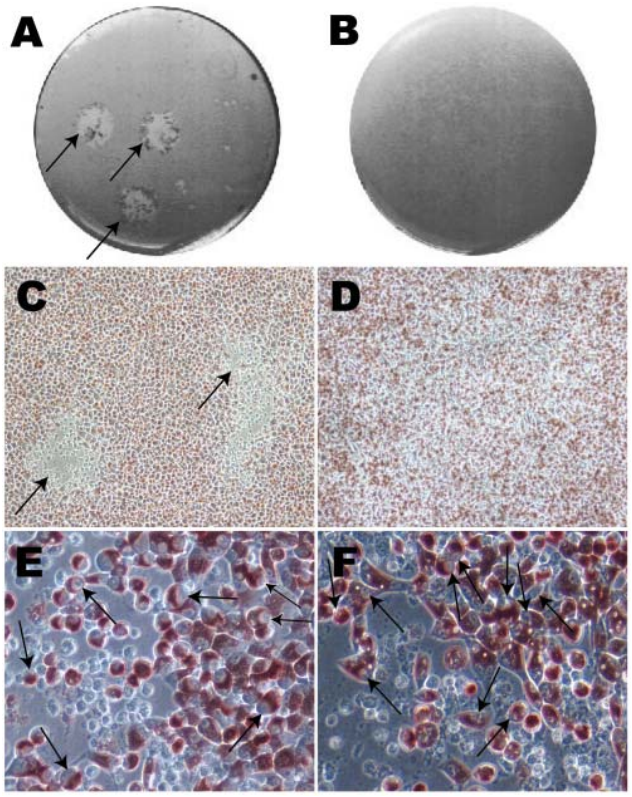

Figure 1. Photographs and optical microscopy views of the wells showing plaques formed by *Chlamydia trachomatis* F/IC-Cal3 (A, C, E) and no plaque formed by clinical F persistent strain (B, D, F). A) Single well showing 2 distinct plaques (indicated by arrows). B) Well showing no plaque morphology. C) and D) Optical microscopy image showing plaque areas with little or no neutral red staining (arrows) surrounded by viable cells stained red (magnification ×100). Higher magnification (×400) showed numerous cells that had been infected by reference strain F/IC-Cal3 (E) and the clinical persistent F strain (F).

lected. A blunt-ended transfer pipette was used to punch a hole ≈1–2 mm in diameter through the gels over the plaque. The contents were placed into a microcentrifuge tube containing CMGH, sonicated and added to shell vials containing McCoy monolayers for propagation. Centrifugation of shell vials at 2,400 × *g* for 1 h at 35°C was required to successfully grow each clonally segregated strain. Strains were propagated and purified using gradient ultracentrifugation as previously described (*2*,*18*–*20*). The pellet was resuspended in SPG, and stored at –80°C.

Shotgun Harvest, Isolation, and Propagation of Single Clonal Populations for Clinical Strains

Because no visible plaques formed for the clinical strains, except for clinical H, the plates were inspected under 100× and 400× light microscopy. Wells were selected for our shotgun harvest as shown in the diagram (Figure 2). Ten spots per well were numbered where the infections were observed under microscopy. Each spot was harvested (≈2–3 wells × 10 spots per well = 20–30 harvests) using a sterile, blunt-ended transfer pipette.

IAO and FAO were carefully removed, and the wells were stained using FITC-conjugated *C. trachomatis* LPS-MAb (Virostat). Only harvested areas that corresponded to a confined group of infected cells with a clear margin from uninfected cells were selected, sonicated, inoculated, propagated in shell vials and flasks purified and stored as above. The original clinical samples were also independently propagated in shell vials as described previously (*2*,*18*–*20*) for comparison with growth in the plaque assay.

Preparation of Genomic DNA and Sequencing of *ompA* and 16S rRNA for Each Clone

Purified culture was used for genomic DNA extraction according to High Pure Template Preparation Kit package insert (Roche Diagnostics**,** Indianapolis, IN, USA). PCR was performed and reagents, thermocycling profile, and sequencing were used according to previously described protocols (*21*). Table 2 (*22*) shows the primers used for PCR and sequencing to identify the strain-type of each clone. Multiple sequences were aligned by using MegAlign software (DNASTAR, Madison, WI, USA) and compared with public sequences (*21*,*23*). A variant was defined as having ≥1 nucleotide difference(s) from the sequence of the reference strain for either *ompA* or 16S rRNA genes.

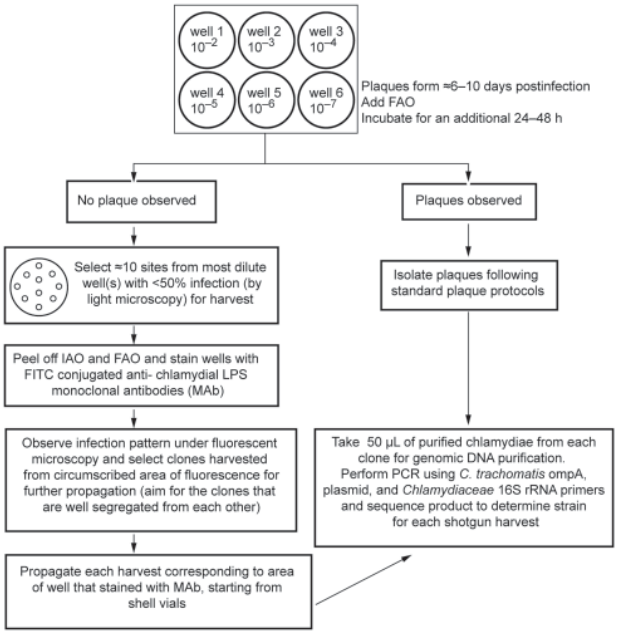

Figure 2. Diagram of the cell culture and shotgun harvest assay for *Chlamydia trachomatis* clinical strains propagated in McCoy cells. Serial dilutions of each clinical sample were used for inoculating wells 1 to 6. IAO, initial agarose overlay; FAO, final agarose overlay.

Table 2. PCR and sequencing primers used for determining strain types of clonal isolates from reference strains and clinical samples

Primer	Sequence (5′→3′)	Location	Ref
CTompA-F	GTCCCGCCAGAAAAAGATAG	–60 to –41	This study
CTompA-seqF	ATAGCGAGCACAAAGAGAGC	–44 to –25	This study
VB3	CATCGTAGTCAATAGAGGCAT	817 to 797	(*22*)
MVF3	TGTAAAACGACGGCCAGTGCCCGTGCAGCTTT	561 to 611	(*22*)
CTompA-B	ACGGATAGTGTTATTAACAAAGAT	1261 to 1225	This study
CTompA-seqB	GTAAAACGACGGCCAGT	562 to 596	This study
C16SrRNA-F	CAGTCGAGAATCTTTCGCAAT	359 to 380	This study
C16SrRNA-seqF	AAGGCTCTAGGGTTGTAAAGCACTTT	419 to 444	This study
C16SrRNA-B	TACTGGCCATTGTAGCACGTGTGT	1230 to 1253	This study
Plasmid-PF5	AGACTTGGTCATAATGGACTT	1022 to 1002	This study
Plasmid-seqPF5	AGACTTGGTCATAATGGACTT	1022 to 1002	This study
Plasmid-PB5	TTGTCTCGGATTTTAAAAAATGT	588 to 566	This study
FCabortus	GGTATGTTTAGGCATCTAAAA	172 to 192	This study
RCabortus2	GGCCATTGTAGCACGTGTGTA	1248 to 1228	This study

Phylogenetic Construction of *ompA* Nucleotide and Amino Acid Sequence Alignments

Nucleotide and amino acid alignments and phylogenetic analyses of the 19 reference strains and clonal variants were performed by using MEGA 3.1 (Center for Evolutionary Functional Genomics, Tempe, AZ, USA) as described (*21*,*23*). Briefly, neighbor-joining trees were calculated using the Kimura 2-parameter model that assumes that nucleotide frequencies and rates of substitution do not vary among sites. For amino acids, neighbor-joining trees were calculated using the gamma distance model that considers the dissimilarity of substitution rates among sites. We used bootstrap analysis (1,000 replicates) to determine confidence intervals for each branch.

Results

Plaque Formation by Reference *C. trachomatis* Strains

Figure 1, panel A, shows typical plaque formation for F/IC-Cal3. Higher inocula resulted in plaques that fused and, therefore, were not suitable for harvest. These findings are similar to those of others who have used plaque- or focus-forming assays for clonal segregation of laboratory-adapted chlamydial strains (*16*,*24*).

Table 1 shows the day p.i. that plaques were visualized and the number of isolated clones and nucleotide polymorphisms with respect to reference strain sequences. All reference strains formed mature plaques ≈1–2 mm in diameter. Experimentally mixed infections of D/UW-3 and E/Bour resulted in 13 D, 9 E and 3 D/E clones, and 9 D and 0 E clones. The 3 D/E clones were identified as mixed based on electropherograms where 2 peaks were observed in a single nucleotide position that corresponded to D and E sequences for ≈20 nucleotide positions. These 3 mixed infections were further plaque-purified as above and yielded single clonal populations of D or E, which validated our plaque assay for isolating clonal populations.

Detection of Clonal Populations of *C. trachomatis* Clinical Strains by Shotgun Harvest

The clinical strains Ja, K, F, and G showed no plaques, while persistent strain K showed signs of <0.5-mm plaques at 10 days p.i., and strain H showed typical plaques at 7 days p.i. A longer growth period up to 20 days p.i. did not result in distinct plaque formation for Ja, K, F, or G. Figure 1, panels B and D, show a well with an inoculum of 1.25×10^3 at day 10 p.i. where no plaques were visualized.

Approximately 20–30 regions of 2 mm each (Figure 3, panel A) from 2–3 wells per isolate were harvested for each of the clinical strains after light microscopic examination (Figure 3, panels B and C). On the basis of fluorescent microscopic examination of the wells after removal of the agarose layers and staining with FITC-conjugated LPS-MAb to detect segregated areas of chlamydial infection (Figure 3, panel D), 11 clinical Ja, 11 clinical K, 5 clinical H, 5 clinical F, and 7 clinical G harvests were selected for propagation.

Notably, the size of the inclusion body was much smaller for clinical strains than for reference strains. Figure 1, panel E shows typical large inclusion bodies formed by reference F/IC-Cal3 compared with tiny and occasional medium-sized inclusion bodies at day 10 for persistent clinical strain F (Figure 1, panel F). Similar results were observed for persistent clinical strains G and H when compared with respective reference strains. In contrast, acute clinical strains Ja and K had inclusion bodies that were intermediate in size (data not shown). When original clinical samples were propagated in shell vials, inclusions remained small and the rate of growth was similar as for the plaque assay.

Mixed clinical G and F strains yielded 12 G clones (92.31%), 1 F clone (7.69%), and no mixed clones based on sequencing. Figure 3, panel A, represents 1 well after 10 random areas were harvested since no plaque was visible (Figure 3, panel B). Figure 3, panel C, represents a microscope photo where chlamydial inclusions are difficult to visualize due to their small size. Figure 3, panel D, is a

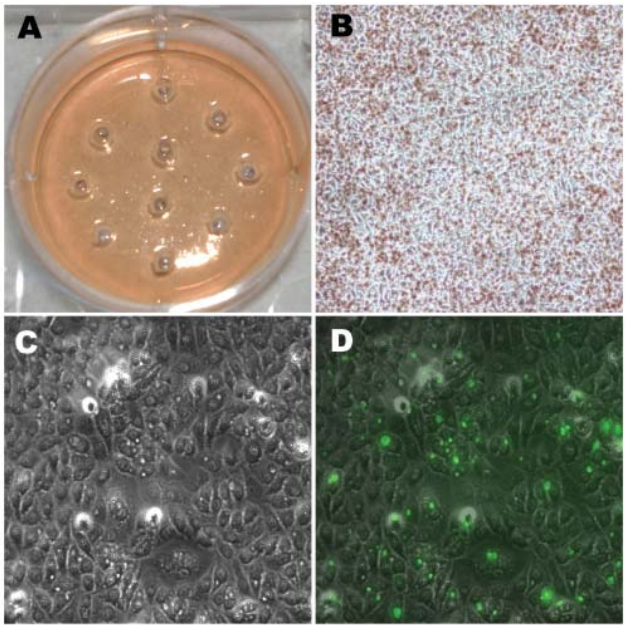

Figure 3. Photographs of light and fluorescent microscopy showing the shotgun cell culture harvest method for isolating clonal populations of clinically persistant strains. A) 1 well in a 6-well plate after harvesting 10 random areas using sterile pipettes; no plaques could be visualized in any well unlike the situation for the reference strains in Figure 2. B) The same well before harvesting infected areas (magnification ×100). C) Light microscopy view of an area with infected cells containing small inclusion bodies after agarose overlays had been removed (magnification ×400). D) Fluorescent microscopy view of the same field as in C (magnification ×400); infected cells were stained with *Chlamydia trachomatis*–specific lipopolysaccharide antibodies. Arrows denote small fluorescing inclusion bodies within the cell cytoplasm.

fluorescent image of Figure 3, panel C, displaying small and medium-sized inclusion bodies.

Sequence Analyses of *ompA* and 16S rRNA *C. trachomatis* Clonal Populations

A total of 30 chlamydial *ompA* genotypes were identified from the plaque assay and shotgun harvest based on sequence analyses using BLAST and MegAlign as we have described (*21*,*22*) (Table 1). Three reference strains showed mixed infections: Ba/Apache-2 with new Ba *ompA* genotypes, Ba_1, Ba_2, and Ba_3 (1 clone each), F/IC-Cal3 with F-III (*5*), L_2/434 with L_2' (*9*), and A/SA-1 with *C. abortus* S26/3 (*25*), which were clonally segregated into 14 A and 4 *C. abortus* clones (Table 1). All 4 *C. abortus* clones had the same sequences for *ompA* and 16S rRNA.

The *C. abortus*–specific primers (Table 2) were used to amplify another seed stock of A/SA-1, *C. abortus* (from our plaque assay), *C. trachomatis* strain A/Har-13, and *C. caviae* and *C. muridanum*. For the *C. abortus*–specific PCR amplification, only A/SA-1 and *C. abortus* samples were positive, while the rest were negative.

Phylogenetic Analyses of *ompA C. trachomatis* Reference and Clonal Populations

Phylogenetics of *ompA* nucleotide and amino acid sequence alignments were performed to evaluate divergence of 5 clonal variants of Ba/Apache-2 and F/IC-Cal3. The trees showed the clustering of the 5 clonal variants with their respective parental strains (Figure 4, panels A and B).

Discussion

Plaque- and focus-forming assays have been developed to isolate individual clonal populations of reference strains of *C. trachomatis* (*12*,*15*,*16*) and *Chlamydophila pneumoniae* (*24*). The first methods for *C. trachomatis* used L (*15*) and McCoy cells (*16*). More recently, flow cytometry has been used to segregate cells infected with *C. trachomatis*, *C. caviae*, and *Chlamydia suis* (*12*). However, these techniques have focused on laboratory-adapted clinical and reference strains and have not used nonpropagated or nonlaboratory-adapted clinical samples.

The novel shotgun harvest assay that we developed was successful in segregating clonal populations of *C. trachomatis* strains and variants that were devoid of plaque-forming characteristics. Consequently, our method is an important advance in reliably detecting and purifying clonal isolates from clinical samples. We also modified the plaque protocol of Matsumoto et al. (*16*), which allowed us to use lower concentrations of reference strains, ensuring widely separated or single plaques. Most important, our methods showed sample collections that contained mixtures of new and emerging strains and variants based on *ompA* and 16S rRNA sequences.

The most remarkable mixed infection was for reference strain A/SA-1 in which *C. abortus* was identified. *C. abortus* was not a likely contaminant because A/SA-1 was an original isolate**.** PCR of another seed stock of A/SA-1 was positive for *C. abortus*, and *C. abortus* had not previously been propagated in our laboratory. The original sample was obtained from the conjunctiva of a trachoma patient in Saudi Arabia in 1957. This finding was unexpected because *C. abortus* has not been described among trachoma-endemic populations. Although *C. abortus* may be responsible for zoonoses in pregnant women, it resides in a unique niche, the placenta, compared with *C. trachomatis* (*26*). Thus, an explanation for our findings is that *C. abortus* is now capable of crossing species or niche barriers. Indeed, we recently identified mixed conjunctival infections with *C. trachomatis*, *Chlamydophila psittaci*, and/or *C. pneumoniae* in 35% of infected persons residing in a trachoma-endemic region of Nepal (*27*). The findings were statistically

unlikely to have occurred by chance. Additionally, infection with *C. pneumoniae* or *C. psittaci* was significantly associated with trachomatous inflammation, a precursor for scarring. With mounting evidence for widespread interstrain recombination among intracellular bacteria such as *Chlamydiaceae* (*8,10,21–23,28*), the A/SA1 coinfection with *C. abortus* along with those described above are likely the tip of the iceberg in terms of the prevalence of mixed *Chlamydiaceae* infections and the possibility for recombination that may result in diverged tissue tropism (*21,23*). We are currently examining samples from other trachoma-endemic populations for coinfection with *C. abortus* and other *Chlamydiaceae* species.

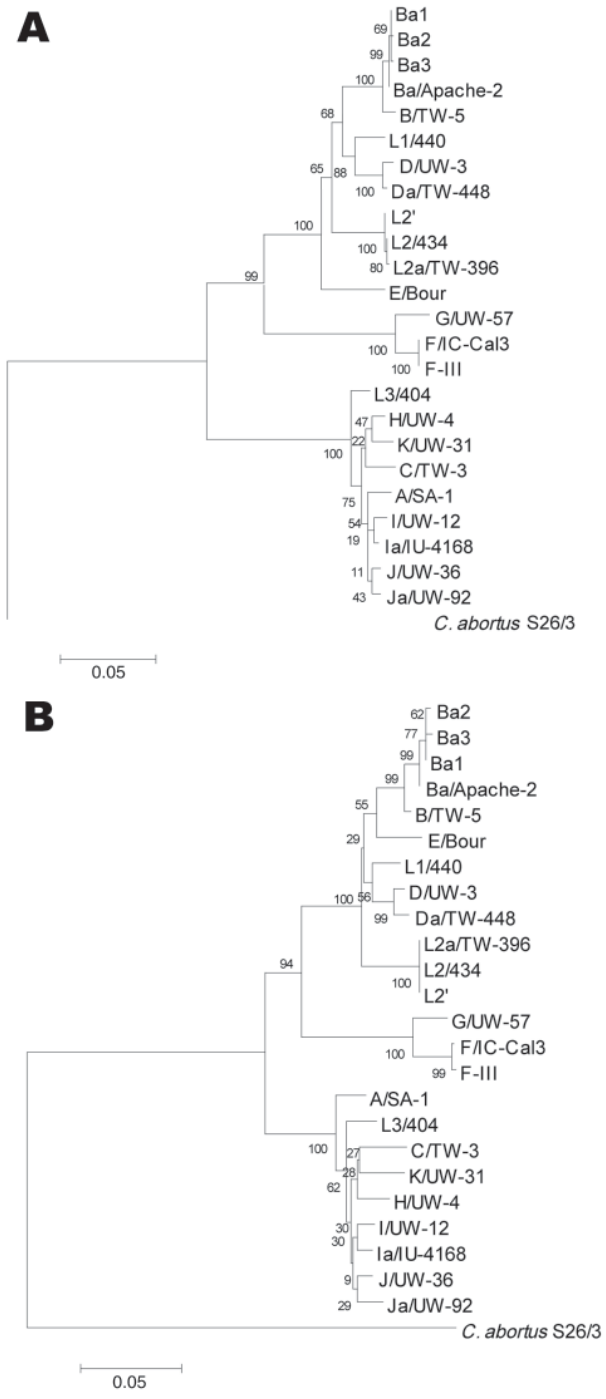

Figure 4. Neighbor-joining trees representing evolutionary relatedness of the 19 reference strains, *Chlamydophila abortus* and 5 clonal variants based on *ompA* nucleotide (A) and amino acid (B) sequence alignments. The trees were constructed from ClustalW 1.8 alignment (www.ebi.ac.uk/Tools/clustalw2/index.html), and the values at the nodes are the bootstrap confidence levels calculated from 1,000 bootstrap resamplings. See Materials and Methods for details.

Reference strain Ba/Apache-2 also comprised clonal populations of 3 previously unrecognized *ompA* genotypes, Ba_1, Ba_2 and Ba_3, that were distinct from publicly available Ba *ompA* sequences (*6,7,29*). The C662T mutation among our clones encoded a nonsynonymous P221L substitution in a constant region (CR) between variable segments (VSs) II and VSIII of MOMP, which contains 5 CRs and 4 VSs. This change from a proline, an imino amino acid with unique "kink," to a nonpolar leucine on CRIII might disrupt the mid-portion ß-strand transmembrane of MOMP (*30–32*). Furthermore, the E225K in Ba_2 occurs in VSIII where the subspecies-specific epitope for LGV and A–K strains (*32*) is located, likely changing polarity of the epitope from a negative to a positive charge. These mutations, then, may lead to adaptive structural and/or functional changes for MOMP.

The presence of mutations in Ba_1, Ba_2, and Ba_3 suggests that these have occurred under immune selection in vivo, because growing reference strains in vitro has not shown detectable mutations (*3,14*), although in theory this could occur. On the basis of phylogenetic reconstructions (Figure 4), the clonal variants likely represent natural diversity arising from the respective parental strain. Also, the ability of Ba strains to either mutate specific protein regions or recombine may facilitate their invasion of other mucosal sites. Urogenital Ba infections do occur, and we have previously described a Ba/D recombinant that was isolated from the genital tract (*8*).

Notably, most of the *ompA* mutations were located within CRs and encoded for nonsynonymous substitutions, the majority of which encoded for nonconservative amino acids with altered properties. For instance, *ompA* genotype F-III contains a nonconservative G90E substitution. G90E is located in CRII next to VSI, which may decrease membrane hydrophobicity and disrupt the >0.5 nonpolar or hydrophobic index requirement for the MOMP spanning region (*32*). In 2 separate studies, we identified F-III variants as statistically significantly associated with pelvic inflammatory disease (PID) (*5,33*). The F-III mutation may explain, in part, the association with PID. However, additional studies will be required to delineate these associations.

In our experimentally mixed infections, recovery of separate clones of D/UW-3 and E/Bour, and of clinical G and F validated each assay (Table 1). The greater number of clones for D/UW-3 (52%) than for E/Bour (36%), and for G (92.31%) than for F (7.69%) might indicate different growth rates and timelines for plaque formation and characteristics of each strain (*15*,*16*). It is also possible that 1 strain produces byproducts of growth that are inhibitory for coinfecting strains. Nevertheless, these data emphasize the importance of selecting multiple wells of low inocula for plaque or shotgun harvests to identify all strains that are present. Additionally, mixed infections may occur where some strains cause plaque formation and others do not, which stresses the importance of the shotgun harvest even when the morphologic features of plaque are present.

In the present study, we analyzed clones by sequencing *ompA*, the plasmid, and 16S rRNA to enhance strain categorization. The plasmid was evaluated because its absence has been reported to correlate with reduced or no plaque formation (*34*). However, all of our clones contained the plasmid, which is consistent with other studies (*35*–*37*). The lack of classic plaque formation for clinical isolates likely stems from their slow growth and lack of adaptation to conventional cell culture. This was borne out by their slow growth in shell vials and flasks, experiments which were performed separately from the plaque assay. Clinical strains may exit the cell without cellular disruption, facilitating subsequent rounds of infection and lack of plaque formation. Beatty recently showed,that EBs could be released without lysis and also be retained by host cells (*38*). However, our clinical H formed plaques similar in morphology to reference strains. The presence of a complete toxin gene, as in H/UW-4 and J/UW-36 (*39*), may have contributed to clinical H plaque formation. H/UW-4 has been shown to produce more cytotoxicity than D/UW-3, which contains a partial toxin gene, and *C. muridanum*, which contains a full-length gene (*40*). Although all 19 reference strains formed classic plaques morphology, some have no toxin (LGV strains) or a partial gene, which suggests that plaque formation reflects adaptation to culture that has occurred over decades instead of the effects of the toxin. Further experiments will be required to determine the genetic factors involved in plaque formation.

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Molecular Epidemiology of Eastern Equine Encephalitis Virus, New York

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Perpetuation, overwintering, and extinction of eastern equine encephalitis virus (EEEV) in northern foci are poorly understood. We therefore sought to describe the molecular epidemiology of EEEV in New York State during current and past epizootics. To determine whether EEEV overwinters, is periodically reintroduced, or both, we sequenced the E2 and partial NSP3 coding regions of 42 EEEV isolates from New York State and the Eastern Seaboard of the United States. Our phylogenetic analyses indicated that derived subclades tended to contain southern strains that had been isolated before genetically similar northern strains, suggesting southern to northern migration of EEEV along the Eastern Seaboard. Strong clustering among strains isolated during epizootics in New York from 2003–2005, as well as from 1974–1975, demonstrates that EEEV has overwintered in this focus. This study provides molecular evidence for the introduction of southern EEEV strains to New York, followed by local amplification, perpetuation, and overwintering.

Eastern equine encephalitis virus (EEEV; genus *Alphavirus*: family *Togaviridae*) is maintained in an enzootic cycle between ornithophilic mosquitoes and birds. The virus causes disease in some avian hosts and in incidental hosts, such as horses and humans; case-fatality rate in humans is ≈33% (*1*). Virus activity has been detected in North and South America. In the United States, EEEV has been detected along the Gulf of Mexico and the Atlantic Seaboard as well as in inland foci near the Great Lakes, including upstate New York. The EEEV virion contains a single-stranded, positive-sense RNA genome of ≈12 kb. The 5′ end of the genome encodes 4 nonstructural proteins: NSP1, NSP2, NSP3, and NSP4. The structural proteins are encoded in the 3′ third of the genome and are translated from a subgenomic RNA, 26S, resulting in 5 protein products: C, 6K, E1, E2, and E3 (*2*). Previous sequencing studies analyzed genetic relationships of EEEV strains in the Western Hemisphere and compared strains distributed across widespread geographic regions (*3*–*6*). EEEV has 4 distinct genetic lineages; lineage I consists of highly conserved strains from North America, and lineages II–IV encompass strains from Central and South America (*3*).

Outbreaks of EEEV in New York have been observed periodically since 1952, when the virus was first detected in pheasants (*7*). Disease in humans and/or horses has been noted on Long Island, in the lower Hudson Valley, and in central upstate New York; the last known human case in New York occurred in 1983 in Onondaga County (*8*). Most EEEV activity in New York has occurred in counties bordering Oneida Lake in central upstate New York (Figure 1). Most of the activity in this region has been concentrated in the Big Bay–Toad Harbor Swamp complex in Oswego County and Cicero Swamp in Onondaga County (*8*). *Culiseta melanura* (Coquillett), the main enzootic vector of EEEV, breeds abundantly in these swamps (*9*). Localized epizootics in the counties of Oswego and Onondaga have been documented in a transmission focus during 1971–1977, 1982–1983, and 1990–1991 (*8,10–13*) and from 2003 to the present (2007; D.S. Young et al., unpub. data). Between these epizootic periods, EEEV was undetectable in horses and birds and only infrequently detected in mosquito pools (D.S. Young et al., unpub. data) (*8*). From 1992 through 1997 in upstate New York, EEEV was detected in 18 mosquito pools from Onondaga County (1994) and 3 mosquito pools from Oswego County (1996); no equine or avian cases were detected (D.S. Young et al., unpub. data). From 1998 through 2002, EEEV was not detected in

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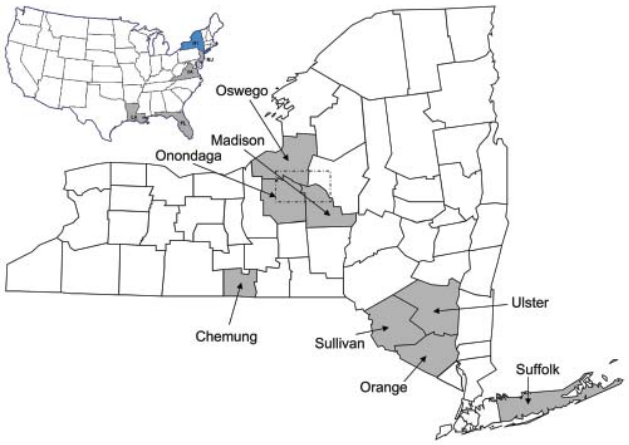

Figure 1. New York counties where eastern equine encephalitis virus (EEEV) strains have been located (shaded). Dotted box indicates focus of most EEEV activity. Inset shows locations of eastern equine encephalitis virus strains sequenced in this study. New York State (NY) highlighted in blue; New Jersey (NJ), Virginia (VA), Florida (FL), Louisiana (LA) highlighted in gray. US map courtesy of www.theodora.com/maps, used with permission.

mosquitoes or vertebrates in New York. However, in 2003, EEEV activity increased across New York with the emergence of the current epizootic (2003–2007) in the Onondaga and Oswego Counties region.

Patterns of localized perpetuation, overwintering, and extinction of EEEV in transmission foci are poorly understood. To determine whether EEEV overwinters locally in temperate regions such as upstate New York or whether annual reintroduction is required to reinitiate the transmission cycle, we compared nucleotide sequences comprising the entire E2 coding region and part of the NSP3 coding region. We examined 35 strains isolated in New York during 1971–1975 and 2003–2005 and 7 strains collected along the Eastern Seaboard of the United States during 2002–2003. Using these data, we described the molecular epidemiology of EEEV strains collected during the current and past epizootics in New York.

Materials and Methods

EEEV Detection and Isolation

Isolates from New York State and the Eastern Seaboard were sequenced for this study (online Appendix Table, available from www.cdc.gov/EID/content/14/3/454-appT.htm). Strains originating outside of New York were isolated from avian serum samples, which were collected during a study conducted by the US Geological Survey and stored at –80°C until inoculation onto cell culture. All EEEV strains from within New York were collected from mosquito, avian, and equine samples that were submitted to the Wadsworth Center's Arbovirus Laboratories as a part of surveillance efforts by the New York State Department of Health. EEEV strains isolated during 1971–1975 were obtained from our archives.

To obtain mosquito-derived EEEV strains, mosquitoes were collected from May through October by local county health department staff, who used standard miniature light or gravid traps. Mosquitoes were identified to the species level, and pooled samples of 10–50 mosquitoes in 2-mL microfuge tubes were submitted to the Arbovirus Laboratories for analysis. Tubes contained a steel ball-bearing (Daisy Brand, Rogers, AR, USA), and to each tube we added 1 mL of mosquito diluent (20% heat-inactivated fetal bovine serum [FBS] in Dulbecco's phosphate-buffered saline with 50 μg/mL penicillin/streptomycin, 50 μg/mL gentamicin, and 2.5 μg/mL amphotericin B). Pools were homogenized by using a mixer mill (Retsch, Haan, Germany) at 24 cycles/s for 30 s and centrifuged for 4 min at 6,000 rpm at room temperature. The clarified homogenate was transferred to a new microcentrifuge tube and stored at –80°C until testing.

To obtain vertebrate-derived EEEV strains, samples of horse brains were submitted by the Wadsworth Center's Rabies Laboratory, and samples of avian kidney, heart, and brain were submitted by the New York State Department of Environmental Conservation Wildlife Pathology Unit. Each horse brain was excised into 3 separate 1–3 mm^3 sections, which were pooled for testing for each horse. Avian tissues were tested by excising the same-size portion from 1 of the tissues or by pooling sections of all 3 tissues. Excised tissues were placed in 2-mL microfuge tubes containing a ball-bearing and 1 mL of BA-1 virus diluent (M199 with Hanks' salts and L-glutamine; [Mediatech, Herndon, VA, USA] in sterile distilled water with 0.05 M Tris(hydroxymethyl)aminomethane, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/mL penicillin, 100 U/mL streptomycin, 1 μg/mL amphotericin B, and 20% FBS). Hydrogen chloride was added to the diluent to bring the pH to 7.4. Samples were homogenized on the mixer mill at 24 cycles/s for 4 min and centrifuged for 5–8 min at 10,000 rpm at 4°C.

Virus was isolated by inoculating 100 mL of supernatant from mosquito pools, vertebrate tissues, or avian serum onto confluent monolayers of African green monkey kidney (Vero) cells grown in 6-well plates. Plates were incubated for 1 h at 37°C in 5% carbon dioxide, with gentle rocking every 15 min. To each well, 3 mL of maintenance medium (1× minimum essential medium with Earle's salts [Invitrogen, Carlsbad, CA, USA], 2% heat-inactivated FBS, 1% 100× L-glutamine, 0.15% sodium bicarbonate, 1% penicillin–streptomycin, 0.1% amphotericin B, and 0.1% gentamicin diluted in sterile distilled water) was added, and plates were returned to the incubator and observed

daily. If cytopathic effect was observed, the infecting virus was identified by either immunofluorescence assay (*14*) or reverse transcription PCR (RT-PCR) by using One-Step RT-PCR kit and protocol (QIAGEN, Valencia, CA, USA) as directed by the manufacturer. Primer sequences and cycling parameters are described elsewhere (*15*). Target bands were examined under UV light after electrophoresis on a 1.5% agarose gel. Samples that were positive for EEEV were stored at –80°C until use.

RNA Extraction and RT-PCR

RNA was extracted from the Vero cell culture supernatant after a single passage, the original sample (no passage), or isolates with an unknown passage history by using the RNeasy kit (QIAGEN) as directed by the manufacturer. The entire E2 coding region was amplified in 2 separate reactions. To produce overlapping fragments, primers EEE8460 (5′-AGAATCCACACGAAACACTCACCA-3′) and EEE9200c (5′-ATCCGTGCAGGTGGTTGTATGGTC-3′) were used for the first reaction, and primers EEE9105 (5′-TCCACAGTGCCAAGGTGAAAA-3′) and EEE9887c (5′-CTGCAAGTGGGATAAGCGTCTG-3′) were used for the second reaction. The partial NSP3 coding region was amplified by using primers EEE4836 (5′-CAGAGCGAGTTTACAGATTACG-3′) and EEE5477c (5′-AACGGCGAACGACTGAA-3′). Sample RNA (5 μL) was added to 45 μL of One-Step RT-PCR master mix (QIAGEN) prepared according to the manufacturer. Several drops of mineral oil were added on top of each reaction. Samples were reverse transcribed for 30 min at 55°C and heat inactivated at 95°C for 5 min. To eliminate residual RNA, RNase was added to each reaction after reverse transcription. Samples were amplified by PCR according to the following thermocycler conditions: 94°C for 10 min; 39 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s; and 72°C for an additional 10 min. PCR product (40 μL) was added to 4 μL of BlueJuice loading dye (Invitrogen, Carlsbad, CA, USA), and loaded onto an agarose gel containing 0.4 μg/mL of ethidium bromide. DNA underwent electrophoresis and was examined under UV light. Samples were sequenced on either an ABI 3100 or ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the Wadsworth Center Molecular Genetics Core facility. (Sequencing primers are available from the authors upon request.)

Phylogenetic Analysis

Trace files were compiled by using the SeqMan module of Lasergene (DNAstar, Madison, WI, USA), with a minimum of 2-fold base-call redundancy required for all sequences. Consensus sequences for each strain sequenced in this study and reference strains obtained from GenBank were aligned by the ClustalV method (*16*) in the MegAlign module of Lasergene. Two phylogenetic trees were produced for the E2 coding region analysis: the main E2 tree, which included all strains included in this study, and the E2 subset tree, which contained only strains sequenced in this study and only those sequences from GenBank for which both E2 and NSP3 sequence data were available. The main E2 tree was generated by maximum likelihood in PAUP version 4.0b10 (Sinauer Associates, Sunderland, MA, USA) by using the HKY85+G model with relevant parameters estimated from the data. The robustness of the branching pattern was estimated by performing 1,000 neighbor-joining bootstrap replicates under the maximum-likelihood substitution model, also using PAUP; these values are presented on the maximum likelihood tree. The E2 subset and NSP3 trees were generated by neighbor-joining analysis with 1,000 bootstrap replicates by using the Kimura 2-parameter model in MEGA2 (*17*). Nucleotide sequences of newly sequenced strains were deposited in GenBank (see online Appendix Table for accession numbers). Nucleotide diversity (π) and the Tajima D statistics were computed by using DnaSP (*18*).

Results

Virus Strains

We sequenced 42 EEEV strains, which represented various geographical locations, hosts, and isolation dates (online Appendix Table). Of the 42 strains, 29 were from central upstate New York. Of those 29 strains, 13 were isolated during the 1970s epizootic, and the remainder were isolated during the current epizootic. The remaining strains sequenced were from various locations in the southeastern United States (Louisiana, Florida, and Virginia), New Jersey, and New York outside the central upstate focus (counties of Suffolk, Orange, Sullivan, Ulster, and Chemung) (Figure 1).

E2 Phylogenetic Analysis

Phylogenetic analysis of the E2 coding region demonstrated that all isolates sequenced in this study belonged to lineage I (Figure 2), and showed strong spatiotemporal clustering. The 3 strains isolated from Oswego County in 1971 (NY71a, NY71b, and NY71c) clustered together as a result of almost identical E2 coding regions. All 8 strains isolated from Oswego County in 1974 (NY74a–h) and the only strain isolated in 1975 (NY75) grouped together strongly and formed the Oswego74 clade (Figures 2–4). Strong clustering was also evident among strains isolated mainly from Onondaga County during 2003–2005 (the Onondaga03 clade, Figures 2–4). Of the 16 strains in this clade, 13 had identical E2 coding regions (data not shown). Both NY04g and NY04j grouped together and were isolated in close geographic proximity in Sullivan and Ulster Counties, respectively, in the lower Hudson Valley.

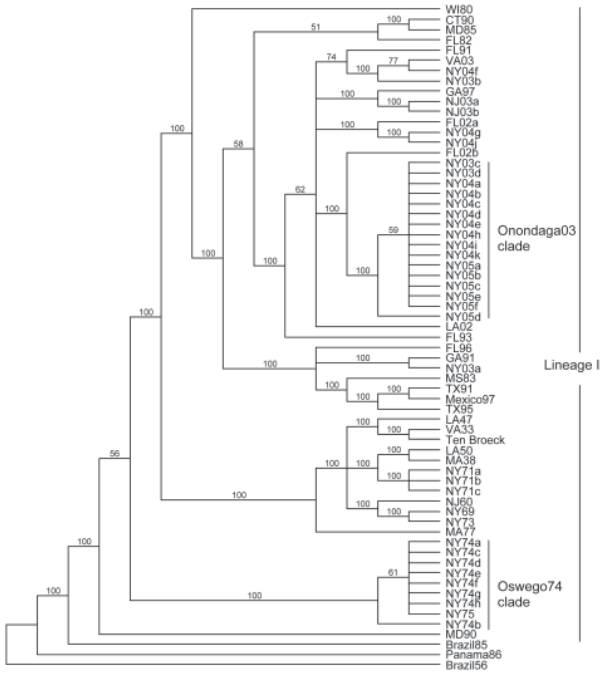

Figure 2. Maximum-likelihood phylogenetic tree of eastern equine encephalitis virus strains, based on the complete E2 coding sequence. Numbers at the nodes indicate bootstrap confidence estimated by 1,000 neighbor-joining replicates on the maximum-likelihood tree. The tree was rooted with lineage II (Brazil56), III (Panama86), and IV (Brazil85) strains.

Sequence Diversity and Phylogenetics

Examination of π, a measure of sequence diversity, confirmed the close relationships of sequences sampled during spatiotemporally defined epizootics. The π values for the Oswego74 and Onondaga03 clades were 0.00035 and 0.00030, respectively. The π value for the entire set of US strains included in our analysis was 0.01058, ≈30× greater than intraepizootic values. The Tajima test failed to reject neutrality in either the Oswego74 or Onondaga03 clade because of extremely low genetic diversity: only a few mutations were present in each grouping. Thus, the EEEV collected during New York epizootics is generally characterized by a high degree of sequence conservation with little genetic variation among spatially and temporally related strains.

However, spatiotemporal conservation was not absolute. NY73, isolated from a horse in Onondaga County in 1973 (NY73) was most genetically similar to NY69, isolated from a pheasant in Suffolk County on Long Island in 1969. Similarly, a strain isolated from a horse in Chemung County, New York (NY04k), fell into the Onondaga03 clade, which further demonstrated occasional relaxation of the otherwise strong time-space clustering of the strains studied. Consideration of strains from outside of New York provided additional instances in which the pattern of spatiotemporal clustering was broken. Well-supported subclades frequently contained southern progenitor strains that had been isolated years before they appeared in New York or New Jersey (Figures 2–4). Examples of this trend include the following: VA03 linked with NY03b and NY04f, GA97 linked with NJ03a and NJ03b, and FL02a linked with NY04g and NY04j. In addition, strain FL02b, isolated from an ovenbird (*Seiurus aurocapillus*) collected in Florida during 2002 was highly similar to the Onondaga03 clade.

To evaluate the possibility that analysis of different coding sequences would yield different results, we studied the NSP3 coding sequences of all strains for which sequence data were available or further sequencing was possible. GenBank sequence data for the NSP3 coding region was limited; therefore, another phylogenetic tree for the E2 coding region was produced by using a subset of lineage I strains for which the NSP3 sequences were available (Figure 3). This E2 subset tree was used for comparisons with the NSP3 tree to determine whether the trees shared similar topology. Phylogenetic analysis of the NSP3 coding region produced similar overall topologies (Figure 4); both trees

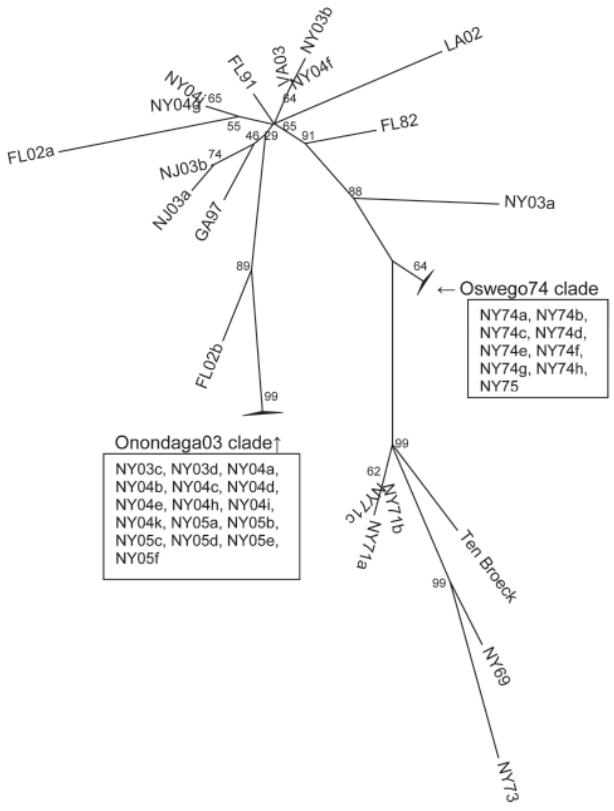

Figure 3. Phylogenetic tree of subset of lineage I eastern equine encephalitis virus strains, unrooted neighbor-joining analysis of E2 coding region. Strains included are identical to those used in the NSP3 coding region analysis.

recognized the major clades (Oswego74 and Onondaga03) and most of the minor subclades.

Discussion

Samples that were newly sequenced for this study were selected to include relevant arthropod and vertebrate hosts (mosquitoes, birds, and horses) and were drawn from archives and ongoing arbovirus surveillance efforts. Additional sequences used in the analyses were obtained from GenBank. Strains collected from the current (2003–2005) epizootic were sequenced from primary field-derived material when possible. This was done to minimize the likelihood that adaptation to tissue culture, which has been observed for other alphaviruses (*19*), could bias our results. Because our analyses did not show strong passage-history–dependent clustering, it seems likely that the sequences from strains passed once in Vero cells are accurate representations of wild-type sequences. Further, strong sequence conservation among strains collected from taxonomically diverse hosts suggests that the source of the virus (mosquitoes or horses) was unlikely to result in sequence changes that might bias our conclusions.

Previous studies provided preliminary evidence that EEEV overwinters in upstate New York (*5*,*20*). The strongest such evidence was derived from RNA sequences and fingerprints that showed strong clustering of 11 strains collected in 1990 and 1991 (*5*). It has been suggested that EEEV may have been relatively isolated in upstate New York for up to several years and that the virus may have overwintered from 1990–1991 (*5*,*20*). The studies we present here document genetic conservation of strains throughout 3 transmission seasons and over 2 winters, which supports the observation that EEEV may overwinter in a relatively isolated upstate New York focus. Despite the accumulating molecular epidemiologic evidence for EEEV overwintering, the precise mechanisms are poorly understood. One potential overwintering mechanism is latent or chronic infection of wild birds. In such a scenario, springtime viral recrudescence might reinitiate the transmission cycle each new season (*21*,*22*). However, the results of a serologic survey of wild birds in upstate New York during 1986–1990 failed to support this notion, showing no consistent evidence for the current or recent infection of after-hatch year birds with EEEV early in the transmission season (*23*). Transovarial transmission in mosquitoes also has been hypothesized as a means for overwintering, but it has not been convincingly demonstrated naturally or experimentally (*24*–*26*). The main epizootic vector, *Cs. melanura*, overwinters in the larval stage (*27*), so the virus would need to perpetuate in these larvae. As an alternative, predatory birds could acquire the virus by feeding on infected prey, perhaps enabling the virus to persist through winter without mosquitoes. A previous study describing the isolation of *West Nile virus* (WNV; *Flaviviridae: Flavivirus*) from a hawk in New York in winter (*28*) supports this idea. However, conclusive evidence for this theory does not yet exist. Moreover, despite several ecologic studies of EEEV in upstate New York, the mode of long-term persistence in enzootic transmission cycles remains obscure. Accordingly, the molecular epidemiologic studies described here were undertaken to determine more convincingly whether EEEV overwinters locally in upstate New York.

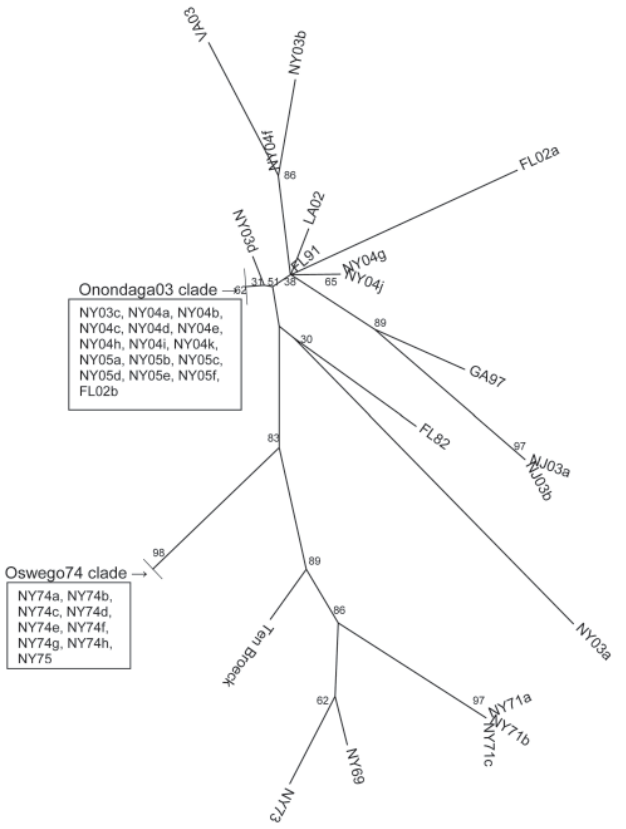

Figure 4. Phylogenetic tree of NSP3 coding region of subset of lineage I eastern equine encephalitis virus strains, unrooted neighbor-joining analysis.

Sequence data from the E2 and NSP3 coding regions of EEEV strains collected during 2 independent multiyear epizootics, 1 in the 1970s and 1 in the 2000s, enabled us to use a molecular approach to examine whether EEEV overwinters in temperate regions. We observed strong spatiotemporal clustering of EEEV strains, including several strains that were identical in their E2 coding region, collected in a single focus over the course of several years. For example, 16 EEEV strains isolated during 2003–2005 form the Onondaga03 clade, which strongly suggests that the virus overwintered there. The probability that this highly conserved genotype was reintroduced in each of 3 consecutive years seems quite low. The Oswego74 clade also

supports overwintering of EEEV. Collectively, these data indicate that EEEV was perpetuated locally through several winters in upstate New York during elevated epizootic activity periods, with 1 dominant genotype circulating in the focus.

The history of EEEV activity in New York suggests that transmission dynamics are not uniform and that periods of relative intensity punctuate interepizootic periods, when virus is undetectable or detectable only infrequently in mosquito pools (D.S. Young et al., unpub. data) (*8*,*29*). Phylogenetic analyses suggest that epizootics occur after reintroduction of novel EEEV genotypes from southern progenitor strains. For example, the Onondaga03 clade groups strongly with the strain FL02b, which was isolated from an ovenbird in Florida in 2002. The ovenbird resides in Florida and Central America in the winter and migrates north to Canada and the northern United States, including upstate New York, in the summer (*30*). The current epizootic therefore appears to be the result of introduction of a southern EEEV strain similar to FL02b in 2003. Although the results presented here cannot determine the precise mode of EEEV transport along the Eastern Seaboard (e.g., trade winds have also been suggested as a mechanism for moving infected mosquitoes [*31*,*32*]), it seems likely that migratory birds are involved in virus trafficking to at least some degree.

The E2 and NSP3 phylogenetic trees demonstrate that some subclades contain southern strains isolated years before genetically similar northern strains. Such is the case with subclades GA97, NJ03a, NJ03b and VA03, NY03b, NY04f and in the E2 trees only (FL02a, NY04g, NY04j). This pattern provides evidence for regular reintroduction of EEEV into enzootic areas of New York State and New Jersey. The subclades VA03, NY03b, NY04f suggest that northward migrating birds brought the virus from the South into New York in 2003. However, the low number of samples from the Eastern Seaboard limits definitive conclusions. For this study we included only 7 samples isolated from the Eastern Seaboard in 2002 and 2003. Sequencing additional southern strains isolated in 2004 or later, to characterize the genetic relationships between northern and southern strains in greater depth, would be beneficial.

A defining feature of the collection of EEEV sequences analyzed here is the genetic conservation within and between epizootics. We observed values of genetic diversity in the Onondaga03 and Oswego74 clades that were surprisingly similar and very low (0.00030 and 0.00035, respectively). These values are ≈10-fold lower than π observed in a sample of WNV sequences collected during 1999–2003 in Suffolk County, NY (0.00241) (*33*). The causes for the strikingly different patterns of genetic diversity observed in these 2 viral systems could include 1) increased movement of WNV-infected birds or mosquitoes compared with EEEV-infected birds or mosquitoes, leading to more frequent introduction of novel genotypes, 2) a higher replicase error rate, and 3) relaxed selective constraint in hosts or vectors of WNV relative to EEEV. Regardless of cause, the basic evolutionary dynamics of these RNA viruses appear to differ markedly.

Overall, our data support the previous findings of Weaver et al. (*5*,*20*) and provide new insights into the ecologic and evolutionary dynamics of an ongoing EEEV epizootic. We provide evidence that the virus is introduced from southern progenitor strains which, if they become established, overwinter in upstate New York; few new genotypes were successfully introduced into the epizootic focus. Our results also highlight the relative spatiotemporal genetic conservation of the virus. To facilitate a more detailed understanding of patterns of perpetuation and spread of this important zoonotic pathogen, future monitoring of EEEV activity should focus on sampling along the entire Eastern Seaboard.

Acknowledgments

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Transmission of Equine Influenza Virus to English Foxhounds

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We retrospectively demonstrated that an outbreak of severe respiratory disease in a pack of English foxhounds in the United Kingdom in September 2002 was caused by an equine influenza A virus (H3N8). We also demonstrated that canine respiratory tissue possesses the relevant receptors for infection with equine influenza virus.

Influenza A viruses are divided into subtypes according to the serologic reactivity of the surface glycoproteins hemagglutinin (H1–H16) and neuraminidase (N1–N9). Aquatic birds are regarded as the natural reservoir for influenza A viruses; a few mammalian hosts are infected by a limited number of virus subtypes. The first evidence of the H3N8 subtype, which currently circulates in horses, crossing species barriers was reported after an outbreak of respiratory disease among racing greyhounds in Florida in 2004. Isolation of virus from 1 case and detection of specific antibodies in other cases identified equine influenza virus as the cause of the outbreak (*1*). This information led us to reexamine an outbreak of severe respiratory disease that occurred in a pack of 92 English foxhounds in the United Kingdom in September 2002.

The Study

The outbreak was signaled by a sudden onset of coughing. Some hounds became lethargic and weak; in some, these signs progressed to loss of consciousness. One hound died and 6 were euthanized. Postmortem examination of the hound that died (case 1) and 1 that was euthanized (case 2) showed subacute broncho-interstitial pneumonia; virus was suspected as the cause. When they were puppies (≈8 weeks of age), the hounds had been inoculated with commercially available vaccines against the major canine respiratory and enteric viruses. Postmortem tissue samples submitted to a canine infectious diseases laboratory were negative for known canine viral pathogens (e.g., canine herpesvirus, adenovirus, parainfluenza virus). The diagnosis as to the cause of the pneumonia, returned in 2002, was "unknown, suspected viral etiology."

In January and March 2005, serum samples were obtained from the hounds affected by the respiratory disease outbreak in 2002 (pack 1). Serum samples were obtained from another 3 packs of foxhounds in the same region of the United Kingdom during December 2004 through February 2005. Samples were collected from 31–33 hounds (equivalent numbers of males and females) in each pack, ranging in age from 9 months to 9 years. The serum was screened for antibodies by using the single radial hemolysis assay (*2*). None of the samples contained antibodies to the strains that were included in the assay to control for nonspecific reactivity: equine H7N7 subtype strain A/equine/Prague/56 and the human influenza virus strain A/Puerto Rico/8/34 (H1N1). Antibodies to the H3N8 subtype strains A/equine/Newmarket/1/93 and A/equine/Newmarket/2/93 were, however, detected in 9 of the samples obtained during the first visit to pack 1 (Table). Of these, 8 were from hounds that had survived the outbreak in 2002; however, 1 was from a hound (no. 22) born after the outbreak in another part of the United Kingdom, which suggests that the 2002 outbreak might not have been the only incident of equine influenza to have infected hounds in the United Kingdom. Another 3 positive serum samples were obtained during a second visit to pack 1, and a repeat sample from hound no. 22 again had positive results. The specificity of the antibodies for equine influenza A (H3N8) strains was confirmed by hemagglutination inhibition assays that included human influenza (H3N2) strain A/Scotland/74 (data not shown).

An immunohistochemical test to detect influenza A virus that used equine influenza–specific rabbit polyclonal antiserum was applied to formalin-fixed paraffin-embedded (FFPE) tissues from the 2 hounds that were examined postmortem in 2002 (*3*). Immunostaining of lung tissue showed positive staining in areas of pneumonic change; infected cells had the morphology of epithelial cells and macrophages (Figure 1). Immunostaining of visceral tissues (lung, liver, spleen, myocardium, intestine, pancreas, and oropharynx) was negative.

Deparaffinization of the FFPE lung tissue from the 2 hounds was performed as described previously (*4*) with a few modifications. RNA was extracted from the sample pellets obtained using the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Ten different primer pairs designed to amplify short (<250-bp) products from the matrix hemaglutinin and neuraminidase genes were used (details available from the authors on request). Reverse transcription–PCR (RT-PCR) was carried out by using the QIAGEN OneStep RT-PCR Kit. Only 1 primer pair (forward: 5′-AGGCAGGATAAGCATATACT-3′ and reverse: 5′-GTGCATCTGATCTCATTACA-3′, amplifying nucleotides 735–871 of

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Table. Antibody levels of English foxhounds involved in 2002 respiratory disease outbreak, United Kingdom*

Hound no.	Date sampled, 2005	Sex	Year born	Influenza virus strain	
				A/equine/Newmarket/1/93	A/equine/Newmarket/2/93
1	Jan 21	F	2002	<10	<10
2	Jan 21	M	2002	48	<10
2	Mar 9			46	<10
3	Jan 21	F	2001	<10	<10
4	Jan 21	F	2001	<10	<10
5	Jan 21	F	2002	47	21
5	Mar 9			64	36
6	Jan 21	M	2002	<10	<10
7	Jan 21	M	1999	<10	<10
8	Jan 21	M	2001	<10	<10
9	Jan 21	M	1998	43	<10
10	Jan 21	M	1999	76	54
11	Jan 21	M	1999	<10	<10
12	Jan 21	F	2002	55	28
13	Jan 21	M	1997	<10	<10
14	Jan 21	F	2003	<10	<10
15	Jan 21	M	2001	51	18
16	Jan 21	M	1999	<10	<10
17	Jan 21	F	2002	<10	<10
18	Jan 21	M	2002	13	11
19	Jan 21	M	1999	<10	<10
20	Jan 21	M	2001	<10	<10
20	Mar 9			<10	<10
21	Jan 21	F	2001	<10	<10
22	Jan 21	M	2003	52	25
22	Mar 9			71	40
23	Jan 21	M	2001	51	27
23	Mar 9			55	20
24	Jan 21	F	1999	<10	<10
24	Mar 9			<10	<10
25	Jan 21	F	2002	<10	<10
26	Jan 21	F	2000	<10	<10
26	Mar 9			<10	<10
27	Jan 21	F	1999	<10	<10
28	Jan 21	F	2000	<10	<10
29	Jan 21	F	2002	<10	<10
30	Jan 21	M	2002	<10	<10
30	Mar 9			<10	<10
31	Jan 21	F	1998	<10	<10
32	Jan 21	F	1999	<10	<10
33	Jan 21	M	2003	<10	<10
34	Mar 9	NK	NK	14	<10
35	Mar 9	NK	NK	<10	<10
36	Mar 9	NK	NK	99	54
37	Mar 9	NK	NK	<10	<10
38	Mar 9	NK	NK	<10	<10
39	Mar 9	NK	NK	<10	<10
40	Mar 9	NK	NK	83	33

*Measured by single radial hemolysis (mm^2) in serum samples. NK, not known.

the hemagglutinin gene) yielded an amplification product, which was purified by using PCR Kleen Spin Columns (Bio-Rad, Hercules, CA, USA) and sequenced by using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). A BLAST search with the 74-bp nt sequence obtained from this amplicon confirmed that the virus shared 100% identity with the equine influenza (H3N8) strains Newmarket/1/93 (*5*) and Newmarket/5/03 (*6*). This region contains 2 phylogenetically informative sites. Lysine at position 261 indicates that the virus belongs to the American lineage (*5*); this is supported by the presence of isoleucine at position 242 because all

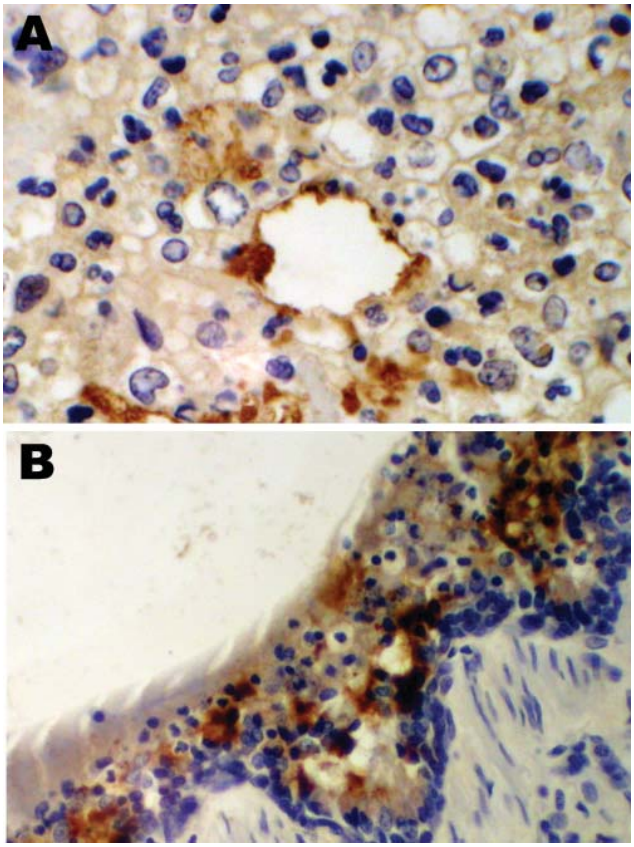

Figure 1. Immunohistochemical staining for equine influenza A virus (brown stain) in sections of respiratory tissue from English foxhounds involved in 2002 respiratory disease outbreak, United Kingdom. A) Case 1, showing focal staining of an apparently necrotic bronchiole in an area of pneumonia; magnification x100. B) Case 2, showing a large amount of staining throughout the epithelium and inflammatory cells present in the brush border; magnification x200; hematoxylin counterstain.

European lineage strains isolated since 1998 have valine at 242.

Conclusions

An important factor in interspecies transmission is the ability of the hemagglutinin protein of the virus to bind to certain receptors on the host cells before the virus is internalized. Although all influenza A viruses recognize cell surface oligosaccharides with a terminal sialic acid, their receptor specificity varies; it is thought that species-specific differences in the distribution of linkages on respiratory epithelial cells influences the ability of influenza A viruses to transmit between species. Respiratory tract tissue samples were obtained within 2–4 hours of death from a horse and a greyhound, each euthanized for reasons other than this study, and rinsed extensively to remove surface mucous. The tissues were stained by immunofluorescence by using the lectins Sambucus nigra (SNA, specific for SAα2,6 galactose(Gal)/N-acetylgalactosaminide) and *Maackia amurensis* (MAA, specific for SAα2,3) as previously described (*7*). The MAA lectin bound strongly to the equine tracheal epithelium (Figure 2, panel A), which confirms the finding that the NeuAc2,3Gal linkage preferentially bound by equine influenza viruses is found on sialyloligosaccharides in the equine trachea (*7*). The MAA lectin also bound strongly to the canine respiratory epithelium (Figure 2, panel B) at all levels of the respiratory tract examined (distal, medial and proximal trachea; primary and secondary bronchi), which suggests that receptors with the required linkage for recognition by equine influenza virus are available on canine respiratory epithelial cells, although further subtle differences in receptor specificity may exist. The SNA lectin, specific for SAα2,6Gal, which did not bind to the equine tracheal epithelium, showed some binding to the canine epithelium (data not shown).

Because the hounds infected in 2002 were housed near horses, it is possible that the virus was transmitted from infected horses by the usual (aerosol) route. However, during

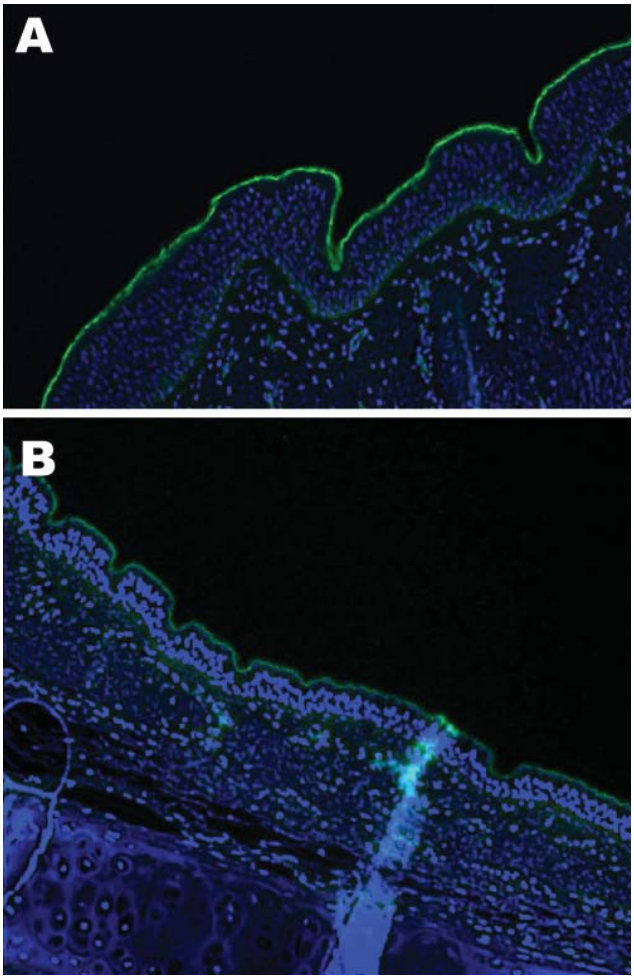

Figure 2. Lectin staining for α2,3 sialic acid linkages on A) equine trachea and B) canine trachea; magnification x200; cell nuclei counterstained with Hoechst 33342 solution.

the week before onset of clinical signs, the hounds had been fed the meat of 2 recently euthanized horses from independent sources. That viral antigen expression was confined to the lungs indicates a respiratory rather than oral route of infection. It is possible that eating respiratory tissue from an infected horse led to inhalation of sufficient virus particles to initiate a respiratory infection. Consumption of infected bird carcasses has been implicated in the transmission of highly pathogenic avian influenza virus of the H5N1 subtype to tigers and leopards (*8*) and a dog (*9*) and was demonstrated experimentally by feeding virus-infected chicks to domestic cats (*10*).

Although the mechanism remains unclear, we have demonstrated transmission of equine influenza virus to dogs in the United Kingdom, independent of that in the United States. We have also shown that canine respiratory tissue displays the relevant receptors for infection with equine influenza virus.

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Dr Daly recently joined the University of Liverpool's Virus Brain Infections Group. Her research interests are zoonotic viral infections; she is currently conducting research on the immunopathology of Japanese encephalitis virus.

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Screening Pneumonia Patients for Mimivirus[1]

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Acanthamoeba polyphaga mimivirus (APM), a virus of free-living amebae, has reportedly caused human respiratory disease. Using 2 newly developed real-time PCR assays, we screened 496 respiratory specimens from 9 pneumonia-patient populations for APM. This virus was not detected in any specimen, which suggests it is not a common respiratory pathogen.

Investigation of a suspected Legionnaire's pneumonia outbreak in 1992 led to the isolation of a new microorganism from a water cooling tower in Bradford, England. This pathogen was thought to be a bacterium because it resembled small gram-positive cocci; however, in 2003 it was correctly identified as a virus (*1*). *Acanthamoeba polyphaga mimivirus* (APM), named for its ameba host and bacteria-mimicking characteristics, is a double-stranded DNA virus with the largest viral genome described to date (1.2 Mb) (*2*). *Mimiviridae* is the newest member of the nucleocytoplasmic large DNA virus (NCLDV) group, which also contains *Poxviridae*, *Iridoviridae*, *Asfarviridae,* and *Phycodnaviridae* (*1*). APM encodes specific translation proteins that are more commonly associated with cellular organisms than with viruses (*2*).

Other ameba-associated microorganisms from environmental sources, such as *Legionella pneumophila*, are known to cause outbreaks of acute pneumonia in immunosuppressed and elderly persons, although person-to-person transmission is uncommon. Whether APM is similarly responsible for individual cases or outbreaks of respiratory disease has yet to be conclusively determined. Previous studies have reported serologic evidence of APM infection in 7.1% to 9.7% of patients with community- or nosocomially acquired pneumonia *(3,4)*. APM DNA was also amplified by a nested PCR assay from a bronchoalveolar lavage specimen of a 60-year-old patient receiving intensive care for hospital-acquired pneumonia (*3*). In this study, we used newly developed real-time PCR assays to screen pneumonia patients from a variety of epidemiologic settings for APM infections.

The Study

Real-time PCR assays for APM were developed from multiple primers and probes designed for conserved regions of class I NCLDV genes L396 and R596, class III NCLDV gene L65, as well as the R656 gene, from the published APM genome sequence (GenBank accession no. NC_006450) by using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). All probes were labeled at the 5′ end with 6-carboxy-fluorescein and quenched at the 3′ end with Black Hole Quencher-1 (Biosearch Technologies, Novato, CA, USA). Different primer and probe combinations were evaluated, and the 2 PCR assays that gave the best performance were selected for further studies (Table 1). Assays were performed by using the iQSupermix Kit (Bio-Rad, Hercules, CA, USA) in 25-μL reaction volumes. Amplification was performed on an iCycler iQReal-Time Detection System (Bio-Rad) by using the following cycling conditions: 95ºC for 3 min for 1 cycle; 95ºC for 15 s and 55ºC for 1 min for 45 cycles each. Total nucleic acid was extracted from all specimens by using either the NucliSens Automated Extractor (bioMérieux, Boxtel, the Netherlands) or the automated BioRobot MDx (QIAGEN, Valencia, CA, USA) according to the manufacturers' instructions. Each clinical specimen was also tested for the human ribonuclease P gene to measure nucleic acid integrity as previously described (*5*).

For PCR-positive controls, recombinant plasmids containing APM DNA (kindly provided by Didier Raoult, Unite des Rickettsies, Universite de la Mediterranee, Marseille, France) were constructed. Primer pairs bracketing the L396 and R596 genes were used to amplify 1,560-bp and 879-bp full gene regions, respectively, using 300 nmol/L of forward primers 396 F (5′-TTA ATC ATC TTC CAA AAA ATT TAA TTC-3′) and 596 F (5′-ATG TCG TTA TCA AAA CAA GTA GTT CC-3′), and 300 nmol/L of reverse primers 396 R (5′-ATG GCG AAC AAT ATT AAA ACT AAA A-3′) and 596 R (5′-CTA ATT TTC AAT ATA GTG CGT AGA TTC TA-3′). These PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN) and then cloned into a pCR-II TOPO vector by using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids were then isolated by using the QIAprep Spin Miniprep Kit (QIAGEN) and quantified by UV spectroscopy. Standard curves were prepared from serial 10-fold dilutions of the quantified plasmid in nuclease-free water containing 100 μg/mL of herring sperm DNA (Promega, Madison, WI, USA).

The L396 and R596 real-time PCR assays could detect as few as 10 copies of plasmid DNA per reaction with amplification efficiencies of 99.6% [slope –3.33 and r^2 = 0.99] (Figure, left panels) and 99.2% [slope –3.34 and r^2 = 1.00] (Figure, right panels), respectively. No amplification

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[1]Dare et al., *Acanthamoeba polyphaga mimivirus* real-time PCR assays, was presented at the 23rd Annual Clinical Virology Symposium, Apr 29–May 2, 2007, in Clearwater Beach, Florida, USA.

Table 1. Primer and probe sequences for *Acanthamoeba polyphaga mimivirus* real-time PCR assays

Target	Primer/probe	Gene region	Sequence (5′→3′)
APM-396	Forward	Helicase	ACC TGA TCC ACA TCC CAT AAC TAA A
	Reverse		GGC CTC ATC AAC AAA TGG TTT C
	Probe		ACT CCA CCA CCT CCT TCT TCC ATA CCT TT
APM-596	Forward	Thiol oxidoreductase	AAC AAT CGT CAT GGG AAT ATA GAA AT
	Reverse		CTT TCC AGT ATC CCT GTT CTT CAA
	Probe		TTC GTC ATA TGC GAG AAA ATG CTA TCC CT

was obtained by either assay with pooled total nucleic acid extracts from respiratory samples from healthy humans or from other common DNA respiratory viruses, including adenovirus, human bocavirus, or herpesviruses.

The real-time PCR assays were used to test respiratory specimens from 496 pneumonia cases representing 9 distinct patient populations, which consisted of hospitalized pneumonia patients from population-based pneumonia surveillance studies in Thailand and the United States, transplant recipients with pneumonia, and isolated pneumonia outbreaks in either retirement homes for the elderly or familial clusters (Table 2). Of the 496 specimens tested, no positive results were obtained for APM DNA by either assay.

Conclusions

We developed a rapid method of screening samples for APM DNA by using 2 sensitive and specific real-time PCR assays designed to target conserved NCLDV class I genes. With only 1 APM sequence published (NC_006450) (*2*), little is known of APM strain variation; therefore, use of assays that target different genes increases the likelihood that genetic variants of APM will not be missed. A suicide-nested PCR method for APM detection has been reported (*3*); however, the quicker turnaround time and lower risk for amplicon contamination makes the real-time PCR method more attractive for screening large numbers of samples.

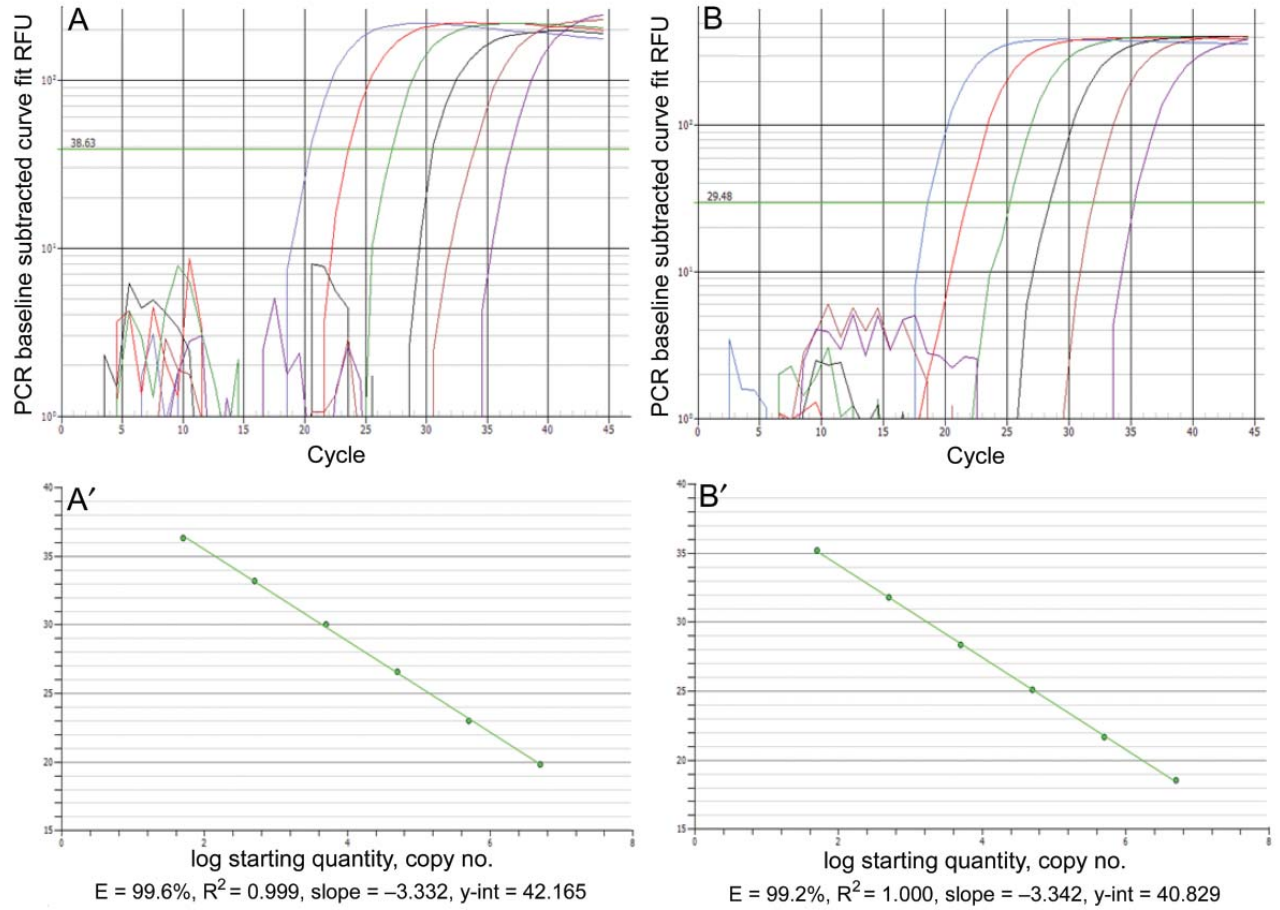

Figure. Real-time PCR amplification plots and standard curves for *Acanthamoeba polyphaga mimivirus* (APM)-396 (A, A′) and APM-596 (B, B′). Linear amplification was achieved over 6 logs for both assays over 5×10^6 to 5×10^1 copies of plasmid DNA. RFU, relative fluorescence units.

Table 2. Characteristics of 496 pneumonia patients tested for *Acanthamoeba polyphaga mimivirus* DNA*

Setting or population	Sample size	Age group	Sample type	Location	Period	Other causes
Community-acquired pneumonia cases	124	Children<5 y	Nasal swabs	Urban USA	Oct 2000–Sep 2001	None detected
	120	Adults, children	NP swabs	Rural Thailand	Sep 2003–Aug 2004	None detected
Nosocomially acquired pneumonia outbreaks	23	Geriatric	NP/OP swabs	Retirement center, USA	Sep 2003	20% rhinovirus
	24	Geriatric	NP/OP swabs	Retirement center, USA	Jul–Aug 2002	20% rhinovirus
	24	Geriatric	Nasal swabs	Retirement center, USA	May 2004	50% hMPV
Community-acquired pneumonia outbreak	5	Adults, children	BAL, sputum, ET aspirate	Familial cluster, USA	Nov 2004	None detected
Bone marrow transplant recipients	42	Adults	NP aspirate	USA	Jan–Apr 2001	60% other respiratory viruses
	45	Adults	Nasal wash, NP swabs	USA	2003	10% influenza and picornaviruses
Lung transplant recipients	89	Adults	NP swabs	Canada	2002–2003	30% other respiratory viruses

*NP, nasopharyngeal; OP, oropharyngeal; hMPV, human metapneumovirus; BAL, bronchoalveolar lavage; ET, endotracheal.

A seroprevalence study of APM among Canadian patients with community-acquired pneumonia identified APM antibodies in 9.7% of 376 patients compared with 2.3% of 511 healthy controls (*3*). However, seropositivity may reflect exposure to APM antigen rather than active infection, and the potential for nonspecific cross-reactions with the serologic assays used may have inflated the true prevalence of APM infection (*6*). In a separate report, a laboratory-acquired APM infection was linked to acute pneumonia by seroconversion in a technician in Marseille, France, thus providing evidence that this virus can occasionally cause clinical disease (*7*). However, using sensitive real-time PCR assays, we failed to detect APM DNA in 496 respiratory specimens from 9 epidemiologically varied pneumonia patient populations.

If we assume an APM prevalence of 0.2% (1 case in the study sample), the estimated probability of obtaining our results by chance, based on binomial analysis, would be 0.37. Most of the specimens we tested were from the upper respiratory tract, whereas the only reported APM PCR–positive sample was from a lower respiratory bronchoalveolar lavage specimen (*3*). Moreover, the patient populations sampled may not represent those at highest risk for APM infection. Nevertheless, our study supports the findings of an Austrian study that failed to detect APM in 214 nasopharyngeal specimens from hospitalized children with respiratory symptoms (*8*).

Our study did not detect APM in a large collection of specimens from patients with pneumonia, which indicates that this virus is not a common cause of severe acute respiratory disease. Because APM is an ameba-associated pathogen like *Legionella*, exposures to APM are most likely to occur from environmental sources. Further studies of more epidemiologically appropriate populations may be necessary to adequately access the importance of APM as a potential human respiratory pathogen. The real-time PCR assays described here will help facilitate these studies.

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Protective Effect of Maritime Quarantine in South Pacific Jurisdictions, 1918–19 Influenza Pandemic

Melissa A. McLeod,* Michael Baker,* Nick Wilson,* Heath Kelly,† Tom Kiedrzynski,‡ and Jacob L. Kool§

We reviewed mortality data of the 1918–19 influenza pandemic for 11 South Pacific Island jurisdictions. Four of these appear to have successfully delayed or excluded the arrival of pandemic influenza by imposing strict maritime quarantine. They also experienced lower excess death rates than the other jurisdictions that did not apply quarantine measures.

Recent pandemic plan development by many countries suggests the international concern about pandemic influenza (*1*). However, no work has been published to date to inform such planning by evaluating islands' border control practices to prevent the arrival of pandemic influenza. Yet border control is potentially easier to study for islands than for states with porous land borders, and for many island states with limited health and economic resources border control may provide the only practical defense against the introduction of pandemic influenza.

The Study

We aimed to identify the features that distinguished successful from unsuccessful border control attempts to exclude pandemic influenza from South Pacific Island jurisdictions (including the "continental" island of Australia) during the 1918–19 influenza pandemic. Jurisdictions were defined as countries, territories, or states within federal systems that had the capacity to implement their own border control measures. Although island jurisdictions in the Pacific are widely dispersed geographically, it appears that nearly all were at some risk for the spread of pandemic influenza from ship-borne contact. The details of ship-borne spread of this pandemic in the Pacific have been well documented (*2*,*3*). Indeed, we have only been able to identify 1 area in the South Pacific that had no reported arrival of the pandemic in the 1918–1922 period, i.e., the geographically remote Lau and Yasawa Islands (in the Fiji Group) (*2*).

Data on quarantine, pandemic arrival, and pandemic-attributable health effects were accessed through a systematic search of Medline, Embase, Australasian Medical Index, and Web of Science. Archival data were accessed directly from the National Archives (in Wellington, New Zealand, and Canberra, Australia) and from government departments and websites for New Zealand; Australia; the Secretariat for the Pacific Community Headquarters in Noumea, New Caledonia; and the World Health Organization.

Our literature search identified 35 articles and documents that included information on the use of border control in 11 of 25 South Pacific Island jurisdictions. An additional 21 archival documents were reviewed. Four jurisdictions in this region met our definition of strict maritime quarantine (monitoring all passengers and crew for at least 1 day before disembarking was permitted). These jurisdictions were American Samoa (5 days' quarantine) and Continental Australia, Tasmania, and New Caledonia (all 7 days' quarantine). All of these jurisdictions delayed the arrival of the pandemic by implementing their own full maritime quarantine (*2*–*7*) (Figure), although in the case of New Caledonia the quarantine was imposed by Australia. In each of these jurisdictions, local health officials credited the success in delaying influenza to strict maritime quarantine.

While it was in force, the maritime quarantine used by American Samoa from November 23, 1918, appeared to exclude pandemic influenza (*2*). Once influenza did reach this jurisdiction in 1920, no recorded deaths were attributed to influenza (in a population of ≈8,000) (*8*). In contrast, influenza spread rapidly through Western Samoa (now named Samoa). The impact was amplified by a lack of medical assistance and by food shortages in the area. Western Samoa had the worst death rate for any country or territory recorded in the 1918 pandemic, losing 19%–22% of its population (*2*).

Continental Australia implemented a maritime quarantine in October 1918. The arrival of influenza was delayed until early January 1919 (*14*), 3 months after the pandemic had appeared in New Zealand, where no systematic form of border control was in effect. The Australian island state of Tasmania instituted a strict maritime quarantine beginning January 27, 1919, once the Australian state of New South Wales had reported cases of pandemic influenza (*4*). Pandemic influenza did not penetrate into Tasmania until August 1919, and when it did, the chief health officer noted that it was a milder infection than experienced on mainland Australia. The resulting death rate for Tasmania of 0.81/1,000 population (*6*) was one of the lowest recorded worldwide.

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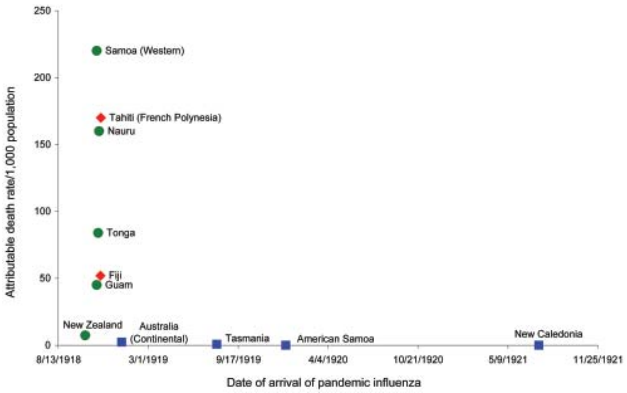

Figure. Comparison of attributable mortality rate from pandemic influenza versus time of arrival of influenza into South Pacific Island jurisdictions for the pandemic beginning in 1918. Sources for mortality data with wave-specific crude mortality rates per 1,000 population (r) from pandemic influenza: American Samoa (r = 0) (*7*,*8*); Australia (Continental) (r = 2.4) (*9*); Fiji (r = 52) (*2*); Guam (r = 45) (*8*,*10*); Nauru (r = 160) (*3*); New Caledonia (r<10) (*11*); New Zealand (r = 7.4) (*12*); Samoa (r = 220) (*2*); Tahiti (r = 190) (*13*); Tasmania (r = 0.81) (*6*); and Tonga (r = 840) (*2*). Sources for date of pandemic influenza arrival data (where different from the source of the mortality data detailed above): Australia (Continental) (*5*). Blue square, strict maritime quarantine; red diamond, incomplete maritime quarantine; green circle, no border control.

New Caledonia was protected from the pandemic until 1921 by the strict 7-day quarantine of outbound vessels from Australian ports that began in late 1918 (*11*,*15*). Visiting ships from Sydney and Wallis Island were the eventual source of an influenza outbreak that began on July 17, 1921 (*11*).

Partial quarantine (as defined by the routine release, without quarantine, of asymptomatic passengers) proved unsuccessful in both Fiji and Tahiti in French Polynesia in 1918 (*2*,*16*). The other island jurisdictions that were identified as using no measures of border control (see circles in Figure) experienced the arrival of pandemic influenza at similar times.

The Figure also shows the death rates attributed to pandemic influenza per 1,000 total population compared with the date of the first recorded cases (for those jurisdictions for which date of first case and mortality data in the second wave of the 1918 pandemic were available). The jurisdictions of Australia, Tasmania, New Caledonia, and American Samoa appear to have benefited from a lower death rate resulting from delay in the arrival of influenza. Also, the lower death rates in some of these countries may have been partly attributable to such factors as preexisting levels of immunity, various socioeconomic characteristics of the populations (e.g., differing levels of poverty), and demographic factors (e.g., crowding and rurality). Unfortunately, limitations of available historical data prevented exploring these issues.

Conclusions

Strict maritime quarantine appears to have been a successful method for delaying and excluding influenza for at least 4 South Pacific Island jurisdictions in the influenza pandemic that began in 1918. Some of these apparent benefits of maritime quarantine may have been attributable to minimal ship contact and geographic remoteness, but these explanations are unlikely given that there were ultimately few places protected in this way in the Pacific. The reasons for the lower mortality rates in jurisdictions that achieved successful delay are unclear. Viral attenuation over time is 1 possibility, although good supportive data for this and other explanations are lacking.

Nevertheless, the use of border control for the future protection of islands from pandemics must take into consideration the different nature of 21st-century societies, such as contact as a result of regular air travel. Island jurisdictions need to continue to undertake pandemic planning for effective border control (potentially with the assistance of larger nations or regional and international agencies). Because some of these jurisdictions involve widespread archipelagos, planning for within-country border control, especially for those populated islands with no airports, is also desirable.

Further modeling studies that are specific to the characteristics of island jurisdictions are also needed to better determine the probability that border control can succeed in the modern era. Nevertheless, now that influenza transmission is better understood, modifications could be made to enhance traditional border control measures to minimize disruptions. For example, in the event of a future pandemic, islands could potentially still trade by ship or plane if they did not allow crews to disembark and if they instituted effective infection control with ongoing surveillance of workers who handle cargo.

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Dolphin Morbillivirus Epizootic Resurgence, Mediterranean Sea

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In July 2007, >100 striped dolphins, *Stenella coeruleoalba*, were found dead along the coast of the Spanish Mediterranean. Of 10 dolphins tested, 7 were positive for a virus strain closely related to the dolphin morbillivirus that was isolated during a previous epizootic in 1990.

An epizootic caused by a newly recognized member of the genus *Morbillivirus*, the dolphin morbillivirus (DMV), killed thousands of striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea during 1990–1992 (*1*–*4*). The first affected dolphins were detected in the Gulf of Valencia (Spanish Mediterranean) in July 1990, but the die-offs soon extended to other regions (*2*). Results of a serologic survey of *S. coeruleoalba* from the Gulf of Valencia and adjacent waters indicated that only adult dolphins harbored antibodies against DMV and that seroprevalence in this age class had decreased from 100% in 1990–1992 to 50% in the study (*5*). This finding suggests that the virus was not endemic, that the dolphins were losing their humoral immunity, and that the population was susceptible to new epizootics. The possibility of new epizootics was also supported by population data (*6*). The density of striped dolphins estimated in the Gulf of Valencia (0.49 dolphin/km^2) in 2001–2003 was again close to the maximum reported for this species in the western Mediterranean (*6*). This high population density was likely to favor the propagation of morbillivirus infections (*7*).

The Study

A new outbreak of die-offs among striped dolphins was detected in the Gulf of Valencia in early July 2007. At the time of writing, unusual die-offs had also been recorded in dolphins from the southern coasts of the Spanish Mediterranean, Balearic Islands, Catalonia, and the Ligurian Sea. Between July and October 2007, >100 dolphins had been found stranded along the Spanish Mediterranean coast. Carcasses were in different states of decomposition. Some dolphins were stranded alive; all had neurologic symptoms and died after being rescued.

In the Gulf of Valencia, the number of stranded animals during July through August 2007 was similar to that recorded in 1990 during the same months (Figure 1, panel A). Stranding rate was also similar during each episode, with an initial low rate at the beginning of July, ≈2 weeks with no stranded dolphins reported, and then a sharp increase of stranding in mid-August (Figure 1, panel B). The most apparent difference between each episode was the size of animals collected: the mean size ± SD of dolphins collected in 1990 and 2007 was 180.9 ± 28.6 cm (n = 34) and 159.9 ± 40.9 cm (n = 17), respectively. This difference is statistically significant (Student *t* test, $t = 2.14$, 49 df, $p = 0.037$).

We examined 10 dolphins (5 adults and 5 juveniles) immediately after their death and collected samples of brain, lung, spleen, liver, and lymph nodes for histologic and molecular studies. Immunohistochemical examination for DMV antigen was performed. A monoclonal antibody to canine distemper virus (MoAb CDV-NP, VMRD, Inc., Pullman, WA, USA), known to react with DMV, was used as primary antiserum at a dilution of 1:200. The secondary antibody, a biotinylated goat anti-mouse immunoglobulin serum, was used at the same dilution. Finally, the avidin-biotin peroxidase complex was incubated at a dilution of 1:100. Sections were counterstained with hematoxylin.

Lesions comprised multifocal bronchiolo-interstitial pneumonia with syncytial cells and nuclear inclusions in the alveolar epithelium and syncytia, as well as lymphoid depletion of the cortical area of the lymph nodes. In 1 dolphin, diffuse pyogranulomatous pneumonia of probable bacterial origin, with disseminated foci to many organs (e.g., brain, heart, lymph nodes, spleen), was found concomitantly with DMV infection. For 5 dolphins, immunostaining was observed in epithelial cells and syncytia in the lungs (Figure 2, panel A), lymphoid cells, neurons, and/or bile duct epithelium. The staining pattern was similar to that described for animals affected by the 1990 epizootic (*8*).

Tissues from the 3 dolphins analyzed by immunohistochemistry were also examined for morbillivirus nucleic acid by reverse transcription–PCR (RT-PCR). We used nested sets of universal morbillivirus primers (UPN1: 5′-ACAAACCNAGRATTGCTGAAATGAT-3′ [genome position 844–869]; UPN2: 5′-CTGAAYTTGTTCT-GAAYTGAGTTCT-3′ [position 1057–1081]) based on the conserved N terminus of the morbillivirus N gene. Tissue samples from 1 dolphin tested positive by N1/N2, and the product was sequenced in its entirety. PCR with

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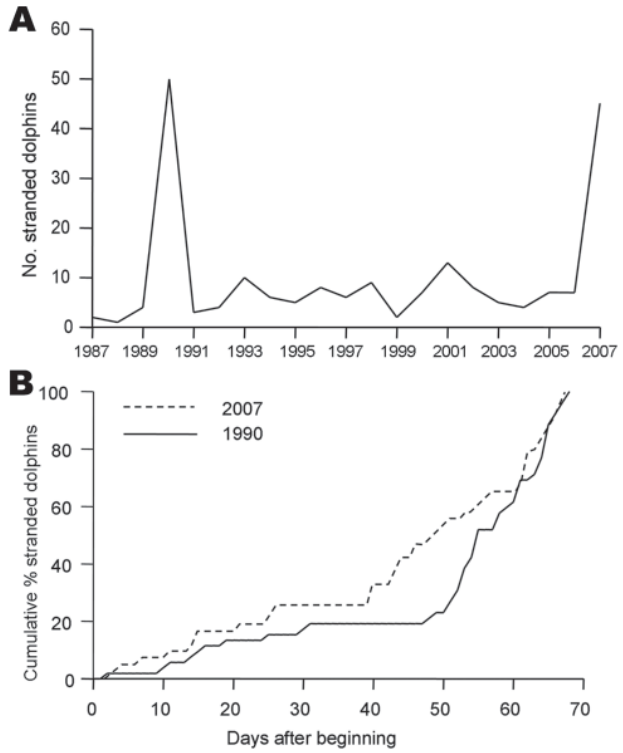

Figure 1. Stranding patterns of Mediterranean striped dolphins, *Stenella coeruleoalba*, in the Gulf of Valencia and adjacent waters (Valencian Community) (514 km of coastline). A) Records of *S. coeruleoalba* stranded in July–August each year from 1987 to 2007. Note peaks of stranding in 1990 and 2007. B) Cumulative percentages of dolphins found dead during July–August, 1990 and 2007 epizootics. Day 0 corresponds to June 23.

nested primers (N1a: 5′-ACTATYAARTTYGGNATN-GAACNATGT-3′ [position 906–932]; N2a: 5′-CTGCAC-TRAAYTTGTTYTGRAYNGAGT-3′ [position 1044–1071] confirmed the other samples as positive.

The N1/N2 sequence (European Molecular Biology Laboratory accession no. EU124652) was aligned to the same region for each of the morbilliviruses with ClustalW (www.ebi.ac.uk/clustalw) (the primer sequences having been removed); phylogenetic studies were performed with the MEGA (version 4) package (www.megasoftware.net). This alignment indicated that this strain was very closely related to the virus that caused the epizootic in 1990 (Figure 2, panel B). Overall, 7 of the 10 dolphins (4 of 5 adults; 3 of 5 juveniles) examined during this study were positive for DMV by immunohistochemistry or PCR. PCR products spanning other viral genes are currently being generated and sequenced to develop a more detailed phylogenetic analysis of the virus currently circulating.

Conclusions

These results show that DMV is again circulating in the Mediterranean population of striped dolphins. The new epizootic resembles the previous die-offs in 1990: both epizootics began in approximately the same region and at the same time and, according to the stranding patterns (Figure 1, panel B), have followed a similar course of infection. However, in the current epizootic, younger animals were

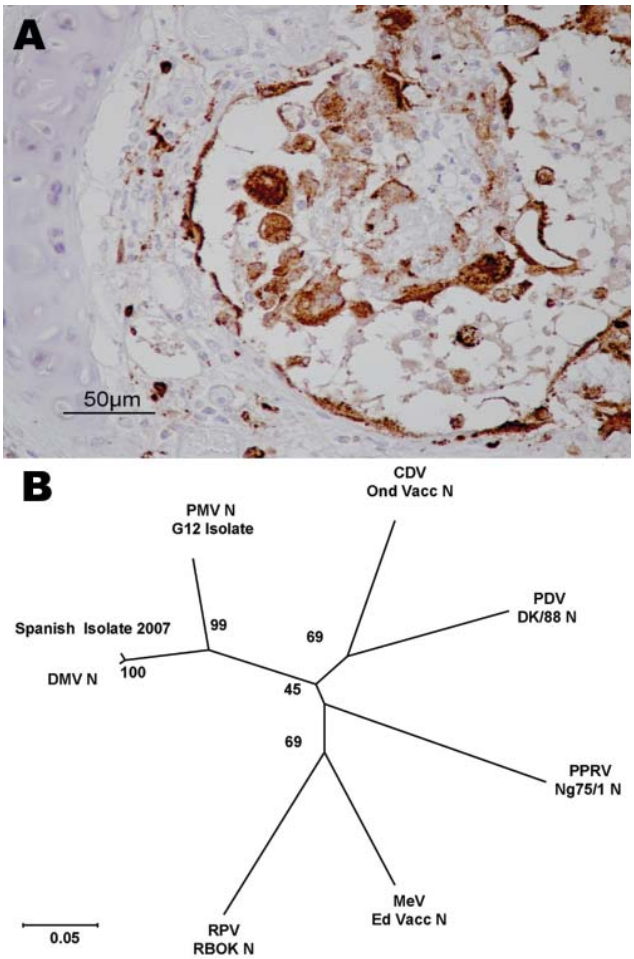

Figure 2. Evidence concerning the identity of the morbillivirus infecting Mediterranean striped dolphins in 2007. A) Immunohistologic staining of lung tissue with a MoAb anti CDV-NP, counterstained with hematoxylin. Epithelial bronchiolar cells and cells in the bronchiolar lumen are positively stained. B) Phylogenetic analysis of the N1/N2 region of the morbillivirus N genes. The number indicated at each node represents the bootstrap value after 10,000 replicates. The evolutionary distances were computed with the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Details of each isolate used for this study and accession numbers for each of the sequences are as follows: DMV 2007 isolate (EU124652), PMV G12 isolate (AY949833), PPRV Nigeria/75/1 Vaccine (X74443), PDV DK/88 strain (X75717), RPV RBOK vaccine strain (Z30697), DMV isolate (AJ608288), CDV Onderstepoort vaccine strain (AF305419), and MV Edmonston vaccine strain (AF266288). DMV, dolphin morbillivirus; PMV, porpoise morbillivirus; PPRV, peste des petits ruminants virus; PDV, phocine distemper virus; RPV RBOK, rinderpest virus RBOK strain; CDV, canine distemper virus; MV, measles virus.

apparently more severely affected by the disease. Although these results are based on stranded dolphins and stranding rates do not necessarily correlate directly with death rates (*9*), we are comparing data from the same area for the 2 epizootics. We are also aware that we used a surrogate of age (i.e., standard length) for these comparisons. However, standard length correlates with sexual maturity (*10*) and has been repeatedly used as proxy for age (*5*). Finally, diseased dolphins are still being detected in late 2007; more juveniles than adults are affected.

These observations further indicate that DMV did not persist as an enzootic infection in the Mediterranean striped dolphins after the 1990–1992 epizootic, that adult dolphins that had survived the first epizootic still had some immunity against the virus, and that unpredictable epizootics may recur. The relatively high density of striped dolphins, their gregarious behavior, and the decreasing level of specific immunity will likely favor the propagation of the virus among the entire Mediterranean population. For this reason, abnormally high die-offs might be expected in other areas of the Mediterranean Sea in the coming months, and measures should be taken to recover and analyze the carcasses.

Recurrent morbillivirus epizootics in marine mammals were described in harbor seals, *Phoca vitulina*, from northern Europe (*11–14*) and bottlenose dolphins, *Tursiops truncatus*, from the western Atlantic and Gulf of Mexico (*15*). Two major questions need to be answered concerning these phenomena: 1) what was the source of infection or reinfection, and 2) did environmental stress (e.g., pollutants, adverse weather, fisheries) precipitate the epizootics (*9*)? In the case of the Mediterranean striped dolphin, the answer to the first question is far from clear, but that both epizootics began in a region close to the Gibraltar Straits, where contacts with infected cetaceans of Atlantic origin could have occurred, is perhaps not coincidental. With regard to the second question, striped dolphins killed by the disease in 1990 had particularly high polychlorinated biphenyls levels, and water temperatures were abnormally high during the winter before the epizootic (*2*). The role of these environmental factors in the 2007 epizootic remains to be more fully investigated. Recurrent epizootics with high die-offs among all age classes will probably have a negative affect on the population dynamics of Mediterranean *S. coeruleoalba*.

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Gastroenteritis Outbreak at Holiday Resort, Central Italy

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During the summer of 2003, a gastroenteritis outbreak spread throughout a holiday resort in central Italy. Fecally contaminated groundwater and seawater were leaking into the non–drinking-water system, which was found to be connected to the drinking-water system of a large resort. This contamination had a primary role in the onset of the outbreak and spread of the infection.

Gastrointestinal infections are the most common diseases among resort guests (*1*). The role of drinking water in transmission, which involves mostly leakage from non–drinking-water or sewage systems or cross-connection between water supply and wastewater systems (*2*–*4*), has been well documented in the literature.

Several episodes of acute gastroenteritis had been observed in coastal holiday resorts of central Italy before 2003. Our study's aim was to identify and eliminate the source of infection. The investigation was conducted from June to September 2003 at a resort where the greatest number of cases had been observed. We describe the epidemics, the activities to trace the sources of infection, and the results achieved.

The Study

We conducted a survey at an Italian holiday resort that had a history of gastroenteritis epidemics. The resort can accommodate 4,080 persons and is organized into 2 different areas: 1 for cabins and 1 for campers or tents. Bathrooms, showers, laundry facilities, and a sports center with swimming pools are provided (Figure 1). The last stretch of a small river (the Salinello River) runs on one side of the village.

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Our study included an epidemiologic survey (tracing the infection sources of the outbreak) and an environmental survey to check the hypothesized origin of infection. The epidemiologic investigation was a matched case–control study; a case-patient was defined as any person at the resort who reported at least 3 episodes of diarrhea or vomiting within a 24-hour period (*5*). Each case-patient was paired with a randomized control patient of the same age group

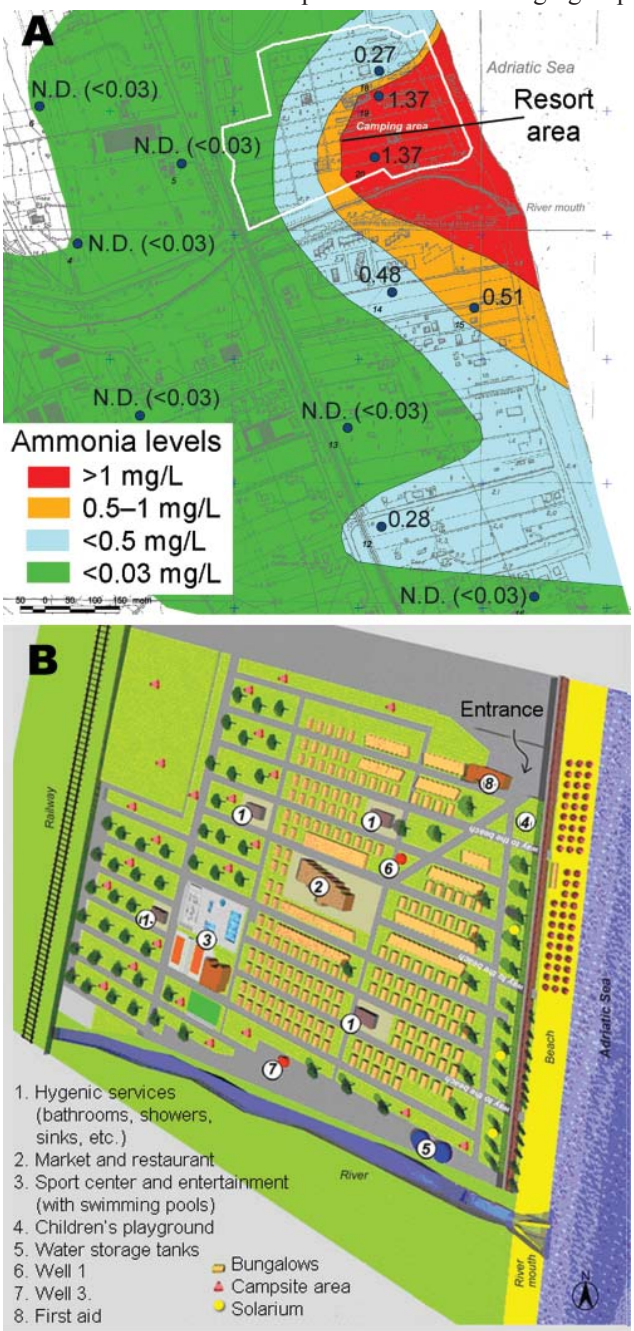

Figure 1. A) Geographic distribution of ammonia residues, central Italy, 2003. Large dots indicate location of wells tested. N.D., not detectable. B) Map of resort area, showing areas of water storage and use.

who had stayed in the resort during the week before the case-patient's onset of symptoms. Data collected from case-patients and control patients included type of accommodation in the resort, contact with other infected persons, and exposure to possible risk factors in the 3 days before symptom onset. We analyzed selected risk factors with univariate statistical techniques (*6*) and stepwise multivariate logistic regression (*7*).

Stool samples were taken and tested for *Campylobacter* spp., *Escherichia coli* O157, *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Vibrio cholerae*, *Clostridium perfringens* toxin, *Bacillus cereus* toxin, norovirus (*8*,*9*), and rotavirus (*10*,*11*). For confirmation of norovirus and strain genotyping, samples were further assayed by open reading frame 1 reverse transcription–PCR (RT-PCR) by using biotin-labeled JV12 ad JV13 primers and reverse line blot hybridization (RLBH) (*12*).

We determined that a gastroenteritis epidemic occurred from July 1, 2003, to September 4, 2003 (Figure 2). Overall, 183 case-patients were identified (169 guests and 14 resort employees); 123 (67%) belonged to 2–4 case-family clusters. Approximately two thirds of case-patients had symptoms within 6 days of arrival. We selected and interviewed 181 controls (128 guests and 53 resort employees) for the study.

Sea bathing, use of cabin and shared toilets, and showers supplied with non–drinking water were significantly associated with the disease, as shown in Table 1. Stool samples were collected from 19 patients from July 15, 2003, through September 4, 2003, mainly at the beginning of the epidemic. Of these, 13 samples (68%) were positive for norovirus (2 by PCR, 6 by ELISA, and 5 by using both techniques). After the identification of norovirus as the putative agent, the physician involved discontinued active sample collection. Although the low amount of DNA amplified did not allow strain characterization by sequencing, norovirus was confirmed in 9 samples by RLBH with specific probes and genotyped as Birmingham (4 strains), Lordsdale (1), and Leeds (2). Two samples were not identified.

Campylobacter sp. was isolated in 1 of 14 samples tested, and 3 of 8 samples were positive for rotavirus. Results of all other microbiologic tests were negative.

In the environmental survey, we evaluated the layout and condition of the water pipelines supplying drinking water and the groundwater collected from 2 wells; these wells supplied water to showers, laundry facilities, public toilets, and irrigation pipelines. Water samples were then tested for fecal or pathogen contamination. We used the fluorescein test (*13*) to check for connections between systems conveying drinking water and non–drinking water within the resort. One hundred grams of sodium fluorescein were added to the 100-m^3 tanks for non–drinking water (10^{-3} g/L), and passage of colored water to the drinking-water pipe system was monitored at 7 supply points by on-the-spot and laboratory spectrophotometric measurements. Eight activated carbon fluorescence traps were positioned for 4 days; absorbed fluorescein was then determined by spectrofluorimetry.

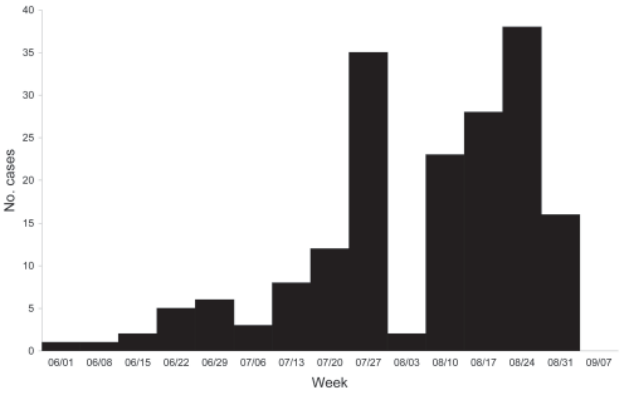

Figure 2. Epidemic curve of cases studied, central Italy, 2003.

Table 2 shows the results of microbiologic tests conducted on the water samples. Results were assessed according to the reference limits established by Italian law for regulation of drinking water (*14*).

Noroviruses were present in the 3 non–drinking-water samples and 2 of 3 sea samples examined, identified as either genotype Lordsdale or Leeds. The on-site fluorescein test detected fluorescein in 2 bungalows, and subsequent analysis of fluorescence traps showed that fluorescein was in a fountain supplied by the drinking-water system and in 2 other bungalows.

The environmental survey also included a study of the chemical pollution of groundwater wells near the resort and analyses of concentrations of ammonia, nitrites, and chloride by geographic distribution. Chloride, ammonia (Figure 1), and nitrite levels were statistically significantly higher in wells near the seashore and mouth of the river than levels in the inner wells. The opposite trend was shown for nitrates, which showed a decreasing concentration gradient from inland toward the sea, likely because of the difference in primary activities from agriculture to industry and tourism.

We proposed several prevention measures to eliminate connections and leaks between non–drinking-water and drinking-water systems and to ensure maintenance of pipelines that were put in place during 2004–2005. In 2004, 120 cases were reported to health services and all were related to the holiday resort; the number of cases decreased to 1 in 2005 and to 0 in 2006.

Conclusions

Laboratory testing of stool samples from case-patients showed that norovirus, rotavirus, and *Campylobacter* spp.

Table 1. Risk factors and food items associated with the presence of gastroenteritis in resort guests, case–control study, central Italy, June–September 2003*

	Univariate statistical analysis				Multivariate logistic regression	
Risk factor	OR	95% CI	χ^2	p value	Coefficients	95% CI
Sea bathing	6.62	2.92–15.00	24.51	<0.01	4.76	1.99–11.42
Use of toilets and showers in cabins and chalet	3.40	1.95–5.90	19.53	<0.01	3.44	1.89–6.24
Use of cabin and villa showers supplied with drinking water	3.11	1.78–5.42	16.52	<0.01	–	–
Use of shared shower facilities supplied with nondrinking water	2.96	1.68–5.20	14.77	<0.01	2.49	1.32–4.68
Use of non–drinking water for various purposes (e.g., laundry, washing dishes, oral hygiene)	2.63	1.46–4.72	10.82	<0.01	–	–
Use of drinking water distributed to cabins and villas	2.15	1.24–3.72	7.54	<0.01	–	–
Use of swimming pools	2.11	1.21–3.69	6.99	<0.01	–	–
Use of bottled drinking water	0.36	0.09–1.39	2.39	>0.05	–	–
River bathing	3.05	0.31–29.81	1.02	>0.05	–	–
Use of common toilets	1.38	0.81–2.33	1.44	>0.05	–	–
Use of drinking water collected at hygienic services (e.g., bathrooms, showers, sinks) and fountains	1.55	0.77–3.11	1.51	>0.05	–	–
Use of mineral water for cooking	1.63	0.61–4.37	0.96	>0.05	–	–
Use of ice	0.79	0.44–1.44	0.57	>0.05	–	–
Use of drinking water collected at hygienic services (e.g., bathrooms, showers, sinks) and fountains for cooking	1.22	0.70–2.11	0.49	>0.05	–	–
Use of drinking water collected at permanent facilities (cabins and villas)	1.19	0.52–2.69	0.17	>0.05	–	–
Water massage at swimming pool	0.85	0.28–2.61	0.08	>0.05	–	–
Consumption of food items						
Sterile canned food	6.18	0.04–862.34	3.54	>0.05	–	–
Cooked vegetables	2.82	0.23–34.85	3.70	>0.05	–	–
Cooked eggs and egg preparations	1.97	0.04–106.07	0.61	>0.05	–	–
Pasta and cooked cereals	1.73	0.32–9.48	2.15	>0.05	–	–
Salami	1.18	0.14–9.68	0.13	>0.05	–	–
Milk and dairy products	1.00	0.2–5.04	0.00	>0.05	–	–
Cooked meat preparations	0.76	0.16–3.57	0.62	>0.05	–	–
Pizza, sandwiches, etc.	0.49	0.1–2.53	3.88	>0.05	–	–
Salads, fruits, raw vegetables	0.29	0.06–1.47	12.22	>0.05	–	–
Cooked fish preparations	0.25	0.03–1.93	10.20	>0.05	–	–
Croissants	0.18	0–26.86	2.97	>0.05	–	–

*OR, odds ratio; CI, confidence interval; –, not performed.

were possibly involved. However, the specific clinical signs, age of case-patients, and occurrence of secondary cases support our conclusion that norovirus was the most likely cause of the outbreak.

The rapid spread (approximately two thirds of case-patients had symptoms within 6 days after arrival) and the high rate of infection suggested a common source of infection, which caused the simultaneous exposure of a large number of resort guests. The twin peaks observed in the epidemic curve are likely related to guest turnover between July and August, when the lowest number of new cases was recorded. Persistence of the source probably caused the prolonged duration of the epidemics.

The case-control study identified sea bathing and use of shared toilet and shower facilities in cabins as statistically significant risk factors. Nygard et al. (*15*) have reported that use of shared shower facilities efficiently spreads infection. The observed fecal contamination in the sea and in the well water used to supply showers supports our conclusions. Tests conducted on well-water samples showed coliforms, enterococci, and *Salmonella togba*, which suggested specific fecal contamination.

The correlation between infection and use of cabin toilet and shower facilities may partly be due to contamination of these areas by infected persons, a theory that is supported by the high frequency of family clusters of infection. Fecal contamination of drinking-water systems may have arisen through connections with the non–drinking-water system in the resort or because of damaged pipework; fluorescein tracer was found in water from bungalow taps and drinking-water fountains.

The groundwater involved in contamination of both drinking-water and non–drinking-water systems was probably polluted by the river flowing into the sea nearby and by

Table 2. Microbiologic analysis of water used within holiday resort, central Italy, case-control study, June–September 2003

Test	Results, positive/no. samples examined (%)		
	Drinking water	Non–drinking water*	Swimming pool water
Microbial count at 36°C*	4/49 (8)	8/20 (40)	0/7
Microbial count at 22°C†	4/49 (8)	9/20 (45)	0/7
Clostridium perfringens‡	0/49	5/20 (25)	Not tested
Total coliforms§	0/49	8/19 (42)	Not tested
Escherichia coli¶	0/49	0/20	0/7
Enterococcii#	2/49 (4)	7/20 (35)	0/7
*Pseudomonas aeruginosa***	9/49 (18)	5/20 (25)	1/7 (14)
Salmonella spp.††	0/13	1/17 (6)	0/5
Vibrio cholerae‡‡	Not tested	0/9	Not tested
Coagulase-positive staphylococci (*S. aureus* and other species)§§	Not tested	Not tested	3/7 (43)
Norovirus antigen¶¶	Not tested	3/3 (100)	Not tested

*Limit of acceptability: drinking water = 100 CFU/mL; swimming pool water = ≤100 CFU/mL.
†Limit of acceptability: drinking water = 100 CFU/mL; swimming pool water = ≤200 CFU/mL.
‡Limit of acceptability: drinking water = 0 CFU/mL.
§Limit of acceptability: drinking water = 0 CFU/100 mL.
¶Limit of acceptability: drinking water = 0 CFU/mL; swimming pool water = 0 CFU/100 mL.
#Limit of acceptability: drinking water = 0 CFU/100 mL; swimming pool water = 0 CFU/100 mL.
**Limit of acceptability: drinking water = 0 CFU/100 mL; swimming pool water = ≤1 CFU/100 mL.
††Limit of acceptability: drinking water = absent/100 mL;.swimming pool water = absent/100 mL.
‡‡Limit of acceptability: drinking water = absent/100 mL.
§§Limit of acceptability: swimming pool water = ≤1 CFU/100 mL.
¶¶Limit of acceptability: drinking water = absent/sample volume examined.

seawater infiltration; the concentration gradient of chemical indicators of organic pollution (ammonia) increased from the coast toward the inland area.

All well water samples and 2 seawater samples were positive for norovirus. Because 2 of the 3 genotypes (Lordsdale and Leeds) found in patients were also identified in these samples, we confirmed massive environmental contamination with human stools and the possible etiologic role of norovirus in this outbreak. Why the Birmingham genotype (most common in patient samples) was not found in the environmental samples is not clear, although the differential stability of viral strains in water and variation in the environmental shedding of different strains with time are possible explanations.

Acknowledgments

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Dr Migliorati is the head of the Laboratory for Food of the I.Z.S.A. & M. His main research interests are the epidemiology and surveillance of food-borne zoonotic agents, especially *Salmonella* spp., *L. monocytogenes*, and *Campylobacter* spp.

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Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Sequence Type 398 in Pigs and Humans

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Methicillin-resistant *Staphylococcus aureus* sequence type 398 (ST398 MRSA) was identified in Dutch pigs and pig farmers. ST398 methicillin-susceptible *S. aureus* circulates among humans at low frequency (0.2%) but was isolated in 3 human cases of bacteremia (2.1%; p = 0.026). Although its natural host is probably porcine, ST398 MRSA likely causes infections in humans.

Nasal *Staphylococcus aureus* carriage has increased in pig farmers, and specific lineages of *S. aureus* are shared by farmers and their animals (*1*,*2*). In addition, rates of nasal carriage of methicillin-resistant *S. aureus* (MRSA) by veterinary personnel working with pigs is high (*3*–*5*). The pig-related MRSA appears to be clonal and was identified by multilocus sequence typing (MLST) as sequence type 398 (*2*,*6*,*7*). Such resistant bacterial strains can spread from animals to the environment, which may facilitate the colonization of persons who are not involved in animal husbandry (*8*). The porcine MRSA strain has been isolated from humans with invasive and superficial infections, and familial outbreaks of colonization and cross-colonization have been documented (*2*,*6*,*7*).

We sought to determine whether the clinical effect of the porcine ST398 MRSA strain can be substantiated by the existence of genetically homologous, methicillin-susceptible *S. aureus* (MSSA) strains among healthy or infected persons. The international MLST database (www.mlst.net) (*9*) listed only 1 ST398 MSSA nasal carriage isolate from a patient in Cape Verde. In addition, 1 ST398 MRSA strain was isolated from a woman living in Groningen, the Netherlands, without further clinical and epidemiologic data available. ST398 MSSA nasal carriage isolates were also identified in several pig farmers in a study by Armand-Lefevre et al. (*1*). We describe the population genetic analysis of Dutch community-based and nosocomial MSSA isolates in comparison with pig- and pig farmer–derived ST398 MRSA isolates, performed by *spa*-sequencing and amplified fragment length polymorphism (AFLP) analysis (*10*,*11*).

The Study

Most of the ST398 MRSA strains studied were collected at the Dutch Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). A total of 20 strains were isolated from the nares of pigs in several slaughterhouses (RIVM 21–40) (*12*), whereas 18 additional strains were detected during in-hospital screenings for MRSA carriage among Dutch farmers from independent farms (RIVM 1–8, 10–12 and 14–20). In addition, 8 clinical and carriage isolates were obtained from the Veterinary Medical Diagnostic Centre in Utrecht (Table).

Amplified fragment length polymorphism (AFLP) analysis was performed as described previously (*10*). A total of 147 marker fragments per strain were scored, and a binary table with marker absence [0] or presence [1] was constructed. A total of 30 fragments with differential occurrence, when genetically heterogeneous MSSA and ST398 MRSA fingerprints were compared, were reamplified and sequenced (Applied Biosystems, Foster City, CA, USA). Fragments were sequenced for 3 independent strains, and the consensus was analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast). Typing of the staphylococcal chromosome cassette (SCC*mec*) and the presence of the Panton-Valentine leukocidin (PVL) genes was performed by PCR.

We embedded the genetic fingerprints of the 46 pig-related MRSA isolates in the population structure of *S. aureus* as obtained before (*10*,*11*). These studies include high throughput AFLP fingerprints of 829 nonclinical *S. aureus* human carriage isolates and 146 and 77 (including 2 MRSA isolates) clinical isolates of human and animal origin, respectively. All carriage strains were isolated from volunteers living in the Rotterdam region, where pig farms are absent.

Sequencing of the repetitive region of the protein A gene *spa* was performed for all ST398 MRSA isolates (*13*).

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Table. Survey of demographic and clinical data of the ST398 pig-associated MRSA (n = 46) and ST398 MSSA (n = 6) strains, the Netherlands*

Isolate no.	Source	Infection	Site of sampling	City	*mec*A	PVL	*spa* type	SCC*mec*
RIVM-1	Human	No	Nose	Putten	Pos	Neg	t011	IVa
RIVM-2	Human	Yes	Urine	Rosmalen	Pos	Neg	t108	V
RIVM-3	Human	No	Nose	Gendringen	Pos	Neg	t108	V
RIVM-4	Human	Yes	Wound/abscess	Helden	Pos	Neg	t108	V
RIVM-5	Human	No	Sputum	Amsterdam	Pos	Neg	t011	V
RIVM-6	Human	Yes	Urine	Zeeland	Pos	Neg	t108	V
RIVM-7	Human	No	Throat/nose	Bennekom	Pos	Neg	t898	III
RIVM-8	Human	No	Sputum	Kootwijkerbroek	Pos	Neg	t011	V
RIVM-10	Human	Yes	Wound/abscess	Galder	Pos	Neg	t567	III
RIVM-11	Human	Yes	Wound/abscess	Olburgen	Pos	Neg	t108	V
RIVM-12	Human	No	Sputum	Sas van Gent	Pos	Neg	t011	IVa
RIVM-14	Human	Yes	Nose	Siebengewald	Pos	Neg	t108	V
RIVM-15	Human	No	Sputum	Grijpskerke	Pos	Neg	t034	V
RIVM-16	Human	No	Sputum	Harskamp	Pos	Neg	t108	V
RIVM-17	Human	Yes	Wound/abscess	Haarlem	Pos	Pos	t034	III
RIVM-18	Human	Yes	Sputum	Heeswijk dinther	Pos	Neg	t108	III
RIVM-19	Human	Yes	Perineum	Veenoord	Pos	Neg	t571	III
RIVM-20	Human	Yes	Wound/abscess	Roggel	Pos	Neg	t108	V
RIVM-21	Pig	No	Nose	Venray	Pos	Neg	t108	V
RIVM-22	Pig	No	Nose	Heeze-leende	Pos	Neg	t108	V
RIVM-23	Pig	No	Nose	Barneveld	Pos	Neg	t011	IVa
RIVM-24	Pig	No	Nose	Dalfsen	Pos	Neg	t108	V
RIVM-25	Pig	No	Nose	Gemert-bakel	Pos	Neg	t108	V
RIVM-26	Pig	No	Nose	Venray	Pos	Neg	t108	V
RIVM-27	Pig	No	Nose	Venray	Pos	Neg	t011	V
RIVM-28	Pig	No	Nose	Alphen-chaam	Pos	Neg	t011	IVa
RIVM-29	Pig	No	Nose	Hoogeveen	Pos	Neg	t1254	IVa
RIVM-30	Pig	No	Nose	Skarsterlân	Pos	Neg	t1254	IVa
RIVM-31	Pig	No	Nose	Someren	Pos	Neg	t011	V
RIVM-32	Pig	No	Nose	Someren	Pos	Neg	t1255	V
RIVM-33	Pig	No	Nose	Zundert	Pos	Neg	t108	V
RIVM-34	Pig	No	Nose	Baarle-nassau	Pos	Neg	t108	V
RIVM-35	Pig	No	Nose	Hofvantwente	Pos	Neg	t011	IVa
RIVM-36	Pig	No	Nose	Zwolle	Pos	Neg	t011	IVa
RIVM-37	Pig	No	Nose	Alphen-chaam	Pos	Neg	t011	V
RIVM-38	Pig	No	Nose	Ijselstein	Pos	Neg	t011	IVa
RIVM-39	Pig	No	Nose	Ijselstein	Pos	Neg	t011	IVa
RIVM-40	Pig	No	Nose	Dalfsen	Pos	Neg	t567	III
6302/3	Pig	Yes	Skin	Utrecht	Pos	Neg	t011	IV
6303/6	Pig	Yes	Nose	Utrecht	Pos	Neg	t011	IV
6303/7	Pig	Yes	Nose	Utrecht	Pos	Neg	t011	IV
6303/9	Pig	No	Nose	Utrecht	Pos	Neg	t011	IV
6303/11	Pig	No	Nose	Utrecht	Pos	Neg	t011	IV
6303/1 MRSA1	Human	No	Nose	Utrecht	Pos	Neg	t011	IV
6303/1 MRSA2	Human	No	Nose	Utrecht	Pos	Neg	t011	IV
V0606303/8	Pig	Yes	Nose	Utrecht	Pos	Neg	t011	IV
T0976	Human	No	Nose	Rotterdam	Neg	Neg	t571	–
TE5029	Human	No	Nose	Rotterdam	Neg	Neg	t571	–
TB27855	Human	Yes	Blood	Rotterdam	Neg	Neg	t571	–
TB28395	Human	Yes	Blood	Rotterdam	Neg	Neg	t571	–
TB29854	Human	Yes	Blood	Rotterdam	Neg	Neg	t571	–
TDV0002580	Gorilla	Yes	Ear	Velp	Neg	Neg	t034	–

*MRSA; methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-succeptable *S. aureus*; ST398, sequencing type 398; PVL, Panton-Valentine leukocidin; SCC*mec*, staphylococcal chromosome cassette; pos, positive; neg, negative. All isolates were nontypeable by pulsed-field gel electrophoresis and confirmed as sequence type 398 by multilocus sequence typing.

Data were analyzed by using the Ridom Staphtype software version 1.4 (www.ridom.de/staphtype).

Analysis of the AFLP data was performed as described previously (*10*). Both hierarchical cluster analysis and principle component analysis were performed with Spotfire DecisionSite 7.2 software (www.spotfire.com). We used the Fisher exact test to compare the distribution of strain categories in different phylogenetic lineages. A 2-sided p value <0.05 was considered significant.

The AFLP analysis of the ST398 MRSA strains derived from human and animal sources (n = 46) indicated that these strains are highly clonal. When the AFLP patterns for the ST398 strains were included in the overall population analysis for Dutch MSSA strains from carriage and infection, the distinct cluster was still observed (Figure 1). Few Dutch MSSA strains from the Rotterdam region coclustered with the ST398 pig-related MRSA isolates (Figure 1, panel B). In total, 6 (0.6%) MSSA isolates coclustered with the ST398 MRSA isolates, of which 2 were nasal carriage isolates from healthy persons (Table). Of the 6 strains, 3 were blood culture isolates taken from 3 elderly patients. All 3 patients had nosocomial bacteremia: 1 after inflammatory aneurism of the aorta, 1 during Fournier gangrene, and the last 1 after primary ventricular fibrillation. Epidemiologic data exclude a cluster of nosocomial infections; patients were not in direct contact (data not shown).

After principle component analysis , the ST398 MRSA strains still clustered as a separate group (Figure 2). The AFLP analysis did not distinguish strains from pigs or pig farmers, and only a limited number of polymorphic AFLP fragments were seen. AFLP markers that were positive for the ST398 MRSAs and absent from the other strains, or vice versa, were sequenced. Of 30 fragments analyzed, 9 were ≈100% specific for the pig-associated strains. Another 3 fragments were present in a subset of the pig-associated strains only. Of these 12 fragments, 4 were not homologous with current entries in the GenBank database, including the 10 *S. aureus* full-genome sequences. Of the 12 pig-specific markers, 8 were homologous with known sequences, which suggests that these markers become pig-specific by point mutations in the AFLP primer annealing site(s) rather than by genomic rearrangement. Several of the sequences encode factors were associated with membranes or transport.

The preponderance of types t011 and t108 was confirmed by *spa* sequencing (*12*). These made up >75% of all cases. However, the other types all belonged to the same family of *spa* types, which suggests recent drift in the sequence motifs. The t011 types are primarily associated with SCC*mec* IV and IVa, whereas the t108 type is nearly fully associated with SCC*mec* V. This finding suggests that ST398 MRSA has arisen independently on at least 2 occasions. Finally, SCC*mec* III is found in association with t108, t898, t567, t034, and t571. This finding suggests promiscuous dissemination of this cassette among the ST398 MRSA. Strain RIVM-17 harbored the PVL genes. Apparently, the bacteriophage carrying these genes found its way into the porcine ST398 MRSA lineage.

Conclusions

The massive colonization of Dutch pigs with a single sequence type of MRSA was unexpected (*12*). Molecular strain typing was initially compromised because PFGE failed (*14*). *Spa* gene sequencing (*13*) showed heterogeneity in the ST398 MRSA lineage with types t011 and t108,

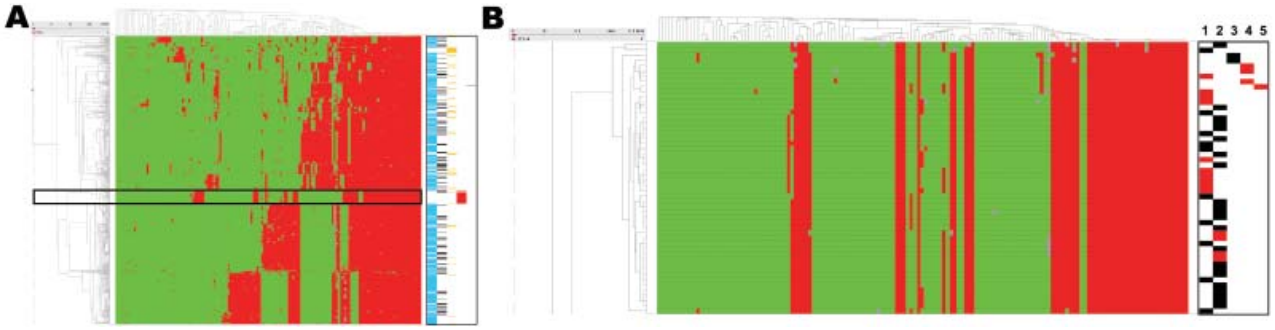

Figure 1. A) Meta-analysis of the amplified fragment length polymorphism data obtained for the pig-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 (ST398 MRSA) and its closely related methicillin-susceptible *S. aureus* (MSSA) strains, carriage MSSA isolates from healthy children and elderly persons, invasive MSSA from hospitalized children and elderly persons, and invasive animal *S. aureus* isolates (including 2 MRSA isolates) (*10,11*). Green and red represent 161,700 binary outcomes generated by high throughput restriction fragment length polymorphism with 147 marker fragments. Marker absence corresponds with green, marker presence corresponds with red, and gray represents ambiguous positions (i.e., weak bands), scored as marker absence in the mathematical analyses. ST398 MRSA strains are boxed. The dendrogram on the left shows the phylogenetic strain clustering; the dendrogram on the x-axis shows marker clustering. Marker groups are cluster specific. Markers on the right are defined as follows: blue, carriage isolates (n = 829); black, bacteremia isolates (n = 146); yellow, animal isolates (n = 77); red, ST398 MRSA isolates (n = 46); pink, reference strains (Mu50/N315). B) Detail highlighting the ST398 isolates. Markers and lanes on the right are defined as follows: black, carriage isolate; red, clinical isolate; 1, ST398 MRSA isolated from humans; 2, ST398 MRSA isolated from pigs; 3, ST398 MSSA human carriage isolates; 4, ST398 MSSA human bacteremia isolates; 5, ST398 MSSA animal clinical isolate.

which are closely related, covering >75% of all isolates. Hence, 1 or 2 new MRSA lineages had been discovered. We found a degree of genetic association between *spa* types and the presence of certain SCC*mec* cassettes, which suggests bacterial evolution and horizontal DNA exchange in the zoonotic reservoir.

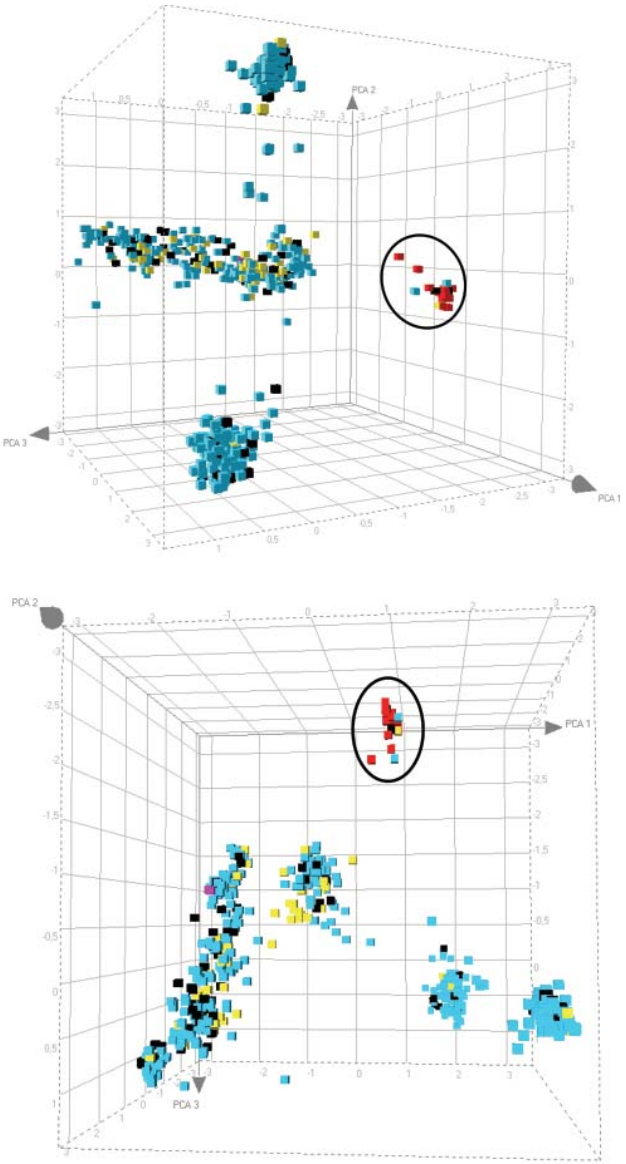

Figure 2. Principal component analysis analysis of the amplified fragment length polymorphism data obtained for the pig-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 (ST398 MRSA) and its closely related methicillin-susceptible *S. aureus* (MSSA) strains, carriage MSSA isolates from healthy children and elderly persons, invasive MSSA from hospitalized children and elderly persons, and invasive animal *S. aureus* isolates (including 2 MRSA isolates). The cubes, plotted in 3-dimensional space, represent all of the strains displayed in Figure 1, panel A. Each axis represents the score calculated for that strain on each principal component. The distribution is shown from 2 different angles. ST398 strains are circled. Blue, carriage isolates (n = 829); black, bacteremia isolates (n = 146); yellow, animal isolates (n = 77); red, ST398 MRSA isolates (n = 46); pink, reference strains (Mu50/N315).

We found that ST398 is rare among Dutch MSSA strains colonizing healthy persons (2 [0.2%] of 829 strains). However, a relatively high number of MSSA isolates homologous to the ST398 MRSA were derived from bacteremic patients (3 [2.1%] of 146; p = 0.026). These 3 bacteremia isolates were not related epidemiologically; they were isolated from different patients in different medical departments over an extended period. This finding suggests that these MSSA strains are quite virulent. The strict segregation of ST398 strains (Figure 1, panel A; Figure 2) corroborates that the strains belong to a separate biotype associated with pigs (*15*).

Our findings pose a warning to public health surveillance: if the ST398 MSSA virulence toward humans would be maintained within the ST398 MRSA lineage from pigs, care should be taken not to introduce this strain into humans. We consider it to be likely that ST398 MRSA from pigs is capable of causing serious infection in humans even though its primary host seems to be pigs.

Dr van Belkum is with the Department of Medical Microbiology and Infectious Diseases, University Medical Center Rotterdam, Rotterdam, the Netherlands. His research interests include MRSA.

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Hemagglutinating Encephalomyelitis Coronavirus Infection in Pigs, Argentina

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We describe an outbreak of vomiting, wasting, and encephalomyelitis syndrome in piglets in Argentina, caused by porcine hemagglutinating encephalomyelitis coronavirus (PHE-CoV) infection. Diagnosis was made by epidemiologic factors, pathologic features, immunohistochemistry, reverse transcription–PCR, and genomic sequencing. This study documents PHE-CoV infection in South America.

Porcine hemagglutinating encephalomyelitis (PHE) is an infectious disease that primarily affects pigs <3 weeks of age (*1*). The disease is caused by PHE coronavirus (PHE-CoV) (*2*), which comprises a single strain and is the only known neurotropic CoV for pigs (*3*–*5*).

PHE-CoV was first isolated in Canada from the brains of suckling piglets with encephalomyelitis (*6*); it was later isolated in England from piglets exhibiting vomiting, anorexia, and depression, where it was named "vomiting and wasting disease" (VWD) (*7*). Neurologic and digestive characteristics of the disease were experimentally reproduced in pigs by using the same field virus isolate (*8*). The infection has been reported in the major pig-raising countries of Europe, Asia, and North America, where it seemed to be endemic with no clinical outbreaks (*7*,*9*,*10*).

Presumptive diagnosis can be made by correlating epidemiologic data, age susceptibility information, and disease course with histopathologic findings (*3*,*10*). For definitive diagnosis, the virus should be isolated and identified (*3*). Currently, immunohistochemical (IHC) tests for PHE-CoV or molecular tools such as reverse transcription–PCR (RT-PCR) enable specific CoV RNA sequences to be detected from infected tissues (*11*,*12*). We describe a PHE-CoV outbreak in an intensively managed farm in Argentina as well as the techniques applied for diagnosis.

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The Study

The farm was a 3-site herd with a total of 6,000 sows. At the time of the outbreak, 55% of breeder stock were gilts or first- or second-parity sows. Site 1 comprised 20 gestation barns and 19 farrowing barns, site 2 (nursery) comprised 9 barns, and site 3 comprised growing and fattening barns.

The outbreak began on August 8 and ended on August 23, 2006. Of 19 farrowing barns, 10 (52.6%) were affected. Total proportion of deaths in pigs that had not been weaned was 16.9% (1,226 dead pigs); an estimated 12.6% of pigs that died had suspected PHE-CoV infection (913 animals).

Clinical signs were observed in pigs ≥4 days of age and consisted of vomiting, listlessness, pallor, and dehydration. Neurologic signs such as abnormal gait, dullness, inability to eat, tremors, and nystagmus were observed in some animals. Vomiting and wasting occurred in 27.6% of pigs ≤1 week of age and gradually declined to 1.6% in pigs 3 weeks of age (mean 13.6%). Twenty-nine percent of weaned pigs housed in nursery barns that received affected animals from site 1 showed wasting (Figure 1, panel A), and the proportion of deaths was 15%–40%. In total (sites 1 and 2), 3,683 piglets died or were euthanized.

Postmortem examinations were performed on 16 affected piglets, 2–11 days of age. Samples submitted for histopathologic examination included brainstem, trigeminal ganglia, tonsils, pyloric gland area of the stomach,

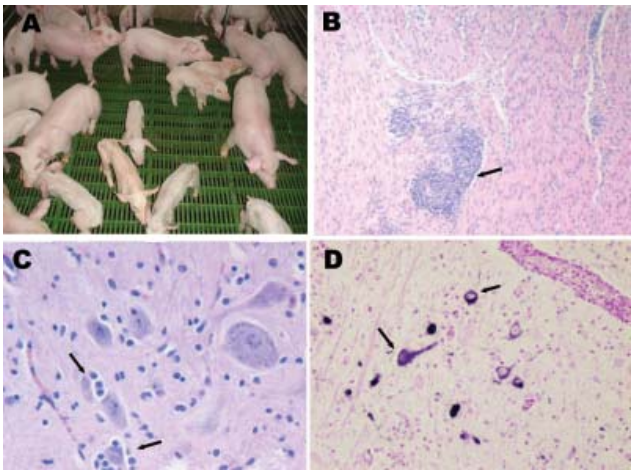

Figure 1. A) Nursery piglets showing clinical signs compatible with porcine hemagglutinating encephalomyelitis coronavirus (PHE-CoV). Nonaffected pigs of the same age are also shown. B) Muscle layer of stomach from affected piglet showing perivascular cuffing (arrow); hematoxylin-eosin stain, magnification ×100. C) Brainstem from affected piglet showing satellitosis (arrows) and gliosis; hematoxylin-eosin stain, magnification x400. D) Brainstem from affected piglet showing positive label of neuron perikarion (arrows); nitroblue-tetrazolium imunohistochemical stain, magnification x400.

jejunum, ileum, lymph nodes, heart, spleen, liver, kidneys, and lung.

Selected paraffin sections of brainstem from 5 piglets that had characteristic microscopic lesions were examined for PHE-CoV antigen by IHC tests with an anti PHE-CoV-67N strain mouse antibody; the samples were diluted 1:1,000 and incubated overnight at 4°C. Samples were then labeled with biotinylated conjugated anti-mouse immunoglobulin G (IgG) goat antiserum at room temperature for 2 h. The color reaction was detected by alkaline phosphatase and 5-bromo-4-chloro-3-indoyl phosphate by using nitro-blue-tetrazolium and true red as cromogens.

RNA was isolated with a commercial kit (RNeasy, QIAGEN GmbH, Hilden, Germany), from brain samples of 7 symptomatic piglets (6–11 days of age) that had nonsuppurative encephalomyelitis, from 1 asymptomatic piglet, and from a PK-15 cell culture suspension inoculated with a pool of tissues from 1 symptomatic piglet. Ribonuclease-A (RNase)–free water was used as negative control. The RT-PCR was performed immediately after RNA isolation by using the specific primer pair for CoV, Cor-FW 5′→3′ (DNA) ACTCAAATGAATTTGAAATATGC, and Cor-RV 5′→3′ (DNA) TCACACTTTGGATAA TCCCA that amplifies a 251-bp fragment of the polymerase gene (*11*). The reaction was performed in a total volume of 50 μL containing 2 μL RNA extract, 10 μL 5× QIAGEN One-Step RT-PCR buffer, 2 μL dNTPs mix (final concentration of 400 μmol/L of each dNTP), 1.8 μL QIAGEN One-Step RT-PCR Enzyme Mix, 4 μmol/L of each primer and RNase-free water to 50 μL. The reaction was conducted in a thermal cycler (PCR Sprint Thermo Electron Corp., Waltham, MA, USA) with an initial reverse transcription at 50°C for 30-s activation at 95°C for 15 s, 40 cycles of amplification (30 s, at 94°C, 30 s at 50°C, and 1 min at 72°C), and a final extension step at 72°C for 10 min.

The amplicons were purified by using the QIAquick PCR purification kit (QIAGEN) and sequenced on a MegaBase 1000 DNA sequencer (GE Healthcare, Chalfont St. Giles, UK). The obtained sequence was analyzed by using NCBI BLAST (www.ncbi.hlm.hig.gov).

Virus isolation was attempted by inoculation of PK-15 and SK-K cells with brains and tonsils from 5 pigs positive for PHE-CoV by RT-PCR. Five blind passages were performed at 7-day intervals. IHC testing was also performed on SK-K cells.

Microscopic changes were observed in samples taken from 5- to 11-day-old affected pigs. The most remarkable changes were perivascular cuffing around Meissner and Auerbach ganglia in the muscle layer of stomach (Figure 1, panel B), ganglioneuritis in the trigeminal ganglion, and nonsuppurative encephalomyelitis (Figure 1, panel C). PHE-CoV–positive neurons were found in the brainstem (Figure 1, panel D) and trigeminal ganglion.

RT-PCR analysis showed a product of the expected size for CoV (≈250 bp) in all analyzed brain samples. No amplification was observed in inoculated PK 15 cells (Figure 2). The relationship between clinical course, lesions, and IHC and RT-PCR results is shown in the Table. A constant 116-nt sequence was obtained for all products amplified from symptomatic piglets and submitted to GenBank (accession no. EF602436). The sequence showed a 95% identity with the complete genome of PHE-CoV strain VW572 (accession no. DQ 011855) and with PHE-CoV RNA-directed RNA polymerase gene (accession no. AF 124988). Detection of amplicons of ≈250 bp with "pancoronavirus" primers in brain samples is highly suggestive of the presence of PHE-CoV. Sequence analysis confirmed this observation. Cytopathic effects were not observed, and PHE-CoV antigens were not detected in inoculated cells.

Conclusions

From an epidemiologic standpoint, the clinical course of the disease (3 weeks), age of affected pigs (<3 weeks), and clinical signs were in agreement with those of VWD caused by PHE-CoV (*3*,*8*,*13*). On PHE-CoV–endemic farms, immune sows apparently provided immunity to their offspring through colostrum (*3*), and clinical disease seldom occurred. In our study, because the gilt pool was so large, a nonimmune subpopulation very likely existed and might have acted as a potential source of virus multiplication. The severity of clinical signs such as vomiting, emaciation, wasting, and death was greater than that previously reported (*10*). Factors that might have enhanced the clinical manifestations of the disease were a nonimmune population and the winter season (*3*,*8*,*10*).

Pensaert (*3*) reported perivascular cuffing and neuronal degeneration at the intramural nervous plexus of the stomach in pigs showing VWD. In addition, Pensaert (*3*)

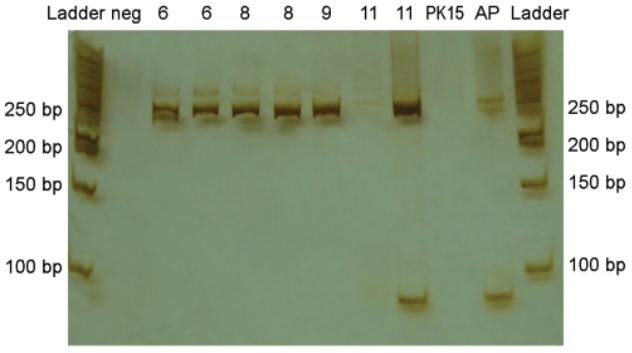

Figure 2. Polyacrylamide gel and silver staining of reverse transcription–PCR products from brains of piglets infected with porcine hemagglutinating encephalomyelitis coronavirus. Amplicons of ≈250 bp were found in brain samples from pigs 6, 8, 9, and 11 days of age. Neg, negative control (water + mastermix); PK15, amplification of PK15 cells inoculated with brain and tonsil from affected piglet; AP, asymptomatic piglet; and Ladder, 50-bp Fermentas.

Table. Clinical signs and results of histopathologic examination, IHC testing, and RT-PCR from piglets affected by PHE-CoV*

Organ	Days old (days with clinical signs)	IHC	RT-PCR	Histopathologic diagnosis
Brain stem	6 (2)	+	+	Encephalitis
Brain stem	8 (4)	+	+	Encephalitis
Trigeminal ganglion	8 (4)	+	+	Focal ganglioneuritis
Brain stem	8 (4)	ND	ND	Encephalitis
Spinal cord	9 (5)	ND	+	Myelitis
Tonsil	9 (5)	ND	ND	Necrotizing tonsilitis
Brain stem	9 (5)	ND	ND	Meningoencephalitis
Medulla oblongata	11(6)	+	+	Meningoencephalitis

*IHC, immunohistochemical; RT-PCR, reverse transcription–PCR; PHE-CoV, porcine hemagluttinating encephalomyelitis coronavirus; ND, not done; +, positive.

described nonsuppurative encephalomyelitis in 70%–100% of animals showing neurologic signs and in 20%–60% of animals with VWD syndrome. In our study, nonsuppurative encephalomyelitis was found in 50% of infected animals. Our IHC results agreed with findings reported by others in which CoV antigen was detected only in neurons (*8*,*14*,*15*). Detection of amplicons of ≈250 bp in brain samples was highly suggestive of PHE-CoV because this is the only known neurotropic CoV for pigs (*4*). In addition, results observed in samples of nervous tissue processed for IHC, RT-PCR, and sequence analysis added further evidence of the precise causal agent of the current outbreak. This report documents the emergence of PHE-Cov in South America.

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Highly Pathogenic Avian Influenza Virus (H5N1) in Domestic Poultry and Relationship with Migratory Birds, South Korea

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During the 2006–2007 winter season in South Korea, several outbreaks of highly pathogenic avian influenza virus (H5N1) were confirmed among domestic poultry and in migratory bird habitats. Phylogenetic analysis showed that all isolates were closely related and that all belong to the A/bar-headed goose/Qinghai/5/2005–like lineage rather than the A/chicken/Korea/ES/2003–like lineage.

Highly pathogenic avian influenza (HPAI) virus (H5N1) has been detected repeatedly in domestic poultry and wild birds since 1997, and it poses a substantial threat to human health (*1*,*2*). Since the end of 2003, influenza virus (H5N1) strains have spread in an unprecedented manner in many Asian countries, and the outbreaks have resulted in >170 human deaths in Thailand, Vietnam, Cambodia, and Indonesia. These outbreaks have also caused serious economic losses in the poultry industry (www.oie.int), including South Korea in 2003—the first official report of subtype H5N1 in South Korea's history (*3*).

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Since the outbreak caused by subtype H5N1 from migratory waterfowl on Qinghai Lake (QH) in May 2005 (*4*,*5*), outbreaks of QH-like avian influenza virus (H5N1) have been reported in the People's Republic of China, Mongolia, Russia, Europe, and Africa, and have been ascribed to the migration of wild birds (*6*,*7*). In contrast to virus found in countries on the western side of Qinghai Lake, the Fujian-like avian influenza virus (H5N1) sublineage has predominated in southern China since late 2005 (*8*), but no outbreaks were reported in Far-Eastern Asian countries such as South Korea and Japan until October 2006. Eventually, in November 2006 and January 2007, outbreaks of HPAI (H5N1) occurred in South Korea and Japan. Here we report the second outbreak of HPAI (H5N1) among poultry in South Korea since November 2006 and its relationship with 2 HPAI virus (H5N1) strains isolated from migratory bird habitats (i.e., in the environment).

The Study

All of the virus strains from domestic poultry used in this study were isolated by the Korean National Veterinary Research and Quarantine Service (NVRQS) in embryonated eggs that were inoculated with tissues and swab specimens collected from the oropharynx and cloaca of affected birds. Two subtype H5N1 strains were isolated in embryonated eggs that had been inoculated with fecal specimens obtained from migratory bird habitats by Chungbuk National University and Chungnam National University. Viral genes were sequenced and analyzed as described (*9*). The full-length sequences for each segment were used in phylogenetic analyses. Gene sequences determined in this study have been deposited in GenBank under accession nos. EU233675–EU233746.

On November 22, 2006, NVRQS confirmed the first case of HPAI (H5N1) at a chicken farm in Iksan, Jeollabuk-Do, in South Korea. The affected flock contained 6,500 chickens and had shown a sudden increase in severe clinical signs and high mortality rate (86%), as reported by farmers and veterinarians (Table). During intensive surveillance in December 2006 within a 10-km radius (the surveillance zone) from the first outbreak farms, we found other HPAI (H5N1)–affected farms at Iksan (chicken farm, 3.4% deaths) and Gimje (quail farm, 4% deaths), Jeollabuk-Do (Figure 1).

On December 21, 2006, the fourth outbreak of HPAI (H5N1) was confirmed in Asan, Chungcheongnam-Do, in breeder ducks that had shown a severe drop in egg production but no deaths. During intensive observation within the surveillance zone from the fourth outbreak farm, another HPAI (H5N1) outbreak was confirmed in Cheonan, Chungcheongnam-Do, on January 20, 2007, in a layer chicken

[1]These authors contributed equally to this work.

Table. History of highly pathogenic avian influenza (H5N1) suspected cases, South Korea, 2006–2007

Date reported	Outbreak no.	Source/ breed*	Age, wk	Region	No. animals/farm	Clinical signs	Isolate
2006 Nov 22	1	Chicken/BB	45	Jeollabuk Iksan	13,000	Depression, death	A/chicken/IS/2006 A/chicken/IS2/2006
2006 Nov 27	2	Chicken/BB	40	Jeollabuk Iksan	12,000	Depression, death	A/chicken/IS3/2006
2006 Dec 10	3	Quail	16–28	Jeollabuk Gimje	290,000	Death	A/quail/KJ4/2006
2006 Dec 21	4	Duck/B	30	Chungcheongnam Asan	9,000	Decrease in egg production	A/duck/Asan5/2006 A/duck/Asan6/2006
2007 Jan 20	5	Chicken/L	32	Chungcheongnam Cheonan	30,000	Depression, death	A/chicken/CA7/2007
2006 Dec 21		Environment		Chungcheongnam Cheonan			A/environment/Korea/ W149/2006
2006 Dec 21		Environment		Chungcheongbuk Cheongju			A/environment/Korea/ W150/2006

*BB, broiler breeder; B, breeder; L, layer.

farm with a 1% mortality rate (Figure 1). Compared with the rates in the first outbreak, the mortality rates in the more recent outbreaks were low (1%–4%) at notification time. This low proportion of deaths could be attributed to the early reporting system between the farmers and NVRQS, when the mortality rate reached ≈1% of the flock on poultry farms, and to the culling of flocks on reverse transcription–PCR confirmation (usually within 1 day) to prevent the spread of the disease. This could have limited the recorded observations when the infecting influenza virus was eliminated before the full extent of its pathogenicity could be manifested, usually after several days of infection.

Conclusions

Great interest has been focused on the role of migratory birds in the spread of H5N1 subtype and the exchange of virus strains between domestic and wild birds in Asia. Therefore, we surveyed avian influenza virus in migratory birds in South Korea to investigate whether the HPAI (H5N1) outbreaks in domestic poultry bore any relationship to bird migration in the same region. During our routine survey for influenza activity in migratory bird habitats, on December 21, 2006, 2 distinct subtype H5N1 strains were isolated from fecal samples from 2 migratory bird habitats—one near the first outbreak farm in Chungcheongnam-Do, and the other from a stream in Chungcheongbuk-Do (Figure 1).

Our phylogenetic analysis of the hemagglutinin (HA) genes of all Korean isolates showed that the isolates belong to the A/bar-headed goose/QH/65/2005 (QH/2005)–like lineage that caused an outbreak among wild birds at Qinghai Lake in China during 2005, rather than the first HPAI (H5N1) lineage (A/chicken/Korea/ES/2003) that infected farms in Korea in 2003 (Figure 2). Notably, the 2 isolates from migratory bird habitats were closely related to the H5N1 subtype poultry virus strains: A/environment/Korea/W149/2006 was similar to the viruses that occurred in Chungcheongnam-Do, and A/environment/Korea/W150/2006 was similar to viruses that affected birds in Jeollabuk-Do. However, all H5N1 subtype virus strains have a series of basic amino acids at the HA cleavage site (PQGERRRKKR/G), which is a characteristic of influenza viruses that are highly pathogenic to chickens (*4*,*5*). The intravenous pathogenicity index score of A/chicken/Korea/IS/2006 was 3.0 in chickens.

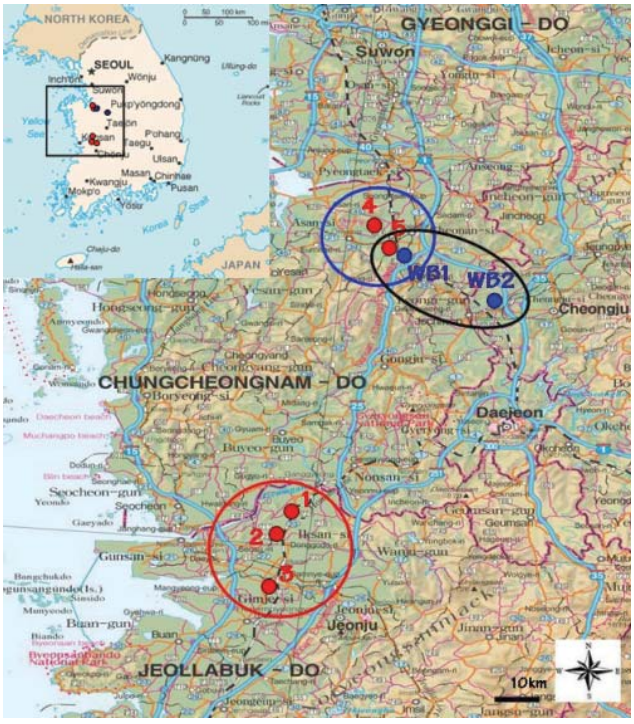

Figure 1. Location of highly pathogenic avian influenza (HPAI) virus (H5N1) outbreaks, South Korea, 2006–2007. Black box on inset shows area of enlargement. Red circle represents outbreaks in Jeollabuk-Do (first, second, and third outbreaks). Blue circle represents those in Chungcheongnam-Do (fourth and fifth outbreaks). Black oval represents regions in which HPAI virus (H5N1) isolates were isolated from migratory bird habitats during this study.

Phylogenetic analysis of the other genes showed a similar evolutionary pattern to the HA gene tree. All 2006–2007 isolates had a 20-aa deletion in the stalk region (residues 49–68) of neuraminidase (NA) compared with the NA of A/goose/Guangdong/1/96. Analysis of the raw sequencing traces showed no mutations in NA genes of all isolates associated with resistance to NA inhibitors. All 2006–2007 Korean HPAI (H5N1) isolates had glutamic acid at position 92 of the nonstructural protein 1, a position that is related to the ability of H5N1/97 virus to escape the host antiviral cytokine response (*12*), and lysine at position 627 in the PB2 protein, which is commonly observed in QH viruses (*6*). Lysine 627 in PB2 is conserved in authentic human influenza viruses and is associated with high virulence of influenza virus (H5N1) strains in mice (*13*). Analysis of membrane (M) 2 protein sequences showed that none of the 2006–2007 HPAI (H5N1) Korean strains were resistant to amantadine.

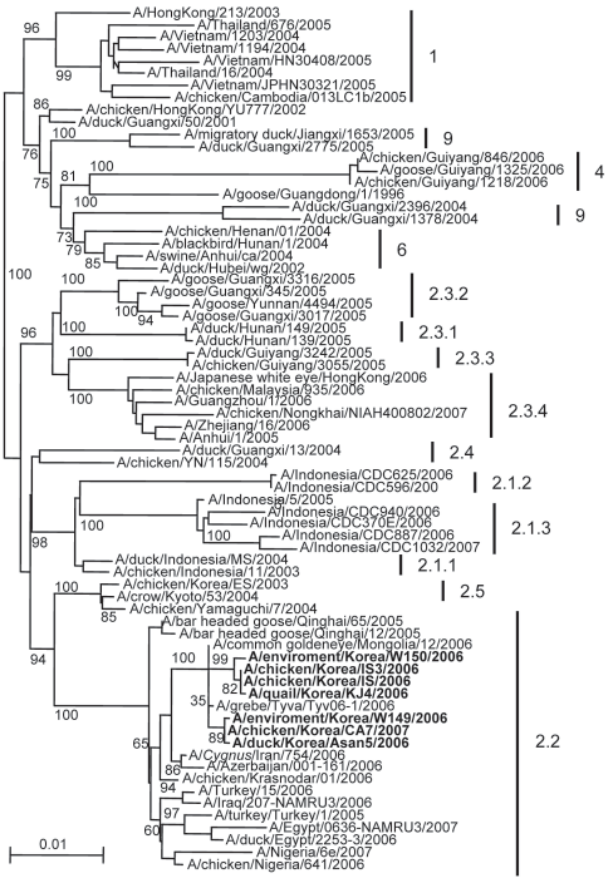

Figure 2. Phylogenetic trees for hemagglutinin (HA) genes of Korean influenza virus (H5N1) isolates from wild birds and poultry farms during 2006–2007. The DNA sequences were compiled and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI, USA). Multiple sequence alignments were made by using ClustalX (*10*). Rooted phylograms were prepared with the neighbor-joining algorithm and then plotted by using NJplot (*11*). Branch lengths are proportional to sequence divergence and can be measured relative to the scale bar shown (0.01-nt changes per site). Branch labels record the stability of the branches >1,000 bootstrap replicates. The tree was produced by referring to the proposed global nomenclature system for influenza virus (H5N1) (www.offlu.net). **Boldface** indicates isolates tested in the current study.

In contrast to the 2003 H5N1 subtype isolates (*3*), all of the 2006–2007 H5N1 subtype isolates were QH/05-like strains. Phylogenetic analysis showed that this sublineage spread wildly through Africa and Europe but not in eastern Asia, until the outbreak reported here. We cannot conclude whether wild migratory birds were the origin of the HPAI virus (H5N1) found in poultry or vice versa in South Korea, because the environmental isolates were obtained after the poultry outbreaks. However, the report of outbreaks of similar HPAI virus (H5N1) strains in Japan on January 13, 2007 (www.oie.int/eng) suggested that migratory birds could be a strong mediator for the spread of HPAI virus (H5N1) in South Korea and Japan, as occurred in 2003, because these 2 countries share similar wild bird migration routes. Therefore, continued monitoring of the domestic and wild bird populations is needed to better understand interspecies transmission and to clarify the importance of avian hosts in the ecology of influenza viruses.

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Mutations in Influenza A Virus (H5N1) and Possible Limited Spread, Turkey, 2006

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We report mutations in influenza A virus (H5N1) strains associated with 2 outbreaks in Turkey. Four novel amino acid changes (Q447L, N556K, and R46K in RNA polymerase and S133A in hemagglutinin) were detected in virus isolates from 2 siblings who died.

Influenza A virus (H5N1) is the predominant candidate for a future influenza pandemic if it develops efficient ability for human-to-human transmission. Since 2003, a total of 109 human deaths have been associated with this strain (*1*). Monitoring the genetic structure of this virus is needed for predicting changes that may confer ability to cause pandemics: pathogenicity, host range, and antigenic drift.

Recently, 2 avian influenza A (H5N1) outbreaks occurred in Turkey. The first outbreak, in Balikesir in northwestern Turkey in October 2005, was limited to poultry. The second outbreak, in Dogubeyazit in northeastern Turkey in November 2005, involved poultry and humans. As of January 2006, a total of 12 human cases, 4 fatal, have been confirmed (*1*).

The Study

We analyzed molecular evolution of the virus genome by sequencing the hemagglutinin (HA), RNA polymerase (PB2), and matrix 2 (M2) genomic segments of 4 chicken and 2 human viral isolates. During the first outbreak, the MYS viral isolate was obtained from a chicken. During the second outbreak, viral isolates SU, 13, and 20 were obtained from chickens in southeastern Turkey, Anatolia, and Istanbul, respectively. Human viral isolates FK and MAK were obtained from 2 siblings who died shortly after the second outbreak. Changes in the viral genomes are summarized in the Table.

Amino acids at positions 16, 13, 18, 20, 28, 55, and 78 in M2 are associated with host specificity; those at positions 31, 34, 26, 27, and 30 are associated with resistance to adamantanes (*2*). All isolates in our study showed greatest homology to the M2 region of influenza A virus A/bar-headed goose/Qinghai /59/05 (H5N1) (GenBank accession no. AAZ16311). None of the isolates had amino acid changes associated with resistance to adamantanes. All chicken and human isolates from the second epidemic had a unique mutation (I42T) in the M2 region. Thus, the only difference between isolates from the 2 outbreaks is a mutation (T42) in isolates from the second outbreak.

The HA sequence of MYS showed 98% homology with that of influenza A virus A/chicken/Kurgan/3/2005 (H5N1) (GenBank accession no. DQ323672) and contained a unique mutation (S133A) near receptor binding residue GVSSAC at positions 134 through 139. HA cleavage sites of all isolates from Turkey contained the sequence PQGERRRKKRGLF, similar to that of influenza A virus A/duck/Novosibirsk/ 56/2005(H5N1) (accession no. ABB17275), which indicates their high virulence. MAK contained mutations D158N and S227N in the HA region. These 2 mutations are important because D158N results in a potential glycosylation site and S227N enhances affinity for human receptors.

Enhanced pathogenic potential of all avian and human influenza virus isolates from the second outbreak was shown by mutation E627K in the PB2 region. In addition, unique amino acid changes found only in human isolates were Q447L in FK and Q447L, R46K, and N556K in MAK.

Conclusions

The Q447L and E627K mutations in FK and MAK virus isolates indicate a common origin of viruses in the 2 siblings. Unique mutations (D158N and S227N in HA and N556K and R46K in PB2) in only the MAK isolate suggest

Table. Mutations in avian and human influenza A virus (H5N1) isolates from Turkey*

Isolate	Mutations		
	HA	PB2	M2
FK†	D158, S227	E627K, Q447L	I28V, I42T
MAK†	D158N, S227N	E627K, R46K, Q447L, N556K	I28V, I42T
MYS‡	D158, S133A, S227		I28V
SU§	D158, S227	E627K	I28V, I42T
13§	D158, S227	E627K	I28V, I42T
20§	D158, S227	E627K	I28V, I42T

*HA, hemagglutinin; PB2, RNA polymerase; M2, matrix 2 protein.
†Human isolates from the second outbreak.
‡Chicken isolate from the first outbreak.
§Chicken isolates from the second outbreak.

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virus evolution in 1 patient. Human-to-human transmission and adaptation of the virus to infect humans have been suggested (*3*). Incomplete knowledge of the history of infections in humans and lack of sample availability limited our study. Unique mutations in PB2, particularly in these human isolates, emphasize the need for clinical, epidemiologic, and molecular studies for further understanding of the global pattern of evolution of influenza A virus (H5N1).

The close location of mutation T42 to viral regions associated with host specificity and resistance to adamantanes and isolation of virus with this mutation from humans should be studied. Although isolates from the FK and MAK group have not been available for comparison, the unique changes should be studied with respect to their ability to confer survival advantage to virus in humans.

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Ciprofloxacin-Resistant *Salmonella enterica* Serotype Typhimurium, China

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We characterized 44 *Salmonella enterica* serotype Typhimurium isolates from Tongji Hospital outpatients in Wuhan, China, May 2002–October 2005. All 31 ciprofloxacin-resistant isolates were also resistant to ≥8 other antimicrobial drugs and carried ≥2 mutations in GyrA and 1 mutation in ParC. Class 1 integrons were identified in 37 isolates.

Salmonellae are a common cause of community-acquired foodborne bacterial gastroenteritis worldwide. The incidence of *Salmonella* infections in the People's Republic of China has not been well documented. However, in the United States, ≈1.4 million persons are infected by *Salmonella* spp. each year (*1*). Although >2,500 serotypes have been reported, *Salmonella enterica* serotype Typhimurium is 1 of the leading serotypes causing salmonellosis worldwide (*2*). Fluoroquinolones such as ciprofloxacin are strongly recommended for treatment of severe *S.* Typhimurium infections in adults (*3*).

In this study, we characterized all *S.* Typhimurium isolates recovered from May 2002 through October 2005 from outpatients of Tongji Hospital, Wuhan, China, a sentinel hospital in the National Center for Surveillance of Antimicrobial Resistance. During the time of this study, Tongji Hospital strictly followed the recommendation for treatment of severe *S.* Typhimurium infections.

The Study

We analyzed stool samples from outpatients who came to Tongji Hospital from the local community for treatment of diarrhea during the study period. A total of 44 *S.* Typhimurium isolates were recovered from the samples. *S.* Typhimurium was identified by using standard biochemical tests and commercial typing antiserum (Statens Serum Institute, Copenhagen, Denmark) according to the manufacturer's instructions. MICs of 15 antimicrobial drugs (Table) were determined by using the broth-microdilution method; susceptibility to streptomycin was measured by using the disk-diffusion method as recommended by the Clinical and Laboratory Standards Institute (*4*). All isolates were further characterized by mutation analysis in the quinolone-resistance determining regions (QRDRs), pulsed-field gel electrophoresis (PFGE), and screening for class I integrons and β-lactamase genes as previously described (*5*–*8*).

Of the 44 isolates, 36 (82%) were resistant to nalidixic acid and 31 (70%) were resistant to ciprofloxacin (Table). Only 3 isolates, recovered in 2002, were susceptible to all 15 tested antimicrobial drugs; 36 (82%) displayed resistance to at least 8 drugs. Of 13 antimicrobial drug–resistant phenotypes identified, the most often observed phenotype (21/44) was resistance to amoxicillin–clavulanic acid, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, sulfamethoxazole, streptomycin, trimethoprim–sulfamethoxazole, and tetracycline (R-type AcAmCCpGNSStSxtT). All isolates were susceptible to cefotaxime and ceftazidime; 5 isolates obtained in 2004 were intermediately susceptible to cefepime (MIC 16 μg/mL) (online Appendix Figure, available from www.cdc.gov/EID/content/14/3/493-appG.htm).

Overall, 8 PFGE strain types (A–H) and 6 clusters (1–6) were identified. All isolates that belonged to clusters

Table. Resistance phenotypes of *Salmonella enterica* serotype Typhimurium isolated from Tongji Hospital outpatients, Wuhan, China, May 2002–October 2005

Antimicrobial agent	MIC, μg/mL*	No. resistant isolates (n = 44)
Phenicols (chloramphenicol)	≥32	33
Penicillins		
Ampicillin	≥32	35
Amoxicillin–clavulanic acid	≥32/16	32
Cephalosporins		
Cefepime	≥32	0
Cefotaxime	≥64	0
Ceftriaxone	≥64	0
Tetracyclines (tetracycline)	≥16	36
Aminoglycosides		
Amikacin	≥64	2
Gentamicin	≥16	35
Kanamycin	≥64	15
Streptomycin†	NA	40
Sulfonamides and potentiated sulfonamides		
Sulfamethoxazole	≥512	39
Trimethoprim-sulfamethoxazole	≥4/76	36
Quinolones and fluoroquinolones		
Nalidixic acid	≥32	36
Ciprofloxacin	≥4	31

*MICs were determined by the broth-microdilution method; results were interpreted in accordance with the interpretive standards of the Clinical and Laboratory Standards Institute (*4*).
†Resistance to streptomycin was determined by disk-diffusion method.
NA, not applied.

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1, 2, and 4 were resistant to ciprofloxacin and to 8–11 other antimicrobial drugs. Two dominant patterns, B and F, were identified and included 16 and 10 ciprofloxacin-resistant isolates, respectively. Among 16 isolates of pattern B, 14 isolates showed the R-type AcAmCCpGNSStSxtT, and 1 was additionally resistant to kanamycin. In pattern F, 4 isolates showed the R-type AcAmCCpGNSStSxtT, and 5 were additionally resistant to kanamycin.

Point mutations in the QRDR of *gyrA*, *parC*, or *parE* were identified in 35 of 36 nalidixic acid–resistant isolates, whereas no *gyrB* mutations and no *qnr* plasmid were found. For 5 nalidixic acid–resistant and ciprofloxacin low-level–resistant isolates, 4 isolates harbored single (D87N) or double (S83F, D87N) mutations in GyrA, and no mutation was found in 1 isolate (ST6). All 31 ciprofloxacin-resistant isolates accumulated a minimum of 3 mutations: GyrA(S83F, D87N), ParC(S80R) (28 isolates) or GyrA(S83F, D87G), ParC(S80R) (3 isolates). Two ciprofloxacin-resistant isolates with PFGE pattern C and 1 isolate with PFGE pattern A2 harbored an additional mutation in ParE (S458P) (online Appendix Figure).

Of 39 sulfamethoxazole-resistant isolates encompassing PFGE clusters 1, 2, 3, and 4, 37 possessed class 1 integrons. All class 1 integron–positive isolates were resistant to 6–12 antimicrobial drugs; 2 distinct class 1 integrons were identified in 37 isolates. Of isolates obtained from 2002 through 2005, 32 contained a 1.9-kb integron gene cassette *dhfrXII-orfF-aadA2*. In 2004 and 2005, 3 and 2 isolates, respectively, contained a 2-kb integron gene cassette $bla_{\text{OXA-30}}$-*aadA1*. None of the 36 ampicillin-resistant isolates contained TEM or SHV enzyme, but OXA-30 gene was detected in 32 isolates, identical in DNA sequence to GenBank AF255921. All 32 isolates harboring OXA-30 enzyme showed MICs to cefepime of 2–16 μg/mL, whereas isolates lacking OXA-30 showed MICs to cefepime of ≤1 μg/mL. In 2004, 5 isolates harboring OXA-30 enzyme with PFGE pattern F showed intermediate susceptibility to cefepime. All ciprofloxacin-resistant *S.* Typhimurium isolates also harbored class 1 integron, β-lactamases, and were phenotypically resistant to 8–11 additional antimicrobial drugs (online Appendix Figure).

Conclusions

We report a high incidence of fluoroquinolone-resistant *S.* Typhimurium isolates from Tongji Hospital outpatients. The MIC variation for ciprofloxacin differed 2- to 4-fold in isolates that had the same QRDR mutation profile, which implies that other mechanisms might partially contribute to the resistance phenotype (online Appendix Figure). After PFGE analysis, *S.* Typhimurium isolates were grouped into 3 ciprofloxacin-susceptible clusters and ciprofloxacin-resistant clusters. Similar distribution patterns have also been observed in isolates from Japan (*9*), which suggests a distinct genetic lineage for ciprofloxacin-resistant isolates that have become dominant. Studies have reported that ciprofloxacin-resistant *S.* Typhimurium isolates were usually resistant to multiple drugs (*9*,*10*). In this study, all ciprofloxacin-resistant *S.* Typhimurium isolates were resistant to 8–11 additional antimicrobial drugs. Among the 32 isolates harboring OXA-30 enzyme in this study, only 5 with PFGE pattern F showed intermediate resistance to cefepime, which suggests different levels of OXA gene expression or the contribution of other unknown mechanisms.

The high incidence of quinonlone-resistant *S.* Typhimurium isolates in this study might be affected by several factors. First, patients infected by antimicrobial drug–resistant *S.* Typhimurium strains had higher rates of hospitalization than did patients infected by susceptible strains (*11*,*12*), and the isolates in this study were from a university-affiliated medical center that usually treats patients with severe illness. Second, US studies have estimated that half of outpatient antimicrobial drugs were inappropriately prescribed for conditions such as viral illness (*13*). In China, inappropriate prescriptions might be even more common because antimicrobial drug prescriptions in hospitals are a source of profit. Although we do not have patient antimicrobial drug–use information, the easy access to antimicrobial drugs raises the possibility that outpatients might have taken fluoroquinolones after the onset of the illness but before the collection of stool specimens. Third, because livestock products are a common source of salmonellosis, the dissemination of ciprofloxacin-resistant *S.* Typhimurium might have been facilitated by the use of fluoroquinolones in livestock production (*2*). Last, use of other antimicrobial drugs, such as ampicillin, gentamicin, or streptomycin, may also contribute to the spreading of fluoroquinolone-resistant *S.* Typhimurium because all the ciprofloxacin-resistant isolates were also resistant to 8–11 additional antimicrobial drugs.

Although fluoroquinolone-resistant isolates were prevalent in Tongji Hospital, ciprofloxacin is still empirically used to treat salmonellosis in adults, due partly to the absence of systematic surveillance programs to actively monitor antimicrobial drug resistance in *Salmonella* spp. Because local data on antimicrobial drug susceptibility are less available, we strongly recommend that hospitals and national and local health laboratories develop and maintain the capacity to perform *Salmonella* culture and in vitro susceptibility testing.

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Geographic Linkage and Variation in *Cryptosporidium hominis*

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UK *Cryptosporidium hominis* isolates have previously shown slight PCR fragment length polymorphism at multiple loci. To further investigate transmission, we conducted a case–control study and sequenced the GP60 locus from 115 isolates. Nine subtypes were identified; IbA10G2 predominated. Having a non-IbA10G2 subtype was significantly linked to recent travel outside Europe.

Cryptosporidium hominis, a human-adapted species of the protozoan parasite *Cryptosporidium*, causes ≈50% of the reported cryptosporidiosis cases in the United Kingdom each year (*1*). Risk factors for *C. hominis* have been identified as traveling abroad and changing diapers of children (*2*). However, studies using multilocus fragment typing of mini- and microsatellite DNA markers have shown that *C. hominis* isolates from the United Kingdom are genetically very similar (*3*,*4*), and no associations between *C. hominis* subtype and risk have been identified (*4*). To explore whether a more detailed examination of genomic DNA could benefit public health, we used sequence analysis of the widely studied and highly variable GP60 gene to reexamine *C. hominis* isolates from a case–control study of sporadic cryptosporidiosis (*2*).

The Study

A total of 115 *C. hominis* isolates were collected and confirmed during a case–control study of human cryptosporidiosis in Wales and northwest England (*2*). To identify subtypes, we analyzed the DNA sequences of an ≈850-bp region of the GP60 gene encompassing the polyserine tract (variable numbers and forms of a repeating sequence of 3 nucleotides coding for the amino acid serine) and the hypervariable downstream region (*5*). We used a nested PCR protocol with primary PCR primers AL3531 and AL3535 and secondary primers AL3532 and AL3534. PCR products were sequenced in both directions.

The microsatellite triplet codons were categorized according to the number of trinucleotide repeats (TCA, TCG, or TCT) coding for the amino acid serine (*6*), and the nomenclature was expanded for subtype family Ia to include the number of repeats (e.g., R1, R2) of the sequence AA(A/G)ACGGTGGTAAGG after the microsatellite region (*7*). Sequence data for representative isolates were deposited in GenBank (accession nos. EU161648–EU161655, EF214734, and EF214735). We then investigated subtypes for relationships with reported exposures by using single-variable analysis performed in SPSS 12.0 version (SPSS Inc., Chicago, IL, USA).

Of 115 *C. hominis* isolates, 14 were not typeable at the GP60 locus (12 did not amplify and 2 gave equivocal reactions); typeability was 87.8%. Nine subtypes were identified but 92 (91.1%) typeable isolates were IbA10G2. Each of the other identified types contained only 1 isolate member except for IgA24, which contained 2. This resulted in a low discriminatory power of 0.171.

More persons with subtypes other than IbA10G2 had a history of recent foreign travel (5/9, 55.6%) than did those with IbA10G2 (27/92, 29.3%), although this was not statistically significant (p = 0.1374 [Fisher exact test], odds ratio [OR] 3.01, 95% confidence interval [CI] 0.59–16.20). However, all 5 case-patients with other subtypes reported travel history outside Europe, 3 to Pakistan (subtypes IaA12R3, IaA22R2, and IaA30R3), 1 to Kenya (IaA25R3), and 1 to New Zealand (IgA24) while only 3 case-patients with IbA10G2 types were known to have traveled outside Europe (to Tunisia and Turkey) (Table 1). All those who reported travel within Europe had subtype IbA10G2.

Four case-patients who had not traveled outside the United Kingdom had non-IbA10G2 alleles, but with the exception of IgA24, these were different from the subtypes found in case- patients who had traveled outside Europe. The relationship between travel outside Europe and GP60 subtypes was statistically significant (p = 0.00008 [Fisher exact test], OR 37.08, 95% CI 4.76–303.65; Table 2). No other epidemiologic associations were present.

Conclusions

Although GP60 sequence typing had very low discriminatory power for UK *C. hominis* isolates, our findings are in agreement with previous findings based on multiple loci that *C. hominis* appears to be highly conserved in the United Kingdom (*3*,*4*). DNA sequencing of a substantial proportion of the GP60 gene, including the microsatellite region, provides higher resolution data than investigating microsatellite length polymorphisms, which may mask differences in sequence (*8*); here, DNA sequencing facilitated identification of a significant link between subtype

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Table 1. Foreign travel history and *Cryptosporidium hominis* GP60 subtypes in sporadic cases, United Kingdom

Foreign travel history	No. subtype IbA10G2	No. other subtypes	Other subtype details
No	65	4	IaA23R4, IbA9G2, IfA12G1, IgA24
Yes	27	5	See below
European travel destinations			
Balearic Islands	3	0	
Canary Islands	5	0	
Cyprus	3	0	
France	4	0	
Greece	2	0	
Spain	6	0	
Non-European destinations			
Kenya	0	1	IaA25R3
Pakistan	0	3	IaA12R3, IaA22R2, IaA30R3
New Zealand	0	1	IgA24
Tunisia	2	0	
Turkey	1	0	
Destination not known	1	0	

and foreign travel outside Europe. Subtype IbA10G2 is very clearly predominant in the United Kingdom. Subtype family Ib and the IbA10G2 subtype have been reported in Europe both in sporadic cases and outbreaks (*9*–*12*) and occur worldwide (*12*). The conclusion of Cohen et al. (*11*), that Ib is the predominant *C. hominis* allele associated with waterborne outbreaks, is explained if this is the most common allele causing human cryptosporidiosis in Europe, as it is in the United Kingdom, and is therefore predominant in human sewage.

In nonindustrialized countries, a greater variety of *C. hominis* subtypes have been reported (*7*,*8*,*13*,*14*). Of the 3 isolates found in case-patients returning from Pakistan, IaA12R3 had been isolated from a patient from Nepal (GenBank accession no. AY167595); IaA22R2 and IaA30R3 had not been reported previously. Subtype IaA25R3 was found in a case-patient returning from Kenya and was homologous to a *C. hominis* reference strain (TU502) of Ugandan origin (GenBank accession no. XM_663000). Notably, of the 4 case-patients with non-IbA10G2 subtypes who did not report foreign travel, 1 had the IgA24 subtype, which matched an isolate from Northern Ireland (GenBank accession no. EF214734), and may well circulate in the United Kingdom; IaA23R4 was homologous to isolates from the United States (GenBank accession no. AF164504) and Canada (GenBank accession no. DQ192510); and IfA12G1 had been identified in Australia (*12*).

C. hominis is highly conserved in indigenous UK case-patients, and subtypes other than IbA10G2 are linked to recent foreign travel outside Europe. It is not possible to predict whether this apparent stability will remain or whether it will be influenced by international travel.

Table 2. Travel outside Europe and *Cryptosporidium hominis* GP60 subtypes in sporadic cases, United Kingdom

Travel outside Europe	No. subtype IbA10G2	No. other subtypes	Total
No	88	4	92
Yes	3	5	8
Total	91	9	100

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Low Frequency of Infection with Avian Influenza Virus (H5N1) among Poultry Farmers, Thailand, 2004

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In Thai provinces where avian influenza outbreaks in poultry had been confirmed in the preceding 6 months, serum from 322 poultry farmers was tested for antibodies to avian influenza virus subtype H5N1 by microneutralization assay. No study participant met the World Health Organization serologic criteria for confirmed infection.

During late 2003 and 2004, highly pathogenic avian influenza virus (H5N1) caused extensive outbreaks and die-offs in poultry flocks in Thailand and several other countries in Southeast Asia (*1*). From January through March 2004, 12 cases, 8 fatal, in humans resulted from infection with influenza virus (H5N1) in Thailand (*2*). In response, the Thailand Department of Livestock Development enlisted government employees to conduct a large-scale cull of poultry in the affected provinces (www.dld.go.th/home/bird_flu/emergency.html). This effort began on January 23, 2004, and resulted in the slaughter of >21 million birds (www.fao.org/ag/againfo/subjects/en/health/diseases-cards/avian_bg.html). Poultry farmers and persons involved in culling are at increased risk for infection (*3*). In May 2004, we conducted a seroepidemiologic investigation of Thai poultry farmers to determine the frequency of avian influenza (H5N1) transmission to humans.

The Study

We conducted a cross-sectional study among poultry farmers and cullers from 1 district in each of the 5 provinces (Chachoengsao, Kanchanaburi, Khon Kaen, Sukhothai, and Suphanburi) where outbreaks of avian influenza (H5N1) among poultry and human infections had been confirmed since January 2004 (Figure). With the assistance of provincial human and animal health authorities, we contacted farmers living in these districts. Informed consent was obtained, and a brief interview was conducted. Because the precise timing of potential exposures could not be determined, a single serum sample was collected from each patient and stored at –20°C until tested under Biosafety Level 3 (BSL-3) conditions. Specimens were tested, according to adapted methods described by Katz et al. (*4*), at the Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University by Microneutralization assay (micro-Nt) for antibody to H5N1 viruses. Before this study, senior laboratory staff from Siriraj Hospital received 2 weeks of on-site training by a visiting scientist from the US Centers for Disease Control and Prevention who had expertise with this assay. The World Health Organization

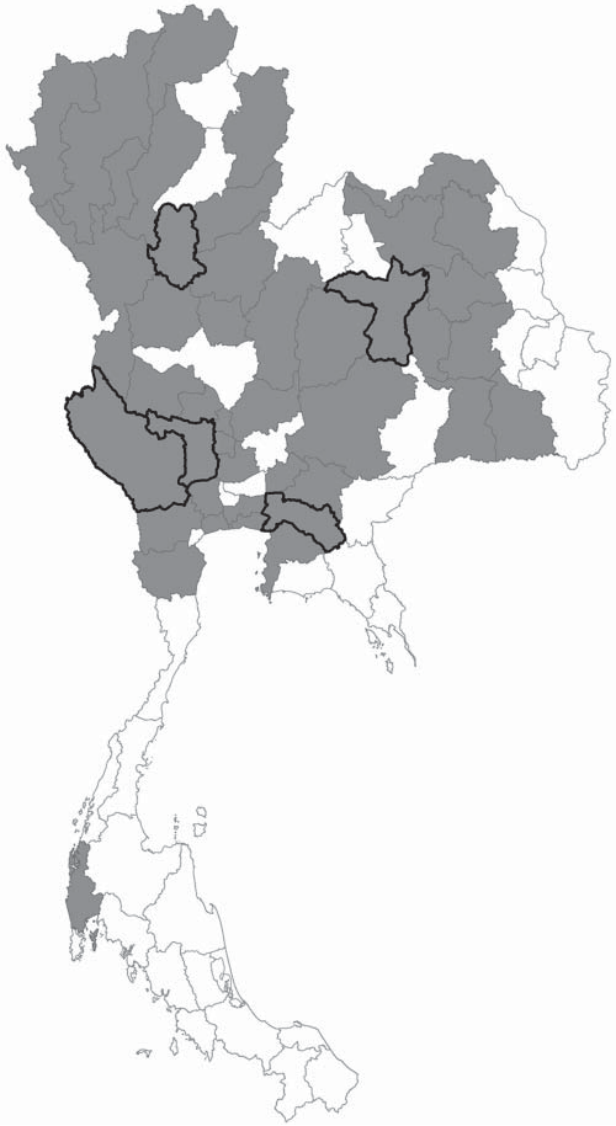

Figure. Map of Thailand. Gray shading indicates provinces with confirmed avian influenza outbreaks; black outlines indicate provinces included in this study.

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(WHO) defines a positive test result as a microneutralization antibody titer for influenza virus (H5N1) of >80 with a confirmatory ELISA or Western blot assay (3,4) (www.who.int/csr/disease/avian_influenza/guidelines/case_definition2006_08_29/en/index.html). Serum samples from persons >50 years of age were excluded from laboratory analysis because the microneutralization assay for antibodies against subtype H5N1 has been reported to be less specific for older persons (*5*).

Of 350 farmers asked to participate, 322 (92%) enrolled in the study, of which 167 (52%) were women, and 28 (8%) persons declined to participate. The mean age of participants was 34 years (range 5–50 years) (Table). Among participants, 188 (58%) reported handling sick or dying poultry, 107 (33%) were involved in culling operations of apparently well poultry in outbreak areas, and 27 (9%) reported only contact with well poultry in the context of routine farming practices. Although no study participant had an anti-H5N1 antibody titer of ≥80, 7 (2.2%) farmers had lower reactive antibody titers. Of these, 4 had titers of 10, 2 had titers of 20, and 1 had a titer of 40. The small number of study participants with anti-H5 antibody titers precluded statistical comparisons to those without reactive antibodies.

Table. Characteristics of 322 persons living on poultry farm in areas where avian influenza (H5N1) infections among poultry and humans had been confirmed since January 2004, Thailand

Variable	No. persons (%)
Province	
Chachoengsao	61 (18.9)
Kanchanaburi	32 (9.9)
Khon Kaen	65 (20.2)
Sukhothai	84 (26.1)
Suphanburi	80 (24.8)
Sex	
M	155 (48.1)
F	167 (51.9)
Age, y*	
≤10	15 (4.7)
11–20	32 (9.9)
21–30	49 (15.2)
31–40	121 (37.6)
41–50	105 (32.6)
Current smokers	67 (20.8)
Chronic illness	74 (23.0)
Type of poultry maintained†	
Layer hen	111 (34.5)
Broiler	42 (13.0)
Fighting cock	88 (27.3)
Backyard chicken	89 (27.6)
Egg-laying duck	7 (2.2)
Meat duck	8 (2.5)
Ornamental birds	3 (0.9)
Type of poultry farm	
Company farm	125 (38.8)
Individual farm (backyard)	197 (61.2)
Observed increased deaths of poultry	231 (71.7)
Living on a mixed swine/poultry farm	24 (7.5)

*Age range 5–50 y; median 35 y.
†Not mutually exclusive.

Conclusions

Poultry farmers and cullers are at increased occupational risk for exposure to avian influenza viruses. However, since 2004, infections have been less commonly reported in cullers, while poultry farmers have made up a large proportion of cases worldwide. A study in Hong Kong Special Administrative Region, People's Republic of China, examined influenza virus (H5N1) transmission and risk factors among poultry workers and government workers involved in culling during the 1997–98 outbreak (*3*). The study concluded that although no hospitalized poultry workers were identified among the 18 patients in that outbreak, 3% of 293 cullers and 10% of 1,525 poultry workers had antibody titers against influenza (H5N1) of ≥80, which suggested that a substantial number of mild or asymptomatic infections had occurred in this occupationally exposed population. In contrast, we found that no poultry workers had microneutralization titers ≥80, whereas 7 (2%) had lower titers that did not meet the WHO definition for seropositivity.

These findings could have several possible explanations. The lower titers may have resulted from cross-reactivity with circulating antibodies after previous human influenza virus infections (*5*,*6*). These low titers could be the result of mild or asymptomatic influenza (H5N1) infections because not all influenza virus infections invariably result in marked antibody responses (*7*). Likewise, these results could reflect the decay of antibody titers over time (*8*). Finally, the Micro-NT assay is a highly specific and strain-sensitive test. Although we used the same virus that was circulating in Thailand at that time, these lower titers could be attributable to infections with another virus variant.

Most human influenza (H5N1) infections have occurred in persons who had had direct contact with sick or dying poultry (*9*–*11*). While human infections with avian influenza (H5N1) continue to be reported, growing evidence indicates that this virus is not easily transmitted from poultry to humans and that mild or asymptomatic infections in humans are not common. A seroepidemiologic investigation in rural Cambodia surveyed 351 participants from 93 households in an area where influenza (H5N1) infections in poultry and a single fatal human case had been documented (*10*). Despite frequent, direct contact with poultry suspected of having influenza (H5N1) infection, none of the Cambodian study participants had antibodies reactive to this subtype. A similar study in Nigeria found that all of 295 poultry workers had negative test results for influenza (H5N1) neutralizing antibodies *(12)*. Studies of healthcare workers suggest that transmission of influenza

virus (H5N1) to hospital staff who cared for infected patients also appears to be uncommon (*13–15*).

Our study provides additional evidence to suggest that influenza virus (H5N1) is not easily transmitted to humans. However, the wide geographic distribution of this subtype, ubiquitous exposures, and the high case-fatality ratio from the infection underscore the importance of adherence to poultry-handling practices recommended by the Food and Agriculture Organization and WHO (www.wpro.who.int/NR/rdonlyres/7693BAF7-13E7-42DB-B92B-004CF5D517E7/0/WHOinterimrecommendation26012004.pdf, www.fao.org/ag/againfo/subjects/en/health/diseases-cards/avian_qa.html#8). Molecular surveillance indicates that the avian influenza virus (H5N1) continues to evolve rapidly (www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf). Additional sero-epidemiologic studies are warranted to monitor for changes in transmissibility and the spectrum of clinical illness.

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Sylvatic Dengue Virus Type 2 Activity in Humans, Nigeria, 1966

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Using phylogenetic analysis of complete virus genomes from human isolates obtained in Nigeria in 1966, we identified sylvatic dengue virus (DENV) strains from 3 febrile patients. This finding extends current understanding of the role of sylvatic DENV in febrile disease and documents another focus of sylvatic DENV transmission in West Africa.

Sylvatic dengue viruses (DENVs) are ecologically and evolutionary distinct ancestral lineages that circulate in forests of Southeast Asia and West Africa between nonhuman primates and arboreal *Aedes* mosquitoes. In Asia, serologic analysis and virus isolations suggest an association between *Macaca* spp. and *Presbytis* spp. monkeys and sylvatic DENV-1, -2, and -4, with *Aedes niveus* as the principal vector (*1*). Sylvatic DENV-3 has not been isolated, but it is believed to exist in Malaysia on the basis of sentinel monkey seroconversions (*1*). In West Africa, only sylvatic DENV-2 has been identified, where it circulates among *Erythrocebus patas* monkeys and sylvatic mosquitoes, including *Ae*. *furcifer*, *Ae*. *vitattus*, *Ae*. *taylori*, and *Ae*. *luteocephalus* (*2*–*6*).

Sylvatic DENV strains are considered the evolutionary progenitors of endemic and epidemic (henceforth called endemic) strains that are transmitted among humans in urban environments throughout the tropics and subtropics by the peridomestic mosquitoes *Ae*. *aegypti* and *Ae*. *albopictus*. Previously, no evidence has shown that sylvatic DENV cycles are involved in outbreaks of human dengue, which involve the genetically and ecologically distinct endemic strains. Available data suggest that sylvatic strains are either confined to forest habitats or produce relatively mild illness, as described in a few documented human sylvatic DENV-2 infections in West Africa (*5*,*6*). Additionally, the small number of documented human infections suggests that sylvatic DENV-2 strains do not produce secondary human infections (spillover epidemics). One possible explanation for confinement of sylvatic DENV strains to the forest is that they do not have contact with the peridomestic vectors *Ae*. *aegypti* and *Ae*. *albopictus*, which are not abundant in enzootic regions.

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Recent reports have shown that the gallery forest–dwelling mosquito *Ae*. *furcifer* is highly susceptible to sylvatic DENV infection (*2*) and disperses from the forest into villages in eastern Senegal (*3*). This finding suggests that this species may act as a bridge vector for exchange between forest and peridomestic habitats. Furthermore, the ability of *Ae*. *aegypti* and *Ae*. *albopictus* to transmit sylvatic DENV (*2*), as well as the lack of evidence that any adaptation of sylvatic DENV is needed to replicate efficiently in humans (*7*), suggests that transfer between forest and human habitats could occur regularly.

The Study

Given the above evidence, we hypothesized that unrecognized spillover epidemics may be caused by sylvatic DENV-2 strains in West Africa. We therefore examined isolates of DENV recovered from febrile patients, ranging from 3 months to 38 years of age, who were seen at the outpatient department of the University College Hospital, Ibadan, Nigeria, from August 1964 through December 1968 (*8*). Complement fixation and neutralization tests (with the Hawaii strain of DENV-1 and the Trinidad-1751 strain of DENV-2 as reference strains) classified 14 of 32 original isolates as DENV-2 (*8*). Three of these 14 isolates were obtained from the University of Texas Medical Branch World Reference Center for Emerging Viruses and Arboviruses; the other 11 are not known to exist in any virus collection (Table).

RNA was extracted from these 3 isolates, after passage in C6/36 mosquito cells, by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Their complete genomic sequences were determined by designing overlapping PCR amplicons. After purification by electrophoresis on 1% agarose gels, both strands were sequenced directly by using the ABI (Roche Diagnostics, Indianapolis, IN, USA) protocols with both the PCR and internal primers to derive a consensus sequence. We then generated a phylogenetic tree, including 22 endemic DENV-2 isolates representing strains from diverse localities throughout the tropics and neotropics, as well as representative strains of DENV-1, DENV-4 (including sylvatic isolates P72–1244 and P75–215, respectively), and DENV-3 strains as outgroups (41 sequences, 10,185 nt in length). Phylogenetic trees were inferred by using Bayesian analysis with 1 million iterations (*9*) as well as maximum parsimony and neighbor-joining implemented in PAUP version 4.0 (*10*) (Figure).

All analyses indicated that all 3 Nigerian isolates were genetically distinct from endemic DENV-2 isolates, including those from West Africa, and fell within the sylvatic DENV-2 clade (Figure). Among sylvatic isolates,

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Table. History of dengue virus type 2 isolates collected from patients with febrile illness in Ibadan, Nigeria, 1964–1968*

Isolate†	Patient age/sex	Source	Passage history‡	Date of collection	GenBank accession no.
IBH319	3 mo/F	Blood	NA	1964 Aug 11	–
IBH10126	8 y/M	Blood	NA	1966 Jun 1	–
IBH11208§	3.5 y/F	Blood	SM5, C6/36 –1	1966 Aug 17	EF105307
IBH11234§	31 y/M	Blood	SM16, C6/36 –1	1966 Aug 18	EU003591
IBH11358	5 mo/F	Blood	NA	1966 Aug 24	–
IBH11444	6 y/F	Blood	NA	1966 Aug 31	–
IBH11449	1.5 y/F	Blood	NA	1966 Aug 31	–
IBH11664§	1.5 y/M	Blood	SM30, C6/36 –1	1966 Sep 12	EF105388
IBH11935A	13 y/M	Blood	NA	1966 Sep 23	–
IBH12541	23 y/M	Serum	NA	1966 Oct 17	–
IBH13028A	24 y/M	Serum	NA	1966 Nov 17	–
IBH24075	1.5 y/F	Blood	NA	1968 Jan 12	–
IBH26489A	5 y/M	Blood	NA	1968 Apr 24	–
IBH26953	3 y/M	Blood	NA	1968 May 15	–

*Available dengue virus isolates were obtained from the University of Texas Medical Branch World Reference Center for Emerging Viruses and Arboviruses.
†A complete inventory of dengue viruses isolated in Ibadan during 1964–1969 is described in the 1969 annual report of the University of Ibadan, Arbovirus Research Project, p. 152–7.
‡NA, not available; SM, suckling mouse; C6/36, *Aedes albopictus* cell line. –1 indicates that the virus isolates were passaged once in C6/36 cultures in our laboratory to obtain high-titer stocks.
§Virus isolates used in this study.

Malaysian and West African sylvatic DENV-2 strains were genetically distinct. All analyses also delineated a chronologic divide among isolates within the sylvatic African DENV-2 clade; all pre-1980 isolates formed a group distinct from all post-1980 isolates. This observation supports recent evidence of rapid sylvatic DENV turnover caused by high nucleotide substitution rates (*11*). These findings also extend the temporal and spatial range in which sylvatic DENV are known to circulate in West Africa. The first documented isolation of sylvatic DENV in West Africa was from a febrile patient in Senegal identified in 1970 (*6*); the sylvatic isolates from the Nigerian patients predate this Senegalese strain by 4 years.

Conclusions

The 1964–1968 DENV-2 activity among humans in Ibadan is epidemiologically interesting. Although we were able to obtain only 3 of the of 10 DENV-2 strains collected in 1966 (Table), their identification as sylvatic strains and isolation from febrile patients suggest the first documented outbreak of sylvatic DENV-2 in humans. We define outbreak as a small, localized group of affected persons; such groups are often confined to a village or a small geographic area. Although no written records on the location of residence or exposure of these patients exist, all resided within the Ibadan city limits (D.E. Carey, pers. comm.). At that time, humans and monkeys living within 4 diverse ecologic zones (rainforest, derived savannah, southern Guinea savannah, and plateau) in Nigeria showed high levels of DENV-2 neutralizing antibodies, which suggested enzootic/endemic DENV-2 and a sylvatic cycle in Nigeria 30 years ago (*12*,*13*). Furthermore, the clinical manifestation of dengue caused by these Nigerian sylvatic DENV-2 strains

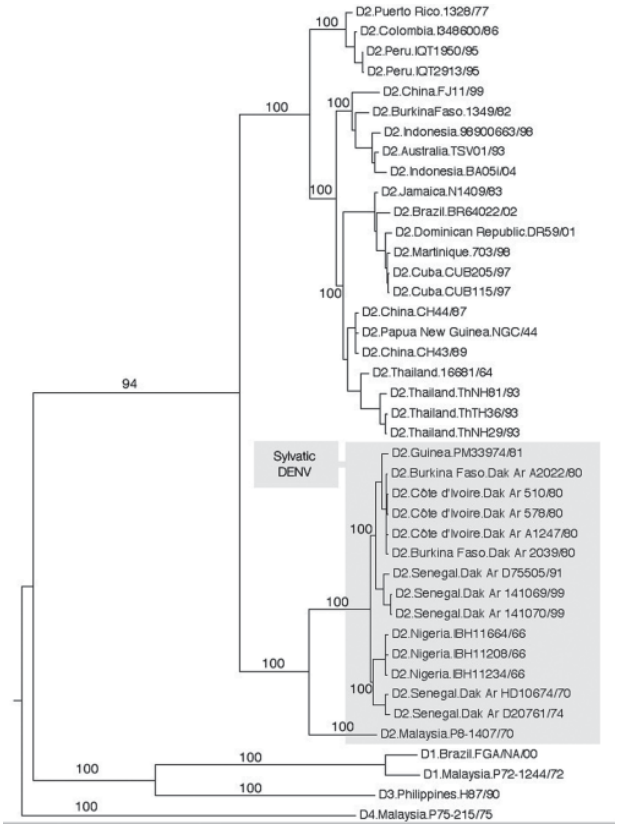

Figure. Phylogenetic relationships of 3 complete coding regions of Nigerian dengue virus type 2 (DENV-2) isolates obtained from febrile patients in Ibadan, Nigeria, during the 1966 rainy season. A total of 15 sylvatic DENV-2 genomes (shaded) were compared with human isolates of DENV-2 and representatives of DENV-1, DENV-3, and DENV-4. Phylogeny was inferred by using Bayesian analysis, and all horizontal branches are scaled according to the number of substitutions per site. Bootstrap values are shown for key nodes.

was indistinguishable from classic dengue fever caused by endemic strains (D.E. Carey, pers. comm.) (*5*,*6*,*14*). Isolation of sylvatic DENV-2 from febrile patients in an urban environment (*8*), the ability of peridomestic *Aedes* spp. mosquitoes (*Ae*. *aegypti* and *Ae*. *albopictus)* to serve as vectors for similar West African strains (*2*), and the lack of evidence that any adaptation of sylvatic DENV is needed to replicate efficiently in humans (*7*) suggest that spillover epidemics occur in urban settings.

Currently, limited availability of reliable epidemiologic information and inability to differentiate clinically or serologically between urban and sylvatic DENV-2 infection prevent an accurate assessment of the true extent of human exposure in West Africa. Thus, further study is needed to elucidate the interactions of sylvatic and urban transmission. Even with functional reporting and surveillance, clinical diagnosis of dengue in West Africa is complicated by cocirculation of several other viruses that cause clinically similar febrile diseases (e.g., chikungunya, o'nyong-nyong, Zika viruses) (*15*). Other obstacles to accurate assessment of the public health effect of sylvatic DENV in Africa include limited access of the population to healthcare facilities, lack of access to viral and serologic diagnostics, popular beliefs that discourage standard medical treatment unless illness is severe, and a lack of surveillance of monkeys. To overcome these obstacles, comprehensive ecologic and epidemiologic studies are needed to assess the roles of nonhuman primates and other vertebrate hosts in the maintenance of sylvatic DENV, the degree and routes of ecologic contact between humans and sylvatic DENV, and the replication and immunologic dynamics of sylvatic DENV in nonhuman primates.

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Leptospirosis-associated Severe Pulmonary Hemorrhagic Syndrome, Salvador, Brazil

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We report the emergence of leptospirosis-associated severe pulmonary hemorrhagic syndrome (SPHS) in slum communities in Salvador, Brazil. Although active surveillance did not identify SPHS before 2003, 47 cases were identified from 2003 through 2005; the case-fatality rate was 74%. By 2005, SPHS caused 55% of the deaths due to leptospirosis.

Leptospirosis, a spirochetal zoonotic disease, is increasingly recognized as an important cause of hemorrhagic fever (*1*–*4*). The classic presentation of severe leptospirosis, Weil's disease, is characterized by jaundice, acute renal failure, and bleeding. However, the 1995 Nicaragua outbreak raised awareness for leptospirosis as the cause of a severe pulmonary hemorrhagic syndrome (SPHS) (*5*). This syndrome, first identified in South Korea and People's Republic of China (*6*), is now reported worldwide (*2*). SPHS is associated with fatality rates of >50% and in certain settings, has replaced Weil's disease as the cause of death among leptospirosis patients (*7*–*10*).

The factors responsible for SPHS and its emergence are not well understood. In Brazil, outbreaks of leptospirosis occur annually in slum communities during seasonal periods of heavy rainfall (*11*). Although SPHS is a frequently observed manifestation of leptospirosis in Rio de Janeiro and São Paulo (*7*,*8*,*12*), the occurrence of SPHS varies according to geographic region. In the city of Salvador (population 2.7 million), active surveillance did not detect SPHS among 1,786 leptospirosis cases identified from 1996 through 2002 (unpub. data). However, in 2003 we identified a patient in whom massive pulmonary hemorrhage and acute respiratory distress syndrome developed; subsequently, the number of cases with similar manifestations unexpectedly increased. We report the investigation of the emergence of SPHS in a setting where it was not previously observed.

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The Study

The Oswaldo Cruz Foundation and State Secretary of Health of Bahia have conducted active surveillance for leptospirosis since March 1996 in the metropolitan region of Salvador. According to health secretary protocols, suspected cases are referred to the state infectious disease hospital. The study team consecutively identified case-patients who were admitted to this hospital and met the clinical definition for severe leptospirosis (*11*). Study participants were enrolled according to informed consent protocols approved by the Oswaldo Cruz Foundation and Weill Medical College of Cornell University. After the first case was identified on May 1, 2003, the study team prospectively identified SPHS from October 2003 through December 2005 by evaluating patients 5 days a week for findings of massive pulmonary hemorrhage (hemoptysis >300 mL or aspiration of fresh blood after endotracheal intubation, which did not clear with suctioning) and respiratory insufficiency (respiratory rate ≥30 per min or use of supplemental oxygen therapy). Medical records of patients hospitalized between January 2000 and September 2003 were reviewed to identify cases that may not have been previously recognized.

A data-entry form was used to extract information from medical charts. Patients or family members were interviewed to obtain information on demographics and risk exposures in the household and workplace. Blood samples were collected during hospital admission, on day 4 or 5 of hospitalization, and 2 weeks after the initial sample was obtained. Laboratory-confirmed diagnosis of leptospirosis was defined as a 4-fold rise in titer between paired samples or a single titer of ≥800 in the microscopic agglutination test (MAT) (*11*) or a positive result on the immunoglobulin M (IgM) ELISA (Bio-Manguinhos, Rio de Janeiro, Brazil) (*13*). The MAT panel included the 19 reference strains recommended by the World Health Organization (Royal Tropical Institute, the Netherlands) and a clinical isolate, *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130 (*11*).

Surveillance identified 47 (10%) SPHS cases among 474 patients who met the clinical definition for severe leptospirosis from 2003 through 2005. Review of medical records did not identify an SPHS case before 2003. Of 47 SPHS cases, 37 (79%) had a confirmed diagnosis of leptospirosis. Single and paired samples were not obtained for testing

from 2 and 8 of the 10 unconfirmed SPHS case-patients, respectively, all of whom died during hospitalization.

Among the 47 SPHS case-patients, 7 (15%) and 20 (42%) had pulmonary hemorrhage and respiratory insufficiency, respectively, at the time of hospitalization (Table 1). Pulmonary hemorrhage was identified in 19 (40%) patients only after endotracheal intubation (Table 2). Except for respiratory insufficiency, hemoptysis, and oliguria, SPHS case-patients had similar clinical manifestations to those of non-SPHS patients at the time of initial evaluation (Table 1). However, 7 (15%) and 16 (34%) of the 47 SPHS patients did not have signs of jaundice and acute renal insufficiency, respectively. Respiratory failure developed in all SPHS patients. Acute lung injury was documented in 25 (76%) of the 33 patients for whom arterial blood gas measurements were obtained (Table 2). Although patients received supportive care with mechanical ventilation (94%), dialysis (53%), and packed erythrocyte transfusion (60%), case-fatality rate for SPHS was 74% and significantly higher than that (12%) for non-SPHS leptospirosis (Table 1). By 2005, SPHS was the cause of 55% of the deaths among leptospirosis patients (Figure).

The annual incidence of SPHS was 0.43 cases per 100,000 population, based on the 32 patients who resided within Salvador. The overall incidence of severe leptospirosis was 4.65 cases per 100,000 population (341 cases). SPHS cases occurred during the same winter period of seasonal rainfall during which non-SPHS leptospirosis cases were identified. All SPHS case-patients were residents of slum settlements from which non-SPHS case-patients were identified before and after the appearance of SPHS. Most SPHS case-patients were adults (mean age 37.6 ± 19.4 years) and male (70%) (Table 1). However, women had higher risk (30% vs. 17%, SPHS vs. non-SPHS leptospirosis; odds ratio 2.87, 95% confidence interval 1.36–5.98) of acquiring SPHS. A case-control investigation did not identify significant risk exposures for acquiring SPHS among leptospirosis cases. Serologic testing found that the high-

Table 1. Characteristics of leptospirosis patients identified during active surveillance in metropolitan Salvador, Brazil, from 2003 through 2005, according to presence of SPHS

	With SPHS		Without SPHS		
Characteristics	No. responses	No. (%) or mean ± SD	No. responses	No. (%) or mean ± SD	p value*
Age, y	44	37.6 ± 19.4	427	34.9 ± 14.5	0.57
Female sex	47	14 (30)	427	55 (13)	0.002
Clinical manifestations					
Days of symptoms before hospitalization	43	6.3 ± 3.6	419	6.1 ± 2.8	0.70
Respiratory insufficiency†	47	20 (42)	427	73 (17)	0.001
Hemoptysis	47	7 (15)	427	27 (6)	0.03
Jaundice	47	40 (85)	427	363 (85)	0.99
Total serum bilirubin, mg/dL	22	17.8 ± 13.4	140	13.9 ± 10.3	0.22
Oliguria	47	22 (47)	427	106 (25)	0.001
Blood urea nitrogen, mg/dL	33	121 ± 78	254	128 ± 79	0.60
Serum creatinine, mg/dL	33	3.9 ± 2.3	254	3.8 ± 2.7	0.64
Hypotension‡	42	12 (28)	387	85 (22)	0.33
Leukocyte count, 10^3 cells/mm^3	45	15.3 ± 8.0	422	14.4 ± 6.5	0.71
Hematocrit, %	23	31.8 ± 7.1	135	33.6 ± 6.1	0.11
Thrombocytopenia§	21	6 (29)	120	32 (27)	0.86
Therapeutic interventions					
Dialysis	47	25 (53)	425	89 (21)	0.001
Packed erythrocyte transfusion	47	28 (60)	424	42 (10)	0.001
Intensive care unit admission	47	44 (94)	424	112 (26)	0.001
Hospital outcome					
Death	47	35 (74)	427	49 (12)	0.001
Days of hospitalization for patients who died	35	3.2 ± 2.5	49	4.8 ± 5.9	0.91
Days of hospitalization for survivors	12	20.8 ± 15.0	378	9.1 ± 8.4	0.02
Confirmed case¶	47	37 (79)	427	351 (82)	0.56
Serovar Copenhageni as the presumptive infecting agent#	23	22 (96)	316	285 (90)	0.71

*The χ^2 and Student *t* tests were used to evaluate for significant differences (p value <0.05) for proportions and continuous data, respectively. SPHS, severe pulmonary hemorrhagic syndrome; SD, standard deviation.
†Respiratory rate ≥30 per min or use of supplemental oxygen therapy.
‡Systolic arterial blood pressure <90 mm Hg or use of vasoactive drugs.
§Platelet count <50,000 cells/mm^3.
¶The microscopic agglutination test (MAT) and immunoglobulin M ELISA were used for laboratory confirmation.
#Proportions are shown for patients who had an MAT-confirmed diagnosis of leptospirosis and highest agglutination titers against *Leptospirosis interrogans* serovar Copenhageni.

Table 2. Pulmonary manifestations of patients with leptospirosis-associated severe pulmonary hemorrhagic syndrome (n = 47)

Characteristics*	No. responses	No. (%) or mean ± SD
Onset of massive hemoptysis		
During hospital admission	47	7 (15)
After hospitalization and before endotracheal intubation	47	21 (45)
During or after endotracheal intubation	47	19 (40)
Chest radiographic examination		
Bilateral alveolar infiltrates	24	21 (88)
Bilateral interstitial infiltrates	24	3 (12)
PaO_2/FiO_2, mm Hg	33	200 ± 155
Acute lung injury†	33	25 (76)
Acute respiratory distress syndrome‡	33	21 (64)

*Findings at initial evaluation. SD, standard deviation.
†Defined as a ratio between arterial partial pressure of O_2 and inspired O_2 fraction (PaO_2/FiO_2) ≤300 mm Hg and the presence of bilateral alveolar or interstitial infiltrates on chest radiograph or bilateral crepitus on physical examination, without clinical evidence of increased left atrial pressure.
‡Defined as PaO_2/FiO_2 ≤200 mm Hg in addition to criteria for acute lung injury.

est agglutination titers were directed against *L. interrogans* serovar Copenhageni in 22 (96%) of 23 and 285 (90%) of 316 SPHS and non-SPHS cases, respectively, which were confirmed by MAT (Table 1). Culture isolation procedures were not implemented from 2001 through 2005, but an isolate was obtained from an SPHS patient in 2006. Serotyping procedures (*11*) identified it as *L. interrogans* serovar Copenhageni.

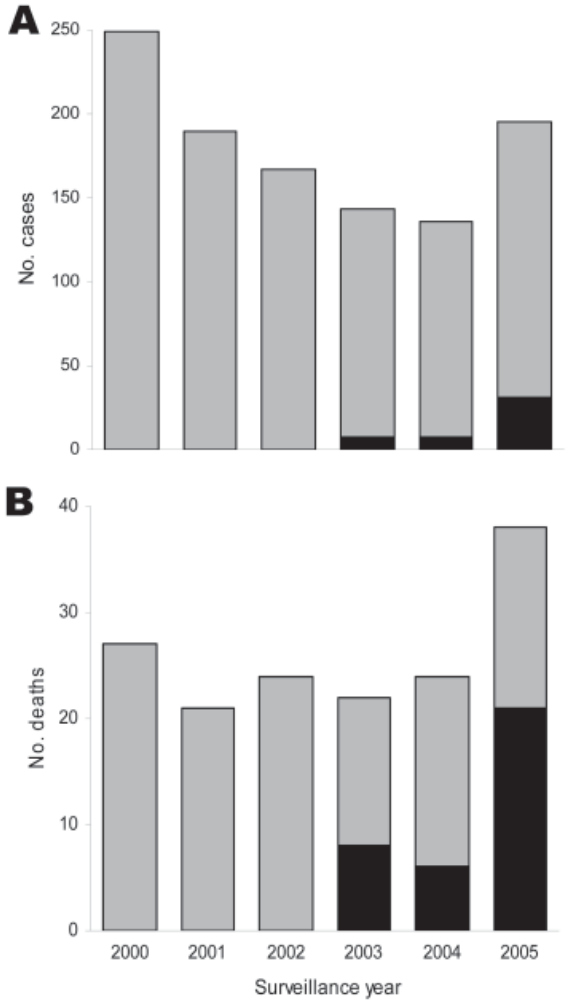

Figure. Active hospital-based surveillance for leptospirosis in Salvador, Brazil, 2000–2005, including total number of suspected leptospirosis cases (A) and deaths (B) among case-patients. Case-patients with and without severe pulmonary hemorrhagic syndrome are shown by black and gray bars, respectively.

Conclusions

There has been growing recognition of the importance of leptospirosis as the cause of SPHS (*1*–*3*). Yet except for a few outbreak situations (*4*–*6*), it remains unclear whether increased reporting represents enhanced detection of an under-recognized manifestation (*3*) or de novo emergence of this disease form. This investigation identified the appearance of SPHS in a region in which urban leptospirosis is endemic and active surveillance was in place. Prior under-recognition of this syndrome was unlikely because clinicians were aware of SPHS's occurrence in other Brazilian cities (*7*,*8*,*12*). Laboratory confirmation of leptospirosis was obtained for 79% of the cases, indicating that SPHS was due to this disease rather than to other causes of hemorrhagic fever. Three years after the first case was identified, SPHS accounted for 19% of hospitalizations and 55% of the deaths from leptospirosis.

Our findings underscore the difficulties in identifying SPHS, even in the setting of heightened surveillance. The fatality rate was 74% among SPHS patients despite aggressive supportive care. Clinical parameters that could differentiate patients at risk of acquiring SPHS were not found during initial evaluation, thereby hampering attempts to implement timely triage procedures. Finally, pulmonary hemorrhage was only identified in 40% of the patients at the time of endotracheal intubation. Recognition of SPHS will therefore need to rely on a high index of suspicion in patients who have acute respiratory insufficiency.

That we did not identify environmental risk exposures for acquiring SPHS suggests a role for pathogen or host-specific factors. In Thailand, the recent sustained outbreak of leptospirosis was due to the widespread introduction of a *Leptospira* clone (*14*). Our findings suggest that the agent

for SPHS in Salvador was *L. interrogans* serovar Copenhageni, which was also the cause of non-SPHS leptospirosis in this setting (*11*,*15*). Further isolation studies and genotyping analyses are needed to determine whether the appearance of SPHS is due to introduction or emergence of a clone that has enhanced virulence, within this serovar. Gender-specific factors, whether risk activities or host-susceptibility determinants, may have contributed to acquiring SPHS because women who with leptospirosis had twice the risk for this disease form. A large proportion of the world's slum population resides in leptospirosis-endemic regions. Research is needed to elucidate the factors responsible for transmission of SPHS so that effective prevention can be identified.

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Pandemic Influenza, Reopening Schools, and Returning to Work

Martin I. Meltzer*

In this issue of Emerging Infectious Diseases, Victoria Davey and Robert Glass present a paper (*1*) in which they consider the question of when to "switch off" community-based interventions designed to reduce the spread of pandemic influenza. These authors attempt to answers questions such as when it would be optimal to reopen schools that have been closed as part of a nonpharmaceutical, communitywide influenza mitigation strategy.

The authors use a mathematical model, previously described in this journal (*2*), to simulate the spread of pandemic influenza throughout a community that represents the US population. This model is similar to another model that was used to examine the effectiveness of closing schools to slow the spread of influenza pandemic (*3*). Both models simulate the spread of influenza by dividing a representative population into households. The models then track each household member with each member having a defined number of random contacts (per day) that are allocated within a network of possible contacts. Once a contact is calculated to have occurred, the probability of influenza transmission is calculated. Also included in the calculations are variables such as influenza incubation and infectiousness periods.

What does the model "say"? Davey and Glass considered what would happen if schools were reopened and community-wide sequestering were halted when influenza cases in a community fall below preset thresholds (e.g., 1, 2, or 3 cases in 7 days). Sequestering strategies would be restarted if the epidemic pandemic resurged and ≥10 cases occurred in a 7-day period. This "pulsing technique" would reduce the number of days needed to sequester schoolchildren and the community by 6% to 32%. The authors maintain that for a given pandemic scenario, the reduction in days sequestered would not notably affect the number of persons infected. The implication is that reduction in days sequestered will reduce the economic impact and social disruption caused by community-wide, nonpharmaceutical interventions.

Are the results "believable"? As with all mathematical models, some potential technical problems exist. First, almost all models that simulate individual person-to-person influenza transmission use 1 or 2 databases that record the probability of influenza transmission. One database was recorded in the early 1970s in Tecumseh, Michigan (*4*), and the other among ≈400 households across France (*5*). Is it reasonable to use these estimates to simulate influenza transmission in every community, town, city, and metropolis in the United States? Furthermore, the researchers who calculated these transmission probabilities did not actually measure the probabilities of who infected whom. These probabilities were calculated by using a statistical technique known as maximum likelihood estimation. Essentially, this is reverse engineering to find the transmission probabilities that best fit the measured data (number of cases over time). However, as others have demonstrated, it is possible to reverse-engineer several transmission probabilities that fit the data (*6*). Would the results calculated by Davey and Glass appreciably change if another set of transmission probabilities was used?

Other assumptions may also be examined. Typically, as with other pandemic models, the authors model different rates of compliance, but each rate is assumed to remain static for the duration of sequestering (e.g., 50% compliance during 40 days of sequestering). In reality, compliance with sequestering may be more fragmented; e.g., teenagers may stay sequestered during the morning but not so much in the afternoon. Other behavior may change during a pandemic; e.g., people may alter the number and duration of contacts, with degree of alteration changing as the pandemic progresses. These changes in behavior could either reinforce or reduce the effectiveness of nonpharmaceutical interven-

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tions (*7*,*8*). Furthermore, compliance can decrease dramatically when an intervention is stopped and then restarted. Crosby provides an excellent example of reduced compliance after an attempt to reintroduce compulsory wearing of facemasks in San Francisco during the 1918 pandemic (*9*).

Are such models useful? Yes, so long as readers accept that the results are illustrative and are not absolutely accurate. The models clearly illustrate the complexities of estimating influenza transmission and the potential success of interventions (i.e., such models require a very large set of variables, many with uncertain values). Perhaps the most useful role of such models is the debate that is stimulated regarding the most appropriate, and most feasible (i.e., most likely to work), set of interventions.

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On *Rickettsia* Nomenclature

Robert F. Massung,* William L. Nicholson,* Marina E. Eremeeva,* and Gregory A. Dasch*

This issue of Emerging Infectious Diseases contains 2 independent reports of *Rickettsia sibirica* infections in Spain and Portugal. The authors identify the agent both as a subspecies (*1*) and as a strain (*2*). This inconsistency reflects a lack of consensus regarding the use of subspecies designations for *Rickettsia* taxa, so appropriate designation of these pathogens as strains or subspecies remains problematic.

Tick-borne typhus of northern Asia (or North Asian spotted fever) was first discovered in the 1930s, and its etiologic agent, *R. sibirica,* was formally described in 1949. In 1993, an isolate designated "*mongolitimonae*" was first discovered in the People's Republic of China (*3*), considered a new species (*4*), and finally described as a new genotype of *R. sibirica* (*5*). A proposal to create 2 subspecies, *R. sibirica sibirica* and *R. sibirica mongolitimonae*, was recently published (*6*). A new prokaryote name must be both effectively and validly published. To become effectively published, a name must meet certain rules, as defined by the International Code of Nomenclature of Bacteria (*7*). To become validly published, the name must then appear on a Validation List published in the International Journal of Systematic and Evolutionary Microbiology (*8*). This provides an orderly system for bacterial names to be properly introduced and published in the scientific literature. Valid and nonvalid names are listed on regularly updated websites (*9*,*10*). Names introduced without being validated have no standing in bacterial nomenclature. That is currently the status of these proposed *R. sibirica* subspecies. Therefore, the use of strain designations is still appropriate.

If, and when, the subspecies names are validated, they are likely to be adopted and routinely used in the literature. Rickettsial taxonomy continues to evolve, and future changes should be determined by critical scientific judgments and general consensus within the scientific community.

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Hospital Resources for Pandemic Influenza

To the Editor: In their November 2007 article, Pandemic Influenza and Hospital Resources, Nap et al. evaluated hospital resources for pandemic influenza in the northern part of the Netherlands (*1*). Their results can be compared with those that I have described for the combined suburban communities of Roswell and Alpharetta, Georgia, USA (*2*). The Netherlands evaluation assumed that antiviral drugs will be available and will reduce hospitalizations by 50% and deaths by 30%. In view of the uncertainty of effective antiviral drugs and timeliness of vaccines, I did not estimate their effects. Nevertheless, several issues warrant comparison.

The plan for the Netherlands has no provisions for urgent care, i.e., parenteral fluids or antimicrobial drugs that are administered to ambulatory patients who are not hospitalized. Nap et al. may not perceive a need for enough beds to handle surge capacity. Allowing for 30% of beds to be used for patients with conditions other than influenza, they report a maximum availability of 232 beds per 100,000 population for pandemic influenza patients, and they estimate use of 72 beds per 100,000 in the pandemic model. In contrast, a maximum of 47 beds per 100,000 are available in Roswell/Alpharetta. Availability of beds in intensive care units, however, is identical for both regions, at 8 beds per 100,000 population.

The Netherlands plan calls for intensified treatment evaluation in 48 hours to withdraw care from patients who have little chance for recovery. Because most patients can be expected to have pneumonia and 2-organ failure (on average), a 50% mortality rate can be expected. In US hospitals, withdrawing care is difficult, even if mortality rates are expected to be 75% or 90% during acute illness with organ failure.

The pandemic influenza resource evaluation from the northern part of the Netherlands provides a useful contrast with at least 1 US hospital. The dramatic difference in bed availability highlights the potential challenges involved in local planning. The surge capacity limits in Roswell/Alpharetta led us to consider an alternative infusion center to provide care during an influenza pandemic.

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Multidrug-Resistant *Acinetobacter baumannii* Osteomyelitis from Iraq

To the Editor: *Acinetobacter baumannii* identified in military settings is commonly multidrug resistant (MDR) (*1–3*). Tigecycline displays *A. baumannii* activity, but clinical experience is limited. We report a case of probable osteomyelitis caused by MDR *A. baumannii* and treated with tigecycline.

A 55-year-old man was transporting soldiers in Iraq when he sustained a grenade injury, in which material entered his anterior thigh and created a large posterolateral hip exit wound and an open left subtrochanteric femur fracture. He was flown to Germany; his wound was debrided, and the fracture was stabilized with an external fixator along with pins to his ilium and proximal and distal femur. A wound vacuum covered the exposed bones within the large soft tissue defect. He was stable upon transfer to our hospital 14 days after the injury; leukocyte count was 16,000/μL (reference range 4.5–11,000/μL), and erythrocyte sedimentation rate (ESR) was 44 mm/h (reference range 0–19 mm/h); blood cultures were not obtained. Plain radiographs showed an open femur fracture with gas in the soft tissue, shrapnel, and a gross deformity of the left iliac wing. ^{111}Indium-labeled leukocyte imaging confirmed increased activity in the left acetabulum, femoral neck, and surrounding soft tissue. Two days after his arrival, the external fixator (except for 1 pin in the distal shaft and 1 in the proximal femur) was removed, and an open reduction and internal fixation (ORIF) of the femur was performed. A cephalomedullary femoral rod and hip screw and 60 tobramycin-impregnated beads were placed into the hip joint; a wound vacuum was placed over the defect. A deep sample of the iliac wing was obtained, ground into a homogenate, placed aseptically on media, and observed for microbial growth; both coagulase-negative *Staphylococcus* and gram-negative rods grew in 1 culture. Both were considered pathogens of probable osteomyelitis based on exposed periosteum. Treatment with vancomycin plus ciprofloxacin was begun. After the gram-negative rods were identified as MDR *A. baumannii*, tigecycline (MIC 1.5) was substituted for ciprofloxacin (MIC>2). *A. baumannii* was susceptible to tobramycin (MIC≤2), intermediate to imipenem (MIC 8), and resistant to

all other agents tested (Microscan, Dade Behring Company, Deerfield, IL, USA). Tigecycline susceptibility was performed by Etest (AB Biodisk, Solna, Sweden); breakpoints were inferred from available literature for *Enterobacteriaceae* (≤2.0 is susceptible) as no current Clinical Laboratory Standards Institute breakpoints are established (*4*). Susceptibility testing for *Staphylococcus* spp. was not performed; tigecycline's role in treating the staphylococci in this setting was not determined because vancomycin was also used.

Postoperatively, leukocyte count returned to normal, wound drainage decreased, and a computed tomographic scan showed appropriate femur alignment with progressive heterotopic bone in the ilium. The patient was transferred to our rehabilitation facility and continued on vancomycin and tigecycline. Two weeks after the ORIF (hospital day 38), the wound vacuum was removed, a split-thickness skin graft was placed, and the patient was discharged. He returned to our infectious diseases clinic 2 weeks later; ESR was 12; tigecycline and vancomycin were stopped after 43 days. The probable osteomyelitis of the femur and ilium was resolved by standard clinical and radiologic parameters.

Tigecycline has displayed activity against many MDR pathogens, including *A. baumannii* in vitro (*4*), although recent investigations have demonstrated resistance and inconsistent susceptibility patterns (*5*). Clinical management of *A. baumannii* bone infections in humans has not been well established. In an experimental animal model of methicillin-resistant *S. aureus,* tigecycline showed adequate bone concentration with microbial clearance in 90% and 100% of patients who received tigecycline and tigecycline plus rifampin, respectively (*6*). This suggests that tigecycline may have also been useful for the coagulase-negative staphylococci identified in this patient and could have been considered as the sole treatment agent.

Tigecycline concentration in bone was also evaluated in an experimental rat model and a single-dose human study (*7*,*8*). The rat model showed an area under the curve in bone ≈250× higher than plasma (*7*). The investigation in humans could not duplicate these results; the discrepancy was attributed to either tight binding of tigecycline to bone or poor extraction methods (*8*). The testing method used in previous animal models was recently adapted for human use and has suggested increased sensitivity (*9*). An assessment of human bone concentrations after multiple tigecycline doses may be necessary to determine the potential role in osteomyelitis management.

Tobramycin bone and surrounding tissue concentrations have been demonstrated after tobramycin-impregnated beads were placed in animals and humans with open fractures or chronic osteomyelitis (*10*). The role of tobramycin beads is not established for osteomyelitis, but use is common. Their contribution to this patient's outcome is difficult to assess because *A. baumannii* was also susceptible to tobramycin.

Cases of *A. baumannii* osteomyelitis have been documented recently, but isolates were susceptible to other agents; none were treated with tigecycline (*3*). The role of tigecycline for osteomyelitis with MDR *A. baumannii* requires further study.

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Dengue Virus, Nepal

To the Editor: *Dengue virus* belongs to the genus *Flavivirus*, family *Flaviviridae*. It has 4 serotypes: dengue virus type 1 (DENV-1), dengue virus type 2 (DENV-2), dengue virus type 3 (DENV-3), and dengue virus type 4 (DENV-4). Dengue virus is maintained in a cycle between humans and *Aedes aegypti*, domestic day-biting mosquitoes. Dengue virus induces clinical illness, which ranges from a nonspecific viral syndrome (dengue fever [DF]) to severe and fatal hemorrhagic disease (dengue hemorrhagic fever [DHF]). DF/DHF occurs primarily in tropical and subtropical areas of the world. Domestic dengue virus infection occurs in >100 countries; >2.5 billion persons live in these areas. Approximately 100 million cases of DF, 500,000 cases of DHF, and several thousand deaths occur annually worldwide (*1*). During the past decades, dengue virus has emerged in southern Asia; DF/DHF epidemics have occurred in Bhutan, India, Maldives, Bangladesh, and Pakistan (*2–4*).

From August through November 2006, the number of febrile patients increased in 4 major hospitals in the Terai region of Nepal: Nepalgunj Medical College, Bheri Zonal Hospital in Nepalgunj, Tribhuban Hospital in Dang, and Narayani subregional hospital in Birgunj. Patients with severe symptoms were referred to Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, for diagnosis and treatment. The clinical features in most patients were consistent with signs of DF, but some patients showed signs (high fever, rash, ecchymosis, epistaxis, positive tourniquet test, liver dysfunction, and thrombocytopenia [platelet count <100,000/mm^3]) consistent with the World Health Organization (WHO) definition of DHF. Ascites and plural effusion developed in 2 patients. Blood specimens were collected from all patients at the time of admission to the local hospitals. Particle agglutination (PA) assay (Pentax Ltd, Tokyo, Japan) (*5*) and immunoglobulin (Ig) M–capture ELISA (Dengue/JE IgM Combo ELISA kit, Panbio Ltd, Brisbane, Queensland, Australia) were performed. Dengue virus–specific IgM was detected in 11 patients who had fever, headache, and rash (Table). Each of these patients had negative

Table. Clinical and laboratory data for 11 patients admitted to hospitals and diagnosed with dengue fever or dengue hemorrhagic fever, Nepal, 2006*

Patient age, y/Sex	Month admitted	Location	Initial diagnosis	Travel history	Clinical signs and symptoms	Selected laboratory and other test results
20/M	Sep	Kathmandu	DF	Yes	Fever, headache, nausea	Hb 15.4 g/dL; TLC 10,500/mm^3; Plt 185,000/mm^3; blood culture for salmonellae negative; ALT 38 IU/L
27/F	Sep	Bardiya	Viral fever	No	Fever, headache, vomiting	TLC 5,600/mm^3; blood culture for salmonellae negative
3/M	Sep	Salayan	Encephalitis	No	Fever, vomiting, convulsions	Widal negative; TLC 4,700/mm^3
13/M	Oct	Sindhuli	Typhoid fever	No	Fever, headache	Widal negative; TLC 4,500/mm^3; blood culture for salmonellae negative; *Brucella* antigen negative; chest radiograph normal
22/M	Oct	Birgunj	DHF	No	Fever, headache, vomiting, ascites	Bil 0.8 mg/dL; ALT 80 IU/L; Plt 22,000/mm^3; chest radiograph normal
55/F	Oct	Dang	DF	No	Fever, headache, muscular pain	Plt 51,000/mm^3; TLC 7,600/mm^3; MP negative; ESR 20 mm/h; Bil 0.7 mg/dL
22/F	Oct	Birgunj	Viral fever	No	Fever, headache, body ache	*Brucella* negative; Widal negative; TLC 5,600/mm^3
13/M	Nov	Dang	DF	No	Fever, headache, rashes	Plt 95,000/mm^3; TLC 4,700/mm^3; Hb 13.1 g%; Bil 0.8mg/dL; ALT 26 IU/L
35/F	Nov	Birgunj	DHF	No	Fever, headache, bruises; tourniquet: positive	Bil 0.81mg/dL; Plt 31,000/mm^3; PT 2 min 30 s (control 14)
40/M	Nov	Birgunj	DF	No	Fever, headache, rashes	ALT 127IU/L; Plt 110,000 /mm^3; PCV 38.8%;TLC 5,500/mm^3; ultrasonography liver size, 16.8 cm
42/M	Nov	Dang	DF	No	Fever, headache, rashes	Bil 0.7 mg/dL; Widal test negative; TLC 6,800/mm^3; Plt 164,000/mm^3

*Blood specimens were collected at time of hospital admission. Diagnosis was confirmed by using immunoglobulin M–capture ELISA. DF, dengue fever; Hb, hemoglobulin;TLC, total leukocyte count; Plt, platelets; ALT, alanine aminotransferase; DHF, dengue hemorrhagic fever Bil, bilirubin; MP, malaria parasites; ESR, erythrocyte sedimentation rate; PT, prothrombin time; PCV, packed cell volume.

results for Japanese encephalitis virus–specific IgM. Of the 11 patients, 10 had no history of travel to India or other dengue-endemic countries. DF or DHF was initially diagnosed for 7 patients, and viral encephalitis, typhoid fever, or viral fever was diagnosed for others without serologic tests. Reverse transcription–PCR and virus isolation were performed at Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, but the dengue virus genome was not detected, and no virus was isolated, likely because sample collection was delayed and the sample was transported to Japan in a deteriorated condition.

DF/DHF have been considered to be a possible public health threat to Nepal because DF/DHF epidemics have occurred recently in India and Pakistan, which reported several thousand cases and >100 deaths (*6*). The first DF case in Nepal was reported in 2004 (*7*). Further, the first DENV-2 strain of Nepal origin was isolated from a Japanese traveler who visited Nepal and in which DF developed after the patient returned to Japan. The isolated DENV-2 (GenBank accession no. AB194882) was 98% homologous with DENV-2 isolated in India (*8*). The prevalence of dengue virus antibody was reported to be 10.4% in the southwestern region of Nepal (*9*). These reports suggest that dengue virus has been circulating in Nepal for several years. Thus, DF/DHF has likely been misdiagnosed and illness caused by dengue virus underestimated in Nepal. In contrast, Japanese encephalitis has been a public health problem in southwestern region of Nepal, and large epidemics have occurred almost every year since 1978 (*10*). Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis.

The emergence occurred in the lowland Terai belt region, which borders the state of Bihar, India. The *Aedes* mosquito is known to persist in this region. The emerging DENV-2 is likely to have been introduced into Nepal from India.

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Human Tuberculosis Caused by *Mycobacterium bovis*, Taiwan

To the Editor: *Mycobacterium bovis* is one of the causative agents of tuberculosis (TB) in humans and animals. Drinking unpasteurized milk, eating undercooked meat, and close contact with infected animals are the main sources of infection for humans. Currently, 119 *M. bovis* spoligotypes are contained in the fourth international spoligotyping database (SpolDB 4) and are categorized into 3 main sublineages corresponding to ST prototypes 482, 683, and 479 (*1*).

Although an *M. bovis* surveillance program for farm animals has been implemented by the Taiwan Council of Agriculture, no surveillance system exists for human TB cases caused by *M. bovis*. To monitor the epidemiology of *M. bovis* in domestic animals, a regular tuberculin skin test (TST) is compulsory for cattle and sheep and optional for deer in Taiwan (*2*). In 2005, screening of *Mycobacterium* spp. infections by TST was performed for 111,412 cattle and 73,396 caprint and ovine herds, of which 188 (0.17%) and 148 (0.2%), respectively, were positive (*2*). We used spacer oligonucleotide typing (spoligotyping) and

mycobacterial interspersed repetitive units–variable number tandem repeat (MIRU-VNTR) methods to investigate human TB caused by *M. bovis* in Taiwan.

During 2004–2005, a total of 3,321 mycobacteria isolates from individual patients were sent to the reference laboratory for strain typing. Of the 3,321 patients, 2,427 (73.1%) were male, 903 (27.2%) were from eastern Taiwan, and 513 (15.4%) were aboriginal persons. The mean age of the patients was 58.7 years: 224 (6.7%) were <25 years of age, 667 (20.1%) were 25–44 years of age, 906 (27.3%) were 45–64 years of age, and 1,524 (45.9%) were ≥65 years of age.

Isolates were screened by using a GenoType kit (*3*) and multiplex PCR (*4*). To differentiate between *M. bovis* and *M. bovis* BCG strains, presence or absence of region of difference 1 was analyzed (*5*). Spoligotyping was performed with a commercial kit (Isogen Bioscience BV, Maarssen, the Netherlands) following the manufacturer's instructions (*6*). Spoligotyping profiles were analyzed by using Bionumerics software, version 4.51 (Applied Maths, Kortijk, Belgium). The resolved spoligotype was designated by comparing it to SpolDB4 (*1*). The MIRU-VNTR assay was performed by using a modified, high-throughput, 15-loci MIRU typing system that we developed (*7*).

Of the 3,321 patient isolates, 3,306 (99.5%) were *M. tuberculosis* and only 15 (0.5%) were *M. bovis*. Mean age of the 15 *M. bovis*–infected patients was 62.2 years (Table). Twelve (80%) patients were male, and 3 (20%) were female. Of these 15 patients, 10 (66.7%) had newly diagnosed TB and 5 (33.3%) had been treated with anti-TB drugs. Most (11/15, 73%) of the patients were from eastern Taiwan, where the reporting rate for TB was the highest among the 4 regions of Taiwan; 60% (9/15) were aboriginal persons. Of the 15 patients, 13 (86.7%) had pulmonary TB, 1 had both pulmonary and extrapulmonary TB, and 1 had extrapulmonary TB. Only 2 patients (cases 2 and 3) had known contact with farm animals. Univariate analysis showed that region (eastern region 1.2% vs. other region 0.2%; odds ratio [OR] 7.4, 95% confidence interval [CI] (2.4–23.4) and ethnicity (aboriginal 1.8% vs. nonaboriginal 0.2%; OR 8.3, 95% CI 3.0–23.5) were associated with *M. bovis* infection but not age and sex. The association between region and *M. bovis* disappeared after controlling for age and ethnicity. Aboriginal ethnicity was the only factor significantly associated with TB caused by *M. bovis* after controlling for age (adjusted OR 12.7, 95% CI 4.2–38.9).

Spoligotyping profiles of the 15 *M. bovis* isolates were typical of *M. bovis* with the absence of spacers 3, 6, 9, 16, 21, and 39–43 (*8*). Although 15 cases were reported separately from different regions of Taiwan, only 1 spoligotyping profile was identical to spoligotype ST 684 of the bovis sublineage. In addition, we identified 2 similar MIRU-VNTR profiles: 523–23232–42533–22 (13/14, 92.9%, cases 1–9, 11–13, and 15) and 523–22232–42523–22 (1/14, 7.1%, case 14). We were unable to obtain sufficient DNA from 1 strain (case 10) for MIRU-VNTR typing.

Aboriginal persons in Taiwan were more likely to have TB caused by *M. bovis* and had a 5-fold higher reporting rate for TB (*9*) than nonaboriginal persons. Environmental and genetic factors may be associated with a higher reporting rate for TB among aboriginal populations (*10*), but the contribution of *M. bovis* infection needs to be investigated. Because only 1 major spoligotype and 2 similar MIRU patterns were found in this case series, spread of a predominant clone in Taiwan is likely. In addition, because of insufficient epidemiologic data, we were unable to determine the proportion of cases caused by reactivation of latent infection and those caused by recent transmission. In our study population, *M. bovis* infection in humans appeared to be predominately indigenous in Taiwan because no imported case was noted. We are now

Table. Characteristics of 15 patients with tuberculosis caused by *Mycobacterium bovis*, Taiwan, 2004–2005

Patient no.	Age at diagnosis, y	Sex	Type of tuberculosis	Ethnicity	Region
1	53	M	Pulmonary	Aboriginal	Northern
2	71	M	Pulmonary	Han	Central
3	76	M	Pulmonary	Han	Central
4	84	M	Pulmonary	Han	Southern
5	71	F	Pulmonary	Aboriginal	Eastern
6	55	M	Pulmonary	Aboriginal	Eastern
7	51	M	Pulmonary	Aboriginal	Eastern
8	65	F	Pulmonary	Aboriginal	Eastern
9	51	M	Pulmonary	Han	Eastern
10	90	M	Pulmonary	Han	Eastern
11	34	M	Pulmonary	Aboriginal	Eastern
12	81	M	Pulmonary	Han	Eastern
13	64	F	Pulmonary	Aboriginal	Eastern
14	44	M	Bladder/urinary	Aboriginal	Eastern
15	43	M	Pulmonary/miliary	Aboriginal	Eastern

genotyping *M. bovis* strains isolated from farm animals to help elucidate the source of infection and transmission of *M. bovis* in Taiwan.

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Marine Mammal *Brucella* Genotype Associated with Zoonotic Infection

To the Editor: Brucellosis is a zoonotic disease that remains endemic to many parts of the world. There are 6 classic *Brucella* species described with different preferred hosts. Human disease is most commonly associated with consumption of unpasteurized dairy products or with occupational exposure for veterinarians, agricultural workers, laboratory workers, meat industry workers, and hunters.

In recent years, it has become clear that novel members of the genus, yet to be formally named, are associated with a variety of marine mammal species, particularly dolphins, porpoises, and seals (*1*). To date there are 3 reports in the literature of naturally acquired infection of humans with *Brucella* species originating from marine mammals (*2*,*3*) One other case, representing infection of a laboratory worker, has also been reported (*4*). Two of the naturally acquired cases were reported in Peru (*2*). One person had consumed raw shellfish and swam in the Pacific Ocean but did not report any direct contact with marine mammals; the second person reported infrequent visits to the coast and no contact with marine mammals but had consumed raw shellfish. An additional naturally acquired case was recently reported from New Zealand, where extensive molecular testing characterized the strain involved as a marine mammal type (*3*). This patient again reported no exposure to marine mammals but did report that he fished regularly, had contact with uncooked fish bait, and consumed raw snapper. The cases in Peru were notable for severe, atypical symptoms; both patients had symptoms of neurobrucellosis. The New Zealand case was associated with spinal osteomyelitis (*3*). In contrast, the laboratory-acquired infection was mild and uncomplicated (*4*).

We have characterized these isolates by a variety of molecular approaches in conjunction with ongoing studies, which examine genetic diversity within *Brucella* species isolated from marine mammals. Multilocus sequence analysis (*5*) showed that all 3 isolates from naturally acquired human infection with *Brucella* species from marine mammals shared an identical genotype (ST27). In previous characterization of 56 *Brucella* isolates from marine mammals, ST27 was found only once. Strain F5/99, originally isolated from an aborted bottlenose dolphin fetus off the western coast of the United States (*6*), shares this genotype. Use of an alternative typing approach, based on restriction fragment length polymorphism analysis of outer membrane protein–encoding genes

(*7*), gave identical findings. Again, all 3 isolates derived from naturally acquired human infection represent an identical genotype. This genotype is shared only by strain F5/99 among a collection of 120 *Brucella* isolates from marine mammals characterized by this method. Finally, isolates were characterized by a variable number of tandem repeats–based typing approach (*8*). When comparing profiles at 6 loci with a relatively slow evolutionary speed, previously shown to be useful for dividing *Brucella* isolates into species groups (*8*), we determined that F5/99 and the 3 naturally acquired human isolates share a unique profile not seen in any of >1,400 isolates of marine or terrestrial *Brucella* species examined to date. In contrast, the strain associated with laboratory-acquired infection (*4*) was not a member of ST27 but belonged to ST23, a genotype that is predominantly associated with porpoises (*9*).

It is clear from these findings that the 3 cases of naturally acquired infection with *Brucella* species originating from marine mammals reported to date were caused by closely related organisms. The particular genotype concerned, ST27, is rare in our collection, having been noted only once in marine mammals. However, this may reflect the fact that most isolates examined to date originated from northern Europe; only 5 isolates in our collection originated from Pacific waters. It is possible that isolates of this genotype are predominantly or exclusively associated with regions other than those extensively sampled to date. Indeed, examination of the literature provides further evidence for the presence of this genotype in marine mammals in the Pacific. BLAST (www.ncbi.nlm.nih.gov/blast) comparison of outer membrane protein sequences from a minke whale isolate originating in the North Pacific (*10*) showed a close match with the equivalent sequence from F5/99.

Although numbers are currently small, the isolation of an identical genotype from all 3 cases of naturally acquired human infection derived from marine mammal *Brucella* species raises the possibility of increased zoonotic potential associated with this genotype. Furthermore, where diagnosis is based on serologic testing alone, it is possible that human infection with marine mammal *Brucella* species may go unnoticed. Members of ST27 may be more pathogenic to man, per se. Alternatively, they may be associated with natural hosts or circulate through intermediaries that make contact with humans more likely. Notably, none of the 3 patients reported direct contact with marine mammals, though all had consumed raw seafood. These findings clearly suggest that more extensive studies of the presence and distribution of marine mammal *Brucella* genotypes, particularly ST27, in waters other than those of northern Europe would be valuable for clarifying the natural habitat of ST27. Furthermore, relevant authorities should be aware of the potential for zoonotic disease caused by this *Brucella* genotype particularly, but not exclusively, where occupation or lifestyle may make exposure more likely.

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Ehrlichia chaffeensis in Child, Venezuela

To the Editor: Human monocytic ehrlichiosis is a tick-borne infectious disease caused by *Ehrlichia chaffeensis* (*1*). Serologic studies have indicated *E. chaffeensis* infection in Latin American countries: Venezuela (*2*), Mexico (*3*), Argentina (*4*), Chile (*5*), and Brazil (*6*). However, no molecular evidence for *E. chaffeensis* has been reported.

In December 2001, a 9-year-old boy was admitted to a hospital in Carabobo, Venezuela, after 3 days of fever (39°C–41°C), malaise, anorexia, headache, abdominal pain, and cutaneous tick-bite lesions. During the 6 weeks before admission, the patient had been exposed to ticks in a rural area (Cojedes, Venezuela). At the time of physical examination, the patient appeared acutely ill with fever (41°C), dehydration, somnolence, conjunctivitis, facial edema, cervical adenomegaly, soft depressible abdomen painful to palpation, and hepatomegaly. Cardiopulmonary examination found regular cardiac sounds with systolic tricuspid murmur and abnormal bilateral respiratory sounds (rhonchi). Skin examination showed multiple tick bites and an erythematous maculopapular rash. Appropriate informed consent was obtained.

Blood values were as follows: leukocytes 6,280 cells/mm^3 (84% neutrophils, 13% lymphocytes, 2% monocytes, 1% eosinophils), platelets 130,000/μL, hemoglobin 12.5 g/dL, glucose 102 mg/dL, blood urea 28.3 mg/dL, creatinine 0.9 mg/dL, aspartate aminotransferase (AST) 20.4 U/L, alanine aminotransferase (ALT) 54.4 U/L, erythrocyte sedimentation rate (Katz index) 15 mm/h, prothrombin time ratio 1.02, partial thromboplastin time –2.8 s. Radiographs of the thorax showed bilateral infiltrate. Echocardiogram showed minor tricuspid insufficiency. Serologic tests were negative for Epstein-Barr and hepatitis B and positive for cytomegalovirus and hepatitis A viruses. Blood and stool cultures were negative. Blood samples were taken 4 and 35 days after illness onset; buffy-coat smears were stained with Dip Quick (Jorgensen Laboratories, Inc., Loveland, CO, USA), and immunologic and PCR tests were performed. Immunoglobulin (Ig) M against dengue virus was present at days 4 and 35 of illness; IgG against dengue was absent on day 4 and present on day 35. PCR and viral isolation tests for dengue virus were negative. Serologic tests for *E. chaffeensis* (indirect immunofluorescence) were also negative on day 4 and positive (256) on day 35. Detection of *Ehrlichia* species–specific DNA was performed by using nested PCR as described (*7*).

Starting on the first day of hospitalization, the patient was treated with doxycycline (14 days) and chloramphenicol (8 days). After 24 hours, malaise, headache, facial edema, and conjunctivitis improved. After 48 hours, fever and rash were gone. After 3 days, his appetite improved; progressively over time, cervical adenomegaly and cutaneous lesions improved. Abdominal pain persisted for 7 days after treatment. Nausea and vomiting started 2 days after admittance; on day 7, vomit was of coffee-ground consistency. All remaining symptoms abated thereafter. The patient had diarrhea during days 3–6 after admittance; hepatomegaly disappeared after 4 days. Ultrasonographic images of the abdomen indicated acute cholecystitis and hepatosplenomegaly; endoscopic examination of the upper digestive tract showed hyperplasia, hyperemia, and linear and pseudomembranous lacerations in the middle and distal thirds of the esophagus (Mallory-Weiss syndrome) and moderate erythema of the stomach. Test results for *Helicobacter pylori* and *Giardia lamblia* were negative.

Laboratory results showed leukopenia and monocytosis on day 5 of illness. Leukocyte count was within reference range thereafter; thrombocytopenia was present until day 7 (99,000/mm^3). ALT was elevated from day 3 and peaked (481 IU) on day 7. AST levels increased on day 5 and peaked (215 IU) on day 7. Both values decreased progressively to reference levels (after 25 days for ALT and 46 days for AST). Lactic dehydrogenase was elevated for 9 days while erythrocyte count, sedimentation rate, and serum glucose, amylase, urea, creatinine, bilirubin, calcium, sodium, and potassium remained within reference limits. The patient was released after 8 days of hospitalization.

The buffy-coat smear performed 4 days after illness onset showed basophilic intracytoplasmic inclusions inside vacuoles of lymphocytes and monocytes, with typical features of morulae reported for human monocytic ehrlichiosis (Figure). Nested PCR analysis was positive for *E. chaffeensis*, and sequencing of the amplified DNA fully confirmed the 16S rRNA targeted sequence.

This report provides molecular evidence of *E. chaffeensis* infection in a patient with acute disease in Venezuela. A previous case of human monocytic ehrlichiosis in a 17-month-old girl in Venezuela has been demonstrated serologically (*2*). *E. canis* in an asymptomatic patient in Venezuela has been demonstrated by PCR and culture isolation (*8*) and was recently demonstrated in symptomatic patients (*9*). Excluding the esophageal lesions (Mallory-Weiss syndrome), our case is compatible with cases reported previously (*10*). The clinical manifestations of ehrlichiosis are similar to those of dengue fever and mononucleosis, both common diseases in Venezuela. The positive anti-dengue IgM and the seroconversion of the IgG together with the negative PCR and isolation results suggest a recent, inactive infection with dengue virus.

According to our findings, ehrlichiosis should be a differential diagnosis for febrile patients who have

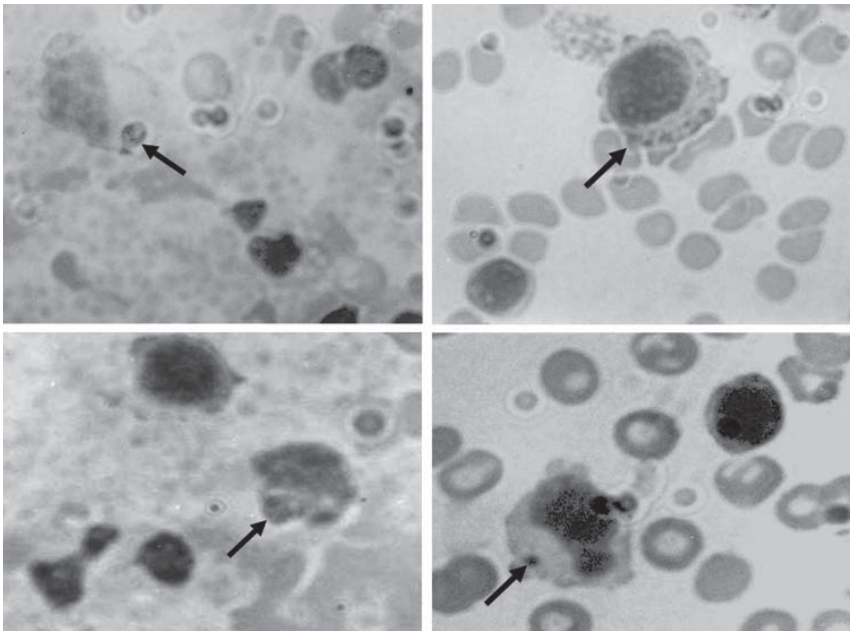

Figure. Peripheral blood smears (buffy-coat preparation) showing variable-sized basophilic inclusions (arrows) in mononuclear cells from a 9-year-old boy with human monocytic ehrlichiosis, Carabobo, Venezuela. Dip Quick (Jorgensen Laboratories, Inc., Loveland, CO, USA) staining; magnification ×1,000.

thrombocytopenia, hepatomegaly, and recent exposure to ticks. Although *Amblyomma americanum*, the main known vector of *E. chaffeensis*, has not been reported in Venezuela, *Rhipicephalus sanguineus* and *A. cajennense* are abundant in rural areas of Venezuela; their ability to be vectors should be investigated.

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We are grateful to Jacqueline Dawson for providing the DH82 cell lines infected with *E. canis* and *E. chaffeensis*. We also thank Guillermo Comach for performing serologic testing, isolation, and PCR techniques for dengue virus.

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Resource Allocation during an Influenza Pandemic

To the Editor: Planning for pandemic influenza is accepted as an essential healthcare service and has included creation of national and international antiviral drug stockpiles and novel approaches to emergency vaccine development (*1*). The effectiveness of these strategies in a pandemic may be substantial but is unknown. More certain is that effective management of severe and complicated influenza will reduce deaths and that demand will exceed available treatment resources (*2*). Appropriate allocation of treatment resources is therefore essential, perhaps more important than any specific treatment such as administering antiviral medication to symptomatic patients. Re-

source allocation requires the following: 1) making clear societal decisions on the goals for healthcare resources; 2) conducting operational research to develop an evidence base to support the achievement of these goals; and 3) developing systems to capture and learn from new information in a pandemic to facilitate modification of the response as the characteristics of the pandemic emerge. Most societies have not yet addressed the first issue fully. In the United Kingdom, a Department of Health consultation on planning critical care during emergencies cites "the underpinning principle of providing the greatest good for the greatest number of people during the course of an emergency" and thus appears to support a capacity-to-benefit approach (*3*). Similarly, triage criteria developed in Canada, based on the Sequential Organ Failure Assessment (SOFA) score, exclude those persons believed to be too ill or otherwise unlikely to benefit from critical care (*4*). Even more important for most severely ill patients, however, will be deciding whether to admit them to the hospital at all. The UK pandemic-planning criteria currently recommend a scoring system for hospital admission based on an assessment of poor outcome rather than on capacity to benefit (*2*). Indeed, age >85 years and severe underlying cognitive impairment, which would rule out admission to critical care in Canada, would strongly favor admission to hospital care in the United Kingdom, the opposite of the situation for a younger cognitively intact person with similar disease severity. If tools are to be developed to support triage at all stages of the patient pathway in a pandemic, societies must consider the ethical issues raised (*4*,*5*), debate them, and take a position on the values that should underpin decision making in a pandemic.

Even when clear societal goals are established, much work remains to ensure that the healthcare community is equipped to steer healthcare resources to deliver these effectively (*6*). Community-acquired pneumonia has been used as a surrogate for influenza to test predictive scoring systems for assessing severity and assisting triage decisions (*7*). Seasonal influenza epidemics would provide the most realistic setting available, in particular, if protocols were in place to test criteria when a relatively severe influenza season occurs. In addition to identifying criteria for setting priorities within influenza management, such testing will need to consider the balance of resources between influenza treatment and treatment of other usual noninfluenza conditions that will require emergency care during the pandemic. Decisions that must be made during a pandemic are complex, varying from when to stop major elective surgery so critical care capacity can be opened up, to how to triage those who have experienced major trauma and those with influenza. These decisions could differ from those same decisions made outside a pandemic, and an adequate evidence base is needed if they are to be of good quality.

The third component of our preparation for optimally deploying standard care in a pandemic is being able to change our approach quickly as new knowledge emerges. In the so-called Spanish influenza pandemic of 1918–19, the unfamiliar clinical course meant that influenza was not even considered when the first cases appeared (*8*), and expectations had to be revised concerning who was most vulnerable and at what stage in their clinical course they were most at risk. Therefore, healthcare professionals must develop and test the public health infrastructure to capture patient factors associated with outcome and treatment response during a pandemic and feed this information back into clinical practice rapidly and reliably, as occurred during the epidemic of severe acute respiratory syndrome (*9*). International collaboration will be important for sharing this work (*10*) and developing useful tools early in a pandemic. Having recognized the risk for pandemic influenza, we must now complement the research into novel influenza treatments by addressing our knowledge gap on how best to use our resources to deliver optimal clinical care in the management of influenza guided by effective clinical surveillance.

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Novel Relapsing Fever Spirochete in Bat Tick

To the Editor: Tick-borne relapsing fever in western North America is a zoonosis caused by spirochetes in the genus *Borrelia* that are transmitted by argasid ticks of the genus *Ornithodoros* (*1*). Human disease occurs in many focal areas and is associated with infections of *Borrelia hermsii*, *B. turicatae*, and possibly *B. parkeri* (*2*,*3*). Although the ecologic parameters that maintain *B. hermsii* and *B. turicatae* differ, human infections usually occur in rustic cabins (*B. hermsii*) and caves (*B. turicatae*) inhabited by ticks and their terrestrial vertebrate hosts (*1*). Recently, Gill et al. (*4*) provided evidence that the argasid bat tick, *Carios kelleyi*, feeds upon humans. Subsequently, Loftis et al. (*5*) used PCR analysis and DNA sequencing to detect in *C. kelleyi* an unidentified *Borrelia* species that was closely related to *B. turicatae* and *B. parkeri*. We report the partial molecular characterization of another novel tick-borne relapsing fever spirochete in *C. kelleyi*, which expands our knowledge for this group of pathogenic spirochetes and their potential vertebrate hosts and tick vectors.

C. kelleyi were collected August 18, 2005, from a house in Jones County, Iowa, built in 1857. Bats had been excluded from the attic since 1992. Nine months before ticks were collected, bats were prevented from roosting under the eaves. DNA was extracted from 31 nymphal *C. kelleyi*, as described previously (*6*). For each tick, regions of the *glpQ*, *flaB,* and *16S rRNA* genes were amplified and sequenced as described (*3*,*7*,*8*). Sequences were assembled by using the SeqMan program in the Lasergene software package (DNASTAR, Madison, WI, USA).

Fourteen (45.1%) of 31 ticks were positive by PCR for ≥1 of the genes tested. Partial DNA sequences were determined from tick no. 16, for which amplicons for all 3 genes were obtained. The partial *flaB* sequence had 4 bases different from the 300-base sequence (98.66% identity) reported previously (GenBank accession no. AY763104) for another *Borrelia* sp. found in *C. kelleyi* (*5*). We constructed a 1,992-bp concatenated sequence that contained 1,273 bp of the *16S rRNA*, 351 bp of *flaB*, and 368 bp of *glpQ*. This concatenated sequence was aligned with homologous, trimmed DNA sequences of the same length obtained from representative full-length sequences determined previously for *B. hermsii*, *B. turicatae,* and *B. parkeri* (*3*,*9*) (Figure). This *C. kelleyi* spirochete was more closely related to *B. turicatae* and *B. parkeri* than to *B. hermsii* but was clearly distinct from all 3 species (DNA sequence identities of 98.89%, 98.75%, and 95.98% to *B. turicatae*, *B. parkeri*, and *B. hermsii*, respectively).

A *glpQ* amplicon from another nymphal tick (no. 3) was sequenced (GenBank accession no. EF688578) and was unique in the database; it was also considerably different from the *glpQ* sequence determined from tick 16, with 325 of 368 bases matching (88.3% identity). The *Borrelia glpQ* sequence from tick 3 had 85.1%–89.1% identity compared with *glpQ* sequences from *B. hermsii*, *B. turicatae,* and *B. parkeri*. This finding suggests the presence of at least 2 relapsing fever group spirochetes in *C. kelleyi* that await further characterization.

We found a novel *Borrelia* in bat ticks that is closely related to, but distinct from, the other known species of tick-borne relapsing fever spirochetes in North America. The human health implications of the new relapsing fever group spirochete are not yet known. The willingness of *C. kelleyi* to feed on humans and the fact that infection with bacteria closely related to true relapsing fever spirochetes occurs in

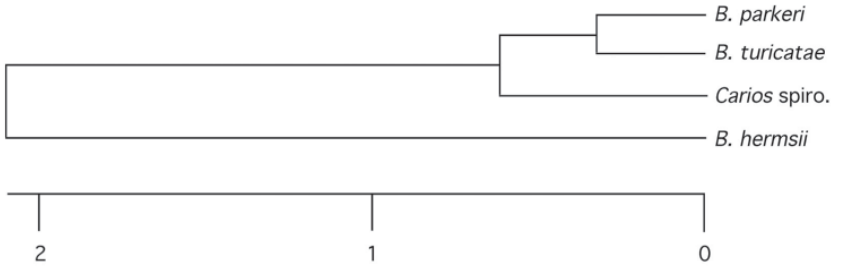

Figure. Phylogram comparing the novel spirochete in the bat tick *Carios kelleyi* with *Borrelia parkeri*, *B. turicatae,* and *B. hermsii* based on the concatenated partial *16S rRNA-flaB-glpQ* DNA sequences in the *Carios* spirochete (1,992 bp total) (produced with ClustalV software from DNASTAR [Madison, WI, USA]). Scale bar represents the number of base substitutions per 100 aligned bases. GenBank accession numbers for the *C. kelleyi* spirochete sequences used to construct the tree are EF688575, EF688576, and EF688577. Spiro, spirochete.

these ticks suggest that human habitation near bats and their associated tick colonies could pose a public health risk. Growth in laboratory animals or culture could help isolate these novel organisms for further studies to establish the distribution and public health implications of this newly identified *Borrelia* sp.

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KI and WU Polyomaviruses in Children, France

To the Editor: Two new members of the *Polyomaviridae* family, provisionally named *Karolinska Institutet virus* (KIPyV) and *Washington University virus* (WUPyV), have been recently discovered (*1*,*2*). These new polyomaviruses were identified by screening human respiratory secretions with molecular tools. KIPyV and WUPyV are genetically related to the BK virus and the JC virus, the 2 known members of the family *Polyomaviridae* that affect humans.

In France, from November 2006 through June 2007, nasopharyngeal aspirates were obtained from 537 children who were <5 years of age and who had acute respiratory tract disease. The aspirates were tested for respiratory syncytial virus (RSV); influenza virus types A and B; parainfluenza virus types 1, 2, and 3; and adenoviruses (AdVs) by direct immunofluorescence assay. The aspirates were also tested for human metapneumovirus (HMPV) by an enzyme immunoassay (HMPV EIA, Biotrin, Lyon, France) and for the human bocavirus (HBoV) by PCR (*3*). Samples were placed on MRC5 cell monolayers for virus isolation.

Nucleic acid extracts were tested for KIPyV and WUPyV DNA by PCR. KIPyV detection was performed by using a nested PCR approach that targeted the VP1 capsid gene as described by Allander et al. (*1*). For WUPyV detection, primers targeted the predicted 3′ end of the large T antigen coding region as described by Gaynor et al. (*2*). The amplification specificity was assessed by sequencing the PCR product; sequences were deposited in GenBank (WUPyV isolates, accession no. AM778536–48; KIPyV isolates, accession no. AM849808–10).

At least 1 type of virus was identified for 271 (50.5%) children. The viruses found were RSVs in 175 (32.6%), HBoVs in 54 (10.0%), HMPVs in 50 (9.3%), rhinoviruses/enteroviruses in 11 (2%), influenza A viruses in 8 (1.5%), human AdVs in 6 (1.1%), and parainfluenza type 3 viruses in 4 (0.7%) samples. Aspirates were not tested for coronaviruses; detection of rhinoviruses/enteroviruses was likely low because cell culture is less sensitive than molecular assays.

A total of 13 (2.4%) samples were positive for WUPyV; of these 4 (30.8%) were co-infected with another virus. The 13 children with samples positive for WUPyV had a median age

of 11.2 (2–48) months and the male/female sex ratio was 2.2. KIPyV DNA was detected in samples from 3 (0.6%) boys (ages 10, 18 and 30 months); 1 of those samples was co-infected with RSV and HMPV.

Sequences of WUPyV and KIPyV isolates varied little from each other and from other GenBank sequences, which suggests that these polyomaviruses are genetically conserved viruses. Clinical characteristics of children infected with WUPyV and KIPyV are retrospectively recorded (Table). All children recovered and were able to return home within 1 to 10 days, with the exception of 1 child. This child had been hospitalized since birth for congenital myopathy; nosocomial acquisition or vertical transmission of the WUPyV is suspected.

Our data are in agreement with the 2 original reports that show that the new KI and WU polyomaviruses may be detected in respiratory secretions from patients with respiratory diseases (*1*,*2*). WUPyV was detected in 2.4% of children <5 years of age who were hospitalized with respiratory tract disease, which is in accordance with the 2% incidence reported by Gaynor et al. (*2*). The 0.6% prevalence observed for KIPyV PCR is also in agreement with data reported from Sweden (*1*) and Australia (*4*). A seasonal change in the presence of WUPyV was not observed; however, all KIPyV isolates were found only during January.

KIPyV and WUPyV were mainly detected in samples from children with lower respiratory tract disease, such as bronchiolitis or atypical pneumonia, and in samples from children with exacerbated asthma. These preliminary data on the likely role of these viruses as respiratory pathogens need to be interpreted with caution. Aspirates were obtained only from those with observed symptoms; no asymptomatic controls were tested. Detection of WUPyV and KIPyV in respiratory samples may simply reflect a respiratory transmission route as previously suggested for BK virus and JC virus (*5*). Another virus was in aspirates from 31% of the children with KI and WU polyomaviruses. Substantial rates of codetection were also reported in the initial descriptions of both WUPyV and KIPyV (*1*,*2*). More recently, Bialasiewicz et al. reported a 25% rate of codetection of KIPyV with another pathogen (*4*). These high rates of co-

Table. Clinical findings for 13 children infected with WU polyomavirus and 3 children infected with KI polyomavirus*

Sex/age, mo	Copathogen	Fever, °C	CRP, mg/L	SaO_2, %	WBC, x 10^3 cells/μL	Chest radiograph findings	Diagnosis	Clinical signs and symptoms
WU polyomavirus								
M/2	–	37.9	8.3	NA	9.4	Hyperinflation	Pneumonia	Cough, dyspnea
M/12	–	39.2	112.0	NA	18.2	Hyperinflation, interstitial infiltrate	Bronchiolitis	Cough, dyspnea
F/20	–	38.7	56.0	NA	13.7	Hyperinflation	Atypical pneumonia	Cough, respiratory distress, diarrhea
F/12	–	37.8	15.0	96	12.3	Hyperinflation	Bronchiolitis	Cough, dyspnea, pharyngitis
M/10	–	39.0	90.3	NA	18.0	Normal	Idiopathic fever	Fever
F/14	–	38.9	20.8	95	11.0	Hyperinflation, interstitial infiltrate	Atypical pneumonia	Cough, wheezing, dyspnea
F/10	RSV	38.0	14.0	NA	17.2	Hyperinflation, interstitial infiltrate	Bronchiolitis	Cough, respiratory distress, dyspnea
M/6	RSV	38.0	NA	89	NA	Hyperinflation	Bronchiolitis	Respiratory distress, wheezing
M/48	HMPV	36.8	15.0	94	13.0	Hyperinflation	Asthma	Cough, respiratory distress
M/24	–	37.0	23.0	98	13.9	Hyperinflation	Asthma	Cough, respiratory distress
M/2	–	38.5	38.5	70	16.0	Normal	Idiopathic fever	Cough, fever
M/11	HBoV	39.0	5.5	94	6.2	Interstitial infiltrate	Atypical pneumonia	Cough, fever
M/10	–	37.0	10.0	97	18.0	Alveolar syndrome	Congenital myopathy	Respiratory distress
KI polyomavirus								
M/10	RSV, HMPV	39.0	NA	NA	NA	Hyperinflation	Bronchiolitis	Cough, wheezing, otitis, diarrhea
M/30	–	39.2	<5.0	NA	11.0	Interstitial infiltrate	Influenza-like pneumonia	Cough, dyspnea, otitis, sore throat
M/18	–	38.9	23.0	96	14.8	Hyperinflation, atelectasis	Asthma	Cough, dyspnea, wheezing

*CRP, C-reactive protein; SaO_2, oxygen saturation; WBC, white blood cells; NA, not available; HMPV, human metapneumovirus; RSV, respiratory syncytial virus; HBoV, human bocavirus.

infection raise questions about the real pathogenic role of these viruses.

As with other polyomaviruses, WUPyV and KIPyV could establish persistent and latent infections with likely asymptomatic reactivations (*5*), and detection of these viruses could also reflect a long-term shedding from previous acute episode. Recently published studies have not shown a pathogenic role for these new polyomaviruses in respiratory tract disease (*6*,*7*); however, more comprehensive studies are needed to elucidate whether both KIPyV and WUPyV have any clinical relevance.

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Milk Replacers and Bovine Spongiform Encephalopathy in Calves, Japan

To the Editor: Milk replacers produced from a specific feed factory in Japan were suspected of being associated with a cluster of bovine spongiform encephalopathy (BSE) infection in calves. We conducted a case–control study to test this association.

In Japan, BSE infection has been confirmed in 32 calves as of the end of May 2007; 13 of these calves were born between December 1995 and August 1996. One BSE-infected calf was born in 1992 and had an atypical BSE phenotype (*1*). Because no BSE-infected calves were born in 1997 and 1998, we considered that those born in 1995 and 1996 formed an independent temporal cluster (Figure). Epidemiologic investigation showed that all 13 calves were fed milk replacers produced by a specific factory. Ten calves were born in Hokkaido, and 3 were born in the Kanto region, which is ≈800 km away from Hokkaido.

In the case–control study, all farms where the 13 BSE-infected calves were born and raised for at least 1 year were defined as case farms. Control farms were defined as dairy farms where no BSE calves had been reported. Candidates for control farms comprised 200 randomly selected farms, which were located in 23 prefectures where the milk replacers were distributed. We used a national cattle identification database for random selection. Veterinary officers from the local government interviewed farmers in November and December 2006 and requested that they complete a questionnaire on farming practices in 1996, including herd size and use of milk replacers and calf concentrates. For the case farms, information previously obtained from outbreaks was used. Of the 200 potential control farms, 154 farms were used as controls. Forty-six farms were excluded; 24 farmers did not respond or could not specify the use of milk replacers; and 22 farms had either closed or farmers did not respond for miscellaneous reasons.

Among the 154 control farms, 36 farms (23%) used the milk replacers from the specific factory, 89 farms (58%) used other milk replacers, and 29 farms (19%) did not use milk replacers. Since 1 case farm lacked

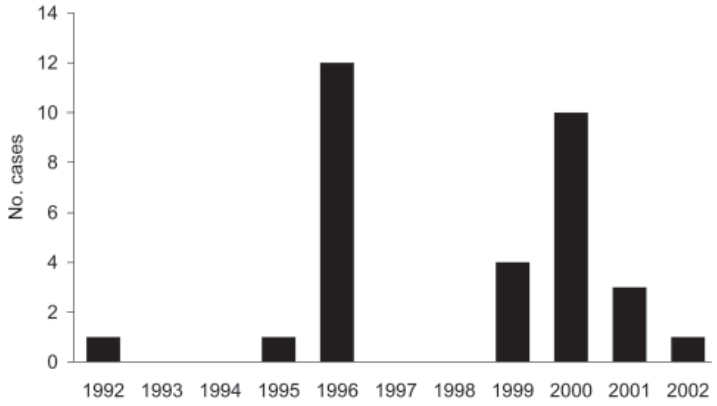

Figure. Number of cases of bovine spongiform encephalopathy by calves' birth year, Japan.

documented evidence about the use of the specific milk replacers, we conservatively assumed that 12 of 13 case farms used the specific milk replacers. We estimated the odds ratio for this risk factor by using logistic regression analysis. Our results indicated that the use of the milk replacers produced by the specific factory was associated with BSE infection (odds ratio [OR] 39.3, 95% confidence interval [CI] 4.9–312.9, p = 0.0005).

The milk replacers produced by the specific factory contained tallow that was produced at domestic rendering factories and imported from the Netherlands. Milk replacers were fed to calves during a relatively short period after birth (an average of 79 and 68 days, for case and control farms, respectively). If 1 production lot of milk replacer became accidentally contaminated with BSE, the exposure would occur in newborn calves within a relatively short period. This contamination may explain why 11 of 13 BSE-infected calves were born within a 2-month period from February 10, 1996, to April 8, 1996.

In Hokkaido, 9 of 10 BSE-infected calves were fed calf concentrates produced in the same feed factory. This proportion was higher than that of the 50 control farms in Hokkaido (22/50, Fisher exact test, p = 0.013). The calf concentrates might have become contaminated with meat-and-bone meal (MBM) because this factory used MBM for other animal feed. Multivariate logistic regression analysis, including this factor and that for the specific milk replacers, did not indicate significant association between the specific calf concentrates and occurrence of BSE (calf concentrates: OR 3.2 [CI 0.8–13.0], p = 0.14; milk replacers: OR 21.7 [CI 2.5–192.6], p = 0.006). The factory that provided the specific concentrates belonged to a company affiliated with the company that produced the milk replacers in question. Given the fact that farmers tend to use milk replacers and calf concentrates from the same company, association of the calf concentrates with the BSE infection may have been masked by the use of specific milk replacers. However, our study is limited by the small number of BSE cases and investigation of events that occurred 10 years ago.

A possible causal association between the feeding of potentially contaminated milk replacers to calves and the occurrence of BSE has been suggested by several epidemiologic studies (*2*–*5*). However, no report shows experimental transmission of BSE by use of tallow or milk replacers (*6*). This lack of evidence in the literature may suggest that the risk of contracting BSE from processed tallow or milk replacers is low (*7*). If MBM is excluded as a source of infection, other transmission mechanisms, such as the feeding of animal fat, may become more important.

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Control of Hepatitis A by Universal Vaccination of Adolescents, Puglia, Italy

To the Editor: The incidence of hepatitis A in Italy has decreased in the past 2 decades because of improved sanitation and better living conditions (*1*). However, large outbreaks occurred in the 1990s in several southern regions of Italy, despite lower rates of infection among the general popula-

tion (*2*–*4*). Person-to-person transmission has been recognized as a major factor in spread of this disease during this period (*5*).

Safe and highly effective hepatitis A vaccines have been available since 1995. Nevertheless, their use has been limited to the Western Hemisphere. Universal vaccination programs have been initiated only in the United States and Israel before 1998 (*6*). In 1998, after a large epidemic of hepatitis A, a vaccination program for toddlers and adolescents was initiated in Puglia in southeastern Italy, which has a population >4 million. This vaccine was offered free to all children 15–18 months of age and to adolescents 12 years of age. Until 2002, a combined hepatitis A plus B vaccine had been used for vaccination of adolescents as part of the national hepatitis B immunization program. In 2003, this hepatitis B vaccination program ended; only hepatitis A vaccines containing 1 antigen are now used. No catch-up vaccination campaign has been planned.

We analyzed disease surveillance and vaccine coverage data for 1991–2006 to evaluate the effect of such a vaccination program on hepatitis A incidence in persons in Puglia during the 9 years after initiation of the program. In the period before the vaccination program was initiated (1989–1997), annual incidence rates of hepatitis A in Puglia ranged from 4.3 to 139.8 cases/100,000. The average annual rate during this period was 49.5 cases/100,000. Two large outbreaks were reported in Puglia, the first in 1992 and the second in 1996–1997 (*5*). During the 9 years after start of the vaccination program (1998–2006), incidence of hepatitis A decreased from 22.8 cases/100,000 in 1998 to 0.7 cases/100,000 in 2006 (Figure). In the same period in other regions of Italy, incidence of hepatitis A was 5 cases/100,000, without any evident annual peak.

Since 2002, annual incidence rates in Puglia have remained at ≤2.8 cases/100,000, lower than those in the rest of Italy. This incidence has been observed in all age groups, without any differences between vaccinated and unvaccinated birth cohorts. Vaccination coverage among children 15–18 months of age was <20% during the period of the vaccination program. Coverage levels in adolescents reached 65% in the third year after the start of the program and then ranged from 57% to 72% (Figure).

Hepatitis A has been a serious public health problem in Puglia. This disease has had a detrimental effect on the local economy, which is based on tourism and trade of food products. However, since the vaccination program was started in 1998, disease incidence has decreased. During the study period, no other alternative prevention measures that could have had an effect on disease control were implemented.

High levels of vaccination coverage have not been achieved since the start of the campaign, and no catch-up vaccination program has been implemented. The decrease in hepatitis A incidence we observed involved all age groups, including those not covered by the vaccination program. This finding may indicate strong herd immunity, which would confirm what has been observed in other countries (*7*–*9*). However, there is uncertainty in interpreting current epidemiologic data. On the basis of available data, we cannot assess whether the current low incidence of hepatitis A in Puglia is caused by vaccination alone or in combination with other factors. We also cannot exclude the possibility that what we observed may have been an interepidemic period and that new episodes may occur in the future.

Our results indicate that local health authorities should be aware of possible increases in the incidence of hepatitis A in Puglia. An urgent catch-up vaccination program may be necessary to prevent future outbreaks. Moreover, a seroepidemiologic survey would be useful for assessing the size of the susceptible population and most vulnerable age groups.

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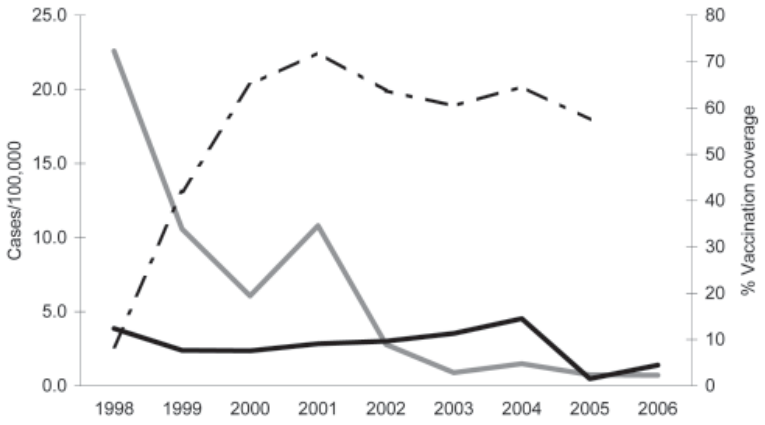

Figure. Incidence of hepatitis A in Puglia, Italy (gray line) compared with the rest of Italy (black line), 1998–2006, and hepatitis A vaccination coverage among adolescents in Puglia (dashed line), 1998–2005.

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Human *Rickettsia sibirica mongolitimonae* Infection, Spain

To the Editor: *Rickettsia sibirica mongolitimonae* has been recently reported as a subspecies of *R. sibirica* (*1*). The first evidence of *R. sibirica mongolitimonae* pathogenicity in humans was documented in France in 1996 (*2*). Since then, 11 more cases in France, Algeria, South Africa, Greece, and Portugal have been reported (*3*–*6*). Because the main clinical manifestations include lymphangitis, the acronym LAR (lymphangitis-associated rickettsiosis) has been proposed (*3*). We report a case from Spain that confirms the broad distribution of this agent in southern Europe.

A 41-year-old man was admitted on June 19, 2007, to the Hospital de Cruces (Baracaldo, Spain) with fever (39°C), malaise for a week, sweating, lumbar and knee pain, disseminated myalgias, and headache. He reported that 20 days before admission he had removed an engorged tick from his right leg while working as a topographer in the Balmaseda Mountains, 30 km from Bilbao. He had also removed several ticks from his body 4 days before the onset of symptoms. Physical examination did not demonstrate relevant findings. There was no inoculation eschar at the tick-bite sites. Rash, lymphadenopathies, and lymphangitis were not observed.

Chest radiograph did not show consolidation or other abnormality. Initial laboratory examination, on June 21, 2007, showed a leukocyte count $5.2 \times 10^3/\mu L$, hemoglobin 14.1 g/dL, platelet count 190,000/μL, erythrocyte sedimentation speed 9 mm/h, urea 38 mg/dL, creatinine 0.9 mg/dL, aspartate aminotransferase 229 IU/L, alanine aminotransferase 170 IU/L, alkaline phosphatase 158 IU/L, gamma-glutamyl-transpeptidase 111 IU/L, total bilirubin 1.3 mg/dL, and C-reactive protein 4.3 mg/dL. Because the patient had been bitten by a tick, acute-phase serum and EDTA-treated blood samples were sent to the Special Pathogens Laboratory (Área de Enfermedades Infecciosas – Hospital San Pedro from La Rioja), where a presumptive diagnosis of rickettsiosis was made. On June 22, 2007, treatment with doxycycline was begun (100 mg/day for 12 days), and his condition rapidly improved.

The early-phase serum yielded low immunoglobulin (Ig) G titer (<64) against *Rickettsia conorii* and *Anaplasma phagocytophilum* antigens, and results of ELISA and Western blotting for Lyme borreliosis were negative. A convalescent-phase serum sample collected 7 weeks later did not contain IgG antibodies against spotted fever group *Rickettsia* species when *R. conorii* antigen was used.

DNA was extracted from the early whole-blood specimen by using QIAamp DNA Blood minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. This DNA extract was used as template in nested PCR assays targeting the spotted fever group rickettsial *ompB* (420 bp) and *gltA* (337 bp) genes (*7*). Quality control included both positive (with *R. conorii* Malish #7 grown in Vero cells) and negative controls that were extracted and PCR amplified in parallel with the specimens. Negative controls consisted of sterile water instead of template DNA. Amplification products of the expected size were obtained. The sequences of these amplicons allowed the identification of *R. sibirica mongolitimonae* with 99.5% and 100% similarity for *ompB* and *gltA*, respectively (GenBank accession nos. DQ097083 and DQ097081).

To our knowledge, *Rickettsia* species have never been detected in ticks or human specimens in Spain. The host ticks of this rickettsia are likely *Hyalomma* species, which are more prevalent in southern Spain. In our region in northern Spain, *Hyalomma marginatum* represented 8% of ticks that fed on humans during 2001–2005, although an increase in this number was recorded last year (data not shown).

In our patient, *Rickettsia*'s pathogenic role was demonstrated by PCR, a technique that has previously enabled us to identify other arthropod-borne *Rickettsia* species (*8*,*9*). This case suggests that *R. sibirica mongolitimonae* infection should be considered in the differential diagnosis of rickettsiosis and tick-bite febrile patients in Spain and confirms the distribution of this rickettsia in southern Europe. According to the literature (*3*), some patients in whom *R. sibirica mongoli-*

timonae infection is diagnosed have >1 eschar, which raises the suspicion that some cases of Mediterranean spotted fever with multiple eschars reported in Spain could be caused by this rickettsial species. More studies about the vectors of this bacteria are needed because studies of *Hyalomma* and *Rhipicephalus* ticks (the suspected hosts) conducted in our area have not demonstrated the presence of this *Rickettsia* species.

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Lymphangitis in a Portuguese Patient Infected with *Rickettsia sibirica*

To the Editor: We report a case of *Rickettsia sibirica* mongolitimoniae strain infection associated with lymphangitis (*1*). A 44-year-old man was admitted to São Bernardo Hospital in Setubal, Portugal, on August 21, 2006. Twelve days previously while on vacation at Troia Peninsula, he noted malaise, insomnia, and dry buccal mucosa. The next day he observed a small erythematous pruritic lesion on the lower right forearm that 2 days later developed into an eschar. He also had fever and sought medical care. After treatment with topical bacitracin, floxacillin, and acetaminophen for 2 days, fever (38.7°C) continued with lymphangitis extending from the right wrist to the elbow. The medication was changed to nimesulide. Three days later a rash developed on the trunk and arms, and lymphangitis extended to the axilla. Fever and chills continued, leading to hospital admission. No history of tick exposure was reported. Physical examination showed blood pressure 128/73 mm Hg, pulse 96/min, and a rubbery, nontender right supraclavicular lymph node ≈1 cm in diameter. Several 5- to 10-mm maculopapular erythematous lesions were observed on the patient's palms. He had inflammation on the right forearm suggestive of lymphangitis and an eschar with surrounding edema and erythema on the dorsal lower right forearm (Figure). Admission evaluation showed platelets 117,000/μL, total bilirubin 0.42 mg/dL, albumin 3.42 g/dL, creatinine 1.1 mg/dL, alanine aminotransferase 244 U/L, aspartate aminotransferase 54 U/L, alkaline phosphatase 1061 U/L, creatine phosphokinase 87 U/dL, lactate dehydrogenase 784 U/L, C-reactive protein 7.1 mg/dL, radiographic pulmonary diffuse reticular pattern, arterial pO_2 68 mm Hg, O_2 saturation 94%, pCO_2 22 mm Hg, and arterial blood pH 7.35. The differential diagnoses included rickettsiosis, pneumonia, and cellulitis. Treatment with vancomycin, ceftriaxone, and 100 mg of doxycycline twice a day was begun. On the day after hospitalization, a heparinized blood sample and 2 skin biopsy samples were collected. Vancomycin and ceftriaxone were discontinued at 48 hours when rickettsial infection was confirmed by PCR on skin biopsy; 48 hours later, the patient was afebrile.

Immunofluorescence assay for antibodies that used *R. sibirica* mongolitimonae strain as antigen demonstrated seroconversion with no antibodies in the serum sample collected on August 21 and immunoglobulin G (IgG) and IgM antibodies at a titer of 256 in serum collected on August 30. DNA was extracted from 1 skin biopsy sample by using a DNeasy Tissue Kit (QIAGEN, Hilden, Germany). The products of nested PCR showed 100% similarity with *gltA* (353/353) and *ompA* (350/350) nucleotide sequences of *R. sibirica* mongolitimonae strain (GenBank accession nos. DQ423368.1 and DQ423367.1) (*1*).

Cutaneous biopsy indicated epidermal and dermal necrosis with extensive lymphocyte- and macro-

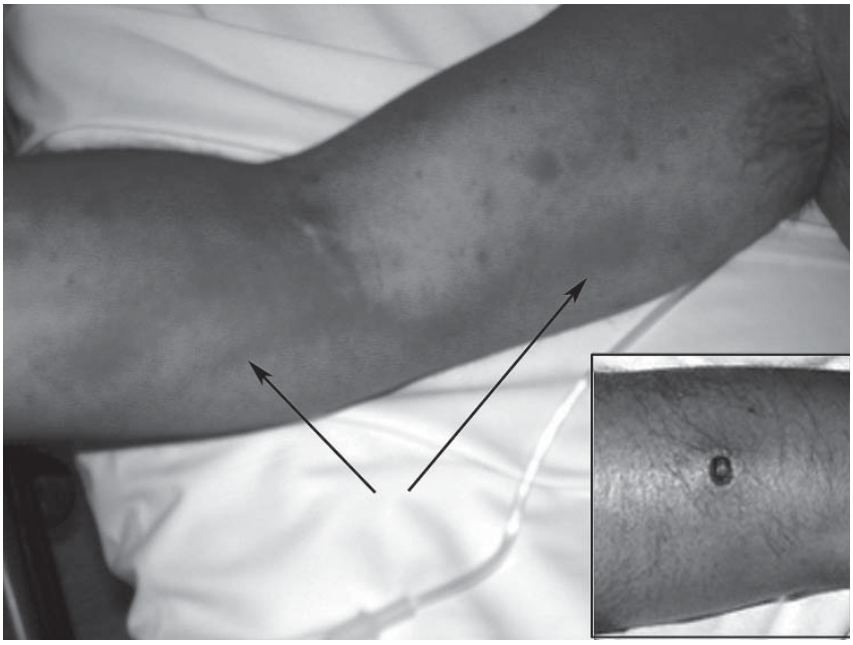

Figure. Lymphangitis extending from the right forearm to the axilla and (inset) eschar on right forearm, caused by *Rickettsia sibirica* mongolitimonae strain. Arrows indicate lymphangitis.

phage-rich inflammatory infiltrates involving the papillary and reticular dermal blood vessels characteristic of rickettsial infection. Relatively scant intracellular organisms were observed in the reticular dermis by spotted fever group rickettsia–specific immunohistochemistry (*2*).

Because of the presence of shared protein and lipopolysaccharide antigens in spotted fever group rickettsiae, distinguishing infections with closely related rickettsiae such as *R. conorii*, *R. africae*, and *R. sibirica* by serologic or immunohistochemical methods is very difficult. However, isolation and/or PCR detection followed by genetic characterization can determine the genotype of the organism to the level of genus, species, and strain. The incidence of *R. sibirica* mongolitimonae strain infection in Portugal is not known because the usual laboratory confirmation by serologic methods does not distinguish these cases from Mediterranean spotted fever.

Both cases of *R. sibirica* infection that have been recognized in Portugal occurred in August during the seasonal peak of Mediterranean spotted fever (*1*). This epidemiology differs from that in other countries (*3–6*). Perhaps differences in seasonal activity, population dynamics, or species of the vectors are the basis for the varying epidemiology. In Portugal, *R. sibirica* has been detected in *Rhipicephalus pusillus* (*1*). *Rickettsia sibirica* mongolitimonae strain was first isolated from a *Hyalomma asiaticum* tick from Inner Mongolia in 1991 (*7*) and subsequently from *H. truncatum* in Niger and from *H. excavatum* removed from a Greek patient (*4*,*8*).

Lymphangitis in some patients with *R. sibirica* mongolitimonae strain infection is a potentially useful diagnostic sign. Nevertheless, half of the patients with reported cases have not had lymphangitis, and infections caused by other *Rickettsia* spp. can also cause lymphangitis (e.g., patients with African tick bite fever and *R. heilongjiangensis* infections) (*1*,*3–6*,*9*,*10*). Thus, the diagnosis of any rickettsiosis should not be based solely on clinical manifestations. The pathogenic role of rickettsiae in lymphangitis remains to be determined. *R. sibirica* may possibly infect the endothelium of the lymphatic vessel along the pathway from the rickettsial portal of entry at the eschar inoculation site to regional lymph nodes. However, rickettsiae have not been observed in lymphatic vessels, and the lymphatic vessel lesion has not been characterized. The possibility of another agent or pathogenic effectors cannot be excluded. Currently, this clinical manifestation is the strongest evidence that rickettsiae may initially spread by a lymphogenous route before hematogenous dissemination.

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The Same Air

Al Zolynas

—for Guy Murchie, The Seven Mysteries of Life

The same air
that moves
through me and you
through the waving branches
of the bronchial tree
through veins
through the heart
the same air
that fills balloons
that carries voices
full of lies and truths
and half-truths
that holds up the wings of butterflies
humming birds eagles hang gliders 747s
the same air
that sits like a dull relative
on humid lakes
in Minnesota in summer
the same air
trapped in vintage champagne
in old bicycle tires lost tennis balls
the air inside a vial in a sarcophagus
in a tomb in a pyramid
buried beneath the sand
the same air
inside your freezer
wrapping its cold arms
around your t.v. dinners
the same air that supports you
that supports me
the same air that moves through us
that we move through
the same air frogs croak with
cattle bellow with
monks meditate with and on
the same air we moan with
in pleasure or in pain
the breath I'm taking now
will be in China in two weeks
my lungs have passed an atom
of oxygen that passed through the lungs
of Socrates or Plato
or Lao-tsu or Buddha
or Walt Disney or Ronald Reagan
or a starving child in Somalia
or certainly you
you right here right now
yes certainly you
the same air
the very same air

Zolynas' books include The New Physics, Wesleyan University Press, 1979; Under Ideal Conditions, Laterthanever Press, 1994 (San Diego Book Award, Best Poetry, 1994); and The Same Air, Intercultural Studies Forum, 1997. Seven of his poems were recently featured in the movie Fighting Words (Indican Pictures, Los Angeles, 2007). A long-time Zen practitioner, he teaches at Alliant International University, San Diego, California, and lives with his wife in Escondido, California.

Cold War, Deadly Fevers: Malaria Eradication in Mexico, 1955–1975

Marcos Cueto, editor

Woodrow Wilson Center Press, Johns Hopkins University Press, Baltimore, Maryland, USA, 2007
ISBN: 978-0-8018-8645-4
Pages: 288; Price: US $45.00

Marcos Cueto is a medical historian who describes the details of malaria eradication efforts in Mexico in the context of the Cold War era authoritarianism. His approach works overall, but occasionally he overreaches.

Mr. Cueto asserts that the politics of the time allowed the medical community to be similarly authoritarian in forcing malaria eradication to be the accepted strategy. He states that the political climate relied on fear-based tactics of spreading anxiety about the communist threat and that similar strategies were used to gain public support for the malaria eradication effort, incorporating military jargon such as "enemy" mosquitoes and "campaigns" against disease into the public health lexicon. These campaigns included a propaganda arm in which pop stars became champions for the cause, to make the public sympathetic to their efforts. At one point, the author likens the strategy of screening persons for asymptomatic malaria parasitemia to the 1950s McCarthy-style witch hunts for hidden communists—stretching the analogy beyond tolerable limits.

A recurring theme in the book is Mr. Cueto's skepticism of new technologies, especially those introduced by other (non-Mexican) national or international organizations. He disparages the adoption of chloroquine, DDT, and smear microscopy as "magic bullet" strategies, claiming they distracted from rather than enhanced control efforts. He suggests that current efforts, which tout the use of long-lasting insecticide-treated bed nets and artemisinin-based combination therapy, are similarly flawed. He never really articulates proven alternatives to the adoption of new technologies, but he vaguely suggests relying on community-based broad public health programs at the grassroots level.

I found the tone of the book a bit cynical and fatalistic. I got the impression that Mr. Cueto believes that all individuals and organizations attempting malaria eradication were doing so not for its own sake but rather as a front for other agendas such as centralizing national power in Mexico, furthering the international interests of capitalist countries, or personal glory. The author uses the fact that the eradication effort failed to support his contention that it was probably a bad idea in the first place.

Fast forward to today when there is a renewed interest in malaria control efforts. Witness the Global Fund to Fight AIDS, Tuberculosis, and Malaria; the Bill & Melinda Gates Foundation; the President's Malaria Initiative; and pop stars such as Bono championing the cause, including the possibility of eradication. Sounds familiar. In light of the current progress of malaria control efforts in Mexico, where most states are now malaria free and the total number of cases has been steadily decreasing, Mexico is well on its way to achieving those original eradication goals. Thus, whether you agree with the author's politics or not, if you are considering getting into the business of malaria eradication, you could benefit by reviewing this very detailed historical account of a malaria eradication effort that was unsuccessful.

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Parasites and Infectious Diseases: Discovery by Serendipity and Otherwise

Gerald Esch

Cambridge University Press, Cambridge, United Kingdom, 2007
ISBN-10: 0521675391,
ISBN-13: 978-0521675390
Pages: 366; Price US $45.00

The author of this book retells several of the famous stories of discovery in the field of vector-borne and parasitic diseases through the eyes of some of the most prominent researchers working in this field today. But Esch does not stop there—he goes on to connect these early stories with more recent watershed contributions as recounted through a series of interviews he references throughout the course of the book. For example, he describes the discovery of the African sleeping sickness agent, *Trypanosoma gambiense*, and the contributions in the early 1900s by Dutton, Castellani, and Bruce. He then moves on to more recent discoveries regarding immunity, antigenic variation, and the role of variable surface glycoproteins. He describes the seminal studies that were performed in this area as recounted through interviews with prominent parasitologists Dick Seed and Keith Vickerman. Through this process, Esch weaves a tapestry of the new and old as it relates to the history of important tropical diseases such as African typanosomiasis, malaria, yellow fever, HIV/AIDS, hookworm, and schistosomiasis, which continue to plague humankind.

The book has a novel organization; the first 100 pages are devoted to a lengthy prologue in which the major content contributors, the disease experts Esch interviewed, are extensively quoted and even venerated, in a casual, entertaining, and well-deserving

manner. Many who peruse this book will be pleasantly surprised by the colorful biographies of well-known parasitologists and vector-borne disease specialists, who some readers no doubt will know as friends, colleagues, collaborators, professors, or even former students.

As the title suggests, there is an effort to locate threads of serendipity woven through the historical tapestry of discovery. The conclusions that emerge unsurprisingly are that serendipity has played a much less important role than one might naively surmise and that the qualities of hard work, rock-solid persistence, a keen mind, and the fortitude to swim against the academic tide have paid generous dividends.

No doubt the attribution of specific recent hallmark contributions by key persons, many of whom are still active in the field today, will be met with some degree of controversy. Nevertheless, the book is interesting, educational, and enjoyable, a "must have" for every library of tropical medicine or medical history. Thumbs up to Esch.

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Henri de Toulouse-Lautrec (1864–1901). At the Moulin Rouge: The Dance (1890). Oil on canvas (115.6 cm x 149.9 cm). Philadelphia Museum of Art: The Henry P. McIlhenny Collection in memory of Frances P. McIlhenny, 1986

Hygeia as Muse

Polynexi Potter*

"A line will take us hours maybe; / Yet if it does not seem a moment's thought, / Our stitching and unstitching has been naught," wrote William Butler Yeats (1865–1939) about the creative process (*1*). For sheer spontaneity, evocativeness, and impeccable draftsmanship, he might have been describing the art of Henri de Toulouse-Lautrec. The artist's speed at work astonished his friends. Having resolved technical problems in his mind, during the gestation of the image or in countless sketches, photographs, and studies from life, he sang and joked during the brief execution of the work.

Lautrec's remarkable legacy seems to have started at birth in Albi, one of the oldest cities in France, into a wealthy family with ties to the Counts of Toulouse (*2*). He was an engaging, rambunctious child with precocious wit. At age 4, he wanted to sign the register at his brother's christening. Reminded that he could not write, he said, "It doesn't matter, I'll draw an ox." That was his earliest known work (*3*). By age 10, he was an inveterate sketcher of people and animals, illustrating everything he touched.

In what would appear an idyllic childhood, signs of trouble, perhaps the underpinnings of aristocratic lineage, started with the death of his young sibling before age 1. Henri, whose parents were first cousins, was also frail. His mother took him out of school and moved to the country, where she devoted herself to his care.

When he was 13, his life began to change: "I fell off a low chair onto the floor and broke my left thigh," he wrote a friend. After a long convalescence, he could only walk lopsidedly "like a duck." Fifteen months later, he fell again: "The second fracture was caused by a fall scarcely heavier than the first" (*3*). Henri, it appeared, had some unknown bone disease, a congenital condition, possibly pycnodysostosis (*4*). Despite the best available care and while the rest of his body continued to grow, the legs atrophied. He supported himself on a cane, which was surprisingly short since his trunk and arms were of normal length. Walking caused him pain and embarrassment.

Though limited by physical disability, he remained upbeat. "I am small but I am not a dwarf," he wrote, "... no urchins have ever bothered me" in the street (*3*). He always wore a hat, even when he painted, "for the light," he said (*3*), although like his signature beard, it may have concealed bone malformations.

He moved to Paris to study with Léon Bonnat, leading portraitist and later professor at the École des Beaux Arts. This apprenticeship turned him away from academic art: "I want to paint like the primitives, whose painting is as simple as that on a carriage door" (*3*). Later, under Fernand Cormon, he met and befriended Vincent van Gogh, Émile Bernard, and other artists, who sought him out for his openness and originality. Aristide Bruant, legendary balladeer and owner of cabaret Le Mirliton, initiated him to Montmartre: "I am against my will leading a truly Bohemian life and am finding it difficult to accustom myself to this milieu" (*3*).

Montmartre, an area on a hill away from the city, developed a unique personality, energetic and provocative, "outside the law" (*3*). Its dance halls, cabarets, cafés, and circuses held unending fascination. He painted them by day and lived in them by night. "From ten o'clock in the evening until half past twelve," reported the newspapers, "the Moulin Rouge [red windmill] presents a very Parisian spectacle which husbands may confidently attend accompanied by their wives" (*3*).

Lautrec lived in Montmartre, except for brief visits to Spain where he studied the work of El Greco and Diego Velásquez; Belgium; and England where he met Oscar Wilde

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and James McNeill Whistler. He exhibited often and was enormously productive, creating before he died at age 36 more than 1,000 paintings and 5,000 drawings. Although he maintained artistic independence, he was well connected. He consulted with Pierre Bonnard, was captivated by the impressionists, collected Japanese prints, and thought an Edgar Degas painting owned by his cousins so compelling, he declared he was always ready to "say his prayers before it" (*3*).

The 1880s and '90s, the Belle Époch in Europe and around the world, saw unprecedented scientific and technological advancements. Literature and the arts, biology, physics, and psychology were transformed. Theater and music adopted new methods, shocking audiences with their frankness. In Paris, the Moulin de la Galette and the Moulin Rouge offered carnival-like entertainment. Celebrated can-can dancer Jane Avril and Louise Weber, dubbed La Goulue (the glutton, for guzzling drinks), attracted huge crowds with decadent performances. Lautrec with his cherry-wood cane, black-and-white checked trousers, and flat-brimmed bowler hat was a regular fixture.

Lautrec was fascinated by human behavior but painted those who interested him: working women; cabaret proprietors; entertainers, whose brilliant if transient careers he observed dispassionately. His works were restrained, as he thought all art should be, but filled with movement. His sparse palette and bold, assured brushstrokes captured the essence of nightlife, the glare of the stage, the shadows of gaiety, the despair and loneliness of crowds, the plight of the working poor, the physical pain of dancers as well as their agility. On one album of lithographs he wrote, "I saw this," a phrase borrowed from Francisco Goya's Disasters of War. The phrase could describe Lautrec's total artistic output, the life of his era, regulated brothels and all.

Color lithography was new and very popular, and Lautrec adopted it as preferred medium. The technique, practiced by many greats (Alphonse Mucha, Pablo Picasso, Jasper Johns) originated in 1796. Based on the principle that oil and water do not mix, it uses both to form a print on a smooth surface. An image drawn with grease chalk onto a stone is moistened; ink is applied, which sticks to the drawing but not to the stone; inked areas are transferred to paper. Each color requires a different stone and separate pass through the press. Lautrec's lithographs, though known as posters, were not today's mass-produced photographic reproductions. They were multiples of small editions, each print individually made, one of a kind, original. "Poster" referred only to large size (*5*).

Publicity posters made by the lithographic technique were a major innovation of Montmartre artists. Lautrec's first effort and his best, Moulin Rouge: La Goulue, was an overnight sensation, pushing him and La Goulue to stardom. The critics took notice. Notoriety encouraged him to continue in the medium, introducing such innovations as spattered paint to simulate the aura of nightclubs and radical placement of figures and objects to achieve unprecedented immediacy. He created more than 300 lithographs, among the finest ever produced.

At the Moulin Rouge: The Dance (on this month's cover) epitomizes bourgeois gaiety in a luxurious establishment. This large multiple figure composition exemplifies Lautrec's vivid contrasting colors and meticulous execution. Dancing on the left is Jacques Renaudin, nicknamed Valentin le désossé (boneless Valentin) because of his rubbery limbs. Gathered in back, left of the waiter, a group of friends, among them Jane Avril, a photographer, some painters; in the center, a professional dancer; mingling with the crowd, colorful women ready to offer their hearts at the right price. The viewer is virtually in the painting.

Amusement, fanatically pursued by the crowds in dance halls and by Lautrec himself, at times, equals living on the edge. For him, excess was compounded by disability and emotional isolation, as well as by the plagues of his time, syphilis (*6*) and possibly tuberculosis: "Visibly, before the very eyes of his friends, he began to burn himself out, slowly at first, then with ever increasing speed" (*3*). He continued to grow as an artist, his style evolving up to the time of death.

The need to gather in large venues to see and be seen and to exchange ideas is not limited to Paris cafés and private clubs. This need, to break barriers, showcase new work, and focus on the problems of humanity, lives on today in scientific conferences. There, ideas mingle with personalities, and bit by bit, solutions are worked out for as the poet put it, "It's certain there is no fine thing / Since Adam's fall but needs much labouring" (*1*).

Hygeia is a capricious muse. She eludes the compromised artist toiling in pain and without physical charm but inspires the globe-trotting scientist gathering in today's venues to blast conventional wisdom and seek solutions to emerging infectious disease.

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Chagas Disease in Immigrants, France

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Complete list of articles in the April issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 10–13, 2008
2008 National STD Prevention Conference: Confronting Challenges, Applying Solutions
Hilton Chicago
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March 16–19, 2008
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Hyatt Regency Atlanta
Atlanta, GA, USA
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March 28–30, 2008
Clinical Infectious Disease Update Course 2008–Eleventh Annual Management Review for the Practicing Physician
The Grand Hyatt
New York, NY, USA
http://www.cbcbiomed.com

April 5–8, 2008
Society for Healthcare Epidemiology of America (SHEA) 18th Annual Scientific Meeting
Buena Vista Palace
Orlando, FL, USA
Abstract submission deadline: January 4, 2008
http://www.shea-online.org

April 8–11, 2008
Genomes 2008 - Functional Genomics of Microorganisms
Institut Pasteur
Paris, France
http://www.pasteur.fr/infosci/conf/sb/genomes_2008

May 5–7, 2008
Eleventh Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/vaccine08

Announcements

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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

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1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
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February 2008

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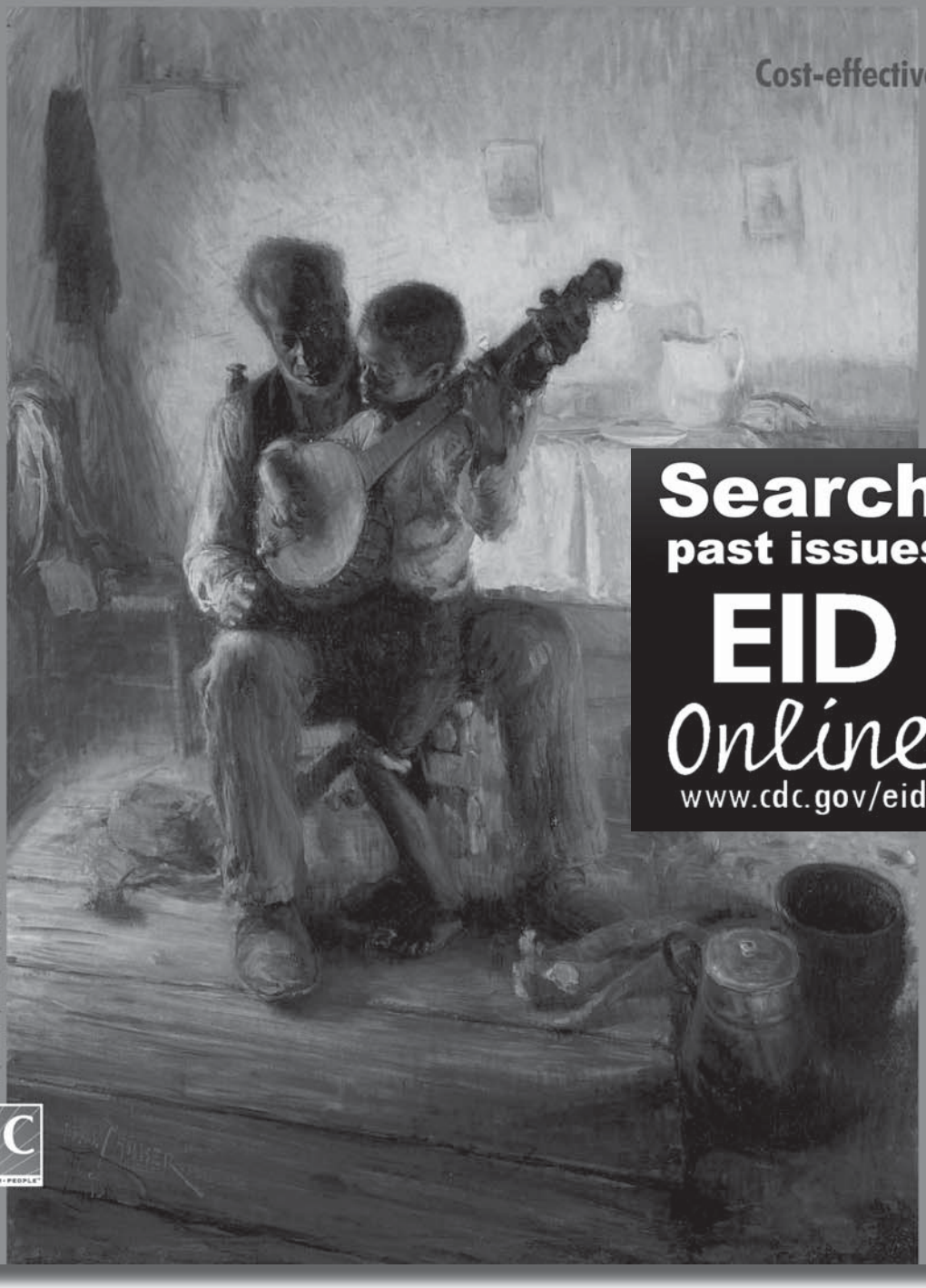

Hampton University Museum, Hampton, Virginia, USA

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

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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.

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