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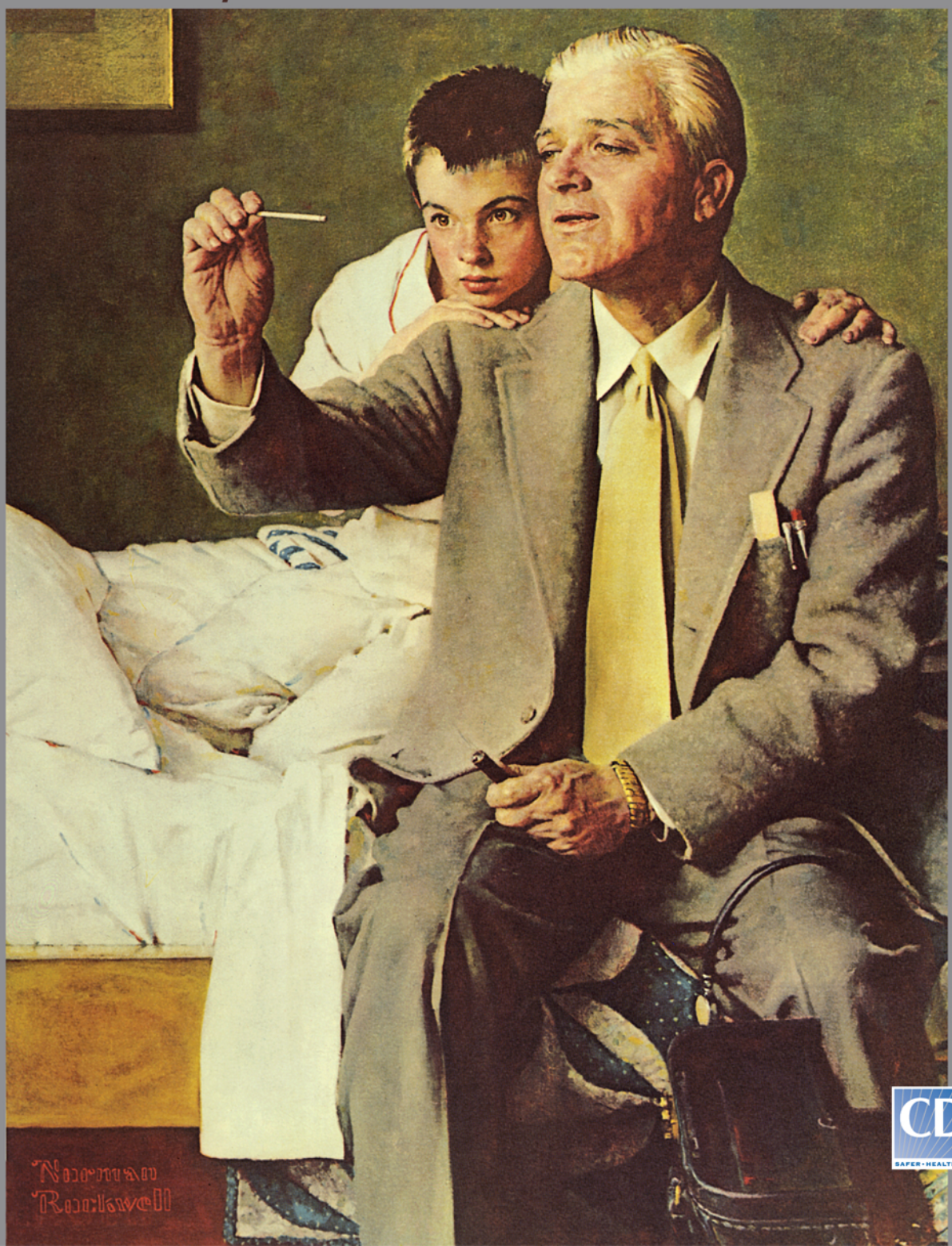

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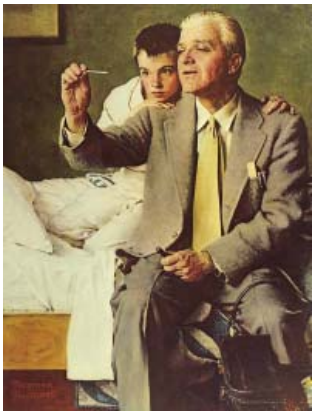

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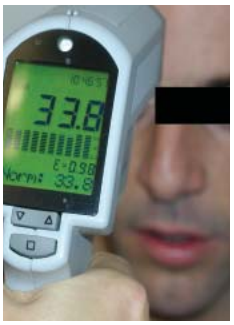

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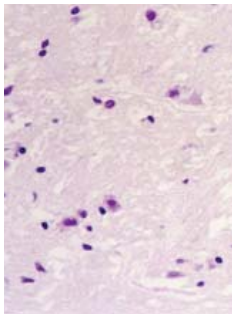

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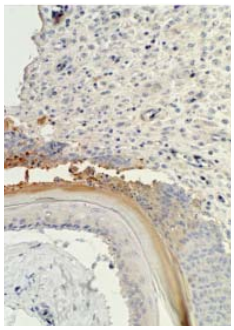

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Bacterial Pneumonia and Pandemic Influenza Planning

Ravindra K. Gupta,* Robert George,† and Jonathan S. Nguyen-Van-Tam‡

Pandemic influenza planning is well under way across the globe. Antiviral drugs and vaccines have dominated the therapeutic agenda. Far less work has been conducted on stockpiling and planning for deployment of antimicrobial drugs against secondary bacterial pneumonia, a cause of substantial illness and death in previous pandemics and epidemics. In the event of a pandemic, effective antimicrobial drug measures are expected to substantially benefit public health. We address issues regarding use of antimicrobial drugs as stocks of individual agents are diminished and the role of resistance surveillance in informing such policy. Furthermore, vaccination with polysaccharide and conjugate pneumococcal vaccines is considered as part of a pandemic strategy. Most illness and death from influenza are likely to occur in developing countries, where neuraminidase inhibitors and vaccines may be neither affordable nor available; thus, compared with industrialized countries, the benefits of treating bacterial complications in developing countries may be substantially greater.

The threat of a pandemic has been raised by the recent emergence of avian influenza virus (H5N1) in Southeast Asia. If an influenza pandemic of the same magnitude and severity as the one in 1918–19 were to occur in the present day, worldwide an estimated 51–81 million persons would die (*1*).

To date, antiviral drugs, principally the neuraminidase inhibitors, and vaccines have dominated the pharmaceutical countermeasures agenda in terms of research and development, stockpiling, and planning for mass deployment. However, the global supply of neuraminidase inhibitors is likely to be limited, and an immunogenic vaccine matched specifically to the pandemic strain would take at least 4–6 months to produce. Effective public health measures are predicted to slow, rather than halt, the spread of infection. Large numbers of influenza cases are therefore likely to occur when a pandemic strain emerges.

Evidence from laboratory, clinical, and epidemiologic studies suggests that bacterial co-infection contributes substantially to the illness and death that occurs in pandemic and seasonal influenza. We consider bacterial co-infection in the context of current preparedness activities and guidelines regarding antimicrobial drug stockpiling and deployment, including reference to existing quinolone stockpiles held by a number of countries. We also discuss the potential role of vaccination against *Streptococcus pneumoniae* in the context of pandemic influenza.

Bacterial Pneumonia and Pandemic Influenza

Ecologic studies have demonstrated temporal relationships between influenza activity and bacterial pneumonia. This association was perhaps most strikingly emphasized by the 20th-century pandemics, which have been comprehensively reviewed by Brundage (*2*). Substantial laboratory evidence for synergism between influenza A and bacterial agents has been reviewed by McCullers (*3*).

Bacterial Pneumonia and Seasonal Influenza

Pandemics are relatively rare; therefore, more data are available about bacterial infections associated with seasonal than pandemic influenza A strains. Secondary bacterial pneumonia is a common cause of death in persons with seasonal influenza; co-infections have been found with ≈25% of all influenza-related deaths (*4*,*5*). Pathogen-specific data are summarized below.

Streptococcus pneumoniae

Of laboratory-confirmed cases of community-acquired pneumonia, ≈30% involve bacterial–viral co-infection (*6*–*8*). *S. pneumonia* is the most common cause of community-acquired pneumonia and bacterial co-infection with influenza A (*9*–*12*). Invasive pneumococcal disease is a term used when the organism is isolated from a typically sterile

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site, such as blood or pleural fluid. This definition therefore underestimates pneumococcal pneumonia where isolation of the organism is not possible (*13*). Notwithstanding, a number of studies have documented the temporal association between influenza and invasive pneumococcal disease, which suggests synergism. Grabowska et al. (*14*) recently used 2 epidemiologic methods based on Swedish surveillance data to estimate the excess cases of invasive pneumococcal pneumonia associated with seasonal influenza at 12%–30%.

HIV-infected children have a 40× greater risk than HIV-noninfected children for invasive pneumococcal disease and account for most cases of invasive pneumococcal disease in certain sub-Saharan African countries (*13*,*15*). HIV-infected children and adults would likely be more severely affected by an influenza pandemic.

Staphylococcus aureus (Methicillin Sensitive and Methicillin Resistant)

A retrospective study of influenza-related childhood deaths in the United States in the 2003–04 season found *S. aureus* to be the most common bacterial agent, accounting for 46% of isolates, >50% of which were methicillin-resistant strains (*5*). Surveillance for severe influenza-related *S. aureus* community-acquired pneumonia in the United States during the 2003-04 season recorded 17 cases (88% methicillin-resistant *S. aureus* [MRSA]) and 5 deaths (4 with MRSA) and a median age of 21 years (*16*); laboratory evidence of influenza infection was available for ≈75%. More recently, 10 cases of severe community-acquired MRSA pneumonia in children (6 of whom died) from 2 southern states in a 2-month period were reported (*17*). For 30% of those patients, MRSA was recovered from sputum only, and 4 had a documented recent history of MRSA skin infection in themselves or in a close contact. Preceding staphylococcal skin disease in persons with staphylococcal pneumonia was described by Goslings et al. (*18*) during the 1957–58 pandemic. In the context of emerging community-acquired MRSA skin infection in persons without traditional risk factors, this association has substantial implications for possible emergence of MRSA pneumonia in a future pandemic (*19*).

Other Pathogens

A recent study from New Zealand (*7*) that aimed to characterize viral causes of community-acquired pneumonia reported viral–bacterial co-infection in 45 (15%) of 304 hospitalized patients. *S. pneumoniae (*67%) and *Haemophilus influenzae* (11%) were the 2 pathogens most commonly associated with influenza A infection; atypical microbes (*Chlamydia pneumoniae, Mycoplasma pneumoniae,* and *Legionella pneumophila*) were also well represented (22%). These figures are generally consistent with other published data; group A streptococci are a rare but serious cause of community-acquired pneumonia (*20*) and have been associated with fatal cases of influenza (*5*).

Stockpiling and Strategic Use of Antimicrobial Drugs

In most modern healthcare systems, which increasingly emphasize just-in-time supply chains, shortages of antimicrobial drugs may occur rapidly unless more are stockpiled. These shortages would limit the treatment of secondary bacterial infections in the middle and the later stages of a pandemic. For this reason a range of antimicrobial drug options have been suggested, taking into account the likely limitations of availability in diagnostics for community-acquired pneumonia and the fact that, because of the sheer number of patients, therapy is likely to be empirical. Clinical management guidelines for pandemic influenza have recommended amoxicillin + clavulanate or doxycycline (*21*); third-generation cephalosporins or respiratory fluoroquinolones (*22*); and second-generation cephalosporins, macrolides, doxycycline, or co-trimoxazole (*23*) as first-line empirical therapies for community-acquired pneumonia associated with pandemic influenza. Dependent on the extent of any stockpile, shortages of these preferred agents might occur first during a pandemic.

In the United States, the emergence of community-acquired MRSA has prompted revision to include vancomycin and other agents as empirical therapy for severe cases (*21*,*22*). The demand created by empirical use of vancomycin in such cases, the limited number of alternative agents, and the limited global production capacity of this drug are likely to lead to its shortage. Other treatment possibilities include linezolid, quinopristin/dalfopristin, and tigecycline.

Fortunately, in the United Kingdom most MRSA isolates are sensitive to doxycycline (95% of respiratory isolates; Health Protection Agency [HPA], unpub. data) and rifampin (97%; HPA, unpub. data); fewer are sensitive to trimethoprim (72%). Less severe MRSA infections treated with these widely available and inexpensive drugs would be expected to respond. Rifampin and cotrimoxazole are widely produced in developing countries, where the prevalence of tuberculosis and HIV infection are high.

Real-time Surveillance of Pathogen Resistance

After a country has committed to acquiring a stockpile of antimicrobial drugs, several important practical and logistic issues arise. The first is deciding on the range of antimicrobial drugs to be stockpiled. After the World Health Organization declares a global pandemic phase 5 alert, antimicrobial drug supplies will be quickly depleted as countries scour the global market to build up stocks. The choice of available agents may be limited by this stage; therefore,

procuring in advance is sensible, although this involves predicting which bacterial agents will be of greatest importance. The UK HPA has developed a program of real-time surveillance of antimicrobial susceptibility for the 3 most likely influenza-related bacterial pneumonia pathogens: *S. pneumoniae*, *H. influenzae*, and *S. aureus*. Contemporaneous data are available for each pathogen, enabling recommendation of antimicrobial drugs on the basis of the proportion of respiratory tract isolates likely to be susceptible at a particular point in time. Such real-time data may be useful for guiding the evolution of pandemic antimicrobial drug treatment policy in order to optimize the use of scarce antimicrobial drugs by drawing on a range of different agents according to national stock availability at the time. The surveillance program may also provide early warning of likely clinical failures caused by emerging resistance.

Size, Storage, and Turnover of Stockpiles

Decisions about pandemic stockpiles, procurements, and size depend primarily on financial considerations. Decision-makers must bear in the mind the need not only to purchase the initial stockpile but also to maintain it, perhaps for a sustained period. In most circumstances, stockpiles of vaccines for influenza virus subtype H5N1 and neuraminidase inhibitors are reserved exclusively for use during or immediately before a pandemic; they are not intended for day-to-day use on the same scale. In contrast, antimicrobial drugs are widely used every day. This difference means that antimicrobial drugs could act as buffer stock (conceptually similar to vendor-managed inventory) in most healthcare systems, rather than a true stockpile. Indeed, the word *stockpile* may be a misnomer in relation to increased stores of antimicrobial drugs because these drugs can be channeled into day-to-day use and replaced through fresh procurement. Thus, over time the amount, proportion, and range of these agents held can be slowly altered. These 2 mechanisms, ongoing interpandemic use and restocking, make such a stockpile far less vulnerable than antiviral drugs to expiration before use and far more responsive to changes in antimicrobial drug sensitivity detected between the date of procurement and the onset of the next pandemic.

Further considerations relate to storage. Whereas antiviral drugs and vaccines essentially need to be held in secure centralized storage (the latter within the cold chain) until eventual deployment, antimicrobial drugs can be held, at least in part, lower down the supply chain by wholesalers and community pharmacies or their equivalent.

Additionally, the proportion of pandemic influenza cases that will progress to bacterial complications needs to be estimated. The difficulty in making such an estimate relates partly to the paucity of contemporary data that specifically describe the incidence of bacterial complications after influenza and partly to the fact that widespread use of neuraminidase inhibitors, rarely used for seasonal influenza, might reduce the development of antimicrobial drug–related complications by 25%–40% (*24*,*25*). Data from the extensive reviews by Brundage and Soper suggest that in the 3 pandemics of the 20th century, bacterial pneumonia developed in 15%–20% of influenza patients (*2*,*26*); some estimates for seasonal influenza are far higher (*27*). It can be argued that in 1918 the primary viral infection was so virulent that it caused the premature demise of some patients who might otherwise have survived long enough for bacterial pneumonia to develop; i.e., the reported frequency of bacterial complications was spuriously low. Coupled with a population clinical attack rate that will most likely lie in the range of 25% to 50%, an antimicrobial drug stockpile is likely to be needed for a minimum of 10% of the population. This figure does not account for wastage, misdiagnosis (if, as is most likely, prescribing is based on clinical suspicion alone), or a higher rate of secondary bacterial complication than expected; it is also based on a strategy of treatment only.

Treatment and Prophylaxis Strategies

Alternative strategies include offering antimicrobial drug prophylaxis at the same time as antiviral treatment to patients with conditions that put them at high risk, such as chronic obstructive pulmonary disease. Antimicrobial drugs (or a prescription for them) could be issued to high-risk patients at the same time as antiviral treatment. The ability to start antimicrobial drug therapy with minimal delay and without the need for repeat consultation if antiviral drugs alone are not effective might be advantageous in an already overstretched health system. Both of these strategies incorporate further uncertainty because the high-risk groups in a pandemic are unknown and may not correspond to those currently recognizable for seasonal influenza; if anything, the high-risk groups are more likely than not to be larger in a pandemic. This might increase the requirement for antimicrobial drug stockpiling to 25% population coverage.

Each country should estimate its own needs. Country-specific factors to take into account include treatment strategy (treatment alone or treatment and prophylaxis), health service configuration, historical use of antimicrobial drugs, physician behavior, inappropriate prescribing linked to misdiagnosis, and the availability of antiviral drugs.

Quinolone Stockpiles

A large number of countries hold stockpiles of quinolones, in particular ciprofloxacin, as a contingency against bioterrorist threats. In the United Kingdom, ciprofloxacin is active against all *H. influenzae* isolates (≈100% of recent UK respiratory tract isolates susceptible; HPA, unpub. data), most methicillin-sensitive *S. aureus* isolates (≈82%),

and atypical organisms. Therefore, if these bacterial pathogens were known or suspected to predominate in influenza-related pneumonia associated with a future pandemic, the use of ciprofloxacin might be justified, and agents effective against MRSA would be reserved for severe cases and those with culture-confirmed MRSA (99% of UK respiratory MRSA isolates, most of which are hospital acquired, are quinolone resistant; HPA, unpub. data).

However, ciprofloxacin activity against *S. pneumoniae* (*28*) is only intermediate, and a significant number of bacterial pneumonias complicating influenza may not respond to empirical treatment. This fact is well supported by evidence from mouse models; more modern "respiratory" fluoroquinolones such as gatifloxacin demonstrate good results (*29*) against *S. pneumoniae*, which was not always so for ciprofloxacin. Therefore, in a pandemic empirical ciprofloxacin use could be justified only if all other more suitable antimicrobial drug supplies were exhausted.

Given ciprofloxacin's weak activity against pneumococci, reserving its use in a pandemic to empirical treatment of persons previously vaccinated against pneumococcal infection, who would be at reduced risk for co-infection with this particular organism, would be reasonable. Theoretical support for this hypothesis comes from the United States, where use of a 7-valent conjugate vaccine since 2000 has resulted in declining invasive pneumococcal disease (*30*) and relatively infrequent influenza-related deaths caused by pneumococci in children (*5*). A strategic approach might involve the use of ciprofloxacin in fully immunized persons.

Pneumococcal Vaccination Strategies

Including a vaccination strategy in pandemic planning would potentially reduce the amount of disease caused by secondary *S. pneumoniae* bacterial pneumonia. We have already described this pathogen's role in community-acquired pneumonia and influenza complications. The public health benefit from vaccination could be substantial.

Pneumococcal polysaccharide vaccine (PPV) is currently recommended in many countries for persons ≥65 years of age and for high-risk groups of all ages. Few specific data exist on the effectiveness of PPV for reducing pneumococcal pneumonia–associated illness and death after infection with influenza A virus. Furthermore, in the context of pneumococcal disease not specifically associated with influenza, use of PPV has protected against invasive pneumococcal disease but not against pneumococcal pneumonia in the absence of bacteremia (*31*). Therefore, on the basis of current evidence, prior PPV administration could not reliably be used to identify persons who could receive empirical ciprofloxacin therapy for bacterial pneumonia as a complication of influenza. It could, however, be used as a large-scale preventive measure against invasive pneumococcal disease in adults.

The protective efficacy of a 9-valent pneumococcal conjugate vaccine (PncCV) against nonbacteremic pneumonia as well as invasive pneumococcal disease has been demonstrated in 37,107 children from South Africa among whom the prevalence of HIV infection was 6.5% (*32*).The vaccine also substantially reduced the incidence of first episodes of invasive pneumococcal disease that were resistant to penicillin or trimethoprim-sulfamethoxazole.

PncCV may have more greatly reduced the incidence of pneumonia in children when a virus was isolated (*33*). This effect was more pronounced when influenza A was isolated; protective efficacy was 41% (95% confidence interval 13%–60%). The study provided indirect evidence of the frequency of pneumococcal superinfection of viral pneumonias in children in this setting. If similar results could be achieved through vaccination before an influenza pandemic, the benefits of preventing pneumococcal complications could be substantial. The introduction of conjugate vaccine in the United States in 2000 has led to a decline in invasive pneumococcal disease in not only children but also adults; reduction was 32% for those 20–39 years of age and 18% for those >65 years (*30*). Therefore, vaccination of children might be the most cost-effective policy. In September 2006, the United Kingdom started vaccinating children from the age of 2 months; early unpublished data (minutes from the Joint Committee on Vaccination and Immunisation meeting on February 14, 2007, available from www.advisorybodies.doh.gov.uk/jcvi/mins140207.htm) suggest that invasive pneumococcal disease in children <2 years of age is already reduced.

The use of PncCV in children and 23-valent PPV in adults as part of a pandemic strategy would be consistent with recommendations resulting from current published data. However, such use may still not allow for ciprofloxacin stockpiles to be reliably targeted for specific populations, given the lack of protection against nonbacteremic pneumoccal pneumonia associated with PPV. If conjugate vaccine were used in all patients (although no convincing data exist to support efficacy of conjugate vaccine in adults), ciprofloxacin might be more reliably targeted at a group more likely to have a nonpneumococcal pneumonia. However, a conjugate vaccine is likely to be expensive and limited in serotype coverage, and approval for its use in adults will take time.

Discussion

Substantial laboratory and epidemiologic evidence shows that influenza A and bacterial pathogens often participate in the pathogenesis of pneumonia. Several issues need to be considered with regard to antimicrobial drug treatment for large numbers of patients who have secondary bacterial infection during a pandemic. Real-time antimicrobial drug–resistance surveillance programs could be incorporated into

preparedness frameworks; information from such networks could result in stockpiling of inexpensive, generically manufactured antimicrobial drugs. Vaccination against pneumococcal disease, particularly vaccination of HIV-infected persons, potentially will save lives in the short term as well as provide protection in the event of a pandemic.

Dr Gupta is an infectious diseases physician undergoing postgraduate specialist medical training. He is currently conducting research on HIV resistance in developing-world settings and has an interest in pandemic influenza preparedness.

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Deaths from Bacterial Pneumonia during 1918–19 Influenza Pandemic

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Deaths during the 1918–19 influenza pandemic have been attributed to a hypervirulent influenza strain. Hence, preparations for the next pandemic focus almost exclusively on vaccine prevention and antiviral treatment for infections with a novel influenza strain. However, we hypothesize that infections with the pandemic strain generally caused self-limited (rarely fatal) illnesses that enabled colonizing strains of bacteria to produce highly lethal pneumonias. This sequential-infection hypothesis is consistent with characteristics of the 1918–19 pandemic, contemporaneous expert opinion, and current knowledge regarding the pathophysiologic effects of influenza viruses and their interactions with respiratory bacteria. This hypothesis suggests opportunities for prevention and treatment during the next pandemic (e.g., with bacterial vaccines and antimicrobial drugs), particularly if a pandemic strain–specific vaccine is unavailable or inaccessible to isolated, crowded, or medically underserved populations.

Many influenza experts, policy makers, and knowledgeable observers believe that a novel influenza A (H1N1) strain directly caused most deaths during the 1918–19 pandemic, often from a hemorrhagic pneumonitis that rapidly progressed to acute respiratory distress syndrome and death (*1–3*). Not surprisingly, plans and resources to respond to the next influenza pandemic focus almost exclusively on the virus, i.e., preventive vaccines and antiviral treatment of infections with a novel influenza strain (*4*). However, healthcare providers, medical experts, and published data from the 1918 period suggest that most deaths were caused by secondary bacterial pneumonias (*5–12*); hemorrhagic pneumonitis that rapidly progressed to death was considered an alarming but uncommon clinical manifestation (*8*,*11–13*).

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Undoubtedly, the 1918–19 pandemic strain of influenza had unique pathophysiologic effects. In the wake of its worldwide spread, the number of deaths was unprecedented. However, contemporaneous reports suggest that the pathophysiologic effects of the virus, in and of themselves, did not directly cause most (or even many) of the deaths during the pandemic. If the pandemic strain was not inherently hypervirulent (i.e., if direct pathophysiologic effects of the virus were necessary but not sufficient to cause death in a large proportion of immunologically susceptible hosts) and if bacterial infections were also necessary causes of most deaths during the pandemic, then preparations for the next pandemic should focus on more than preventing and treating infections with a novel influenza strain alone.

We have identified epidemiologic and clinical characteristics of the 1918–19 pandemic that are not readily consistent with the view that most deaths were caused by the direct effects of an inherently hypervirulent virus and were clinically expressed as rapidly progressing, ultimately fatal pneumonitis. Our alternative hypothesis is consistent with known characteristics and firsthand accounts of the pandemic and contains implications for preparing for the next pandemic.

Epidemiologic and Clinical Characteristics of 1918–19 Pandemic

Disease Usually Mild and Self-limited

The 1918–19 pandemic spread worldwide with remarkable speed. Over several months, a novel strain of influenza virus attacked communities worldwide; most persons were immunologically susceptible. However, most cases followed a mild or self-limited course. Had the pandemic strain been inherently hypervirulent, in the absence of modern lifesaving measures one would expect exceptionally high case-fatality rates for all affected pop-

ulations. Yet during that pandemic, most infected persons had self-limited clinical courses and complete recovery (*3*,*7*,*8*,*11*,*14*). For most affected populations, the case-fatality incidence was <2% and the overall mortality rate was <0.5% (*3*,*7*,*8*,*13*,*15*,*16*).

Clinical Courses of Fatal Cases Highly Variable and Often Prolonged

In most affected populations, <5% of deaths occurred within 3 days of illness onset, median time from illness onset to death was 7–10 days, and significant numbers of deaths occurred >2 weeks after initial symptoms (*5*,*17*–*22*; Figures 1, 2). These findings do not suggest that an inherently virulent virus caused fulminant disease and rapid progression to death in high proportions of infected persons—or even in most fatal cases. In the prominently cited experience of Sydney, Australia, most influenza-related deaths occurred within 3 days of hospital admission (*2*,*23*,*24*); however, only the sickest patients were admitted to Sydney hospitals (*23*). In New South Wales overall, only ≈10% of fatalities occurred within 3 days of illness onset (Figure 1, panel F; Figure 2) (*20*).

Progression to Death, No Difference between Early and Late Pneumonias

If most deaths resulted from primary influenza pneumonias that progressed rapidly, one might expect that fatal pneumonias that developed early in clinical courses would progress more rapidly than those that developed later. However, the findings of Opie et al. suggest that primary influenza pneumonias did not progress unusually rapidly to death. Opie et al. conducted postmortem examinations and documented the clinical courses of 234 fatal cases that occurred during the epidemic at Camp Pike, Arkansas, USA (*5*). They found that the durations of pneumonia before death were similar among those in whom pneumonia developed early (0–2 days) versus later (3–5, 6–8, >8 days) after influenza onset (Figure 3) (*5*).

Mortality and Case-Fatality Rates High for Young Adults and Other Unlikely Groups

During the pandemic, overall mortality and case-fatality rates were higher for young adults, indigenous and other relatively closed populations, and certain military and occupational subgroups than for their respective counterparts. Case-fatality and mortality rates were higher for those 25–40

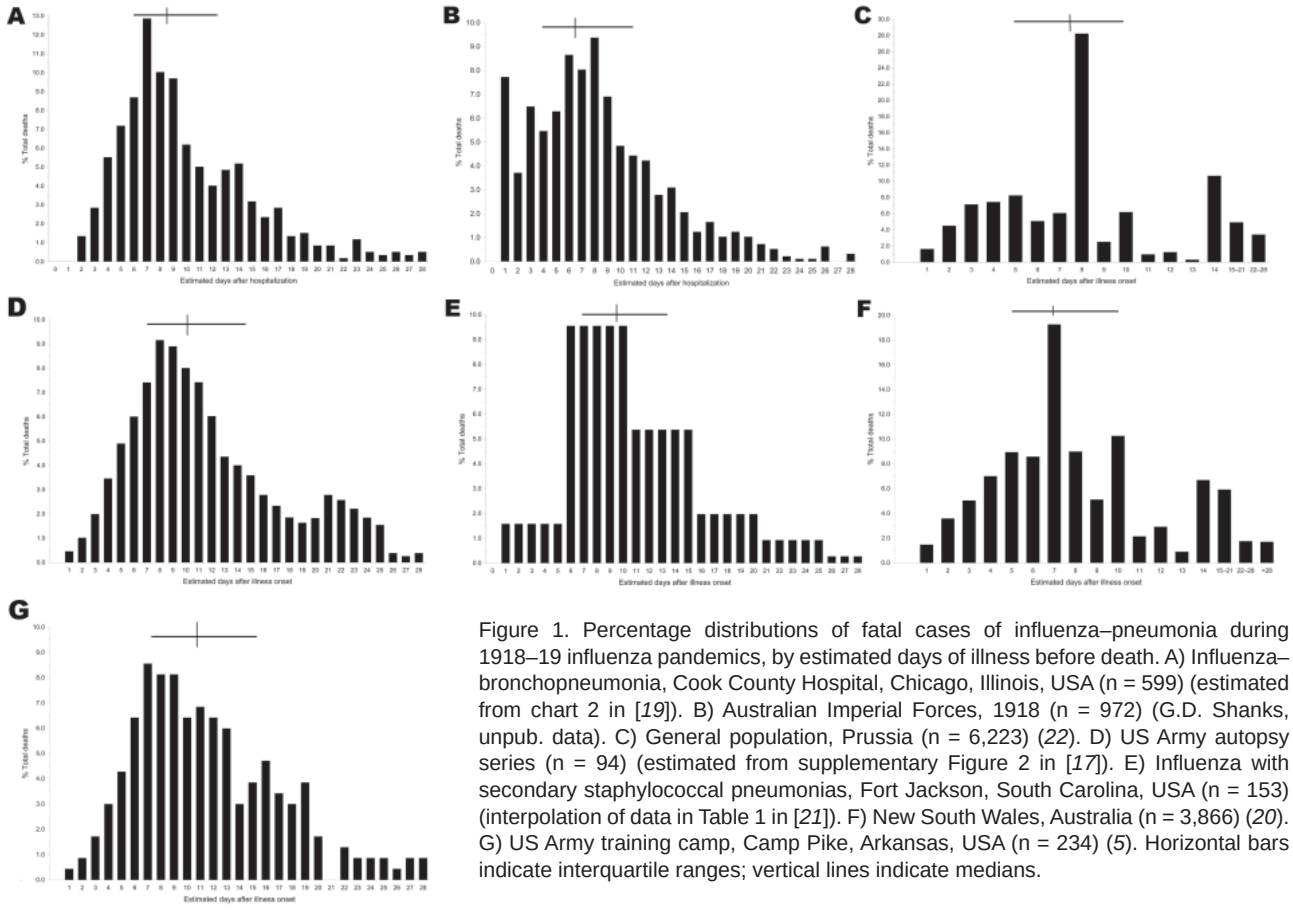

Figure 1. Percentage distributions of fatal cases of influenza–pneumonia during 1918–19 influenza pandemics, by estimated days of illness before death. A) Influenza–bronchopneumonia, Cook County Hospital, Chicago, Illinois, USA (n = 599) (estimated from chart 2 in [*19*]). B) Australian Imperial Forces, 1918 (n = 972) (G.D. Shanks, unpub. data). C) General population, Prussia (n = 6,223) (*22*). D) US Army autopsy series (n = 94) (estimated from supplementary Figure 2 in [*17*]). E) Influenza with secondary staphylococcal pneumonias, Fort Jackson, South Carolina, USA (n = 153) (interpolation of data in Table 1 in [*21*]). F) New South Wales, Australia (n = 3,866) (*20*). G) US Army training camp, Camp Pike, Arkansas, USA (n = 234) (*5*). Horizontal bars indicate interquartile ranges; vertical lines indicate medians.

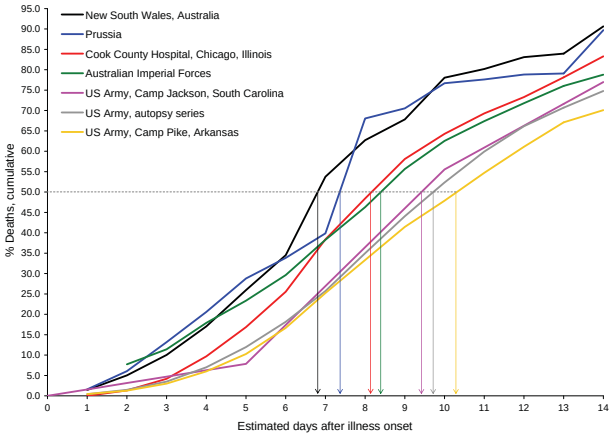

Figure 2. Cumulative percentage deaths from influenza–pneumonia, by days (estimated) from illness onset, among fatal cases during various epidemics, 1918–19 (*5*,*17*–*22*). Vertical arrows indicate median no. days to death.

years of age (particularly men) than for those younger or older (*15*,*16*). Explanations have included aberrant host immune responses to infections with the subtype H1N1 pandemic strain—increasing the risk for "cytokine storm" (*1*)—and higher cardiac stroke volumes in young adults (*24*).

However, at US military training camps, recent arrivals had worse clinical outcomes than their similarly aged, male counterparts who had been in camps longer. For example, during wartime, 60% of all influenza–pneumonia deaths affected soldiers who had been in the service <4 months (total influenza–pneumonia deaths, 34,446; deaths of soldiers with ≤4 months of service, 20,837) (*10*). In the Australian Imperial Forces, mortality rates differed by 50-fold across units of similarly aged soldiers in France and the United Kingdom (G.D. Shanks, unpub. data). US soldiers and Marines who were being transported on ships had similar influenza case rates but higher case-fatality rates (influenza cases 11,385, case rate 8.80/1,000, deaths 733) than the sailors who were permanently assigned to the same ships (influenza cases 2,123, case rate 8.88/1,000, deaths 42) (Figure 4, panel A) (*9*). Among Australians and Americans, sharply higher death rates were reported for civilian miners (*6*,*25*) and military tunnelers (G.D. Shanks, unpub. data) than for their similarly aged counterparts (Figure 4, panel B).

In South Africa, case-fatality rates were >2× higher for "Blacks, Indians, and Coloureds" (influenza cases 2,162,152, deaths 127,745, case-fatality rate 5.9%) than for "Whites" (influenza cases 454,653, deaths 11,726, case-fatality rate 2.6%) (*26*); and the influenza-associated mortality rate was >30× higher for Kimberley diamond miners (influenza deaths 2,564, overall mortality rate 22.4%) (*26*) than for Rand gold miners (influenza cases 61,000, deaths 1,147, case-fatality rate 1.9%, overall mortality rate 0.6%) (*26*). In Rhodesia, influenza-related mortality rate was ≈4× higher in mining compounds (9.2%) than in villages (2.3%) (among mine workers, overall influenza cases 19,471, deaths 2,851, case-fatality rate 14.6%) (*27*).

During the pandemic in New Zealand, death rates were ≈7× higher for indigenous (Maori) populations (influenza deaths 2,160, mortality rate 42.3/1,000) than for other residents (influenza-related mortality rate 4.5/1,000) (*28*). Across other South Pacific islands, death rates were generally higher for indigenous populations than for others. For example, death rates in Fiji were ≈4× higher for indigenous Fijians (influenza cases 5,154, mortality rate 5.7%) than for Europeans (influenza cases 69, mortality rate 1.4%) (*8*). In Guam, where military and indigenous populations were both located, ≈4.5% of the indigenous population, but only 1 sailor assigned to the US Naval base, died (*9*). In Saipan, "practically all of the inhabitants contracted the disease"; however, the mortality rate was reportedly sharply higher for Chamorrans (12.0%) than for Caroline Islanders (0.4%) (*29*). In Western Samoa, an estimated 22% (deaths 7,542) of the entire population died (*8*,*30*).

In various communities of Canada, Sweden, Norway, and the United States, mortality rates were estimated to be 3–70× higher for indigenous than for nonindigenous populations (*8*,*31*). Across British colonial countries of the Caribbean, the difference in mortality rates was >45-fold between the least affected (Bahamas: deaths ≈60, mortality rate ≈0.1%; Barbados: deaths ≈190, mortality rate ≈0.1%) and the most affected (Belize: deaths ≈2,000, mortality rate ≈4.6%); in general, the highest mortality rates in the Caribbean affected East Indian workers, Native Americans, and the poor (*32*).

The findings of sharply different clinical courses and outcomes in subgroups of infected persons of similar ages, sociocultural circumstances, and prior health states belie the importance of host immune intensity and cardiac stroke

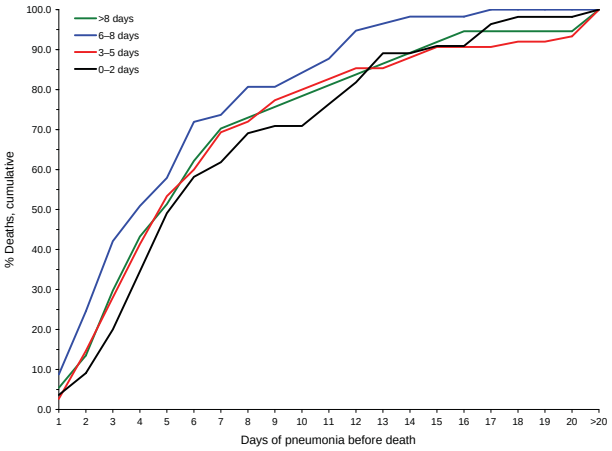

Figure 3. Cumulative percentage deaths by days of pneumonia, in relation to days of illness before pneumonia, among 234 US Army soldiers who died of influenza–pneumonia at Camp Pike, Arkansas, USA, autumn 1918 (*5*).

volume as the definitive determinants of clinical outcomes after infection. Undoubtedly, factors other than the inherent virulence of the virus or the robustness of the host's immune response affected the clinical expressions of influenza infections. In his classic review, E.O. Jordan concluded that "one of the chief reasons for the great variation in case-fatality in different groups is undoubtedly the nature and relative abundance of secondary invaders ... The excessively high mortality in certain army camps, on certain transports and in particular hospitals or barracks seems most readily explicable in this way" (*6*).

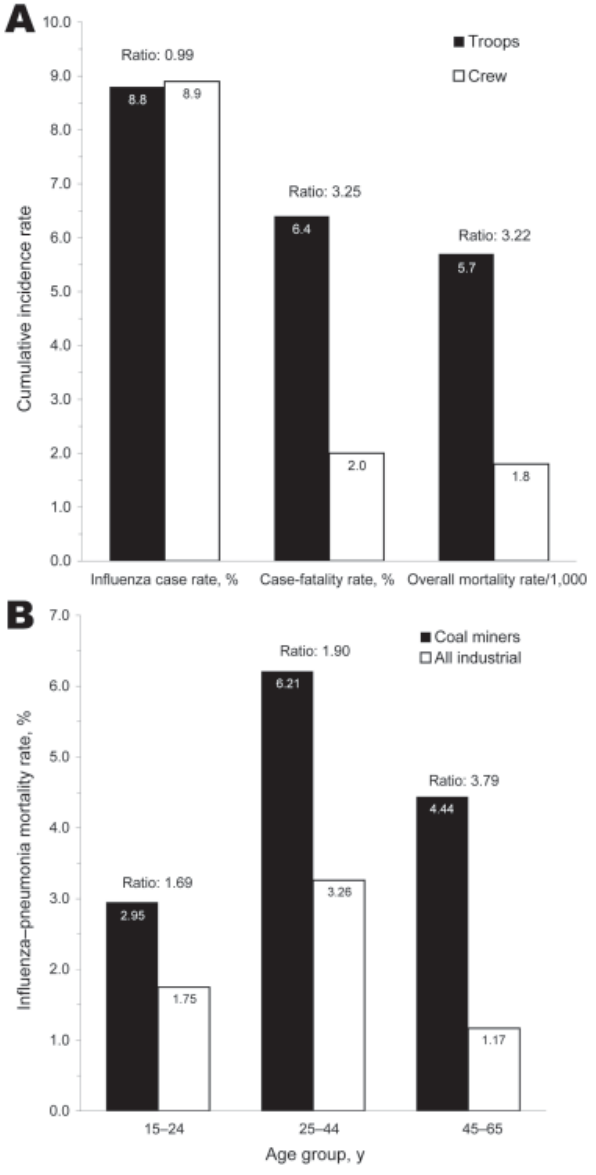

Figure 4. A) Influenza–pneumonia-related morbidity and mortality cumulative incidence rates, in relation to status on troop ships, Cruiser and Transport Service, US Navy, 1918 (*9*). B) Influenza–pneumonia mortality rates for white men, by employment as coal miner versus other industrial occupation, and by age group, October–December 1918 (*6*).

Common Respiratory Bacteria Most Often Recovered from Pneumonia Patients

During the 1918–19 pandemic, the bacteria most often recovered from the sputum, lungs, and blood of pneumonia patients, alive or dead, were common colonizers of the upper respiratory tracts of healthy persons, i.e., *Hemophilus influenzae*, *Streptococcus pneumoniae*, *S. pyogenes*, and/or *Staphylococcus aureus* (*5–13*). During local epidemics, 1 or 2 of these species accounted for most isolates from pneumonia patients (*5–13*). For example, among pneumonia patients at 21 US Army camps in the autumn of 1918, *S. pneumoniae* (especially types III and IV) predominated at 12 camps, *H. influenzae* at 6, and *Streptococcus* spp. at 3 (*5*). *S. aureus* was a major cause of pneumonia among persons with fatal cases at Camp Jackson, South Carolina, USA, and Camp Syracuse, New York, USA (*5*,*12*,*21*).

The bacteria most often recovered from the lungs of patients who died were all common colonizers of the upper respiratory tracts of healthy persons. Types III and IV pneumococci (ubiquitous colonizing strains) were often recovered from the lungs of patients who died during the 1918–19 pandemic but were not considered important pathogens otherwise. Opie et al. concluded, "Every patient with influenza must be considered a potential source of pneumococcus or hemolytic streptococcus infection for his neighbor ... Every person engaged in the care of patients with respiratory diseases must also be regarded as a potential source of danger" (*5*).

Mortality Rates More Strongly Correlated with Pneumonia Rates than with Clinical Case Rates

If the pandemic strain had been inherently hypervirulent and had directly caused most influenza-related deaths, one would expect strong correlations between clinical case rates and mortality rates across affected populations. Yet in affected communities in general, correlations were stronger between mortality and pneumonia rates than between mortality and clinical case rates (*15*,*16*).

In general, age-related mortality rates and pneumonia rates—but not clinical case rates—were W-shaped with sharp peaks for young adults. Influenza-related mortality rates peaked sharply for young adults 25–40 years of age. Data from household surveys throughout the United States suggest that pneumonia case rates also peaked for young adults (Figure 5) (*15*,*16*). In contrast, influenza case rates were highest for school-aged children, plateaued at a lower level for young adults, and continuously declined through older age groups (Figure 5) (*15*,*16*).

After reviewing US household survey data, a senior statistician of the US Public Health Service concluded that "... these relations indicate that the mortality is determined primarily by the incidence of pneumonia. The cause of the high mortality in young adult life evidently lies in the

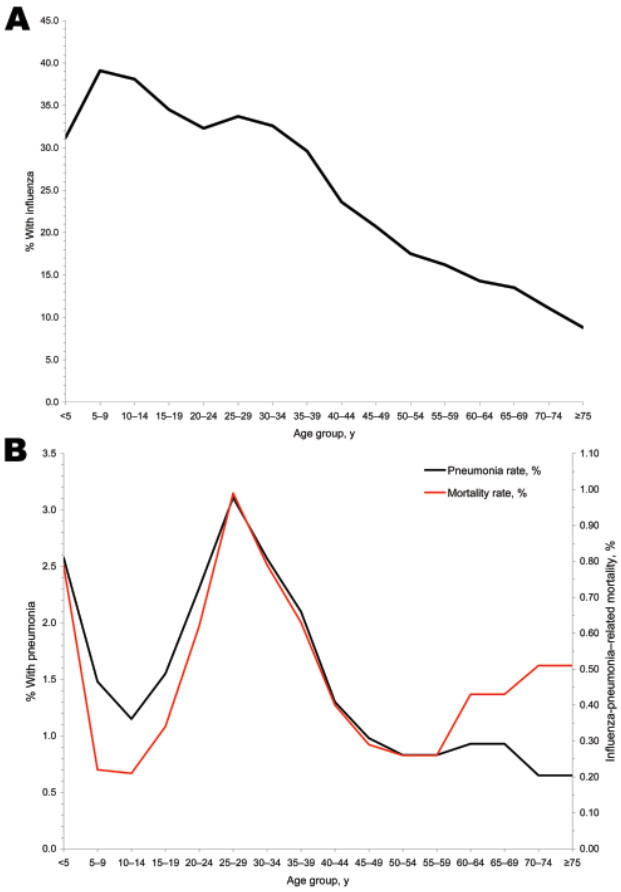

Figure 5. A) Estimated age group–specific influenza case rates (*15,16*). B) Estimated age group–specific pneumonia rates and mortality rates, based on household surveys of 10 communities throughout the United States (*15,16*).

complicating pneumonia. All of the relations ... bear this out ..." (*16*)

Nonpharmaceutical Interventions Associated with Lower Overall Mortality Rates

Systematic analyses of mortality data from large US cities have shown that nonpharmaceutical interventions (e.g., isolation, quarantine, closing schools, banning public gatherings) were associated with lower influenza-related mortality rates during the autumn of 1918 (*33*). Given the rapidity of spread of the pandemic, reductions of mortality rate associated with nonpharmaceutical interventions are unlikely to have been primarily related to reductions of influenza transmission (particularly in large US cities during wartime).

On the basis of their extensive studies in US Army camps during the 1918–19 pandemic, Opie et al. concluded that "Secondary contact infection may be responsible for the development of pneumonia in patients with influenza. ... It is probable that secondary contact infection can be effectively prevented only by individual isolation and strict quarantine of every patient." (*5*) Perhaps the reduction in mortality rate after isolation, quarantine, and other social distancing measures were implemented resulted from decreased exposures of persons with influenza to bacterial respiratory pathogens to which they were transiently highly susceptible.

Firsthand Accounts and Reviews: Most Deaths Caused by Secondary Bacterial Pneumonias

During the pandemic, medical journals contained hundreds of detailed reports of local influenza epidemics. In addition, during and after the pandemic, remarkably detailed reviews of relevant epidemiologic and clinical records and population-based surveys were conducted by government and academic institutions worldwide. Care providers and experts of the day in epidemiology, pathology, bacteriology, and infectious diseases clearly concurred that pneumonias from secondary bacterial infections caused most deaths during the pandemic (*5–14*). In his classic review, Jordan summarized the key factors involved in the production of influenza-related pneumonia during the pandemic as follows:

> "(1) The influenza virus weakens the resistant power of the pulmonary tissues so that various bacteria are able to play the role of secondary invaders; (2) the precise nature of the secondary—and tertiary—invaders is largely a matter of accident, dependent on the occurrence of particular bacteria in the respiratory tract of persons at the time of infection, and in the case of group outbreaks, on their occurrence in contacts; (3) the character of the resulting pneumonia, clinical and pathologic, is largely determined by the nature of the secondary invaders, whether Pfeiffer bacillus, streptococcus, pneumococcus, or other organisms; (4) there seems little doubt that the influenza virus, besides depressing the general pulmonary resistance, also acts directly on the pulmonary tissues, causing capillary necrosis, edema, and hemorrhage; (5) it seems to be true, therefore, that the fatal outcome of influenza pneumonia is determined partly by the degree to which the influenza virus depresses local and general pulmonary resistance, and partly by the virulence and nature of the bacteria which invade the tissues in the wake of the specific virus" (*6*).

Hypothesis

We endorse a sequential-infection hypothesis. This hypothesis is consistent with the known epidemiologic and clinical characteristics of the 1918–19 influenza pandemic, reflects the consensus views of firsthand observers and contemporaneous experts, and incorporates current knowledge regarding the effects of influenza on physical and immune respiratory tract defenses and physiologic interactions between influenza and respiratory bacteria (*12,13,34–36*).

A novel strain of influenza spread rapidly throughout the world in 1918. For most patients, infection with

the virus was clinically expressed as an "influenza-like illness" that was transiently debilitating but rarely fatal. In addition, however, the virus induced aberrant immune responses, including excessive and prolonged production of interferons, proinflammatory cytokines, and chemokines, particularly among young adults (*34*). The pathophysiologic effects included inflammation and destruction of respiratory epithelium; immune cell infiltration of lung tissue with edema and hemorrhage; and ultimately, degradation or destruction of virtually all physical and immune defenses of the lower respiratory tract (*34*). Increased susceptibility of the lower respiratory tract enabled invasion by preexisting or newly acquired colonizing strains of bacteria (*12*,*35*–*38*). The synergistic effects of infection with the virus, aberrant immune responses to the virus, and secondary opportunistic bacterial pneumonias were severe and often fatal.

Finally, for brief periods and to varying degrees, affected hosts became "cloud adults" who increased the aerosolization of colonizing strains of bacteria, particularly pneumococci, hemolytic streptococci, *H. influenzae*, and *S. aureus* (*39*). For several days during local epidemics—particularly in crowded settings such as hospital wards, military camps, troop ships, and mines—some persons were immunologically susceptible to, infected with, or recovering from infections with influenza virus. Persons with active infections were aerosolizing the bacteria that colonized their noses and throats, while others—often in the same "breathing spaces"—were profoundly susceptible to invasion of and rapid spread through their lungs by their own or others' colonizing bacteria.

Implications

Why is it important to determine the major pathophysiologic pathways that led to deaths during the 1918–19 influenza pandemic? After all, the effective prevention and treatment of influenza infections during a future pandemic would prevent all secondary effects, including opportunistic bacterial pneumonias. Yet concerns exist that an effective strain-specific vaccine and effective antiviral drugs may not be produced and distributed to all at-risk populations in time to mitigate the effects of the next pandemic. In the absence of an effective influenza vaccine and antiviral drugs, circumstances during a modern influenza pandemic could resemble those in 1918–19, with the notable exception of the availability of bacterial vaccines and antibacterial drugs. The exclusive focus on the prevention and treatment of a novel strain of influenza virus is risky because it unnecessarily limits options and opportunities for other potentially effective prevention and treatment methods, especially in medically underserved populations in less-developed countries.

We suggest that preparations for the next influenza pandemic should focus on more than preventing and treating influenza virus infections. A modified influenza pandemic plan might include the following components: 1) Before a pandemic, expand indications for and decrease barriers to receipt of vaccination against *S. pneumoniae* (*36*–*38*,*40*). 2) During a pandemic, in communities not yet affected, universally vaccinate with a safe and effective strain-specific influenza vaccine, if available. 3) During local epidemics, treat all serious clinical cases with an antibacterial agent that is effective against *S. pneumoniae, S. pyogenes, H. influenzae, and S. aureus (*including methicillin-resistant *S. aureus*); isolate patients with clinical cases from other patients and as many others as possible (*35*,*37*–*39*). 4) Conduct pandemic-related surveillance that tracks the incidence, nature (e.g., species, affected sites, antimicrobial drug sensitivities), and outcomes of bacterial infections that complicate influenza cases.

Given highly variable colonization and drug-sensitivity patterns across populations and locations, stockpiles of antibacterial drugs should be tailored to their intended uses. Plans for providing medical care should include evidence-based triage and treatment algorithms and home-care treatment guidelines (including prepackaged antiviral and antibacterial drugs) to minimize hospitalizations and maximize home care. Perhaps most important, pandemic-related research activities (including laboratory animal studies, statistical models, and clinical trials) should elucidate the determinants and effects of bacterial pneumonias that occur secondary to influenza. Ultimately, research activities should determine the most effective uses of antibacterial drugs and bacterial vaccines (e.g., indications, agents, doses, and timing for prophylaxis and treatment) in preparation for and during pandemic influenza, particularly for medically underserved and other high-risk populations.

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Diverse Contexts of Zoonotic Transmission of Simian Foamy Viruses in Asia

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In Asia, contact between persons and nonhuman primates is widespread in multiple occupational and nonoccupational contexts. Simian foamy viruses (SFVs) are retroviruses that are prevalent in all species of nonhuman primates. To determine SFV prevalence in humans, we tested 305 persons who lived or worked around nonhuman primates in several South and Southeast Asian countries; 8 (2.6%) were confirmed SFV positive by Western blot and, for some, by PCR. The interspecies interactions that likely resulted in virus transmission were diverse; 5 macaque taxa were implicated as the source of infection. Phylogenetic analysis showed that SFV from 3 infected persons was similar to that from the nonhuman primate populations with which the infected persons reported contact. Thus, SFV infections are likely to be prevalent among persons who live or work near nonhuman primates in Asia.

Human society critically influences the ecologic contexts in which the transmission of infectious agents between species occurs (*1*,*2*). In developing countries, economic growth and new infrastructure have transformed the human–animal interface, facilitating the emergence of previously unrecognized zoonotic diseases. Nowhere is this more evident than in South and Southeast Asia, where the world's densest human populations are situated close to some of the planet's richest reservoirs of biodiversity (*3*). Nonhuman primates figure prominently as potential sources of emerging human pathogens (*4*).

Asian cultures have long traditions of venerating nonhuman primates, and in many Asian communities nonhuman primates (particularly macaques and langurs) are woven into the fabric of everyday life (*5*–*8*). Nonoccupational interspecies contact occurs in urban settings, parks and religious sites, settings where nonhuman primates are kept as pets or performance animals, animal markets, and zoos; it also occurs during bushmeat hunting and consumption (*9*,*10*).

The first 2 contexts listed merit particular attention because they represent nonoccupational situations in which cross-species disease transmission can occur, and they represent settings in which large numbers of humans and nonhuman primates come into contact. Urban nonhuman primates are found in towns and densely populated cities throughout South and Southeast Asia, where their population may reach several thousands (*5*,*11*). This urban niche frequently and increasingly brings them into close contact with humans, as much of their food supply is provided by humans, formally or informally (as when nonhuman primates raid homes or scavenge for refuse). Temple monkeys are free-ranging in parks and religious sites in South and Southeast Asia and have lived commensally with humans for centuries at these sites, some of which have become international tourist destinations.

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Simian foamy viruses (SFVs) comprise a subfamily of simian retroviruses that are ubiquitous in nonhuman primates. Ancient and well adapted, SFVs have coevolved with their nonhuman primate hosts for >30 million years (*12*). Once acquired, SFV infections are lifelong and do not seem to cause disease in their natural hosts (*13*). Nearly all captive and free-ranging macaques have acquired SFV infection by adulthood (*14*,*15*).

Studies have demonstrated that humans who are occupationally exposed to captive or free-ranging nonhuman primates can acquire SFV infection, although the number of known SFV-infected humans is small. At-risk populations include veterinarians; laboratory, temple, and zoo workers; pet owners; and bushmeat hunters (*16*–*20*). SFV prevalence in these populations is 1%–6%. The possibility of human-to-human transmission has been investigated among a small number of SFV-positive persons and their spouses and/or children. To date, no evidence of human-to-human transmission of SFV has been found (*16*,*21*).

Because of the close association of humans and nonhuman primates in Asia, most of which occurs in nonoccupational settings, we examined a large number of persons from several countries for evidence of SFV infection. All participants were asked about their past interactions with nonhuman primates, the species and population of the nonhuman primates with which they interacted, the behavioral contexts of each interaction, and the kinds of injuries, if any, inflicted. Our aim was to detect nonhuman primate–to-human SFV transmission and to learn about the behavioral contexts in which it occurs.

Materials and Methods

Study Sites and Populations

Our data were gathered over 7 years, and our sample totaled 305 persons (172 men, 133 women). Study sites, selected for their known human–nonhuman primate contact, were located in 4 countries in South and Southeast Asia: Thailand, Indonesia, Nepal, and Bangladesh. The seroprevalence of SFV among the nonhuman primates at these sites has been reported (*9*,*15*,*17*).

In Thailand, 211 persons were interviewed and sampled: 8 workers from a zoo in northern Thailand in 2002 and 203 persons at temples, nonhuman primate pet owners, bushmeat hunters, and urban residents from 9 sites in 2004–05. In Indonesia, biological samples and demographic and exposure data were collected from 74 temple workers at 2 sites in Bali: AK in 2000 (n = 56) and UB in 2003 (n = 18). In Nepal in 2003, 9 persons who lived and/or worked at the Swoyambhu Temple in Kathmandu were sampled; this World Heritage site is home to >400 free-ranging rhesus (*Macaca mulatta)*. In Bangladesh, where for decades ≈200 rhesus monkeys have ranged freely in the village of DH, northeast of Dhaka, 11 villagers were sampled and interviewed in 2007.

Protocols for human subject recruitment, biological sample collection, storage and handling, and collection of ethnographic/epidemiologic data have been described (*17*). Questionnaires and laboratory databases were analyzed by using NCSS 2004 (Kaysville, UT, USA) databases. Protocols for obtaining questionnaire data and biological samples were reviewed and approved by the University of Washington Human Subjects Institutional Review Board (02-5676-C06).

SFV Assays

A bioplex whole-virus multiplex flow cytometric assay was used for SFV antibody screening. SFV was conjugated to beads as previously described for simian retrovirus, simian T-cell leukemia virus, simian immunodeficiency virus, and *Cercopithecine herpesvirus 1* (*22*). The results were validated by using plasma from known SFV-positive and SFV-negative monkeys (as determined by immunofluorescence assay). The ELISA using bacterially expressed, purified glutathione S-transferase (GST) and GST-Gag has been described (*23*). For further testing, we conducted a Western blot (WB) assay with SFV-infected or SFV-noninfected cell lysates; the SFV used was isolated from an *M. fascicularis* housed at the University of Washington. Viral bands were detected by using the TMB reagent (3,3′,5,5′-tetramethylbenzidine; Promega, Madison, WI, USA). This assay has been previously described (*15*). Each assay used a strongly positive human serum (HCM2) and negative serum sample from a person who had never been exposed to a nonhuman primate.

Molecular and Phylogenetic Analyses

DNA was extracted from blood samples by using QIAamp Blood Mini Kits (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. For PCRs, the primers and conditions described by Schweizer and Neumann-Haefelin were used for *pol* (*24*), and those by Jones-Engel et al. (*17*) were used for mitochondrial sequences, with the following modifications: an annealing temperature of 52°C was used for 25 cycles in round 1 and for 29 cycles in round 2. For *gag* PCR, the following oligonucleotide primers were used: round 1 forward primer 5′-AGGATGGTGGGGACCAGCTA-3′, reverse primer 5′-GCTGCCCCTTGGTCAGAGTG-3′; round 2 forward primer 5′-CCTGGATGCAGAGCTGGATC-3′, reverse primer 5′-GAG GGAGCCTTTGTGGGATA-3′. The PCR conditions for *gag* and *pol* PCR were identical. All PCR runs included tubes containing water and noninfected human DNA as negative controls. DNA was checked for integrity by using mitochondrial DNA primers. Purified PCR fragments were cloned from round 2 into pCR2.1-TOPO

by using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). For each clone, 3–6 colonies were picked and purified-DNA sequenced. Sequences were analyzed by using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). For *pol,* 425 bp were compared; for *gag,* 1,125 bp were compared. Trimmed sequences were analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and aligned, and neighbor-joining trees (*25*) were estimated by using the Tajima and Nei model (*26*). Bootstrap values (1,000 replicates) are represented as percentages. Positions containing gaps and missing data were not considered in the analysis. Phylogenetic analyses were conducted in MEGA (*27*). Identical results were obtained with MrBayes (*28*) (analyses not shown) under the Hasegawa, Kishino, and Yano substitution model (*28*). In those analyses a search was performed with 1 million generations, and the first 100,000 trees were discarded in the burn-in.

Nucleotide Sequence Accession Numbers

The *gag* and *pol* gene sequences reported here were deposited in GenBank under the following accession nos.: AK04*gag* EU448349, AK04*pol* EU448363, AK19*gag* EU448350, AK19*pol* EU448364, AK23*gag* EU448351, AK23*pol* EU448365, BGH4 *gag* EU450664, HAD3 *gag* EU450665, HAD38*pol* EU448341, HAD3*pol* EU448342, MBG11*gag* EU448344, MBG13*gag* EU448345, MBG14*gag* EU448346, MBG4*gag* EU448343, MBG7-*gag* EU448347, MBG8*gag* EU448348, SFVfasW*gag* EU448357, SM44*gag* EU448353, SM44*pol* EU448358, SM46*pol* EU448359, SM49*gag* EU448354, SM49*pol* EU448360, SM61*gag* EU448355, SM61*pol* EU448361, SM62*gag* EU448356, SM62*pol* EU448362, UB1*pol* EU448366, UB3*gag* EU448352, and UB3*pol* EU448367. SFVmulO is listed under accession no. DQ120937.

Results

Demographic Data (Table 1)

Persons ranged from 18 to 80 years of age. Their context of contact with nonhuman primates was defined as the predominant form of contact at the time of the study. Some persons reported other past contexts of contact. For example, several of the 23 bushmeat hunters, all from the same village in Thailand, had previously worked at a park where free-ranging nonhuman primates were the main attraction, and a few of the temple workers in Bali and Thailand reported having previously owned pet nonhuman primates.

SFV Assays (Table 2)

Of 305 serum samples analyzed, 211 samples from Thailand were initially screened with bioplex at the Washington National Primate Research Center (*22*), and 146 of these samples had negative results. The remaining 65 samples from Thailand and all 94 samples from Nepal, Indonesia, and Bangladesh were screened by ELISA by using GST control antigen and GST-Gag fusion protein. Of these 159 samples, reactivity of 25 exceeded GST background on ELISA, and these were further tested with WB by using SFV-infected or SFV-noninfected tissue culture cell lysates. The major reactive viral protein is the structural protein Gag. Some foamy virus–infected serum samples also react with the viral accessory protein Bet. A total of 8 (2.6%) human samples were confirmed positive by using SFV-infected tissue culture cell or noninfected cell control lysates, which all react with the Gag protein. Gag appears as a characteristic doublet of 68 and 71 kDa (Figure 1, samples 2–9). Antibody to Bet could be detected only in HCM2, HAD3, and NH2. Although reactivity of HMS14 antiserum is weak, this serum was able to neutralize SFV but not the chimpanzee-derived primate foamy virus, which confirmed infection (data not shown). All other human serum samples tested were negative for all viral proteins. Two negative examples are shown in Figure 1: HCJ7, which yielded the same background proteins in noninfected and infected lysate, and BGH1, which did not react with any proteins. Human serum samples were also tested by WB by using GST and GST-Gag protein (*15*). However, because many of the human samples reacted with GST protein, the recombinant protein WB assays were generally inconclusive (data not shown).

Table 1. Demographic and context data for 305 persons who lived and/or worked around nonhuman primates, Asia*

Characteristic	N	% Total population	% (No.) bitten	% (No.) scratched	% (No.) splashed	% (No.) SFV+
Sex						
Male	172	56.4	28.9 (50)	34.8 (60)	25.6 (45)	2.3 (4)
Female	133	43.6	28.4 (38)	28.6 (57)	23.3 (31)	3.0 (4)
Context†*						
Temple	234	76.7	25.6 (60)	40.2 (94)	27.4 (64)	2.1 (5)
Pet	21	6.9	52.4 (11)	42.9 (9)	38.1 (8)	9.5 (2)
Bushmeat hunting	23	7.5	0	4.3 (1)	4.3 (1)	0
Zoo work	8	2.6	75.0 (6)	100.0 (8)	0	0
Urban	19	6.2	57.9 (11)	26.3 (5)	15.8 (3)	5.3 (1)
Total	305	100.0	28.7 (88)	38.4 (117)	24.6 (75)	2.6 (8)

*SFV+, simian foamy virus positive.
†Predominant form of human–nonhuman primate contact at the time of the study.

Table 2. Persons at high risk for SFV, Asia*

Country	No. samples tested	No. ELISA reactive	No. WB positive	No. SFV sequences derived	Total no. confirmed SFV positive
Thailand	211†	15	3	NA	3
Nepal	9	1	1	NA	1
Indonesia	74	8	3	2	3
Bangladesh	11	1	1	1	1
Total	305	25	8	3	8 (2.6%)

*WB, Western blot; SFV, simian foamy virus; NA, not applicable.
†65/213 serum samples were bioplex reactive and further screened with glutathione S-transferase-Gag ELISA.

Prevalence of Bites

No statistical differences in bite exposures were detected between men and women ($\chi^2 = 0.009$, p = 0.924, degrees of freedom = 1) or among age groups ($\chi^2 = 7.678$, p = 0.1043, degrees of freedom = 4). Bites were less common among bushmeat hunters (0%) and persons who lived and/or worked at monkey temples (25.6%) than among those who were exposed to urban (57.9%) and pet monkeys (52.4%). Splashes of body fluids onto mucosa were reported by nearly one fourth (24.9%) of the study population and scratches by 38.4%. Overall, 63.6% of the total population reported being exposed to nonhuman primate body fluids through a bite, scratch, or splash onto mucosa.

Nonhuman Primate Contacts Reported by SFV-positive Persons (Table 3)

Thailand

At the time of sampling, HCM2, a farmer from central Thailand, was 56 years of age. Since 23 years of age, he had trained 8 pig-tailed macaques (*M. nemestrina)* to harvest coconuts. At the time of data collection, he had 3 working *M. nemestrina* that he kept in his compound and transported to the fields on his motorbike. He reported having received several scratches and 2 bleeding bites (hand and arm) over the years. The bites were treated with traditional medicines.

At the time of sampling, HMS14 was 44 years of age. She sold food at a Buddhist temple in northern Thailand and had worked and lived in the area for 30 years. Wild assamese macaques (*M. assamensis)* ranged freely through the temple grounds, commonly entered nearby homes in search of food, and frequently received food from monks and visitors to the temple. HMS14 reported that *M. assamensis* came into her home daily to raid food bins. In 1999, a pet female stump-tailed macaque (*M. arctoides)* was brought to the temple and released. HMS14 had repeated physical contact (but no bites or scratches) with this released pet macaque, which was often present at her food stall. HMS14 reported that on 3 separate occasions in 2004 she was scratched by free-ranging *M. assamensis* and that the scratches were deep enough to bleed.

HMS50, a 43-year-old laborer who had lived in a village in northern Thailand for 33 years, reported that he came to the Buddhist temple several times a week and that *M. assamensis* entered his home, near the temple, a few times a year in search of food. He reported no bites, scratches, or mucosal splashes. He did report that he fed the *M. assamensis* at the temple site.

Indonesia

HAD3, a 58-year-old man, worked at a Hindu temple site in central Bali, where free-ranging long-tailed macaques (*M. fascicularis*) were an attraction for domestic and international tourists. He also reported that he had previously

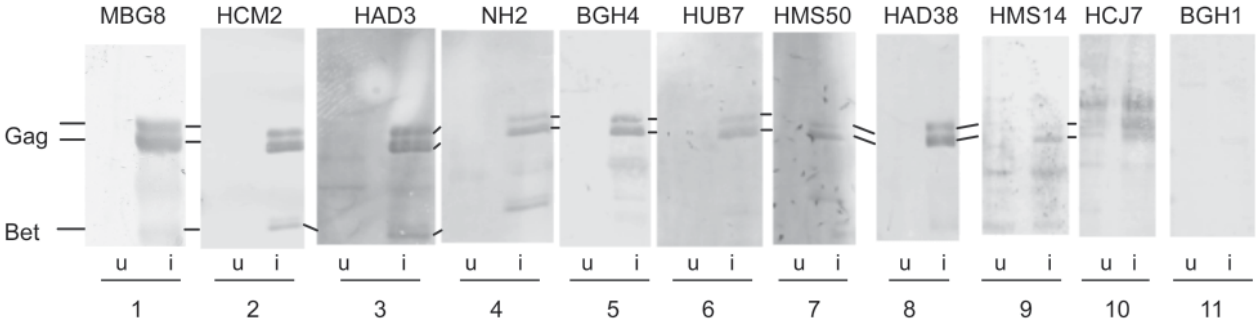

Figure 1. Western blot assays using human serum. Dilutions of human serum (lanes 2–11) or a foamy virus—positive *Macaca mulatta* MBG8 (lane 1) were used to probe filter strips containing equal amounts of lysates from simian foamy virus–infected cells (from *M. fascicularis;* i lanes) or noninfected cells (u lanes). Individual strips were developed by using TMB reagent (3,3′,5,5′-tetramethylbenzidine; Promega, Madison, WI, USA). The positions of the viral proteins Gag and Bet are indicated. Lanes 10 and 11 show the range of reactivity seen with negative serum; lane 10 shows serum with nonspecific reactivity to proteins of approximately the same size as viral proteins; lane 11 shows serum negative for both lysates.

Table 3. Exposure characteristics of SFV-positive persons who had had contact with nonhuman primates, Asia*

Person	Sequence	Sex/age, y	Location	Context of contact	Nonhuman primate contacted	Reported exposures
HCM2	NA	M/56	Southern Thailand	Primate owner, pet	*Macaca nemestrina*	Multiple bites and scratches
HMS14	NA	F/44	Northern Thailand	Village resident, temple and pet	*M. assamensis* and *M. arctoides*	Bleeding scratches
HMS50	NA	M/43	Northern Thailand	Village resident, temple	*M. assamensis*	None
HUB7	NA	M/35	UB, Bali, Indonesia	Temple worker, temple	*M. fascicularis*	>4 bites over many y
HAD38	*gag*	F/32	AK, Bali, Indonesia	Temple worker, temple	*M. fascicularis*	2 bites within 1 y + 1 scratch
HAD3	*gagpol*	M/58	AK, Bali, Indonesia	Temple worker, primate owner, temple and pet	*M. fascicularis*	Multiple bites, scratches
NH2	NA	F/36	Kathmandu, Nepal	Village resident, temple	*M. mulatta*	Severe bite
BGH4	*gag*	F/19	DH, Bangladesh	Village resident, urban	*M. mulatta*	Severe bite 17 y ago

*SFV, Simian foamy virus; NA, not applicable. All persons were confirmed SFV positive by Western blot.

owned 2 pet *M. fascicularis*. He reported having received >5 bleeding bites to his hands from his pet macaques and 1 bleeding bite and multiple scratches from macaques at the temple site. He did not seek medical treatment for the bites or scratches.

HAD38, a 32-year-old woman, had worked as a tourist guide at the same temple site as HAD3. She reported having received 2 bleeding bites and a bleeding scratch from the macaques within 1 year of working at this site. She applied antiseptic to her injuries.

HUB7, a 35-year-old temple worker at a Hindu temple in central Bali, reported that during his 2.5 years of work there he had been bitten 4 times by free-ranging *M. fascicularis*, once each on the hand, arm, leg, and buttock. All bites were severe enough to cause bleeding. He washed each wound with water and sought medical care, which included a tetanus vaccine and antimicrobial drugs, for the bite on his arm. He reported having been scratched only 1 time. He also had touched a pet *M. fascicularis* owned by a family in his village but had never been bitten or scratched by that macaque.

Nepal

NH2, a 36-year-old woman, lived immediately adjacent to the Swoyambhu Temple in Kathmandu and occasionally worked there as a cleaner. She had been bitten 1 time on her middle finger by one of the temple's free-ranging rhesus macaques *(M. mulatta)*. The wound was washed with water, and she was treated with a rabies vaccination and antimicrobial drugs at a local clinic. She denied having ever been scratched.

Bangladesh

BGH4, a 19-year-old housewife, was born in the central Bangladeshi village in which she was sampled. When she was 4 years old, she was severely bitten on her left calf by one of the *M. mulatta* that ranged freely through the village. She did not recall whether she had received any medical treatment. She did not report any other physical contact with nonhuman primates, though she did comment that the local macaques often entered her house in search of food, leaving urine and feces.

Phylogenetic Analyses of SFV Sequences

We derived SFV sequences from the peripheral blood DNA of 3 SFV-infected persons: BGH4, HAD3, and HAD38. We were able to amplify mitochondrial DNA from the DNA sample of another person (HCM2) from which no SFV sequences could be obtained. We have no evidence that DNA obtained from the other 4 human blood samples was of good quality (data not shown). We obtained *gag* sequences from BGH4 (Figure 2, panel A), *gag* and *pol* sequences from HAD3 (Figure 2, panels B, C), and *pol* sequences from HAD38 (Figure 2, panel C). SFV sequences from humans were compared with those from macaques of the group with which the person had been in contact and with those from other macaques of the same species but different geographic origin (Figure 2, panel A, *M. mulatta*; Figure 2, panels B, C, *M. fascicularis*).

SFV from BGH4 clustered most closely with SFV from 4 *M. mulatta* from her village in central Bangladesh (MBG4,11,13,14) and more distantly with 2 performing *M. mulatta* (origin unknown) sampled near her village (MBG7 and MBG8). The virus sequence of BGH4 is equidistant from that of MBG7 and MBG8 and from that obtained from an *M. mulatta* (SFVmulO of unknown origin) housed at the Oregon National Regional Primate Center. SFV *pol* and *gag* sequences from HAD3 (from central Bali) clustered most closely with SFV from AK *M. fascicularis* at the Bali temple site where HAD3 worked, as did HAD38 *pol* sequences. In contrast, the virus sequences from these 2 humans were more distantly related to those from the UB animals, which were also *M. fascicularis* but from another temple site in Bali (≈15 km away). The SFV sequences from HAD3 and HAD38 were even less similar to SFV isolated from *M. fascicularis* from Singapore (SM isolates).

These data suggest that SFV sequences are stable in nonhuman primates and can be used for several macaque

species to mark an individual's geographic origin. Correlation between the SFV sequences isolated from humans and those from the corresponding nonhuman primate populations with which they reported contact was excellent.

Discussion

We found prevalence of SFV infection in the heterogeneous populations studied to be 2.6%. In contrast with previous studies of persons who had occupational exposure to nonhuman primates, the exposure of some of the SFV-infected persons in our study was only through their normal daily routines. Previous research on nonhuman primate–human interaction in South and Southeast Asia describes interspecies contact as a frequent phenomenon in this part of the world (*29*,*30*). Our study takes this line of inquiry a step further, indicating that interspecies contact leads to nonhuman primate–to-human transmission of SFV in a variety of contexts, in several countries, and from multiple macaque species (Figure 3).

Bites from nonhuman primates are thought to be the most likely route of SFV transmission because viral RNA is found at high concentrations in the oral mucosa and saliva of infected animals (*23*). Indeed, 6 of the 8 SFV-infected persons reported having been bitten by a macaque at least 1 time. Although bites were reported by most SFV-positive persons, 2 denied having ever been bitten by a nonhuman primate. Possible explanations are that persons living in a community with a constant presence of nonhuman primates may not regard contacts, even scratches and bites, as notable events or, alternatively, that SFV is transmissible by contact other than bites, such as scratches or contact with nonhuman primate body fluids through breaks in the skin.

Other studies have shown SFV sequences to be highly stable (*12*,*31*). Switzer et al. (*19*) previously reported that they could determine the source chimpanzee of SFV infections in zoo workers by using phylogenetic analyses. We expanded those data to link SFV infections in populations exposed to free-ranging nonhuman primates to animals from their village and, in 1 instance, to differentiate native and introduced macaques solely by their SFV sequences (Figure 2, panel A). The 3 persons from whom SFV sequences were obtained each interacted with a single species of macaque; we did not detect any recombinant viruses, which are more likely to be encountered in persons who come into contact with multiple nonhuman primate species.

A recent review article recapitulates arguments that 2 factors influence the likelihood that disease can be transmitted from an animal reservoir to humans (*32*). First, phylogenetic relatedness suggests that the more closely a species is related to *Homo sapiens*, the more likely it is that transmission to humans can occur. The second factor is interspecies contact, which can be conceived as having 2 dimensions: the duration of contact and the intensity of contact. In general, contacts such as bites, scratches, or mucosal splashing with body fluids have the highest potential for transmitting infectious agents. In this light, the human–nonhuman primate interface in South and Southeast Asia ranks among the most likely contexts for zoonotic transmission.

In South and Southeast Asia, macaque monkeys and humans exhibit higher rates of sympatry than any other human–nonhuman primate overlap, owing in part to the

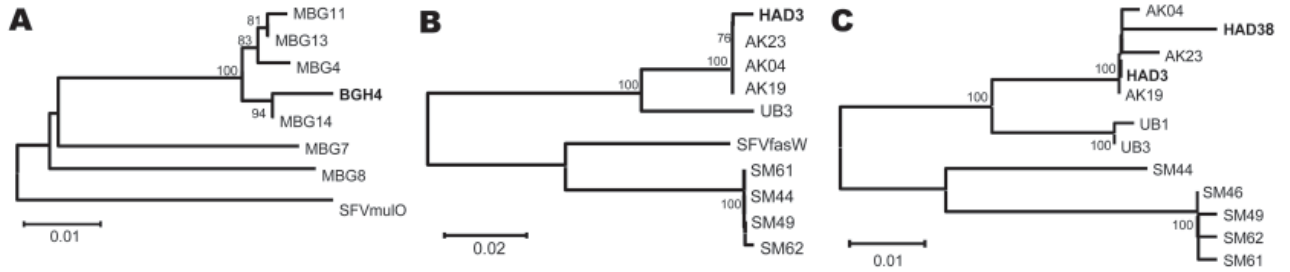

Figure 2. Phylogenetic trees of simian foamy virus (SFV) sequences derived from 3 persons. Human-derived SFV sequences (shown in **boldface**) were compared with those obtained from macaques of the group with which the person had been in contact and to SFV from other macaques of the same species but different geographic origin. Neighbor-joining trees A and B used gag PCR primers (1,124 bp), and C used pol PCR primers (445 bp). A) SFV gag–derived from BGH4 DNA clusters more closely (94% of bootstrap samplings) with gag sequences from 4 *Macaca mulatta* that ranged throughout her village (MBG4, MBG11, MBG13, and MBG14) than with gag sequences obtained from Bangladeshi performing monkeys, *M. mulatta* (MBG7, MBG8), of unknown origin. BGH4 gag is equidistant from gag of MBG7, MBG8, and virus obtained from SFVmulO, an *M. mulatta* of unknown origin housed at the Oregon National Regional Primate Center. B) SFV gag from HAD3, a worker at a Bali monkey temple, grouped with gag from several *M. fascicularis* (AK4, AK19, AK23) found at the same temple (100% of bootstrap samplings). UB3 is also an *M. fascicularis* Bali temple monkey that inhabited a temple ≈15 km away. HAD3-derived gag is less similar to *M. fascicularis* from Singapore (SM) and SFVfasW, an *M. fascicularis* housed at the Washington National Primate Research Center. C) Analysis of pol confirms the relationships (100% of bootstrap samplings) between SFV sequences isolated from humans (HAD3 and HAD38) and those in the corresponding nonhuman primate populations with which they reported contact (AK4, AK19, AK23). HAD3 and HAD38 worked at the same temple site where AK are found. UB1 and UB3 are *M. fascicularis* from a nearby monkey temple. Scale bars indicate number of nucleotide substitutions per site.

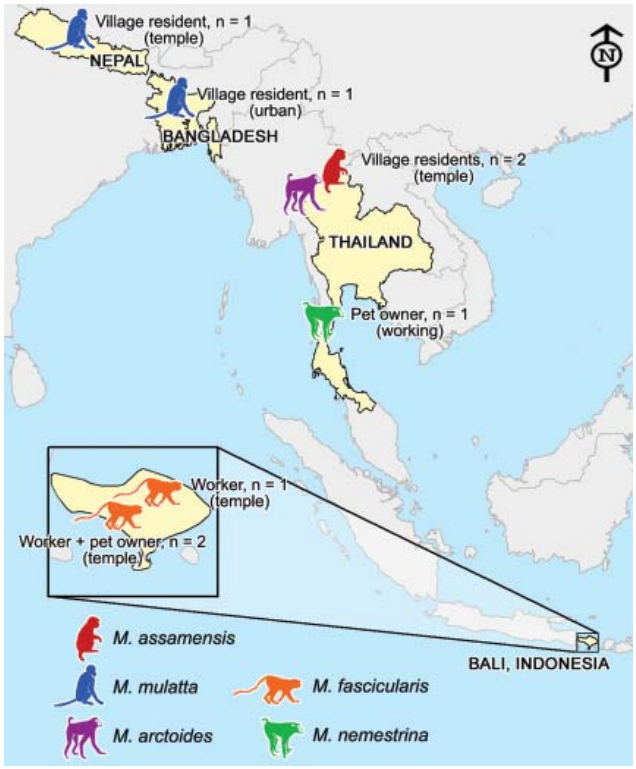

Figure 3. Map of the diverse contexts, countries, and nonhuman primate (*Macaca*) species associated with human infection with simian foamy virus.

major roles that nonhuman primates play in Hindu and Buddhist mythology and folklore. As a result, nonhuman primates are woven culturally and physically into the fabric of everyday life for millions of people. At least 68 temples throughout Thailand are home to populations of free-ranging nonhuman primates (*5*). Villagers in the town of Lopburi contend on a daily basis with >1,000 long-tailed macaques who spill out from the Pra Prang Sam Yot temple. These monkeys and the annual Monkey Buffet Festival (at which a buffet of fruits and vegetables is provided for all of the province's monkeys) are a major tourist attraction. New Delhi, one of the most populous cities in the world, is also home to ≈5,000 free-ranging rhesus macaques. Interspecies contact leading to nonhuman primate bites is a familiar and increasing phenomenon in communities such as these and for their international tourists (*33*,*34*). The 5 major monkey temples in Bali collectively attract up to 700,000 visitors a year, most of whom feed monkeys and thousands of whom are bitten and/or scratched. Engel et al. recently published an analysis that used mathematical modeling to predict the likelihood of a visitor to a Balinese monkey temple becoming infected with SFV (*29*); this model predicted infection for ≈6 of every 1,000 visitors.

Two trends promise to increase human–nonhuman primate contact in South and Southeast Asia: nonhuman primate ecologic resilience and human alterations of the landscape. Because of the first trend, ecologic resilience and high birth rates, many populations of protected (sometimes fed as well) nonhuman primates are increasing rapidly. For example, during the 1990s, population levels of the 3 species of macaques in the Kowloon Hills of Hong Kong increased 100% (*35*). A second trend is habitat loss leading to increased concentrations of nonhuman primate populations in areas more densely populated by humans. In the northern Indian state of Himachal Pradesh, 86% (258,000) of rhesus macaques now inhabit urban areas as a result of habitat loss (*11*). This trend of increased urbanization of nonhuman primates is mirrored throughout Asia (*36*,*37*). In contrast, bushmeat hunting, the most common human–nonhuman primate interaction in Africa, is likely to decrease interspecies contact over time, as wild nonhuman primate populations continue to dwindle. These demographic facts lead us to echo previous calls for a global surveillance network to monitor the emergence of zoonotic disease, with the crucial caveat that such a network focus on areas of highest interspecies contact.

Our data suggest that the number of persons at risk for infection with SFV is much larger in South and Southeast Asia than elsewhere. This finding presents both a challenge and an opportunity for future research. The challenge is to find infected persons and follow the course of infection in addition to taking action to prevent future transmission. The opportunity lies in assembling a large cohort of infected persons, which will enable the use of epidemiologic techniques to learn about the natural course of SFV infection in humans.

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Puumala Hantavirus Excretion Kinetics in Bank Voles (*Myodes glareolus*)

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Puumala hantavirus is present in bank voles (*Myodes glareolus*) and is believed to be spread mainly by contaminated excretions. In this study, we subcutaneously inoculated 10 bank voles with Puumala virus and sampled excretions until day 133 postinfection. Levels of shed viral RNA peaked within 11–28, 14–21, and 11–28 days postinfection for saliva, urine, and feces, respectively. The latest detection of viral RNA was 84, 44, and 44 days postinfection in saliva, urine, and feces, respectively. In contrast, blood of 5 of 6 animals contained viral RNA at day 133 postinfection, suggesting that bank voles secrete virus only during a limited time of the infection. Intranasal inoculations with bank vole saliva, urine, or feces were all infectious for virus-negative bank voles, indicating that these 3 transmission routes may occur in nature and that rodent saliva might play a role in transmission to humans.

Hantavirus, a genus within the family *Bunyaviridae*, contains rodent-borne viruses that cause 2 severe diseases in humans: hantavirus cardiopulmonary syndrome in the Americas and hemorrhagic fever with renal syndrome (HFRS) in Eurasia. HFRS causes ≈150,000–200,000 hospitalizations each year throughout the world (*1*). Puumala virus (PUUV), which is spread in large areas of Europe, causes a milder form of HFRS called nephropathia epidemica (*2*). Since 1989, when the disease became notifiable in Sweden, the largest number of cases was reported during 2007 (2,195) compared with a median 207.5 cases during 1990–2007 (M. Hjertqvist, pers. comm.). The mean incidence of nephropathia epidemica in the 4 northernmost county councils in Sweden was as high as 225.5/100,000 in 2007 (*3*).

PUUV is carried and maintained by infected bank voles (*Myodes glareolus*); transmission is believed to occur by inhalation of virus-containing, aerosolized, rodent excreta (*4*). Infectious PUUV has been detected in saliva, urine, and feces from experimentally infected colonized bank voles (*5*), and excreted PUUV is infectious for up to 12–15 days outside the host (*6*). However, the relative importance of saliva, urine, and feces in transmission of PUUV between bank voles or from bank voles to humans and how levels of virus change over time in different excretions are not known.

In this study, we used real-time reverse transcription–PCR (RT-PCR) to measure levels of shed viral RNA in saliva, urine, and feces of subcutaneously inoculated bank voles until they were killed at day 133 postinfection (PI). To evaluate possible transmission routes for PUUV, we investigated infectivity of different excretions and used a subset of viral RNA–positive saliva, urine, and feces samples to intranasally inoculate virus-negative bank voles.

Materials and Methods

Animals and Virus

Colonized bank voles were maintained in separate cages in biologic safety isolators with food and water provided ad libitum. All handling of animals was in compliance with guidelines of the Swedish Institute for Infectious Disease Control, Stockholm, Sweden. The PUUV strain Kazan wild type (PUUV Kazan-wt) (*7*,*8*) was used for subcutaneous inoculation of bank voles, and Vero E6 cell

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line–adapted PUUV strain Kazan (PUUV Kazan-E6) (*7*) was used in inhibition experiments and as a positive control in the real-time RT-PCR.

Subcutaneous Inoculation and Sample Handling

Bank voles were subcutaneously inoculated with ≈200 bank vole 50% infectious doses of PUUV Kazan-wt diluted in Hanks balanced salt solution medium (Invitrogen, Paisley, Scotland). Animals were sampled for saliva, urine, and feces on days 0, 1, 2, 3, 4, 8, 9, 11, 14, 16, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 133 PI. Serum samples were obtained on day 21 and at the termination of the experiment (day 133 PI). Some animals did not survive until day 133, but they were sampled by using the same procedures until time of death.

Saliva was collected by gently rotating a moistened cotton swab in the mouth of the bank vole. The cotton swab was subsequently placed in a cryotube containing 500 μL dilution medium (Hanks balanced salt solution medium containing 2% HEPES [Invitrogen], 2% fetal calf serum [Sigma-Aldrich, St. Louis, MO, USA], and 1% penicillin-streptomycin [Sigma]). Urine was collected by grasping the scruff of the neck of the animal and holding it over a petri dish to cause urination, as described by Botten et al. (*9*). Urine samples were stored in cryotubes. For feces sampling, bank voles were placed in separate containers until feces could be collected and transferred into cryotubes. All saliva, urine, and feces samples were stored at –70°C until analyzed. Serum samples were stored at –20°C until analyzed for antibodies to PUUV by ELISA.

Intranasal Inoculation

Intranasal inoculation was performed by using subsets of the PUUV RNA–positive excretion samples from subcutaneously inoculated bank voles (pooled saliva samples from bank voles no. 6 [day 21] and no. 7 [day 21]; pooled urine samples from bank voles no. 10 [day 16], no. 1 [day 21], no. 8 [day 28], and no. 7 [day 14]; and feces suspension from bank vole no. 3 [day 21]). A total of 5 μL of saliva, urine, or feces suspension was delivered to each nostril of 14 anesthetized bank voles. Saliva, urine, and feces were administered to groups of 4, 5, and 5 bank voles, respectively. These intranasally inoculated bank voles were sampled for saliva, urine, and feces on days 0, 6, 14, 21, 26, 35, and 42 PI, and a subset of these samples was tested for PUUV RNA by real-time RT-PCR. Animals were bled at days 21 and 42 PI and then humanely killed. All handling of samples was performed as described for the subcutaneous inoculation experiment.

ELISA

To confirm PUUV infection of the animals, a PUUV-nucleocapsid immunoglobulin (Ig) G ELISA (*10*) was performed by using serum from day 21 PI for the subcutaneous inoculation experiment, and from days 21 and 42 PI for the intranasal inoculation experiment. Briefly, 1 μg/mL of rKAZ (*Escherichia coli*–expressed recombinant PUUV Kazan) was coated on a 96-well plate. After washing and blocking, samples to be tested were added to the plate in duplicate at dilution of 1:200. After washing, alkaline phosphatase–conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA) was added to the plate. The plate was washed again and *p*-nitrophenyl phosphate (Sigma-Aldrich) substrate was added; the optical density was determined at 405–620 nm.

Extraction of RNA

Viral RNA was extracted by using the Ex-tract DNA/RNA Extraction Kit (Severn Biotech Ltd., Kidderminster, UK) with procedures described by Boom et al. (*11*) with minor modifications. For saliva samples, 100 μL of sample was transferred into a new tube containing 100 μL of dilution medium, 20 μL of silica particles, and 1 mL of L6 buffer. Urine samples were centrifuged at 1,800 × *g* for 5 min, and 20 μL of supernatants was transferred to a new tube containing 180 μL of dilution medium, 20 μL of silica particles, and 1 mL of L6 buffer. Approximately 50 mg of fecal sample was homogenized in 600 μL of phosphate-buffered saline (PBS) and centrifuged at 1,800 × *g* for 5 min. A total of 200 μL of supernatant was transferred to a tube containing 20 μL of silica particles and 1 mL of L6 buffer.

Tubes were vortexed for 10 s and incubated for 15 min at room temperature on a shaker. After centrifugation at 15,700 × *g* for 45 s, pellets were washed twice with 1 mL of L2 buffer, twice with 1 mL of 70% ethanol, and once with 1 mL of acetone. After acetone removal, the pellet was dried at 56°C for 5–10 min, dissolved in 49 μL of RNase-free water (Invitrogen) and 1 μL of RNaseOUT (Invitrogen), and incubated at 56°C for 15 min. After centrifugation at 15,700 × *g* for 4 min, the supernatant was transferred into a new tube and immediately analyzed for viral RNA by using real-time RT-PCR. PUUV Kazan-E6 (30,000 focus-forming units [FFU]/mL) was used as a positive control.

Real-Time RT-PCR

A real-time RT-PCR targeting the small segment of the PUUV genome was performed by using the QuantiTect Probe RT-PCR Kit (QIAGEN, Hilden Germany). The reaction consisted of 1× QuantiTect Probe RT-PCR master mixture, 300 nmol/L forward primer 983F (5′-GTGCACCAGATCGGTGTCC-3′) (Invitrogen) (*12*), 900 nmol/L reverse primer 1038R (5′-CAATTCAGCCATCCCAGCA-3′) (Invitrogen) (*12*), 150 nmol/L TaqMan MGB probe 1003T (5′-CCTACATGCATTTATG-3′) (Applied Biosystems, War-

rington, UK) (*12*), 0.25 μL of QuantiTect RT mixture, 5 μL of sample RNA (corresponding to RNA from 2 μL urine, ≈5 mg feces, or 10 μL oral swab suspension), and RNase-free water (Invitrogen) to give a final volume of 25 μL. A 96-well plate (Bio-Rad Laboratories, Hercules, CA, USA) was used, and PCR thermal cycling was performed by using an iCycler (Bio-Rad Laboratories) with the following cycling conditions: 50°C for 30 min and 95°C for 15 min, followed by 45 cycles at 94°C for 15 s and 60°C for 1 min. All samples were tested in duplicate.

Evaluation of Real-Time RT-PCR Inhibition by Bank Vole Excretions

Samples of saliva, urine, and feces from uninfected bank voles were prepared similarly to samples from infected bank voles. Saliva was diluted twice in dilution medium, urine was diluted 10 times in dilution medium, and feces was prepared as an 8% suspension in PBS. Uninfected samples were spiked with PUUV Kazan-E6 at 10-fold serial dilutions ranging from 30 to 30,000 FFU/mL. Dilution medium and PBS were used as controls. All samples were extracted and analyzed in duplicate by using real-time RT-PCR.

Results

Inhibition of PUUV Real-Time RT-PCR by Feces

Urine and saliva samples showed cycle threshold values similar to dilution medium and the PBS control for all viral dilutions. However, feces samples spiked with PUUV Kazan-E6 showed cycle threshold values ≈3–6 cycles above control values (Figure 1), which has been shown to correspond to 10–100× lower detection of RNA for all viral dilutions (*13*).

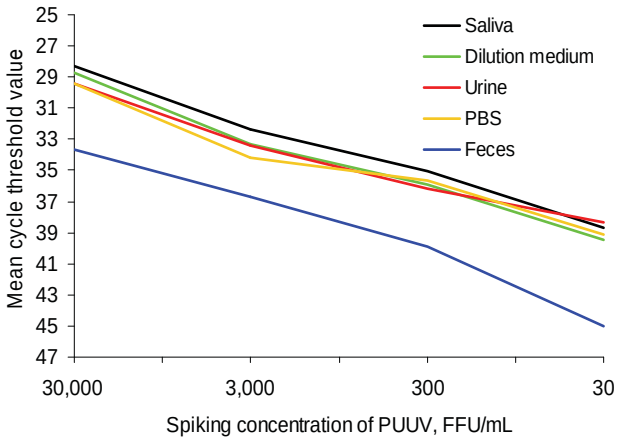

Figure 1. Inhibition of Puumala virus (PUUV) real-time reverse transcription–PCR by feces, but not saliva or urine, of bank voles. Mean cycle threshold values are shown for different solutions spiked with a cell line–adapted PUUV. Cycle threshold values of negative samples were set at 45. PBS, phosphate-buffered saline; FFU, focus-forming units.

Kinetics of Excreted PUUV RNA from Subcutaneously Inoculated Bank Voles

Ten colonized male bank voles were subcutaneously inoculated with PUUV Kazan-wt. All bank voles seroconverted, as shown by an IgG ELISA that used serum samples obtained from animals at day 21 PI. Four bank voles did not survive until day 133; bank voles no. 4 and 9 died after being anesthetized on day 21 PI, and bank voles no. 5 and 10 died of unknown reasons on 112 and 35 days PI, respectively.

Viral RNA was detected in subsets of saliva, urine, and feces samples (Figure 2, Table 1). Cycle threshold values of negative samples were set at 45. Levels of excreted PUUV RNA peaked on days 11–28 PI for saliva, 14–28 PI for urine, and 11–28 PI for feces. The earliest and latest detection of PUUV RNA was found for saliva on days 8 and 84 PI, compared with 11 and 44 days PI for urine and feces. One animal (no. 2) was PUUV RNA negative in all urine samples, and 1 animal (no. 10, which died on day 35 PI) was negative in all feces samples (Table 1). Viral RNA was detected in serum from 5 of the 6 surviving animals at day 133.

Intranasal Transmission of PUUV by Bank Vole Saliva, Urine, and Feces

We tested whether RNA-positive excretion samples also contained infectious virus and whether intranasal inoculation was a possible route of infection for all types of excretions. A subset of the PUUV RNA–positive urine, feces, and saliva samples collected from subcutaneously inoculated bank voles was administered intranasally to 14 virus-negative female bank voles. Seven (2/4 given saliva, 2/5 given urine, and 3/5 given feces) of 14 intranasally inoculated bank voles seroconverted (Table 2).

Saliva, urine, and feces samples were obtained from the 14 intranasally inoculated animals and tested by real-time RT-PCR. PUUV RNA was detected in subsets of saliva, urine, and feces samples from all 3 groups (Table 2).

Discussion

We have shown in controlled experimental conditions how levels of shed PUUV RNA change over time in saliva, urine, and feces from PUUV-infected bank voles. All 3 excretions can transmit virus to other bank voles when administered intranasally, which suggests that all 3 excretion pathways can function as natural transmission routes between bank voles and from bank voles to humans.

In previous studies on PUUV, experimentally infected bank voles seem to excrete infectious virus for a limited time after infection (*5*,*14*). This finding is consistent with

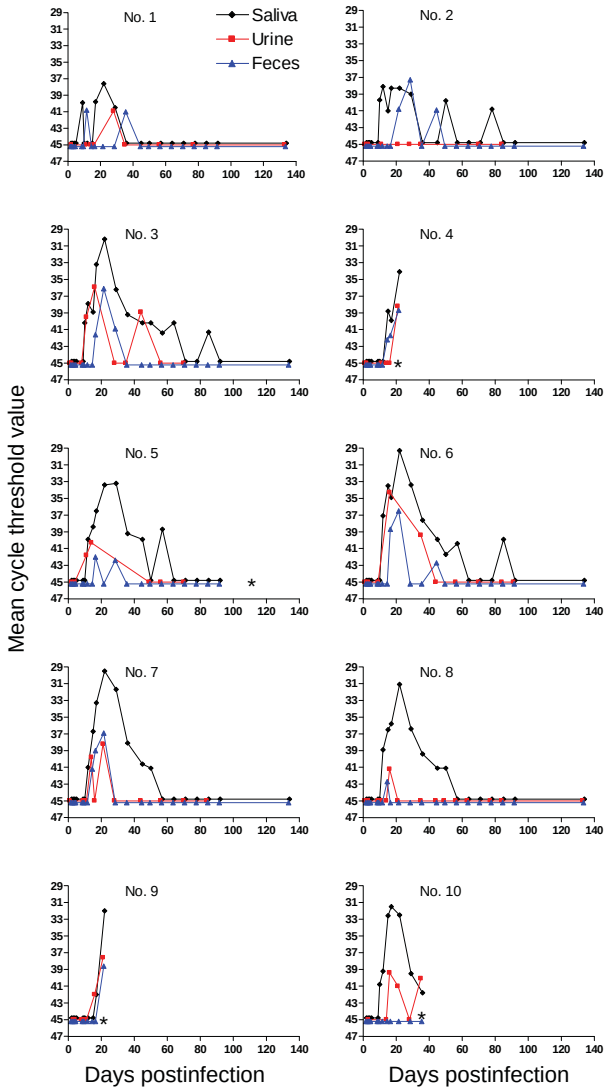

Figure 2. Detection of Puumala virus (PUUV) RNA by real-time reverse transcription–PCR in saliva, urine, and feces of bank voles subcutaneously inoculated with PUUV strain Kazan wild type. Cycle threshold values of negative samples were set at 45. *, bank voles 4 and 9 died on day 21 postinfection and bank voles 5 and 10 died on days 112 and 35 postinfection, respectively.

our real-time RT-PCR data. We observed clear peaks of shed viral RNA in saliva, urine, and feces preceded and followed by levels below detection limits. Viral RNA was detected in blood of 5 of 6 surviving animals on day 133, which suggested that persistently infected bank voles do not normally shed virus during the entire course of infection. Levels of excreted viral RNA decreased below the detection level in some animals, but RNA was detected in subsequent samples (Figure 2).

Similar patterns have been observed for Sin Nombre virus (SNV)–infected deer mice (*Peromyscus maniculatus*). Botten et al. reported an initial peak in SNV RNA levels in lung samples at 21 days PI, followed by a second peak at 60 days PI (*15*). In another report on SNV, Kuenzi et al. found a variation in PCR positivity of blood samples from wild-caught deer mice (*16*). These authors suggested 2 interpretations of the results: either that viral RNA is consistently present in the blood but is near the limits of PCR detectability or viral RNA reappears in blood as a consequence of unknown physiologic events. We believe that similar interpretations can be made concerning levels of PUUV RNA in bank vole excretions. Whether levels of excreted PUUV change as a consequence of external factors, e.g., cold temperatures or social stress, remain to be shown.

A problem when working with biologic material combined with PCR techniques is the effect of inhibitory substances; several inhibitory components in feces have been identified, such as bile salts and polysaccharides (*17*). In the spiking experiments, saliva and urine showed no PCR inhibition because results for excretions were comparable to those of dilution medium and PBS (similar cycle threshold values). In contrast, 10–100× less viral RNA was recovered from spiked feces samples (Figure 1), which indicated that more virus was shed in bank vole feces than we were able to detect. We conclude that saliva contained higher levels of viral RNA than urine did because saliva samples were ≈10–20× more diluted than the urine samples but still showed lower cycle threshold values.

Although real-time RT-PCR is an effective method for measuring levels of RNA, it does not necessarily measure the presence of infectious virions. We therefore tested a subset of real-time RT-PCR–positive excretion samples for infectious virus. Different methods can be used to detect infectious hantavirus and potential transmission routes. Bernshtein et al showed that more bank voles were infected when injected with lung suspension from PUUV-positive bank voles than after intercage transmission (*14*). Injection shows if an excretion contains infectious virus, but in nature a similar event will occur only when saliva is transferred by biting. Especially for urine and feces, intranasal inoculation probably resembles natural transmission. Bank vole saliva, urine, and feces are infectious when injected intramuscularly into virus-negative bank voles (*5*). We show that saliva, urine, and feces are also infectious when given intranasally, which indicates that PUUV in bank vole saliva can be transferred not only by biting. Intranasal inhalation of saliva may also involve ingestion, which may also be a viable route of infection. Ingestion could occur when several bank voles share a common food source. Hooper et al. have recently shown that Andes hantavirus is infectious to hamsters when administered by intragastric injection and speculate that ingestion of contaminated material might be a mode of transmission to humans (*18*).

Table 1. Detection of PUUV RNA over time in excreta from bank voles subcutaneously inoculated with PUUV strain Kazan wild type*

Bank vole no.	Excretion type	Detection of PUUV RNA in excreta by time, d																				
		1	2	3	4	8	9	11	14	16	21	28	35	44	49	56	63	70	77	84	91	133
1	Saliva	–	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
	Urine	NT	–	NT	NT	NT	NT	–	–	–	NT	+	–	NT	NT	–	NT	NT	–	NT	NT	–
	Feces	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
2	Saliva	–	–	–	–	–	+	+	+	+	+	+	–	–	+	–	–	–	+	–	–	–
	Urine	–	NT	–	NT	NT	NT	–	NT	NT	–	–	–	NT	NT	NT	NT	–	NT	–	NT	NT
	Feces	–	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–
3	Saliva	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–
	Urine	–	–	NT	NT	–	NT	+	NT	+	NT	–	–	+	NT	NT	NT	–	NT	NT	NT	NT
	Feces	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–
4†	Saliva	–	–	–	–	–	–	–	+	+	+											
	Urine	–	–	NT	NT	NT	NT	–	–	–	+											
	Feces	–	–	–	–	–	–	–	+	+	+											
5‡	Saliva	–	–	–	–	–	–	+	+	+	+	+	+	+	–	+	–	–	–	–	–	
	Urine	NT	–	NT	–	NT	NT	+	+	NT	NT	NT	NT	NT	–	–	NT	–	NT	NT	NT	
	Feces	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	
6	Saliva	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	–	-	+	–	–
	Urine	–	NT	–	NT	NT	–	NT	NT	+	NT	NT	+	–	NT	–	NT	–	NT	-	–	NT
	Feces	–	–	–	–	–	–	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–
7	Saliva	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
	Urine	–	NT	NT	NT	NT	–	–	+	+	+	–	NT	–	NT	–	NT	–	NT	–	NT	NT
	Feces	–	–	–	–	–	–	–	+	+	+	–	-	–	–	–	–	–	–	–	–	–
8	Saliva	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–
	Urine	–	–	–	NT	NT	–	–	–	+	–	NT	–	–	–	–	–	NT	–	–	NT	–
	Feces	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	—	–
9†	Saliva	–	–	–	–	–	–	–	–	+	+											
	Urine	NT	NT	–	–	–	–	–	NT	+	+											
	Feces	–	–	–	–	–	–	–	–	—	+											
10§	Saliva	–	–	–	–	–	+	+	+	+	+	+	+									
	Urine	NT	NT	–	NT	NT	NT	NT	–	+	+	–	+									
	Feces	–	–	–	–	–	–	–	–	–	–	–	–									

*PUUV, Puumala virus; –, negative; +, positive, NT, not tested (no or insufficient urine volume).
†Died on day 21 postinfection.
‡Died on day 112 postinfection.
§Died on day 35 postinfection.

We have shown that intranasal inoculation of saliva, urine, or feces enables subsequent detection of viral RNA in all types of excretions, which indicates that virions excreted by different routes do not show restricted tropism for particular tissues. When we analyzed serum obtained on day 42 PI from intranasally inoculated bank voles by ELISA, 7 of 14 had seroconverted (Table 2). Only 1 of the animals was positive at day 21 PI (Table 2). This late seroconversion in bank voles may have been caused by relatively low doses of virus in bank vole saliva, urine, and feces samples used for intranasal inoculation. We believe that this information will be useful in future vaccine and infection studies because it indicates that a low level of hantavirus might not induce seroconversion until after 21 day PI. It would be useful to investigate whether a low dose of hantavirus inoculum can induce seroconversion after 42 day PI.

To better evaluate and predict risk for human hantavirus infections, information on factors associated with occurrence and transmission of hantavirus in natural rodent populations is needed. It has been assumed that rodent behavior is required for maintenance of PUUV in the natural reservoir because PUUV infection in relation to bank vole demography shows nonrandom transmission patterns (*19*). PUUV stability outside the host likely plays a role in transmission to other rodents and in the number of human cases (*20*). Hantaviruses have been shown to be stable ex vivo, and Hantaan hantavirus can infect cell culture after being stored for as long as 96 days in medium at 4°C (*21*). Furthermore, PUUV is infectious for bank voles for up to 12–15 days in contaminated cage bedding (*6*). How different excretions contribute to virus stability in the environment and what implications this might have on direct versus indirect transmission among rodent reservoirs remain to be shown. The role of different excretions in transmission of PUUV may vary with the age and density of bank voles and the season. Hypothetically, shedding in saliva might be more efficient for virus transmission in male bank voles living in a high-density area during mating season, when

Table 2. Infection of bank voles with PUUV after intranasal inoculation with saliva, urine, and feces samples*

Inoculum	Bank vole no.	Seropositivity		PUUV RNA in excreta by time, d†		
		Day 21 PI	Day 42 PI	Saliva	Urine	Feces
Saliva	1	–	+	NT	NT	NT
	2	–	–	NT	NT	NT
	3	–	–	NT	NT	NT
	4	+	+	0, 6, 14, **19, 26, 35, 42**	0, **19, 26, 42**	0, 6, 14, **19, 26, 35, 42**
Urine	5	–	+	NT	0, 24, **26, 28**	NT
	6	–	–	NT	NT	NT
	7	–	+	0, 6, 14, 19, 26, **35, 42**	0, 14, 19, 35	0, 6, 14, 19, 26, 35, **42**
	8	–	–	NT	NT	NT
	9	–	–	NT	NT	NT
Feces	10	–	+	NT	6, 14, 24, **26, 35, 42**	NT
	11	–	–	NT	NT	NT
	12	–	+	NT	0, 24	NT
	13	–	+	0, 14, 19, 26, **35, 42**	6, 19, 24	0, 6, 19, 26, **35, 42**
	14	–	–	NT	NT	NT

*PUUV, Puumala virus; PI, postinfection; –, negative; +, positive; NT, not tested.
†Numbers indicate days PI for tested excretions. Days with positive excretions are shown in **boldface**.

many fights occur. In contrast, shedding in feces, which may provide the virus with a more stable environment, may play a more dominant role in transmission in a low-density area during fall or winter.

In conclusion, we studied levels of PUUV RNA in excretions of infected bank voles over a period of 4.5 months. We have shown that bank vole saliva, urine, and feces can cause infection when inhaled by other bank voles, which indicates that all 3 excretions can transfer virus to humans.

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Community Strains of Methicillin-Resistant *Staphylococcus aureus* as Potential Cause of Healthcare-associated Infections, Uruguay, 2002–2004

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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains have emerged in Uruguay. We reviewed *S. aureus* isolates from a large healthcare facility in Montevideo (center A) and obtained information from 3 additional hospitals on patients infected with CA-MRSA. An infection was defined as healthcare-onset if the culture was obtained >48 hours after hospital admission. At center A, the proportion of *S. aureus* infections caused by CA-MRSA increased from 4% to 23% over 2 years; the proportion caused by healthcare-associated MRSA (HA-MRSA) decreased from 25% to 5%. Of 182 patients infected with CA-MRSA, 38 (21%) had healthcare-onset infections. Pulsed-field gel electrophoresis determined that 22 (92%) of 24 isolates were USA1100, a community strain. CA-MRSA has emerged in Uruguay and appears to have replaced HA-MRSA strains at 1 healthcare facility. In addition, CA-MRSA appears to cause healthcare-onset infections, a finding that emphasizes the need for infection control measures to prevent transmission within healthcare settings.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was recognized as a nosocomial pathogen in the 1960s and now represents a substantial proportion of *S. aureus* infections in inpatient and outpatient settings (*1*,*2*). Risk factors for healthcare-associated MRSA (HA-MRSA) are well defined and include hospitalization, surgery, dialysis, residence in a long-term care facility, and use of indwelling catheters or other percutaneous medical devices (*3*,*4*).

During the 1990s, MRSA emerged as a cause of infection among healthy persons in the community who had none of the above HA-MRSA risk factors (*5*–*10*). Community-associated MRSA (CA-MRSA) infections most commonly manifest as skin and soft tissue infections, but more invasive infections, including sepsis syndrome, necrotizing pneumonia, and fasciitis, also occur (*11*,*12*). Outbreaks of CA-MRSA infection have occurred among prisoners, sports participants, military recruits, and healthy full-term newborns (*7*–*9*). In a population-based study in Atlanta and Baltimore, the incidence of CA-MRSA infection was highest among children <2 years old (*11*). Factors that appear to facilitate transmission of CA-MRSA include frequent skin-to-skin contact, crowding, compromised skin integrity, sharing of potentially contaminated items, and lack of personal hygiene.

HA-MRSA and CA-MRSA possess resistance to β-lactam antimicrobial agents, conferred by the staphylococcal cassette chromosome (SCC) *mec* element (*13*). However, CA-MRSA strains are typically less resistant to non–-β-lactam antimicrobial agents (*14*). CA-MRSA strains almost invariably contain a particular SCC*mec* type (SCC*mec* IV, V, or VI), whereas HA-MRSA strains usually contain SCC*mec* types I, II, or III (*15*–*18*). CA-MRSA strains typically possess the Panton-Valentine leukocidin (PVL) toxin, which has been associated with skin abscesses and necrotizing pneumonia (*19*).

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CA-MRSA has been reported worldwide, and several reports describe the entrance of CA-MRSA strain types into healthcare settings (*20*–*22*). However, few articles have documented CA-MRSA emergence in South America (*23*,*24*). The objectives of this investigation were to describe trends in *S. aureus* and MRSA infections in Uruguay, to explore transmission of these strains in healthcare settings, and to characterize CA-MRSA strains circulating in Uruguay.

Methods

Definitions

In this investigation, we defined a case of MRSA infection as illness compatible with staphylococcal disease in a patient from whom a strain of *S. aureus* resistant to oxacillin by disk diffusion was isolated from a clinically relevant site. Because it was suspected that community strains had entered the healthcare setting, epidemiologic risk factor data were not useful in distinguishing community versus healthcare strains. Therefore, microbiologic definitions were used. A MRSA isolate was considered to be an HA-MRSA strain if it was resistant to at least 2 of the following antimicrobial agents: trimethoprim/ sulfamethoxazole (TMP/SMX), ciprofloxacin, gentamicin, rifampin, and tetracycline. A MRSA isolate was considered to be a CA-MRSA strain if 1) antimicrobial susceptibility results were available for at least 2 of the following agents: TMP/SMX, ciprofloxacin, gentamicin, rifampin, tetracycline, and 2) the isolate was resistant to no more than 1 of the agents and was confirmed to be susceptible to at least 2 of these agents.

We considered an infection to be healthcare onset if the MRSA culture was obtained >48 hours after a patient was admitted to the hospital and the patient had no evidence of the infection at the time of admission. A MRSA culture obtained within 48 hours of hospital admission or evidence of infection on admission was considered an indication of a community-onset infection.

Skin disease was defined as a primary skin infection such as abscess, cellulitis, folliculitis, or a skin infection spreading to contiguous tissues. Surgical site infections (SSIs) were not considered to be skin disease.

Assessment of Temporal Trends

To describe trends in *S. aureus* and MRSA infections, we reviewed laboratory records from August 2002 through July 2004 from a large healthcare facility (center A) that provided inpatient, outpatient, emergency, and long-term–care services to nearly 200,000 persons of all ages and socioeconomic levels. Reports of all *S. aureus* cultures, except nasal swabs (to exclude asymptomatic colonized patients), were included. Only the first culture was selected for each patient over the study period. Of the total number of *S. aureus* infections, the percentage due to MRSA was calculated for each quarter year of the study. Similarly, of the MRSA infections, the percentages caused by CA-MRSA and HA-MRSA were calculated for each quarter year. Chi-square tests for trend were calculated by using SAS version 9.1 software (SAS, Cary, NC, USA).

Assessment of Healthcare Transmission

To explore transmission of CA-MRSA strains in hospitals and describe factors associated with transmission, we reviewed medical records of patients with CA-MRSA infections who were hospitalized between January 2003 and August 2004 at 4 facilities in Uruguay, centers A–D. Centers A and B were prepaid health maintenance organizations serving a heterogenous population of all ages and socioeconomic status. Centers C and D were large public referral hospitals serving a population of lower socioeconomic status. Center D was a pediatric hospital. At center A, we identified cases by reviewing laboratory records (including susceptibility data) of clinical *S. aureus* isolates (see Assessment of Temporal Trends). In the other 3 centers, microbiologists and infectious disease physicians provided a list of patients with MRSA infections. We identified the patients who met our microbiologic case definition by reviewing the patients' laboratory records. Demographic and clinical data were abstracted from patient records by using a standardized form. Screening all patients for MRSA was not standard practice in any of the facilities included in this study.

Data collected included age, sex, location of residence (capital city of Montevideo vs. other locations), underlying medical conditions (chronic bronchitis, heart disease or stroke, liver or kidney disease, diabetes, HIV, AIDS, or history of immunosuppression or cancer), infection site (skin vs. non-skin), intensive care unit (ICU) admission, and onset of infection (hospital vs. community).

Data Analysis

We performed multivariable analysis by using logistic regression to determine characteristics independently associated with healthcare-onset CA-MRSA strain type infections. Variable screening was performed by using univariate logistic regression with an α significance level of 0.25. Variables that met the screening criteria were entered in a multivariable model and retained with an α significance level of 0.05. Variables that failed to meet screening criteria were assessed as potential confounders by using β estimate changes of >15% as the criteria. We also assessed effect modification between facility and infection site, facility and age, and infection site and age.

Laboratory Characterization of CA-MRSA Strains

Microbiologists and infectious disease physicians in Uruguay had a list of available isolates from patients infected with MRSA. We selected a convenience sample of 24 isolates from this list to obtain different clinical manifestations (skin and systemic infections), patient characteristics (pediatric and adult), treatment settings (inpatient and outpatient), healthcare institutions, and community and healthcare-onset disease. All isolates were from patients whose disease met our microbiologic definition of CA-MRSA disease. Isolates were sent to the Division of Healthcare Quality Promotion staphylococcal reference laboratory at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). After initially being screened for oxacillin resistance, isolates were tested for antimicrobial susceptibility by broth microdilution, according to the Clinical and Laboratory Standards Institute (*25*). In addition, antimicrobial-susceptibility disk tests were performed to study inducible clindamycin resistance (*25*). A PCR was used to characterize the SCC*mec* resistance complex and to detect the genes encoding the PVL cytotoxin, toxic shock syndrome toxin 1, and enterotoxins A–E and H (*15*,*26*). Pulsed-field gel electrophoresis (PFGE) was performed on restriction digests of chromosomal DNA (by using *Sma*I restriction endonuclease); gels were analyzed with BioNumerics software according to published criteria (*27*).

Results

Trends in *S. aureus* and MRSA Infections (center A)

Of 1,553 *S. aureus* infections at the health maintenance organization facility (center A), 42% were cultured in the hospital setting, 14% in the emergency department, 42% ambulatory care, and 2% from long-term-care service. The patients' median age was 56 years, and 55% were male. The proportion of *S. aureus* infections caused by MRSA remained stable over the 2-year period (χ^2 for trend p = 0.46), averaging 28% (Figure 1). CA-MRSA strains increased from 4% to 23% of all *S. aureus* infections (χ^2 for trend p<0.0001) over the 2-year study period, whereas the proportion caused by HA-MRSA decreased from 25% to 5% (χ^2 for trend p<0.0001) (Figure 2).

CA-MRSA Infections among Patients Hospitalized in 4 Facilities (centers A–D)

Of the hospitalized patients with CA-MRSA in the 4 facilities, 59% were male, 80% were from Montevideo, and 29% had an underlying chronic medical condition (Table 1). Most infections were of the skin (63%), followed by respiratory infections (13%), bacteremias (9%), and SSIs (9%) (Table 2).

Of 182 study patients, 38 (21%) were considered to have healthcare-onset infections. The age distribution of the healthcare-onset and community-onset groups differed with median ages of 59 and 7 years, respectively (Figure 3). Twenty percent of the community-onset group was <2 years of age compared with 3% in the healthcare-onset group. Infection site also differed between the healthcare-onset and community-onset groups; skin infections dominated the community-onset group, whereas most healthcare-onset infections were SSIs or respiratory tract infections (Table 2). ICU admission was more common in the healthcare-onset than in the community-onset group (61% and 19%, respectively, p<0.05) (Table 1).

In multivariable modeling, after the facility was controlled for, age >18 years (odds ratio [OR] 4.8) and non-skin infection sites (OR 5.1) were independently associated

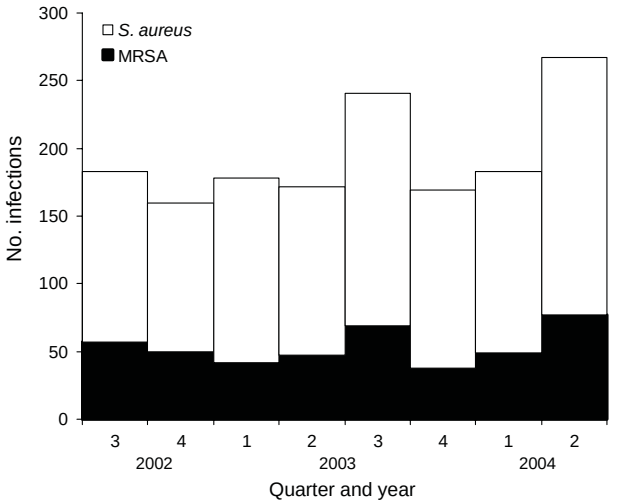

Figure 1. Number of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) infections by quarter and year, center A, August 2002–July 2004. N = 1,553.

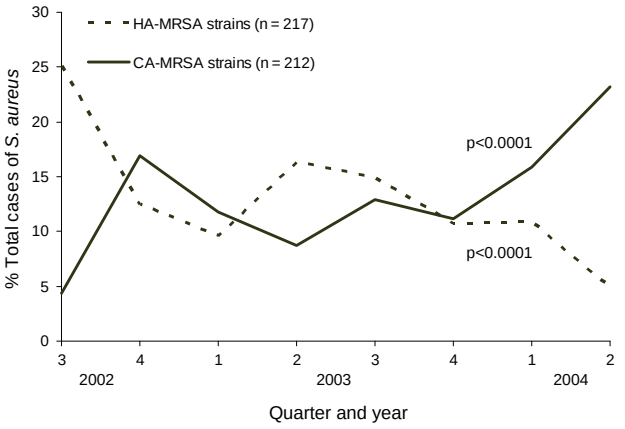

Figure 2. Proportion of *Staphylococcus aureus* due to community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections and healthcare-associated MRSA (HA-MRSA) infections by quarter and year, center A, August 2002–July 2004.

Table 1. Association of factors with healthcare- versus community-onset CA-MRSA, hospitalized patients, centers A–D, Uruguay, 2003–2004*†

Factors	Total no. (%), N = 182	Healthcare-onset, no. (%), n = 38	Community-onset, no. (%), n = 144	Univariate odds of healthcare-onset (95% CI)	Multivariate odds of healthcare-onset (95% CI)†
Age >18 y‡	79 (44)	33 (89)	46 (32)	17.4 (5.8–52.0)	4.8 (1.2–18.7)
Male	107 (59)	26 (68)	81 (56)	1.7 (0.8–3.6)	
Residence outside Montevideo§	31 (20)	5 (15)	26 (21)	0.7 (0.2–1.9)	
Chronic medical condition¶	51 (29)	21 (57)	30 (22)	4.7 (2.2–10.2)	
Infection site, nonskin	68 (37)	31 (82)	37 (26)	12.8 (5.2–31.5)	5.1 (1.7–15.1)
Intensive-care unit admission	51 (28)	23 (61)	28 (19)	6.4 (2.9–13.7)	

*CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; CI, confidence interval.
†Controlling for facility.
‡n = 180; age not available for 2 patients.
§n = 159; location of residence not available for 23 patients.
¶n = 175. Chronic conditions: chronic bronchitis, heart disease or stroke, liver or kidney disease, diabetes, HIV, AIDS, or history of immunosuppression or cancer.

with healthcare-onset CA-MRSA strain type infections (Table 1). Effect modification between facility and infection site, facility and age, and infection site and age was not significant.

Characterization of CA-MRSA Strains

Of 24 isolates selected for molecular typing and toxin testing, 15 (63%) were associated with skin infections, 5 (21%) bloodstream, 3 (13%) respiratory, and 1 (4%) a catheter-site infection. One third of the patients were ≤18 years of age (3 were <2 years of age), and 50% were female. Nineteen (79%) patients were initially seen at hospitals, 3 (13%) at outpatient centers, and 2 (8%) at a prison. Three (13%) were considered to have healthcare-onset infections.

Twenty-two (92%) of the 24 isolates were of PFGE type USA1100, a community strain (*28*). These 22 isolates contained SCC*mec* type IVc and the PVL locus, and 19 of the 22 had indistinguishable *Sma*I PFGE patterns. The other 3 USA1100 isolates were at least 91% related to the homogeneous group. The 3 healthcare-onset infections were of PFGE type USA1100. The 2 non-USA1100 isolates had microbiologic properties consistent with HA-MRSA belonging to PFGE type USA600 and USA800—one from the respiratory tract and the other from a catheter site. Both of these isolates were from adult patients who were considered to have community-onset infections.

Discussion

In summary, in at least 1 healthcare facility in Uruguay, CA-MRSA strains appears to be replacing HA-MRSA strains. Transmission of infections caused by CA-MRSA strain types appeared to be occurring in the hospital as well as the community, and patients whose infections developed in the hospital were older and more likely to have non-skin infections than those with community-onset disease.

In the United States, according to the National Nosocomial Infections Surveillance System, MRSA rates have risen during the 1990s and early 2000s (*1*). Klevens et al. (*2*) demonstrated that the antimicrobial resistance profile of ICU MRSA isolates from this surveillance system changed from 1992 through 2003. MRSA consistent with HA-MRSA strain types decreased from 55% in 1992 to 10% in 2003, while CA-MRSA strain types increased from 4% to 14% during the same period. This resistance pattern change was also described in Europe over an 11-year period (1992–2002) in France and over a 4-year period (1995–1998) in Belgium (*29*,*30*). In our study, although the proportion of *S. aureus* infections caused by MRSA remained stable, we observed a dramatic increase (4% to 23%) in the proportion of MRSA consistent with CA-MRSA strain types at 1 large healthcare institution in Uruguay. Whether the proportion of *S. aureus* due to MRSA remains stable or starts to increase over time should be monitored.

Healthcare-onset infections of MRSA are typically caused by HA-MRSA strain types. However, since the emergence of CA-MRSA strains, nosocomial transmission of CA-MRSA strains has been documented. Saiman et al. (*20*) described nosocomial transmission in an outbreak setting among postpartum women in New York City and in San Francisco. Carleton et al. (*31*) found a proportion of nosocomial MRSA isolates with molecular typing consistent with community strains. In Atlanta, during a 7.5-month prospective study in 2004, Seybold et al. (*21*) found that 20% of nosocomial MRSA bloodstream infections were due to USA300, a CA-MRSA strain type. In our study, 38 (21%) hospitalized patients with CA-MRSA strain type infections met our definition for having healthcare-onset infections.

The virulence and transmissibility of CA-MRSA strains in the hospital, compared with that of HA-MRSA strains, are unknown. CA-MRSA strains are typically susceptible to more antimicrobial agents than HA-MRSA strains, but this situation may change as CA-MRSA strains in the healthcare setting are exposed to high antimicrobial selection pressures. In addition, CA-MRSA strains may

Table 2. Infection type for 182 hospitalized patients with community-onset CA-MRSA infections, centers A–D, Uruguay, 2003–2004*

Infection type†	Total no. (%) infections, N = 269	Healthcare-onset no. (%), n = 45	Community-onset no. (%), n = 224	p value
Skin (any)	169 (63)	10 (22)	159 (71)	<0.0001
Impetigo	29	2	27	
Foliculitis/pustule	3	0	3	
Abscess	65	3	62	
Furunculosis	9	0	9	
Hidradenitis	1	0	1	
Cellulitis	49	2	47	
Abrasion	4	0	4	
Pressure wound	4	0	4	
Trauma wound	3	3	0	
Burn/necrotic lesion	2	0	2	
Respiratory	36 (13)	17 (38)	19 (9)	<0.0001
Pneumonia‡	34	16	18	
Pleuritis	1	1	0	
Pleural abscess	1	0	1	
Bacteremia	24 (9)	3 (7)	21 (9)	0.78
Surgical site	23 (9)	12 (27)	11 (5)	<0.0001
Organ/space	11 (4)	1 (2)	10 (4)	0.70
Septic arthritis	6	1	5	
Osteomyelitis/myositis	5	0	5	
Indwelling devices	3 (1)	1 (2)	2 (1)	0.42
Catheter infection	1	0	1	
Arteriovenous fistula infection	2	1	1	
Otitis	2 (<1)	1 (2)	1 (<1)	0.31
Cerebral ventriculitis	1 (<1)	0	1 (<1)	1.00

*CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*.
†Infections are not mutually exclusive (269 infections in 182 patients).
‡Pneumonia or tracheobronchitis.

have opportunities to exchange genetic material with HA-MRSA strains. CA-MRSA strains commonly contain toxin genes such as the PVL toxin gene, which may cause more serious nosocomial infections (*32*). In our study, 22 of the 24 isolates characterized by PCR had the PVL gene.

Typically, patients with CA-MRSA infections have skin and soft tissue infections. However, this might not be the case for healthcare-onset CA-MRSA strain type infections. Davis et al. (*33*) surmise that CA-MRSA strains exposed to healthcare settings may develop characteristics associated with HA-MRSA strains such as decreased accessory gene regulator function, inducible macrolide-lincosamide-streptogrammin resistance, and non–β-lactam resistance patterns. Whether this translates into a clinical picture more consistent with HA-MRSA infections is unclear. According to our findings, healthcare-onset CA-MRSA strain type infections were more likely than community-onset CA-MRSA infections to be non-skin diseases and to occur in older populations, characteristics common to HA-MRSA disease (*34*).

Based on this study and that of Ma et al. (*24*), description of the Uruguayan CA-MRSA outbreak, USA1100 or multilocus sequence type ST30 appears to be the predominant CA-MRSA strain circulating in Uruguay. This strain was identified in Australia, New Zealand, Europe, the United States, and most recently Brazil (*23*,*31*,*35*). In the United States, ST30 accounted for 41% of CA-MRSA infections in 1 study in San Francisco (*31*). However, more recent data from that city suggest replacement of ST30 by ST8 (USA300) (*36*). Whether replacement of this strain in Uruguay will occur and what its effect on illness and mortality rates would be remain to be seen.

The emergence of CA-MRSA in Uruguay has been rapid and is associated with significant illness and mortality rates (*37*). Although most CA-MRSA strain type infections in Uruguay are of skin or soft tissue, primary pulmonary disease appearing as necrotizing pneumonia has been described as well as skin or soft tissue infections leading to septic pulmonary embolic events (*38*,*39*). Of note, all isolates tested in our study were susceptible to multiple antimicrobial agents, most importantly TMP/SMX. On the basis of these findings, TMP/SMX may be a viable, cost-effective treatment option for many CA-MRSA infections (*40*).

This study was subject to limitations. Because we based our definition of healthcare-onset CA-MRSA strain type infections on time from hospital admission to culture and no evidence of infection on admission, misclassification was possible. Patients admitted to the hospital for non-MRSA diagnoses may have had CA-MRSA strain skin

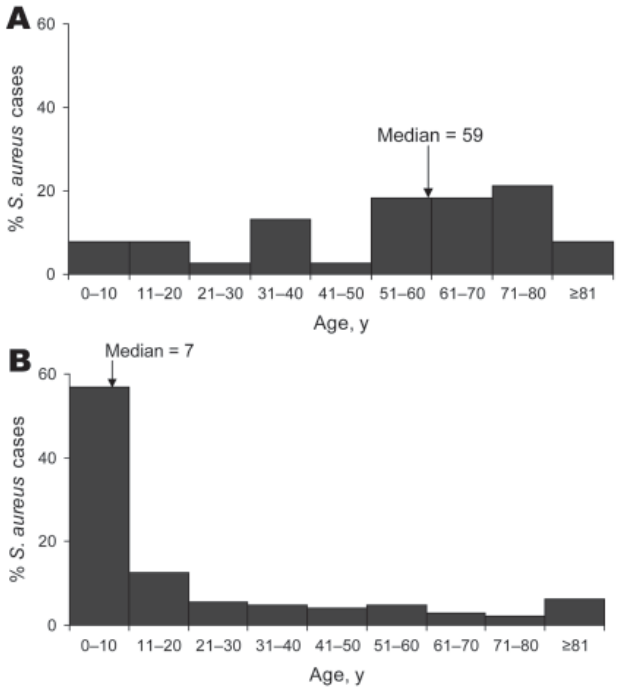

Figure 3. Patient age distribution of A) healthcare-onset versus B) community-onset community-associated methicillin-resistant *Staphylococcus aureus* strain type infections, centers A–D, Uruguay, 2003–2004.

infections on admission that were not documented or cultured within the first 48 hours. These skin infections would have been differentially misclassified as healthcare-onset and would have diluted the relationship between non-skin infections and healthcare-onset CA-MRSA disease, thereby underestimating our associations. In addition, because electronic laboratory records were unavailable in 3 of the 4 facilities, unrecognized CA-MRSA cases potentially affected the distribution of healthcare and community-onset CA-MRSA strain type cases. However, unless cases were systematically excluded, a selection bias was unlikely to have affected factors associated with healthcare-onset disease. Finally, because we suspected that CA-MRSA strains had entered the healthcare setting, we defined CA-MRSA on the basis of susceptibility patterns rather than epidemiologic risk factors. Without PFGE typing all isolates, it is not possible to determine if all included infections were consistent with known CA-MRSA strain patterns. However, of the 24 isolates that were sent to CDC for characterization and met our definition of CA-MRSA, 22 (92%) were consistent with CA-MRSA strain types.

This study describes characteristics of patients with healthcare-onset CA-MRSA strain type infections but does not identify the risk factors involved in nosocomial transmission such as the presence of indwelling catheters, intubation, certain procedures or surgeries, and specific units within the hospital. In addition, we do not know whether infections were acquired in the hospital or whether patients were colonized before arrival and infections subsequently developed in the hospital.

In conclusion, this study describes CA-MRSA emergence in South America. In addition, similar to what is occurring in other countries, it demonstrates CA-MRSA strain type transmission within healthcare settings. Clinicians should be aware that CA-MRSA strains have entered the healthcare setting and cause skin as well as non-skin infections. These infections may respond to a variety of non–β-lactam antimicrobial agents. Adherence to infection control precautions while one is caring for patients with suspected or confirmed CA-MRSA strain infections is critical for preventing transmission and further penetration of CA-MRSA in healthcare settings.

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Systematic Literature Review of Role of Noroviruses in Sporadic Gastroenteritis

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CME ACTIVITY

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Learning Objectives

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

- Describe the frequency of norovirus (NoV) infection worldwide.
- Describe the prevalence of NoV infection among mild-to-moderate community-acquired diarrhea cases.
- Describe the morbidity associated with severe diarrhea due to NoV infection.
- Identify the most common virulent strains of NoV.
- Describe the morbidity and mortality attributed to NoV infection worldwide.

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We conducted a systematic review of studies that used reverse transcription–PCR to diagnose norovirus (NoV) infections in patients with mild or moderate (outpatient) and severe (hospitalized) diarrhea. NoVs accounted for 12% (95% confidence interval [CI] 10%–15%) of severe gastroenteritis cases among children <5 years of age and 12% (95% CI 9%–15%) of mild and moderate diarrhea cases among persons of all ages. Of 19 studies among children <5 years of age, 7 were in developing countries where pooled prevalence of severe NoV disease (12%) was comparable to that for industrialized countries (12%). We estimate that each year NoVs cause 64,000 episodes of diarrhea requiring hospitalization and 900,000 clinic visits among children in industrialized countries, and up to 200,000 deaths of children <5 years of age in developing countries. Future efforts should focus on developing targeted strategies, possibly even vaccines, for preventing NoV disease and better documenting their impact among children living in developing countries, where >95% of the deaths from diarrhea occur.

Despite improved safety of food, water, and sanitation and aggressive promotion of noninvasive interventions (e.g., oral rehydration therapy) and prevention strategies (e.g., increased breastfeeding), diarrhea remains a common cause of illness worldwide. It accounts for ≈1.8 million annual deaths in children <5 years of age (*1*). Reduction of this disease will require targeted prevention and treatment strategies against the common agents causing severe diarrhea.

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Noroviruses (NoVs) and sapoviruses are genetically and antigenically diverse single-stranded RNA viruses that belong to 2 different genera (*Norovirus* and *Sapovirus)* in the family *Caliciviridae* and are collectively referred to as human caliciviruses (*2*). The prototype virus of the NoVs, Norwalk virus, was identified in 1972. However, the inability to cultivate these viruses in routine cell culture and the consequent challenges in developing sensitive nonmolecular diagnostic assays hindered initial efforts to define the epidemiology and assess the impact of disease associated with NoV infection. In the past 15 years, the availability of sensitive molecular diagnostic methods based on reverse transcription–PCR (RT-PCR) has allowed broader examination of the etiologic role of NoVs in epidemic and sporadic gastroenteritis (*3*,*4*).

Since the application of molecular assays, NoVs have been well-documented as the leading cause of epidemic gastroenteritis in all age groups, causing >90% of nonbacterial and ≈50% of all-cause epidemic gastroenteritis worldwide (*5*). Recent studies that used improved diagnostics have demonstrated that NoVs may also fill in the "diagnostic gap" in severe sporadic gastroenteritis among all age groups worldwide (*6*). Much of the misconception of NoV as an infrequent cause of severe sporadic diarrhea might also stem from studies undertaken before the mid-1990s that found low rates of NoV infection because available diagnostics such as electron microscopy and antigen detection assays had poor sensitivity. Recent data are emerging that are debunking these misconceptions, suggesting that the impact of NoV disease may be much greater than previously suspected and the disease may be more severe in some populations (*4*,*6*–*8*). However, because these novel assays are not typically available outside of reference laboratories, the true global prevalence and potential economic impact of NoV disease remain unrecognized (*3*). To further understand the etiologic role of NoVs in sporadic diarrhea, we conducted a systematic review to identify studies that used similar inclusion criteria and molecular assays based on RT-PCR to detect NoVs in fecal specimens from patients with diarrhea.

Methods

We searched MEDLINE, EMBASE, and Google Scholar to identify studies published in English between January 1990 and February 2008. We used the following keywords: *Norwalk*, *norovirus, Norwalk-like virus, human calicivirus, calicivirus, NLV, small round virus,* and *small round structured virus*. We reviewed all abstracts to identify articles that assessed the prevalence of NoV among sporadic cases of diarrhea. To ensure complete capture of all relevant studies, we cross-referenced all articles from the bibliography of the selected articles. After reviewing each article, we selected studies that met the following inclusion criteria: 1) study duration was ≥1 year, and 2) study used RT-PCR to diagnose caliciviruses (NoV and sapovirus) or NoV in patients with diarrhea. We included studies that tested for caliciviruses, even when they did not differentiate between NoV and sapovirus. For these studies, we multiplied the proportion of caliciviruses detected in each study by the mean proportion of caliciviruses that were NoV among studies that differentiated between NoVs and sapoviruses to yield the estimated NoV prevalence in each study. We excluded studies that did not provide a denominator (i.e., the total number of patients with diarrhea in the study population) or that only conducted molecular analysis using a fraction of the fecal samples (online Technical Appendix 1, available from www.cdc.gov/EID/content/14/8/1224-Techapp1.pdf). If the authors presented the data again in another study, only 1 study was included. See online Technical Appendix 2, available from www.cdc.gov/EID/content/14/8/1224-Techapp2.pdf, for a list of all references used in the review but not cited in this article.

We stratified studies into 2 settings: community or clinic-based (mild or moderate diarrhea) and hospital-based including emergency department and inpatients (severe diarrhea). We counted cases in which NoV was detected in the presence of ≥1 other pathogens (i.e., mixed infection) as NoV infection; however, we also present data on mixed infections, when available. Pooled proportions and 95% confidence intervals (CIs) of NoV-positive cases were calculated by using the random effects models (DerSimonnian and Laird method, StatsDirect Ltd, Cheshire, UK). For the studies that included fecal testing on concurrent diarrhea-free controls, the pooled proportions were based on absolute difference in NoV detection rate between cases and controls, thus only including the fraction of cases attributable to NoVs. The Cochran Q statistic and degrees of freedom (df) are presented as a measure of heterogeneity among studies. Analyses were conducted with StatsDirect version 2.5.7 (StatsDirect Ltd).

To calculate the number of outpatient NoV episodes and hospitalizations for children living in industrialized countries (where 23 of 31 studies in our review were conducted), we multiplied the total number of estimated diarrhea episodes in each clinical setting by the pooled proportion attributable to NoV based on the studies we reviewed to yield the number of NoV cases in each setting (*9*). No data exist on estimates of total diarrhea episodes in industrialized countries. Thus, we divided the estimates of outpatient and inpatient rotavirus episodes for industrialized countries, provided by Parashar et al. (*9*), by the proportion of diarrhea episodes attributable to rotavirus (23% and 42%, respectively) in the United States (*10*) and Europe (*11*) to yield the annual number of total diarrhea episodes in industrialized countries. To estimate the proportion of

outpatient (23%) and inpatient (42%) diarrhea episodes attributable to rotavirus, we assumed the midpoint of the proportion of gastroenteritis visits attributable to rotavirus in the United States (19% and 35%, respectively) and 7 European countries (27% and 50% respectively) for each setting, respectively.

To estimate NoV-associated deaths and hospitalizations among children in developing countries, we multiplied global estimates of diarrhea deaths (*1*) and hospitalizations (*9*) by the pooled proportion of NoV among children <5 years of age hospitalized with diarrhea. Data from developing countries were sparse on fraction of NoV-associated diarrhea episodes in the outpatient setting.

Results

Overall, we reviewed 235 studies and identified 31 original studies that met our inclusion criteria (Tables 1, 2) (*6*,*12*–*41*). Of these 31 studies, 20 were conducted in high-income countries, 2 were high-middle-income countries, 5 were low-middle income, and 4 were low-income countries, based on World Bank classification of economies (*42*). The duration of these studies was 1–5 years. Fourteen studies tested only for NoV (*13*,*14*,*19*,*21*,*22*,*27*,*29*,*30*,*35*,*37*–*41*); 17 tested for NoV and sapovirus (*6*,*12*,*15*–*18*,*20*,*23*–*26*, *28*,*31*–*34*,*36*). Among 13 of 17 studies that tested for and presented separate detection rates on both caliciviruses, NoV was detected in 84.5% and 88.5% of the community- and hospital-based studies, respectively (*6*,*15*,*16*,*20*,*23*–*26*, *31*–*34*,*36*). In these 13 studies, 69%–90% of the outpatient cases and 61%–100% of the hospital cases with caliciviruses were identified as NoV.

Illness by Age and Setting

Community- or Clinic-based Cases (i.e., Mild and Moderate Diarrhea)

Among the 13 studies of community- or clinic-based diarrhea cases, NoVs were detected in 5%–36% of cases; pooled proportion was 12% (95% CI 9%–15%; Cochran Q 335; df 12) (Figure 1). Five studies enrolled both adults and children, and 8 studies focused only on children, each with varying age ranges (overall range 0–13 years). In the 8 studies that assessed mixed infections, 0.4%–6.5% (median 1.1%) of the gastroenteritis cases were also positive for another viral or bacterial pathogen (Table 1).

Hospitalizations (i.e., Severe Diarrhea)

Twenty-three studies [hospital-based (n = 21); emergency department–based (n = 1); both (n = 1)] evaluated NoV disease among hospitalized diarrhea case-patients in whom the proportion of NoV disease ranged from 3% to 31%; pooled proportion was 11% (95% CI 8%–14%). Most (n = 19) of these studies of severe diarrhea cases focused on children <5 years of age, and the pooled proportion of NoV disease in these studies was 12% (95% CI 10%–15%) (Figure 2). In the 5 studies that assessed for multiple enteric pathogens, mixed infections were detected in 0%–24% (median 3.7%) of the gastroenteritis cases (Table 2).

Most (>95%) of the world's diarrheal deaths occur in low-middle– and low-income countries (*43*). In our review, 7 of 19 studies assessing prevalence of severe NoV disease among children <5 years of age were conducted in these countries (*23*,*25*,*28*,*33*,*34*,*36*,*39*). Pooled proportions

Table 1. Summary of studies examining prevalence of NoV in persons with severe sporadic AGE, using RT-PCR for studies >12 months' duration, community and outpatient clinics*

Ref	Country	Study duration, mos	Age group, y	No. AGE cases	No. NoV positive (single and mixed)	% NoV positive	No. mixed pathogens	% Mixed pathogens	No. control patients	% Control patients positive for NoV
(*6*)†	England	12†	All	2,422	871	36.0	–‡	–	2,205	16.2
(*12*)§	France	26	<13	414	49	11.8	27	6.5	50	0.0
(*15*)	Netherlands	12	All	709	114	16.1	–	–	669	5.2
(*16*)	Netherlands	36	All	857	43	5.0	–	–	574	1.0
(*13*)¶	Hong Kong	12	All	995	92	9.2	–	–	–	–
(*14*)	Australia	17	All	638	73	11.4	7	1.1	–	–
(*33*)¶	India	36	<5	500	38	7.6	5	1.0	173	4.0
(*18*)§¶	Chile	31	<5	274	15	5.5	–	–	–	–
(*17*)§	Finland	21	<2	1,477	264	17.8	13	0.9	47	0.0
(*20*)	Japan	12	<11	557	106	19.0	12	2.2	–	–
(*21*)	Japan	12	<11	402	58	14.4	–	–	–	–
(*19*)	Japan	12	<5	752	139	18.5	3	0.4	–	–
(*34*)¶#	Tunisia	15	<12	380	49	12.9	13	3.4	–	–

*NoV, norovirus; AGE, acute gastroenteritis; RT-PCR, reverse transcription–PCR; Ref, reference; –. not assessed in the study. Studies were of >12 months' duration.
†Using RT-PCR, tested stored specimens from a previous study that used electron microscopy for NoV detection; enrollment was staggered over 29 months with 70 clinics, each enrolling patients for 12 months.
‡Tested for mixed pathogens but did not provide pathogen-specific results.
§NoV prevalance in these studies was estimated by multiplying the calicivirus prevalance by average proportion of caliciviruses determined to be NoV (84.5%) among outpatient studies reporting NoV and sapovirus results separately.
¶O'Ryan et al. (*18*) included clinic-, emergency department–, and hospital-based patients; Lau et al. (*13*), Monica et al. (*33*), and Sdiri-Loulizi et al. (*34*) included community/clinic- and hospital-based patients. Only community and clinic data are presented here.
#Age- and setting-specific data were obtained through personal communication with the author.

Table 2. Summary of studies that examined prevalence of NoV in persons with severe sporadic AGE, emergency department visits and hospitalizations, by using RT-PCR for >12 months*

Ref	Country	Study duration, mo	Age group, y	No. AGE cases	No. NoV positive (single and mixed)	% NoV positive	No. NoV positive (mixed)	% Mixed	No. control patients	% Controls positive for NoV
Emergency department										
(*22*)	Spain	12	<14	363	16	4.4	1	0.3	–	–
(*18*)†‡	Chile	25 and 17§	<5	248	23	9.3	–	–	80	1.3
Hospital										
(*35*)	Italy	12	<3	365	93	25.5	35	9.6	–	–
(*23*)	Malawi	12	<5	398	26	6.5	12	3.0	–	–
(*24*)	Vietnam	12	<15	1,339	72	5.4	0	0.0	–	–
(*25*)	Thailand	12	<5	105	8	7.6	–	–	–	–
(*36*)	Thailand	20	<5	248	35	14.1	–	–	–	–
(*26*)	Australia	60	<5	1,233	108	8.8	–	–	–	–
(*13*)‡	Hong Kong	12	All	735	123	16.7	–	–	–	–
(*37*)	South Korea	24	<5	962	132	13.7	18	1.9	–	–
(*33*)‡	India	36	<5	350	53	15.1	26	7.4	173	4.0
(*27*)	Germany	12	<16¶	217	45	20.7	–	–	50	4.0
(*38*)	Japan	24	Children	500	66	13.2	2	0.4	–	–
(*18*)‡	Chile	25 and 17§	<5	162	8	4.9	–	–	50	0.0
(*39*)	Madagascar	13	<5	237	14	5.9	0	0.0	–	–
(*27*)†	Peru	24	<5	233	72	30.7	56	23.9	248	11.4
(*29*)	Spain	12	<5	656	79	12.0	36	5.5	–	–
(*30*)	Australia	36	<5	360	9	2.5	–	–	–	–
(*34*)‡#	Tunisia	27	<12	252	61	24.2	12	4.8	–	–
(*40*)	Brazil	12	<5	318	65	20.4	14	4.4	–	–
(*31*)	South Africa	48	All	1,296	32	2.5	–	–	–	–
(*41*)	South Korea	12	<5	762	114	15.0	–	–	–	–
(*31*)	USA	24	<4	1,840	131	7.1	–	–		–

*NoV, norovirus; AGE, acute gastroenteritis; RT-PCR, reverse transcription–PCR; Ref, reference; –, not assessed in the study.
†NoV prevalence (total and mixed alone) in these studies was estimated by multiplying the calicivirus prevalence by average proportion fo caliciviruses determined to be NoV (88.5%) among hospital-based studies reporting NoV and sapovirus results separately.
‡O'Ryan et al. (*18*) included clinic-, emergency department–, and hospital-based patients; Lau et al. (*13*), Monica et al. (*33*), and Sdiri-Loulizi et al. (*34*) included community/clinic- and hospital-based patients. Only emergency department and hospitalization data are presented here.
§Included 2 hospitals with 25- and 17-month enrollment periods.
¶98% (213 of 217) of the case-patients were <5 y of age.
#Age- and setting-specific data were obtained through personal communication with the author.

for NoV-associated childhood hospitalization were 12% (95% CI 8%–17%) among low-middle and low income in comparison to 12% (95% CI 9%–16%) for high- and high-middle–income countries.

Studies with Concurrent Controls

Concurrent diarrhea-free controls were enrolled in 9 of 31 studies, and NoVs were detected in 0%–16% (median 4%) of the controls (*6*,*12*,*15*–*18*,*27*,*28*,*33*). On the basis of the difference in detection rate between cases and controls in these studies, we estimate that the fraction of cases attributable to NoV in these studies was 4%–20% (median 12%) for mild to moderate diarrhea and 2%–26% (median 11%) for severe diarrhea.

Strain Characterization

Overall, 19 studies characterized the NoV strains by using nucleotide sequencing (*13*,*14*,*17*,*19*–*21*, *23*,*24*,*26*,*27*,*32*–*36*,*38*–*41*). Among NoV cases, strains belonging to NoV genogroup (G) II were the most common (range 75%–100%). With the exception of 2 studies, the overwhelming majority of the NoV strains belonged to the GII.4 cluster (*21*,*23*). In Malawi (1 of the 4 low-income countries in our analysis), GII.3 strains were detected in 69% of the samples that were sequenced (*23*). Similarly, in 2006, novel GII.3 strains were identified in 44% of the samples in a clinic-based study in Japan (*21*). Through genetic characterization, the authors demonstrated that these GII.3 strains likely were recombinant strains that appeared over a period of 4 months.

Estimated Prevalence of NoV Disease in Children

We estimate that each year NoVs cause ≈900,000 episodes of gastroenteritis that require a clinic visit and ≈64,000 hospitalizations among children <5 years of age residing in high-income countries (Table 3). If one assumes that the proportion of annual childhood diarrhea-associated hospitalizations (≈124 million) and deaths (≈1.8 million) in developing countries approximates the overall proportion of children (12.1%) with severe NoV illness in our review, NoVs may cause up to 1.1 million hospitalizations and 218,000 deaths each year in children in developing countries.

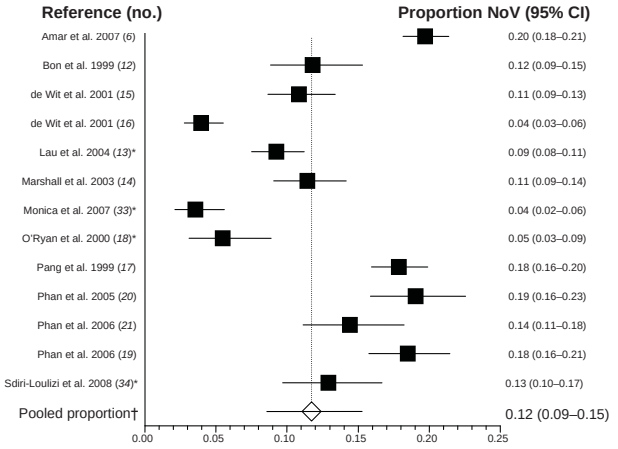

Figure 1. Summary of studies assessing proportion of norovirus (NoV)-positive fecal samples among persons with community and outpatient cases of sporadic diarrhea (all ages). *Lau et al. (*13*), O'Ryan et al. (*18*), Monica et al. (*33*), and Sdiri-Loulizi et al. (*34*) included outpatient and emergency department/hospital patients, but only outpatient data are included in this figure. †Pooled proportion calculated by using the random effects model (DerSimonian and Laird method, StatsDirect Ltd, Cheshire, UK). For studies that included controls, prevalence of NoV among controls was subtracted from prevalence of NoV among case-patients. CI, confidence interval.

Discussion

This systematic review of studies that used RT-PCR for detection of NoVs in fecal specimens clearly indicates that these viruses play an important role in the cause of both mild and severe gastroenteritis worldwide. In 1979, Greenberg et al. demonstrated that virtually all children in various countries worldwide acquired antibodies to NoVs by 5–15 years of age (online Technical Appendix 2). However, despite strong evidence that NoV infection was ubiquitous, detection rates in children hospitalized with diarrhea were low in early studies that used electron microscopy and antigen assays (*4*). The advent of conventional RT-PCR for the diagnosis of NoVs has substantially changed our understanding of their epidemiology. Among all reported studies that used conventional RT-PCR, NoVs were detected in ≈12% of children <5 years of age with severe diarrhea, which suggests that these viruses are the second most common cause of severe childhood gastroenteritis, following rotavirus. In addition, although some studies suggest that NoV infections in the community are slightly less severe than rotavirus infections, data also exist to suggest that these childhood infections may be similar in severity, which may particularly apply to hospitalized children (online Technical Appendix 2). On the basis of the pooled detection rates of NoV in our review, we would estimate that in the United States alone NoVs may account for >235,000 clinic visits, 91,000 emergency room visits, and 23,000 hospitalizations among children <5 years of age (*10*). Limited data from developing countries are available to make firm estimates, but NoV disease may cause >1 million hospitalizations and 200,000 deaths each year among children <5 years of age.

Although these figures provide a preliminary indication of the substantial magnitude of illness from NoV disease, they may underestimate the true extent of disease. Evidence suggests that detection of NoVs in fecal specimens by conventional RT-PCRs may be limited by factors such as low virus concentrations in feces, improper specimen storage, inefficient viral RNA extraction, presence of fecal reverse transcriptase inhibitors, and use of different primers (online Technical Appendix 2). In addition, NoVs are extremely genetically diverse and none of the reported conventional RT-PCR assays is able to detect all strains (online Technical Appendix 2). These hypotheses are supported by the findings of an evaluation of children with gastroenteritis in Peru in which both RT-PCR testing of fecal specimens and serologic assays were used to assess NoV infection, and serologic testing was found to increase the rates of NoV detection from 35% with fecal testing alone to 55% by use of either assay (*28*). A recent validation study comparing state-of-the-art real-time RT-PCR with conventional RT-PCR found that the sensitivity of real-time RT-PCR was greater

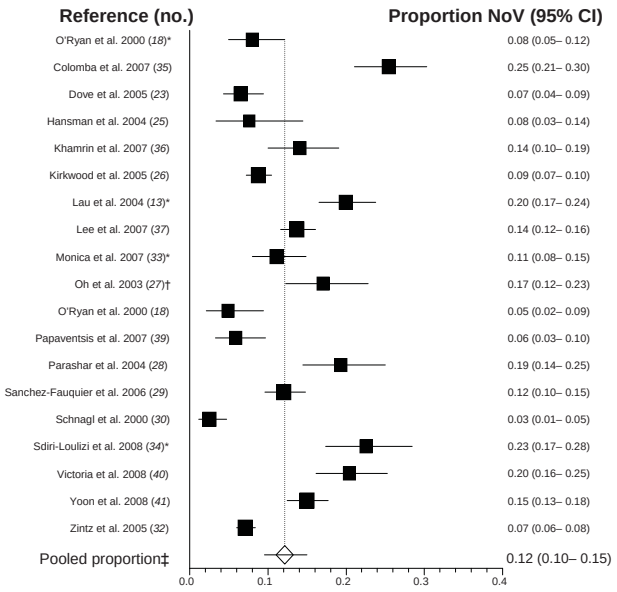

Figure 2. Summary of studies assessing proportion of norovirus (NoV)-positive fecal samples among hospitalized and emergency department cases of children <5 years of age who had sporadic diarrhea. *Lau et al. (*13*), O'Ryan et al. (*18*), Monica et al. (*33*), and Sdiri-Loulizi et al. (*34*) included outpatient and emergency department/hospital patients, but only inpatient data are included in this figure. †Oh et al. (*27*), 98% (213 of 217) of the case-patients were <5 years of age. ‡Pooled proportion calculated using the random effects model (DerSimonian and Laird method (StatsDirect Ltd, Cheshire, UK). For studies that included controls, prevalence of NoV among controls was subtracted from prevalence of NoV among case-patients. CI, confidence interval.

Table 3. Estimates of annual number of episodes of norovirus-associated diarrhea among children <5 years of age in industrialized and developing countries, by setting

Setting	Annual no. diarrhea-associated events*	Pooled proportion of episodes attributable to noroviruses, %	Total no. norovirus episodes	Annual incidence per 100,000 children†‡
Industrialized countries†				
Outpatient	7,743,000	11.7	906,000	1,665
Inpatient	531,000	12.1	64,200	118
Developing countries‡§				
Inpatient	9,015,000	12.1	1,091,000	197
Deaths	1,800,000	12.1	218,000	39

*Refer to Methods section of article.
†Industrialized countries' incidence based on UNICEF 2003 population estimates of 54,425,000 children <5 y of age in developing countries (www.unicef.org/sowc05/english/Developingregional.html).
‡Developing countries' incidence based on UNICEF 2003 population estimates of 552,742,000 children <5 y of age in industrialized (www.unicef.org/sowc05/english/Industrializedregional.html).
§Data from developing countries were sparse on fraction of norovirus-associated diarrhea episodes in the outpatient setting.

than that of the conventional method, especially for samples containing low NoV concentrations or RT-PCR inhibitors (online Technical Appendix 2). The broader application of real-time RT-PCR assays to diagnose NoV among children hospitalized with gastroenteritis should provide better estimates of the true prevalence of disease.

Some factors could have led us to overestimate the extent of sporadic NoV disease. Our estimates of sporadic NoV disease prevalence are based on a review of active surveillance data of all diarrhea cases and do not exclude cases originating from an outbreak. In a few studies, NoVs were detected in patients who were co-infected with another pathogen, and only a limited number of studies enrolled concurrent healthy controls, thus making it difficult to determine the fraction of diarrhea cases truly attributable to NoV. Among all studies testing for co-infections, however, the median rate of detecting another pathogen in addition to NoV was low (2%). This finding, combined with the fact that most studies only assessed for NoV among samples that previously tested negative for other bacterial and viral pathogens, suggests that our overall pooled proportion attributable to NoVs is unlikely to be much lower. In addition, for the studies that enrolled diarrhea-free controls, we subtracted control prevalence from the case prevalence of NoV disease when calculating the overall pooled estimate. Lastly, other unmeasured factors underestimating disease prevalence that could not be accounted for, such as inefficient primers, low virus shedding, delays in specimen collection, and lack of a sensitive case-definition are also likely to exist in these studies.

The heterogeneity in the NoV literature is evident and should be considered when interpreting the results of this review. Our systematic approach and strict inclusion criteria likely reduced heterogeneity but do not eliminate biases in the original studies, diversity in study design and population, and publication bias. Nonetheless, the findings of this review suggest that NoVs are a frequent cause of mild and severe sporadic gastroenteritis among children in high- and middle-income countries. In addition, hospital studies in our review only assessed patients admitted with diarrhea. Because of the highly infectious nature of NoVs, substantial additional health and economic effects would also occur from nosocomial disease and outbreaks in healthcare facilities, as previously identified by Lopman et al (*7*). NoVs are also a frequent cause of severe illness and death from diarrhea among children in developing countries, although firm conclusions cannot be made because of limited data. Systematic evaluations that use broadly reactive, state-of-the-art diagnostic assays, with concurrent evaluation of healthy controls and examination of potential co-infection, are needed to fully understand the role of NoVs in the etiology of sporadic childhood gastroenteritis. These evaluations are especially necessary in developing countries, where diarrhea remains a leading cause of childhood death, causing >1.8 million annual deaths (*1*).

The increasing evidence documenting the magnitude of the NoV disease prevalence provides support for considering targeted interventions, such as vaccines, for reducing the extent of this illness among young children. However, if one considers that NoV frequently causes both sporadic and epidemic gastroenteritis and can affect all age groups, some other potential targets for vaccination may include elderly persons in nursing homes, who are vulnerable to severe complications, and military recruits, in whom sporadic and epidemic NoV disease is known to incur substantial illness and financial costs from work disruption (*7*; online Technical Appendix 2). The development of vaccines against NoVs will likely be challenging because the immunity to these viruses and the diversity and evolution of circulating strains are incompletely understood (online Technical Appendix 2). However, genotype II, cluster 4 NoV strains appeared to be by far the most prevalent strains among the studies we reviewed, and these strains may be the primary targets for vaccine development.

Carefully designed epidemiologic studies that evaluate NoV prevalence in children with diarrhea and a suitable comparison group and that use sensitive molecular assays will help further define target groups that would benefit from

vaccines and other interventions. A particularly pressing need exists for better quantifying the extent of severe norovirus disease among children in developing countries and identifying prevention strategies to help reduce the prevalence of deaths from diarrhea in the poorest countries.

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Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007

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Zika virus (ZIKV) is a mosquito-borne flavivirus first isolated in Uganda from a sentinel monkey in 1947. Mosquito and sentinel animal surveillance studies have demonstrated that ZIKV is endemic to Africa and Southeast Asia, yet reported human cases are rare with <10 cases reported in the literature. In June 2007, an epidemic of fever and rash associated with ZIKV was detected in Yap State, Federated States of Micronesia. We report the genetic and serologic properties of the ZIKV associated with this epidemic.

Zika virus (ZIKV) is a mosquito-transmitted virus in the family *Flaviviridae* and genus *Flavivirus*. It was initially isolated in 1947 from blood of a febrile sentinel rhesus monkey during a yellow fever study in the Zika forest of Uganda (*1*). The virus was subsequently isolated from a pool of *Aedes africanus* mosquitoes collected in 1948 from the same region of the Zika forest; a serologic survey conducted at that time showed that 6.1% of the residents in nearby regions of Uganda had specific antibodies to ZIKV (*1*,*2*).

Over the next 20 years, several ZIKV isolates were obtained from *Aedes* spp. in Africa (*Ae*. *africanus*) and Malaysia (*Ae*. *aegypti*), implicating these species as likely epidemic or enzootic vectors (*3*–*5*). Several ZIKV human isolates were also obtained in the 1960s and 1970s from East and West Africa during routine arbovirus surveillance studies in the absence of epidemics (*6*–*8*). Additional serologic studies in the 1950s and 1960s detected ZIKV infections among humans in Egypt, Nigeria, Uganda, India, Malaysia, Indonesia, Pakistan, Thailand, North Vietnam, and the Philippines (*5*). These data strongly suggest widespread occurrence of ZIKV from Africa to Southeast Asia west and north of the Wallace line.

In 1977, ZIKV infection was confirmed among 7 patients in central Java, Indonesia, during an acute fever study (*9*). Data on these 7 ZIKV cases and several previously reported human infections indicated that clinical characteristics of infection with ZIKV included fever, headache, malaise, stomach ache, dizziness, anorexia, and maculopapular rash; in all cases infection appeared relatively mild, self-limiting, and nonlethal (*6*,*8*–*10*).

In April 2007, an epidemic of rash, conjunctivitis, and arthralgia was noted by physicians in Yap State, Federated States of Micronesia (*11*). Laboratory testing with a rapid assay suggested that a dengue virus (DENV) was the causative agent. In June 2007, samples were sent for confirmatory testing to the Arbovirus Diagnostic Laboratory at the Centers for Disease Control and Prevention (CDC, Fort Collins, CO, USA). Serologic testing by immunoglobulin (Ig) M–capture ELISA with DENV antigen confirmed recent flavivirus infection in several patients. Testing by reverse transcription–PCR (RT-PCR) with flavivirus consensus primers generated DNA fragments, which when subjected to nucleic acid sequencing, demonstrated ≈90% nucleotide identity with ZIKV. These findings indicated that ZIKV was the causative agent of the Yap epidemic. We report serologic parameters of the immune response

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among ZIKV-infected humans, data on estimated levels of viremia, and the complete coding region nucleic acid sequence of ZIKV associated with this epidemic.

Methods

Analysis of Patient Samples

Details of the epidemic, including clinical and laboratory findings for all patients, will be reported elsewhere (M.R. Duffy et al., unpub. data). A subset of ZIKV-infected patients for whom acute- and convalescent-phase paired serum specimens had been collected was analyzed by using several serologic assays to evaluate the extent of cross-reactivity to several related flaviviruses. Patients were classified as primary flavivirus/ZIKV infected or secondary flavivirus/ZIKV probable infected. Primary flavivirus/ZIKV–infected patients were those in whom acute-phase serum specimens (<10 days) had no detectible antibodies (by IgG ELISA and plaque reduction neutralization test [PRNT]) to any of the heterologous flaviviruses tested (Tables 1, 2) and were either IgM-positive in their acute-phase specimen or IgM and IgG positive for ZIKV in a convalescent-phase specimen (seroconversion). Secondary flavivirus/ZIKV probable–infected patients were those who had detectable antibodies to ≥1 heterologous flaviviruses in their acute-phase specimen and were also IgM positive for ZIKV in their acute-phase specimen, or IgM and IgG positive for ZIKV in their convalescent-phase specimen. The designation "ZIKV probable" was used because secondary flavivirus infections demonstrate extensive cross-reactivity with other flaviviruses, and in some cases, higher serologic reactivity to the original infecting flavivirus ("original antigenic sin" phenomenon). Thus, in secondary flavivirus infections shown in Tables 1 and 2, serologic data alone is insufficient to confirm ZIKV as the recently infecting flavivirus. However, these secondary flavivirus/ZIKV probable infections were likely recent ZIKV infections because ZIKV was the only virus detected during the epidemic in Yap, a relatively small and isolated island (*11*).

Serologic Testing

Acute- and convalescent-phase serum samples were tested by IgG ELISA with ZIKV antigen as described for detection of IgG to arboviruses (*12*). Samples were also tested by IgM ELISA as described with the following viral antigens: ZIKV, DENV 1–4 mixture, yellow fever virus (YFV), Japanese encephalitis virus, and Murray Valley encephalitis virus (*13*). Testing for IgM to West Nile virus (WNV) and St. Louis encephalitis virus was performed by using a microsphere immunoassay (*14*). Ratios of

Table 1. IgG and IgM testing with heterologous flaviviruses of patients infected with ZIKV, Yap State, Micronesia, 2007*

		IgG	IgM					
Patient	Days after onset	ZIKV	ZIKV	DENV	YFV	JEV	MVEV	WNV
Primary flavivirus ZIKV								
822a	5	1.5	23.2	1.3	1.4	1.7	1.1	–
822b	10	1.2	39.5	1.2	1.0	2.4	1.2	–
822c	24	3.3	13.1	2.7	0.63	1.8	1.3	–
830a	2	1.1	1.3	4.4	0.48	4.4	2.9	–
830b	21	1.8	16.3	1.9	0.63	1.3	1.6	–
849a	3	1.5	4.5	0.92	0.95	1.2	0.66	–
849b	18	3.0	18.2	2.2	1.0	2.7	1.5	–
862a	6	1.9	25.4	1.7	1.1	1.8	1.0	–
862b	20	2.6	15.4	2	1.1	2.3	1.1	Eq
Secondary flavivirus ZIKV (probable)								
817a	1	5.9	1.4	1.7	0.8	1.7	0.7	–
817b	19	5.7	8.1	5.1	2.1	1.7	1.0	–
833a	1	3.4	1.7	3.7	1.0	2.8	1.3	–
833b	19	8.2	3.1	2.3	0.9	2.5	1.3	–
844a	2	3.8	3.8	6.8	2.0	21.5	0.7	–
844b	16	8.5	12.7	14.9	7.0	42.9	1.6	–
955a	1	5.0	1.8	3.7	1.0	3.4	2.4	Eq
955b	14	26.6	10.9	3.4	0.8	1.7	4.0	Eq
968a	1	4.0	1.7	1.3	0.6	1.2	1.2	–
968b	3	12.3	20.4	2.9	0.8	0.9	2.0	–
839a	3	1	0.92	3.4	0.7	2.7	2.1	–
839b	20	4.9	17.2	2.2	2.1	1.9	1.8	–
847a	5	0.9	0.94	4.1	4.1	2.3	1.3	–
847b	8	14.1	21.5	1.4	3.3	1.1	2.6	–

*Ig, immunoglobulin; ZIKV, Zika virus; DENV, dengue virus type 1–4 mixture; YFV, yellow fever virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; WNV, West Nile virus; –, negative. Eq, result in equivocal range of the assay. IgG and IgM testing was conducted by ELISA except for WNV, which was tested by microsphere assay; ELISA values are patient optical densities divided by negative control optical densities; <2, negative; 2–3 equivocal; >3 positive.

Table 2. Neutralization testing with heterologous flaviviruses of patients infected with ZIKV, Yap State, Micronesia, 2007*

Patient	Days after onset	$PRNT_{90}$ titer									
		ZIKV	DENV1	DENV2	DENV3	DENV4	JEV	YFV	WNV	SLEV	MVEV
Primary flavivirus ZIKV											
822a	5	320	<10	<10	<10	<10	<10	<10	<10	<10	<10
822b	10	2,560	10	10	10	10	<10	<10	<10	<10	<10
822c	24	5,120	10	10	10	10	<10	<10	<10	<10	<10
830a	2	<10	<10	NT‡	NT	NT	NT	NT	NT	NT	NT
830b	21	2,560	<10	<10	<10	<10	<10	<10	<10	<10	<10
849a	3	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
849b	18	10,240	<10	<10	<10	<10	<10	20	<10	<10	<10
862a	6	320	<10	<10	<10	<10	<10	<10	<10	<10	<10
862b	20	2,560	10	10	<10	<10	<10	<10	<10	10	<10
Secondary flavivirus ZIKV (probable)											
817a	1	80	80	160	320	160	<10	<10	<10	40	40
817b	19	10,240	2,560	20,480	5,120	5,120	20	320	160	1,280	640
833a	1	160	320	80	40	20	<10	<10	<10	<10	<10
833b	19	81,920	20,480	5,120	5,120	1,280	<10	<10	80	320	320
844a	2	20	1,280	640	320	160	<10	<10	5	20	20
844b	16	10,240	40,980	10,240	5,120	1,280	5	<10	160	640	640
955a	1	40	1,280	640	160	320	<10	<10	<10	20	20
955b	14	163,840	81,920	20,480	10,240	5,120	10	<10	640	2,560	1,280
968a	1	80	320	320	80	40	<10	<10	<10	40	20
968b	3	10,240	640	640	160	160	<10	<10	10	40	20
839a	3	<10	<10	10	<10	<10	<10	40	<10	<10	<10
839b	20	10,240	40	320	80	80	<10	640	40	80	80
847a	5	<10	<10	<10	<10	<10	<10	640	<10	<10	<10
847b	8	2,560	40	320	160	40	<10	1,280	80	320	320

*$PRNT_{90}$ titer, 90% plaque reduction neutralization test titer; ZIKV, Zika virus; DENV, dengue virus; JEV, Japanese encephalitis virus; YFV, yellow fever virus; WNV, West Nile virus; SLEV, St. Louis encephalitis virus; MVEV, Murray Valley encephalitis virus; NT, not tested (sample depleted).

patient optical density values to negative control values (P/Ns) were calculated for IgG and IgM ELISAs. Values >3 were considered positive, and values 2–3 were considered equivocal. Neutralizing antibody titers were determined by using a PRNT with a 90% cut-off value (*15*).

Real-Time RT-PCR

Two real-time primer/probe sets specific for the ZIKV 2007 strain were designed by using ZIKV 2007 nucleotide sequence data in the PrimerExpress software package (Applied Biosystems, Foster City, CA, USA). Primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA) with 5-FAM as the reporter dye for the probe (Table 3). All real-time assays were performed by using the QuantiTect Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) with amplification in the iCycler instrument (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Specificity of the ZIKV primers was evaluated by testing the following viral RNAs, all of which yielded negative results: DENV-1, DENV-2, DENV-3, DENV-4, WNV, St. Louis encephalitis virus, YFV, Powassan virus, Semliki Forest virus, o'nyong-nyong virus, chikungunya virus, and Spondweni virus (SPOV).

Sensitivity of the ZIKV real-time assay was evaluated by testing dilutions of known copy numbers of an RNA transcript copy of the ZIKV 2007 sequence. Copy numbers of RNA were determined by using the Ribogreen RNA-specific Quantitiation Kit (Invitrogen) and the TBE-380

Table 3. Description and performance characteristics of Zika virus real-time RT-PCR primer/probe sets*

Primer	Genome position†	Sequence (5′ → 3′)	Sensitivity, no. copies	Specificity‡
ZIKV 835	835–857	TTGGTCATGATACTGCTGATTGC		
ZIKV 911c	911–890	CCTTCCACAAAGTCCCTATTGC	100	ZIKV
ZIKV 860-FAM	860–886	CGGCATACAGCATCAGGTGCATAGGAG		
ZIKV 1086	1086–1102	CCGCTGCCCAACACAAG		
ZIKV 1162c	1162–1139	CCACTAACGTTCTTTTGCAGACAT	25	ZIKV
ZIKV 1107-FAM	1107–1137	AGCCTACCTTGACAAGCAGTCAGACACTCAA		

*RT-PCR, reverse transcription–PCR; ZIKV, Zika virus.
†Based on ZIKV MR 766 GenBank accession no. AY632535.
‡ZIKV specificity indicates a positive result with ZIKV only and no reactivity with dengue virus-1 (DENV-1), DENV-2, DENV-3, DENV-4, West Nile virus, St. Louis encephalitis virus, yellow fever virus, Powassan virus, Semliki Forest virus, o'nyong-nyong virus, chikungunya virus, and Spondweni virus.

mini-fluorometer (Turner Biosystems, Sunnyvale, CA, USA). RNA transcripts ranging from 16,000 to 0.2 copies were tested in quadruplicate to determine the sensitivity limit and to construct a standard curve for estimating the genome copy number of ZIKV in patient samples. All serum samples obtained during the epidemic were tested for ZIKV RNA by using this newly designed real-time RT-PCR. Concentration of viral RNA (copies/milliliter) was estimated in ZIKV-positive patients by using the standard curve calculated by the iCycler instrument (Table 4). All RT-PCR–positive specimens were placed on monolayers of Vero, LLC-MK2, and C6/36 cells to isolate virus; no specimens showed virus replication.

Table 4. Results of quantitative real-time RT-PCR of samples from ZIKV-positive patients, Yap State, Micronesia, 2007*

Patient	Days after onset	ZIKV real-time RT-PCR			
		Ct-860†	Ct-1107†	Result	Estimated copies/mL‡
824	1	34.3	34.7	+	11,647
939	2	32.0	32.4	+	67,817
947	2	34.3	33.9	+	21,495
949	2	35.1	35.1	+	8,573
969	1	29.4	29.3	+	728,800
037	1	32.1	32.5	+	62,816
830a	2	30.7	30.0	+	426,325
847a	5	34.8	34.7	+	11,647
950a	0	32.2	32.7	+	53,894
943	3	37.6	35.6	+	5,845
952	1	29.3	29.5	+	625,280
958	11	29.9	30.3	+	338,797
970	1	35.5	34.8	+	10,788
42	0	32.9	33.6	+	27,048
941	3	31.1	38.0	+	930
964	0	38.3	37.6	+	1,263
063a	2	37.5	38.0	+	930

*RT-PCR, reverse transcription–PCR; ZIKV, Zika virus; Ct, crossing threshold; +, positive.
†Ct values with primer set 835/911c/860-FAM or 1086/1162c/1107-FAM. Values <38.5 are positive.
‡Estimated by testing quantitated dilutions of ZIKV RNA transcripts and standard curve calculation generated by the iCycler instrument (Bio-Rad, Hercules, CA, USA; see Methods).

Nucleic Acid Sequencing and Phylogenetic Analysis

RNA was extracted from patient samples that demonstrated the highest concentration of ZIKV RNA determined by the real-time assay, and for which sufficient sample volume was available (patients 824, 037, 830a, and 958). Briefly, RNA was extracted from 150 μL of serum by using the QIAamp Viral RNA Mini Kit (QIAGEN), and RNA was eluted with 75 μL of RNase-free water. A series of RT-PCRs was performed with each RNA preparation by using primer pairs designed to generate overlapping DNA fragments that spanned the entire polyprotein coding region of the virus. Primers were designed by using the ZIKV MR 766 prototype virus coding region sequence (GenBank accession no. AY632535) and the PrimerSelect software module of the LaserGene package (DNASTAR Inc., Madison, WI, USA). Several primers initially failed to amplify because of sequence mismatches between ZIKV MR 766 and ZIKV Yap 2007. Therefore, primers were redesigned by using newly generated DNA sequence data, and a "genome walking" approach was used to derive complete coding region sequence data. The complete list of amplification and sequencing primers is available upon request.

All RT-PCRs were performed with 10 μL of RNA by using the OneStep RT-PCR Kit (QIAGEN) following the manufacturer's protocol. DNAs were analyzed by 2% agarose gel electrophoresis, and bands of the predicted size were excised from the gel and purified by using the QIAquick Gel Extraction Kit (QIAGEN). Purified DNAs were subjected to nucleic acid sequence analysis with sequencing primers spaced ≈500 bases apart on both strands of the DNA fragments by using the ABI BigDye Terminator V3.1 Ready Reaction Cycle Sequencing Mixture (Applied Biosystems). Nucleotide sequence was determined by capillary electrophoresis by using the ABI 3130 genetic analyzer (Applied Biosystems) following the manufacturer's protcol. Raw sequence data were aligned and edited by using the SeqMan module of LaserGene (DNASTAR Inc.). Because of insufficient sample volume, no patient RNA was sufficient to generate DNA that included the entire coding region. Therefore, DNA data obtained from 4 patients was combined to generate a consensus sequence heretofore designated the ZIKV 2007 epidemic consensus (EC) sequence (GenBank accession no. EU545988).

The complete coding region of ZIKV 2007 EC or the nonstructural protein 5 (NS5) gene subregion was aligned with all available flavivirus sequences in GenBank by using the Clustal W algorithm within the MEGA version 4 software package (www.megasoftware.net). Phylogenetic trees were constructed by using either the complete coding region or the NS5 region because a large number of NS5 sequences were available in GenBank and trees for the NS5 region have been constructed (*16*). Additional ZIKV strains from the CDC/World Health Organization reference collection (strains 41662, 41524, and 41525) isolated from *Aedes* spp. mosquitoes collected in Senegal in 1984 were also amplified by RT-PCR in the NS5 region and subjected to nucleic acid sequencing as described above and included in the NS5 region analysis. Trees were constructed from coding region data or from NS5 data by MEGA 4 from aligned nucleotide sequences. We used maximum parsimony, neighbor-joining, or minimum evolution algorithms with 2,000 replicates for bootstrap support of tree groupings. All trees generated nearly identical topology; only the neighbor-joining NS5 tree is shown (Figure 1).

Results

Serologic Analysis

Tables 1 and 2 show results of analysis for IgG and IgM and PRNTs of all acute- and convalescent-phase paired specimens obtained during the epidemic. Specimens were divided into primary and secondary infections on the basis of antibody testing results of acute-phase specimens. IgM antibody response in primary flavivirus/ZIKV–infected patients was specific for ZIKV. However, all of these patients showed some limited degree of cross-reactivity with heterologous flaviviruses. Patient 830a showed IgM-positive results with DENV and Japanese encephalitis virus, whereas all patients showed equivocal results (P/N 2–3) with several of the flaviviruses tested, suggesting low levels of cross-reactivity. $PRNT_{90}$ results also showed that the neutralizing antibody response among primary flavivirus/ZIKV–infected patients was highly specific. Most convalescent-phase PRNT titers for heterologous flaviviruses were negative and rarely exceeded 10 (20 in 1 instance; patient 849b).

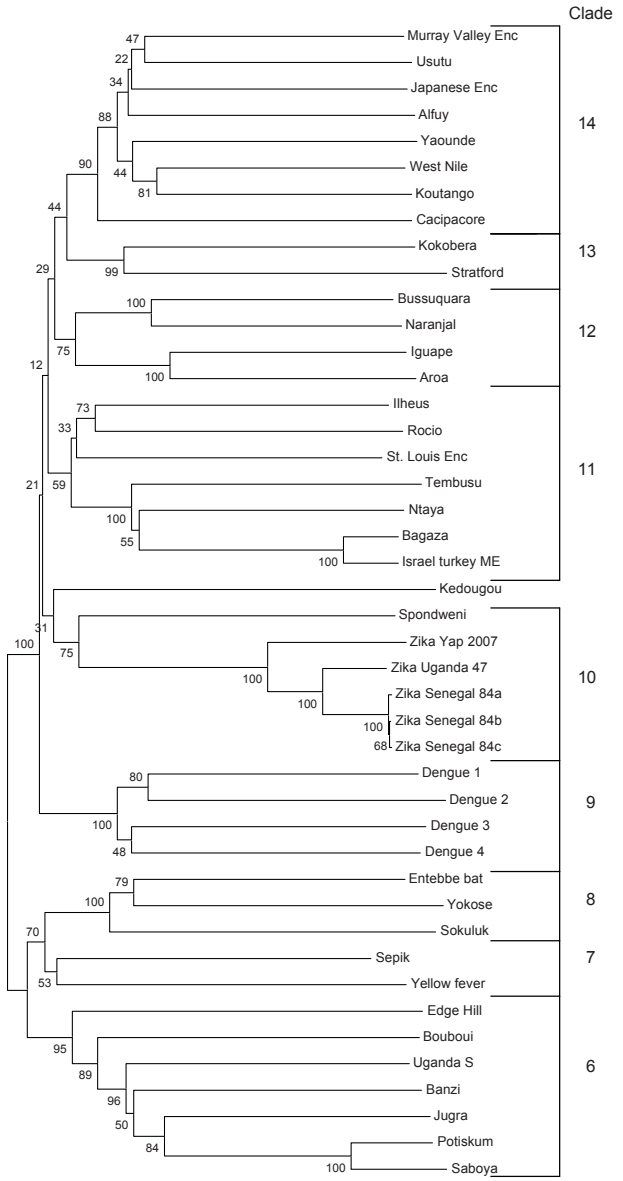

Figure 1. Phylogenetic tree constructed from nucleic acid data from flavivirus nonstructural protein 5 region by the neighbor-joining algorithm in MEGA (www.megasoftware.net). Numbers to the left of the nodes are bootstrap percentages (2,000 replications) for clades. Clade numbers correspond to clades identified by Kuno et al. (*16*). Enc, encephalitis; ME, meningoencephalitis.

Most patient specimens from the Yap epidemic tested were secondary flavirius infections as determined by criteria described for antibody to flavivirus in acute-phase specimens. A subset of these patients for whom acute- and convalescent-phase specimens were available was tested for reactivity against heterologous flaviviruses; results are shown in Tables 1 and 2. In contrast to primary flavivirus/ZIKV–infected patients, secondary flavivirus–infected patients showed a high degree of serologic cross-reactivity with other flaviviruses. Six of 7 patients were positive for IgM against ≥1 of the heterologous flaviviruses tested, and all demonstrated low levels of cross-reactive IgM as shown by a P/N value in the equivocal range. $PRNT_{90}$ results showed that among secondary flavivirus/ZIKV–probable patients, the neutralizing antibody response was higher to ZIKV and more cross-reactive, a finding commonly observed among secondary flavivirus infections. A ≥4-fold $PRNT_{90}$ titer between ZIKV and heterologous flaviviruses was observed in only 3 of the 7 patients. In all other cases, the PRNT difference between ZIKV and other flaviviruses tested was ≤2-fold; in 2 patients (817b and 844b) the PRNT titer was higher for 1 of the heterologous flaviviruses. The PRNT result for the acute-phase specimen from patient 847 suggests previous vaccination with YFV. The convalescent-phase specimen from patient 847 showed a high titer to YFV, a demonstration of the previously described "original antigenic sin" phenomenon observed among flaviviruses (*17*).

Real-Time RT-PCR

A real-time RT-PCR was developed by using newly derived sequence data obtained from several ZIKV-infected patients. All acute-phase specimens obtained during the Yap epidemic (n = 157) were tested in this assay with 2 unique primer/probe sets. Seventeen samples were positive, 10 were equivocal, and 130 were negative (data not shown). The equivocal designation indicates that a particular sample was positive by only 1 of the 2 primer sets or showed crossing thresholds >38.5, which suggests either a false-positive result or a sample with low levels of ZIKV RNA below the defined cut-off of the assay. Table 4 shows estimated viral concentrations of the 17 ZIKV-

positive specimens. The viral RNA concentrations were ≈900–729,000 copies/mL. Most (15 of 17) of the ZIKV-positive samples were from specimens collected ≤3 days after onset; however, 1 specimen (patient 958) collected on day 11 after onset was positive with an estimated titer of ≈339,000 copies/mL.

Nucleic Acid Sequence and Phylogenetic Analysis

Several RT-PCR–positive serum specimens were selected, and RNA was amplified by RT-PCR to generate DNA sequence data for the complete coding region. Because of limited specimen volume, the complete coding region genome sequence was only obtainable by combining sequence data from DNA fragments generated from 4 patients. Thus, the designation EC sequence is used to indicate that the sequence was derived from multiple patients during the epidemic. The exact contribution of sequence data from each patient is available upon request. However, the following points should be noted. Approximately 96% of the complete coding region was obtained from 3 patients; sequence data from the fourth patient was used primarily to fill in short gaps in the data. Second, ≈50% of the coding region data was derived from a complete overlap of data from ≥2 patients; in these overlap regions the sequence identity between different patients was ≈100%. Only 2-nt differences between patients were noted within the overlapping regions, strongly suggesting that 1 ZIKV strain circulated during the epidemic.

Percentage identity over the entire coding region of ZIKV 2007 EC sequence, when compared with the prototype ZIKV (MR 766, isolated in 1947), was 88.9% and 96.5% at the nucleotide and amino acid levels, respectively. Phylogenetic trees constructed from the complete coding region of all available flaviviruses generated by a variety of methods (neighbor-joining, maximum-parsimony, or minimum-evolution) showed the same overall topology, with the ZIKV prototype and 2007 EC virus placed in a unique clade (clade 10) within the mosquito-borne flavivirus cluster previously described by Kuno et al. (*16*). Alignment with phylogenetic tree construction by neighbor-joining, maximum-parsimony, or minimum-evolution algorithms was also performed for the NS5 region of all available flaviviruses because extensive sequencing and phylogenetic analysis have been conducted for this region (*16*).

Three additional ZIKV strains isolated from Senegal in 1984 and sequenced in this study were also included in a tree. This NS5 tree demonstrated similar topology to the complete coding region tree, with all ZIKVs placed within a unique clade (clade 10) along with SPOV. Figure 1 shows the NS5 tree with only mosquito-borne flaviviruses (cluster) displayed. This NS5 tree also shows that within the Zika/Spondweni clade there appear to be 3 branches among ZIKVs: Nigerian ZIKVs, prototype MR766, and 2007 Yap virus. Percentage identity among these ZIKVs confirms the tree topology, in which ZIKV 2007 EC is most distally related to East and West African ZIKV strains (data not shown).

The predicted amino acid sequence of ZIKV 2007 EC contains the Asn-X-Ser/Thr glycosylation motif at position 154 in the envelope glycoprotein, found in many flaviviruses, yet absent by deletion in the prototype ZIKV MR 766. This region of the prototype virus, along with 3 ZIKVs isolated from Senegal in 1984, was sequenced (Figure 2). Included in this alignment is a ZIKV isolate from GenBank (accession no. AF372422). Sequencing confirmed that prototype ZIKV MR766 has a 4-aa (12-nt) deletion when compared with ZIKV 2007 EC virus and ZIKVs from Senegal.

Discussion

Historically, ZIKV has rarely been associated with human disease, with only 1 small cluster of human cases in Indonesia reported (*9*). We report a widespread epidemic of human disease associated with ZIKV in Yap State in 2007. ZIKV epidemics may have occurred but been misdiagnosed as dengue because of similar clinical symptoms and serologic cross-reactivity with DENVs. Our serologic data indicate that ZIKV-infected patients can be positive in an IgM assay for DENVs, particularly if ZIKV is a secondary flavivirus infection. If ZIKV is the first flavivirus encountered, our data indicate that cross-reactivity is minimal. However, when ZIKV infection occurs after a flavivirus infection, our data indicate that the extent of cross-reactivity in the IgM assay is greater. Therefore, if ZIKV infections occur in a population with DENV (or other flavivirus) background immunity, our data suggest that extensive cross-reactivity in the dengue IgM assay will occur, which could lead to the erroneous conclusion that dengue caused the epidemic. Whether this cross-reactivity has occurred is open to speculation. However, reexamination of specimens from dengue epidemics may provide an answer. In addition, use of virus isolation or RT-PCR for laboratory diagnosis of dengue infections would also prevent this misinterpretation. Therefore, use of virus detection assays

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                          1321
Zika MR766 1947 Uganda    ATGATTGGA-----------TATGAAACTGACGAAGATAGAGCG
Zika AF372422             ATGATTGTTAATGAT-----------------GAAAACAGAGCA
Zika 41662 Senegal 1984   ATGATTGTGAATGACACAGGACATGAAACTGACGAAAACAGAGCA
Zika 41524 Senegal 1984   ATGATTGTGAATGACACAGGACATGAAACTGACGAAAACAGAGCA
Zika 41525 Senegal 1984   ATGATTGTGAATGACACAGGACATGAAACTGACGAAAACAGAGCA
Zika 2007 EC Yap          ATGATCGTTAATGACACAGGACATGAAACTGATGAGAATAGAGCG

                          ENV-151                              ENV-164
Zika MR766 1947 Uganda    MetIleGly-----------TyrGluThrAspGluAspArgAla
Zika AF372422             MetIleValAsnAsp-----------------GluAsnArgAla
Zika 41662 Senegal 1984   MetIleValAsnAspThrGlyHisGluThrAspGluAsnArgAla
Zika 41524 Senegal 1984   MetIleValAsnAspThrGlyHisGluThrAspGluAsnArgAla
Zika 41525 Senegal 1984   MetIleValAsnAspThrGlyHisGluThrAspGluAsnArgAla
Zika 2007 EC Yap          MetIleValAsnAspThrGlyHisGluThrAspGluAsnArgAla
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Figure 2. Alignment of nucleotide and amino acid sequences adjacent to the envelope (ENV)–154 glycosylation site of Zika virus strains. Dashes indicate deletions. EC, epidemic consensus.

in dengue epidemics should be a component of laboratory testing algorithms.

Levels of viremia among ZIKV-infected patients were relatively low. Unfortunately, measurement of concentration of infectious ZIKV was not possible because a virus isolate was not obtained from any patient during the epidemic. Absence of a ZIKV 2007 isolate also precluded use of a ZIKV 2007 isolate to generate a standard curve in the RT-PCR, which in turn could have estimated the concentration of infectious virus within patients. An estimation of the number of genome copies circulating in ZIKV-infected patients was calculated by using an RNA transcript and provides some indication of infectious virus concentration in ZIKV-infected patients. If one assumes a ratio range of 200–500 genome copies per infectious virus particle, a range reported for several flaviviruses, then the copies/milliliter values in Table 4 would be in the range of ≈2–3,500 infectious virus particles/mL, with only 4 specimens in which ZIKV exceeded 1,000 infectious units/mL (*18*,*19*). These findings may partially explain why ZIKV was not isolated, especially if one considers that shipping samples to our laboratory took ≈1 week, and shipping conditions were not conducive to virus isolation. These concentration estimates are also consistent with those of a study in which a ZIKV-infected human volunteer showed low viremia; virus was isolated only on day 4, and the volunteer was unable to infect *Ae*. *aegypti* mosquitoes that fed on the patient during the acute stage of disease (*10*).

Although generation of a complete coding region nucleic acid sequence by using a combination of patient samples from the epidemic is an unconventional approach, it was performed out of necessity because of limited volumes of patient samples. However, the extent of agreement among overlapping regions confirms that the sequence obtained accurately represents the virus associated with the epidemic. Nucleic acid sequence of ZIKV 2007 showed divergence (11%) from the prototype strain (MR766) isolated in 1947. However, the predicted amino acid sequence is fairly conserved (96%), which is likely the result of the selective pressure maintained on the virus because replication occurs in vertebrate hosts and arthropod vectors.

Phylogenetic trees based on the complete coding region or the NS5 region confirm results of a study in which ZIKV was classified in a unique clade among the mosquito-borne flaviviruses and most closely related to SPOV (*16*). The NS5 mosquito-borne flavivirus tree (Figure 1), which includes additional ZIKV isolates, confirms these relationships and suggests that there are 3 subclades among ZIKV isolates that reflect geographic origin. Senegal ZIKVs and prototype virus from Uganda may represent West and East African lineages, respectively. The 2007 ZIKV is distantly related to these 2 African subclades and may represent divergence from a common ancestor with spread throughout Southeast Asia and the Pacific. Human ZIKV cases were detected in peninsular Malaysia in 1980, which confirms that ZIKV was active in this region before 2007 (*9*). Additional sequence analysis of other temporally and geographically distinct ZIKV strains is needed to further elucidate relationships among these viruses.

Of particular interest is an additional 12 nt in the envelope gene (corresponding to 4 aa) in our ZIKV isolate that were not present in the ZIKV prototype virus (Figure 2). This difference is noteworthy because these 4 aa correspond to the envelope protein 154 glycosylation motif found in many flaviviruses and associated in some instances with virulence. This glycosylation motif is also absent because of a 6-aa deletion in the ZIKV isolate obtained from GenBank (accession no. AF372422); however, the geographic and temporal origins of this virus were not available. Loss of the envelope protein 154 glycoslyation site has been observed in some flaviviruses, and in the case of Kunjin virus has been shown to occur during passage. However, with Kunjin virus, the glycosylation site motif was lost because of a 1-base mutation, rather than a deletion, that altered the N-X-S/T sequon (*20*). Loss of this glycosylation site by a 4-aa deletion has also been observed in several lineage-2 WNV strains when compared with all other WNV strains (*21*).

The glycoslyation motif in WNV may be lost during extensive mouse brain passage; however, no direct evidence exists to support this hypothesis (*21*). This process may occur in ZIKV; the glycoslyation motif in MR 766 may have been present in earlier passages of prototype MR766 and lost during extensive mouse brain passage. However, earlier passage strains of MR766 were not available for investigating this hypothesis. Alternatively, the presence or absence of this glycosylation motif may represent an ancient evolutionary event with subsequent divergence of 2 ZIKV types with or without the E-154 glycosylation site amino acids. Sequence data derived from 3 additional ZIKV isolates from Senegal showed that glycosylation is intact in these isolates, which suggests evolutionary divergence. More extensive sequence analysis of available ZIKV strains of various temporal, geographic, and passage histories may provide some insight into this issue.

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Dr Lanciotti is chief of the Diagnostic and Reference Laboratory within the Arbovirus Diseases Branch at CDC. His primary research interests are laboratory diagnosis of arbovirus infections and diagnostic test development and support for public health laboratories worldwide.

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Interepidemic Rift Valley Fever Virus Seropositivity, Northeastern Kenya

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Most outbreaks of Rift Valley fever (RVF) occur in remote locations after floods. To determine environmental risk factors and long-term sequelae of human RVF, we examined rates of previous Rift Valley fever virus (RVFV) exposure by age and location during an interepidemic period in 2006. In a randomized household cluster survey in 2 areas of Ijara District, Kenya, we examined 248 residents of 2 sublocations, Gumarey (village) and Sogan-Godud (town). Overall, the RVFV seropositivity rate was 13% according to immunoglobulin G ELISA; evidence of interepidemic RVFV transmission was detected. Increased seropositivity was found among older persons, those who were male, those who lived in the rural village (Gumarey), and those who had disposed of animal abortus. Rural Gumarey reported more mosquito and animal exposure than Sogan-Godud. Seropositive persons were more likely to have visual impairment and retinal lesions; other physical findings did not differ.

Rift Valley fever (RVF) is a mosquito-borne zoonosis that is expanding its range in Africa and the Middle East. Economic effects can be catastrophic for meat and dairy producers, e.g., high illness and mortality rates among affected livestock herds (*1*,*2*) prompting World Organization for Animal Health–mandated international embargoes of livestock exports. These epidemics are even more devastating for pastoral nomads and local herders; many adult animals can die, affecting the next crop of newborns and the survival of locals who are economically and physically dependent on milk and meat during the epidemic. During large RVF outbreaks, extensive numbers of human infections occur as well, leading to substantial healthcare challenges in resource-limited settings. RVF symptoms in persons are typically fever, myalgia, and malaise; in a noteworthy minority of cases retinitis, encephalitis, hemorrhagic fever, and death occur. Overall mortality rate is ≈1% (*3*,*4*).

RVF is caused by the phlebovirus, Rift Valley fever virus (RVFV), which was originally isolated in Kenya and is endemic to other countries of East Africa, South Africa, and the Senegal River valley (*3*,*5*–*7*). The virus, introduced repeatedly into Egypt since the 1970s, and most recently into the Arabian peninsula (Yemen and Saudi Arabia) in 2000 (*8*–*10*), is embedded in ecosystems by vertical transmission in certain floodwater *Aedes* mosquito species (*1*). Consequently, RVF outbreaks are strongly linked to excessive rainfall and local flooding. The most recent Kenyan Rift Valley fever outbreak occurred during El Niño rains from November 2006 through April 2007 (*11*,*12*). The largest RVF outbreak in Kenya took place in an El Niño–related flooding period in 1997–1998 (*13*). Even within different climate zones, RVFV transmission may vary considerably as a function of fine-scale differences in local environment.

Evidence of prior RVFV infection can be tested by ELISA for anti-RVFV immunoglobulin (Ig) G (*14*,*15*). Earlier studies have shown that RVFV seroprevalence in Kenyan populations has been as high as 32% in high-risk areas during epidemics (*13*). During interepidemic periods, observed community RVFV seroprevalence rates have ranged from 1% to 19% in different settings within Kenya (*16*).

Because RVF outbreaks typically occur in remote locations under extreme weather conditions, relatively little is known about the underlying health status of at-risk communities. Likewise, debate continues regarding the likely

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dominant mode of animal-to-human transmission during combined epizootics and epidemics. RVFV reemergence, caused by floodwater mosquitoes, is followed by widespread amplification in high-risk animal populations and progressively greater prevalence among animals. When epizootic conditions are right, additional mosquito species will feed on viremic animals and subsequently transmit RVFV to humans, creating a potential epidemic. Humans can also become infected through exposure to infectious animal tissues or bodily fluids such as abortus, birthing fluids, milk, or blood. Among pastoral nomads and other herders in the semiarid regions of Africa, family members could be differentially exposed depending on traditional gender-specific duties, thereby altering the risk-modifying effects of age or gender. Specific types of animal exposure that are the most risky, and important nonanimal exposures have not yet been elucidated. Knowing which forms of exposure provide the greatest RVFV transmission risk may be useful for endemic or epidemic public health education and for targeting interventions (such as animal vaccination) that can decrease infection or illness during an epidemic. The goals of this study were to 1) determine the baseline human population health status in an area that has suffered repeated RVF outbreaks; 2) identify which animal and nonanimal exposures are associated with RVFV seropositivity; 3) evaluate whether seropositivity, exposures, and risks differ among town and village settings in a high-risk region of northeastern Kenya; and 4) assess whether interepidemic human RVFV transmission occurs.

Materials and Methods

Location

Our study was a location-stratified household-based cluster sampling of human populations residing in 2 areas near Masalani Town, Ijara District, situated in a semiarid region of Northeastern Province, Kenya. The study was performed in March and April 2006, ≈8.5 years after the previous RVF outbreak of 1997–1998, and well before the floods during the fall of 2006 that were associated with the most recent RVF epizootic/epidemic. On the basis of our study objectives, the balanced sampling frame for selection of the planned 250 participants was divided between a rural village, Gumarey (centered at 1° 40′12″S, 40°10′48″E), and a town, Sogan-Godud (centered at 1°41′24″S, 40°10′12″E). Both are sublocations defined within the Kenya Census and are located within 500 m of each other and within 10 km of the Tana River, which is prone to flooding during periods of excessive rainfall. Flatness of the local terrain, combined with poor drainage, makes the area a prime environment for RVFV transmission during floods, as evidenced by ongoing RVF outbreaks. Gumarey has a largely seminomadic pastoralist population, and local homes are traditional grass huts. Sogan-Godud is a larger town with more permanent tin-roofed dwellings and stores (Figure 1).

Population

Study recruitment was begun after consultation and approval by local administrators and religious leaders. After an initial demographic census was conducted to determine the current local population and its distribution, 270 survey participants were selected by randomized cluster sampling of households in the 2 designated subsections of Masalani town. Children <1 year of age and those residing in the area <2 years were excluded. All adult participants provided informed consent. Parents provided informed consent for participating children; children >7 years of age provided individual assent. The study sample comprised a locally representative ethnic mix of >99% Somali or Bantu and <1% Indian or other Asian. Participating households were sampled by using a probability proportionate to size approach. Nonparticipating households were substituted for

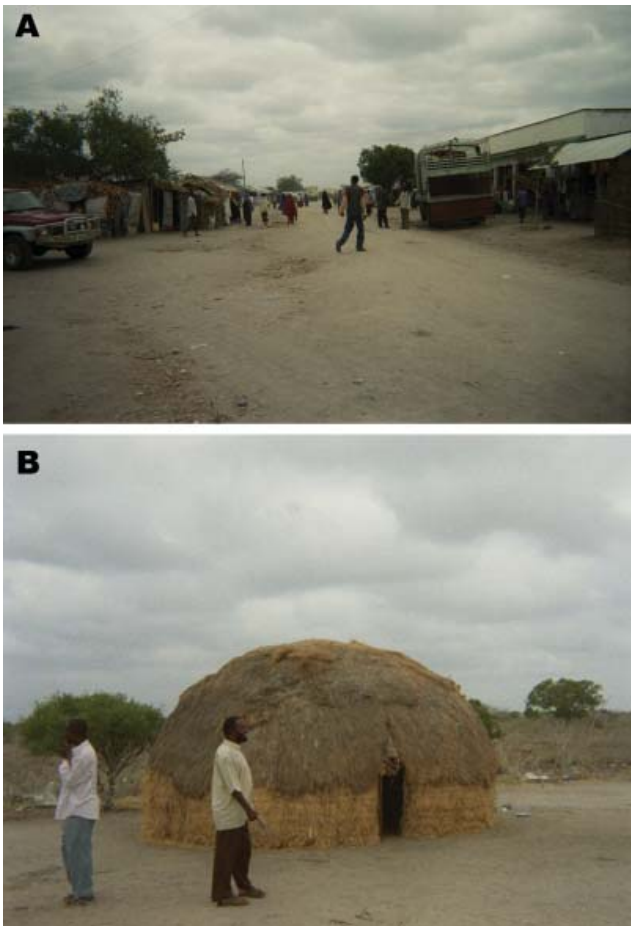

Figure 1. Photographs depicting differences between sublocations in northeastern Kenya. Sogan-Godud (A) has more permanent dwellings and stores with tin-roofed buildings. Gumarey (B) has more semipermanent traditional dwellings and animal grazing areas.

by using additional, randomly-selected households chosen according to sampling rules established at the outset of the survey.

Examination Procedures

Study participants received a structured interview regarding housing, animal exposure, motor function, visual function, and recent or remote RVF-related symptoms (questionnaire in online Technical Appendix 1, available from www.cdc.gov/EID/content/14/8/1240-Techapp1.pdf; accompanying parents served as proxies for children when necessary). Participants also received a complete physical examination, a vision test and indirect ophthalmoscopic examination for signs of current or previous retinal inflammation, and phlebotomy (i.e., 5 mL venous blood samples from persons ≥5 years of age and 1 mL from children <5).

Laboratory Testing

The primary measure of RVFV exposure was seropositivity, indicated by serum anti-RVFV IgG detection using ELISA. Specimens were screened for the presence of anti-RVFV IgG by ELISA by using lysates of Vero cells infected with the MP-12 strain (vaccine strain) of RVFV as the test antigen and lysates of mock-infected cells as the internal control antigen. This assay has been established and validated in previous survey studies (*15,16*). Serum samples diluted 1:100 were read at 405 nm; those with an optical density (OD) value (corrected for reactivity on normal cell antigen) > mean + 2 standard deviations for control serum and absolute value >0.2 were deemed positive. Each sample was run in duplicate, and OD values were averaged. Any OD discrepancy between duplicate tests was resolved by repeat testing. Pooled RVFV-positive serum samples were used as the positive plate controls, and pooled RVFV-negative North American serum samples were used as the negative plate controls. Serologic screening was performed at the Division of Vector-Borne Diseases in Nairobi and confirmed at Case Western Reserve University; correlation of results was excellent. Confirmatory plaque reduction neutralization test (PRNT) was performed at University of Texas Medical Branch at Galveston to assess the risk of false-positive results secondary to ELISA cross-reactivity with related viruses. Confirmatory testing using PRNT was performed on all positive samples (n = 33) and an age- and location-matched set of negative samples (n = 33) (*17*). All ELISA-positive samples had PRNT titers ≥80; most had titers of 320. All but 1 ELISA-negative sample had titers <10. This apparently false-negative sample had a PRNT titer of 80 on repeated testing.

Statistical Analysis

Initial univariate analysis was conducted to describe demographic variables (online Appendix Table 1, available from www.cdc.gov/EID/content/14/8/1240-appT1.htm) Bivariate analysis was based on χ^2 test (or Yates correction to the χ^2 test where appropriate) of several potential predictors of RVFV seropositivity (online Appendix Table 1) as well as bivariate comparisons between villages (online Appendix Table 2, available from www.cdc.gov/EID/content/14/8/1240-appT2.htm). After initial bivariate analysis of RVFV-seropositivity outcomes, predictor variables were further tested for association with RVFV seropositivity by using multivariable logistic regression. Data for all 248 participants were modeled by using predictor variables that had been determined by bivariate analysis to be associated with RVFV seropositivity (online Technical Appendix 2, available from www.cdc.gov/EID/content/14/8/1240-Techapp2.pdf). Logistic models were also constructed by village to determine local predictors of RVFV seropositivity (online Appendix Table 3, available from www.cdc.gov/EID/content/14/8/1240-appT3.htm). Individual predictors were tested for multicolinearity by using χ^2 test. Hosmer-Lemeshow goodness-of-fit χ^2 values were calculated for all logistic models and indicated that model predictors sufficiently described the observed data (online Technical Appendix 2 and online Appendix Table 3). All bivariate analysis and logistic modeling was initially performed by using R software version 2.3.1 (www.r-project.org/index.html) and confirmed by using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Ethical Considerations

This study was performed under a human research protocol approved by the Human Investigations Review Board of University Hospitals of Cleveland and the Ethical Review Committee of the Kenya Medical Research Institute. It is registered as Clinical Trial NCT00287014 and available from www.clinicaltrials.gov.

Results

Survey Results

A total of 270 potential participants were invited to participate; they were selected by randomized cluster sampling of 66 households in the 2 designated administrative sublocations of Masalani Town in Ijara district. Of this selected sample, 248 (91.9%) completed all study procedures, including serum testing (online Appendix Table 1).

The final study cohort comprised 248 participants, of whom 33 (13%, 95% confidence interval [CI] 9.3–18.1) were RVFV seropositive. Of the 248, 122 (49%) were from Gumarey, and of these, 25 (20%, 95% CI 14.0–29.2) were seropositive, and 126 (51%) were from Sogan-Godud, and of these, 8 (6%, 95% CI 2.7–11.8) were seropositive (Figure 2). Of all samples, 118 (47.6%) were from children ≤15 years of age, and 4 of the 118 (3.4%) were seroposi-

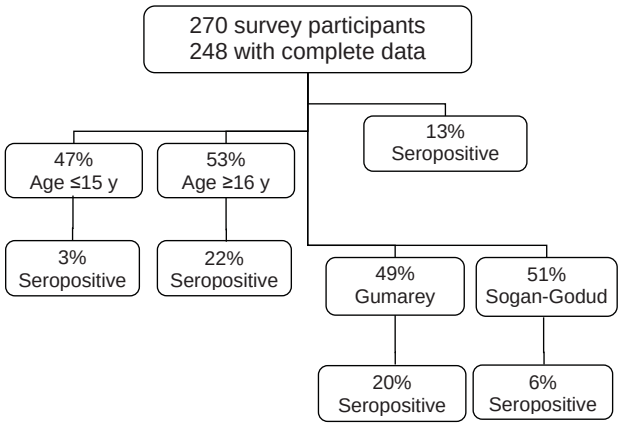

Figure 2. Flowchart of northeastern Kenya Rift Valley fever virus study participants.

tive. These 4 youngest seropositive participants were 4, 12, 13, and 14 years of age, and all were long-term permanent residents of the study area. Of the 130 adults in the sampled cohort, 29 (22.3%) had positive anti-RVFV IgG results; the oldest was 81 years of age.

Links between Past Exposure and Seropositivity

Many exposures, both nonanimal and animal, were associated with RVFV seropositivity (online Appendix Table 1). In bivariate statistical analyses, RVFV seropositivity varied significantly according to the following factors: age (participants >15 years of age were more at risk, p = 0.0001), gender (male participants were more at risk, p = 0.011), location (those from Gumarey were more at risk, p = 0.001), home flooding (p = 0.024); contact with a dead human body (p = 0.0001); contact with cattle (p = 0.012); and involvement in sheltering (p = 0.003), butchering (p = 0.0001), skinning (p = 0.0001), cooking (p = 0.005), milking (p = 0.0001), birthing livestock (p = 0.0001), or disposing of an aborted animal fetus (p = 0.0001).

Other reported exposures varied significantly between the 2 sublocation groups. Those from Sogan-Godud were more likely to have used mosquito nets (odds ratio [OR] 5.2, p = 0.0001) and mosquito coils (OR 8.2, p = 0.0001) to reduce insect exposure. Those from Gumarey were more likely to have had goat contact (OR 2.6, p = 0.046), had cattle contact (OR 4.7, p = 0.0001), consumed raw milk (OR 4.1, p = 0.0001), sheltered livestock (OR 2.6, p≤0.002), butchered livestock (OR 1.5, p = 0.0001), birthed livestock in the home (OR 2.1, p = 0.005), disposed of a livestock fetus (OR 1.7, p = 0.005), or to have had direct contact with human remains (OR 2.1, p = 0.026) (Figure 3; online Appendix Table 2).

The final logistic model to predict RVFV seropositivity included age, location, gender, and disposal of an aborted animal fetus (online Technical Appendix 2). In multivariable logistic regression models used to predict adjusted odds of RVFV seropositivity, location was significant when age and gender were controlled for; those residing in Gumarey were at 4 times the risk of those in Sogan-Godud (adjusted OR 4.15, 95% CI 1.59–10.87). Seropositivity also varied by gender when age and location were controlled for; male participants had >3 times the risk of women participants (20% vs. 9%; adjusted OR 2.78, 95% CI 1.18– 6.58 for male participants vs. female participants), but this difference did not remain significant within sublocation analysis. After age, gender, and location were controlled for, those who had disposed of an aborted animal fetus, were 3 times more likely to be seropositive (72.7% vs. 35.7%, adjusted OR 2.78, 95% CI 1.03–7.52). Age and location, but not gender, were associated with disposal of an aborted animal fetus, such that those who were older or who were from Gumarey were more likely to dispose of an abortus (online Technical Appendix 2).

Subgroup analysis by village showed significant predictors of RVFV seropositivity in Gumarey to be an ill family member, disposal of an aborted fetus, and gender (online Technical Appendix 2). Displacement by flood was also associated with RVFV seropositivity in Gumarey but could not be included in the model because every seropositive participant was displaced by floods and this factor was overdetermined. Male participants were >3 times as likely to be seropositive compared with female participants: (adjusted OR 3.45, 95% CI 1.17–10.19). Disposal of an aborted animal fetus (adjusted OR 15.12, 95% CI 4.445–51.35) and presence of an ill family member (adjusted OR 18, 95% CI 1.35–246.97) were also associated with RVFV seropositivity. In Sogan-Godud, the logistic model to predict seropositivity included age, such that the odds of seropositivity increased 5% for every 1-year increase in age

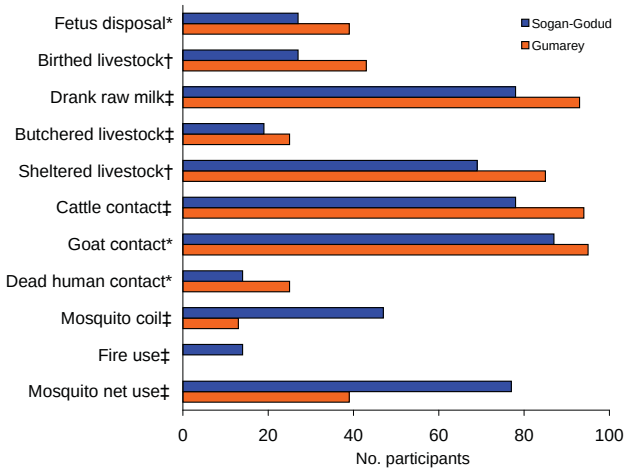

Figure 3. Exposures between northeastern Kenyan villages differed; Gumarey had more animal exposure and Sogan-Godud had more mosquito control. *p<0.05; †p<0.01; ‡p≤0.001 (by χ^2 test).

(adjusted OR 1.05, 95% CI 1.019–1.091) (online Technical Appendix 2).

Children ≤15 years of age had a much lower risk for RVFV seropositivity than those >15 years of age. The adjusted OR for seropositivity (calculated from the overall logistic model) was 1.05; 95% CI 1.03–1.07 per year of age (Figure 4). This difference persisted at the sublocation level with those children in Sogan-Godud still with significantly lower risk than adults.

Symptom History and Physical Examination Findings

Symptoms and signs reported on the survey questionnaire included fever, malaise, myalgia, chills, backache, eye pain, headache, rash, red eyes, photophobia, poor appetite, flushing, nausea, vomiting, meningismus, poor vision, epistaxis, hematemesis, hematochezia, bruising, confusion, vertigo, stupor, and coma. Of these, a past history of myalgias (OR 6.03, p = 0.0001), backache (OR 3.86, p = 0.003), eye pain (OR 2.28, p = 0.034), red eyes (OR 2.75, p = 0.008), meningismus (OR 2.97, p = 0.004), poor vision (OR 2.74, p = 0.008), and coma (OR 14.55, p = 0.005) were statistically associated with RVFV seropositivity in the study population (online Appendix Table 4, available from www.cdc.gov/EID/content/14/8/1240-appT4.htm). Upon physical examination, no nonocular finding was specifically associated with RVFV seropositivity.

Ophthalmologic Findings

Of the 18 identified cases of substantial retinal disease in the survey population, 7/18 (38.9%) were seropositive compared with 11/18 (61.1%) who were seronegative (p = 0.003, χ^2 8.75). All participants with eye disease were ≥21 years of age, and all seropositive participants with eye disease were ≥50 years of age. The OR of late eye disease associated with RVFV exposure (seropositivity) was 4.99 (p = 0.003) (online Appendix Table 5, available from www.cdc.gov/EID/content/14/8/1240-appT5.htm). Measured visual acuity ranged from 6/5 to 6/60 (equivalent to 20/17–20/200) in the seronegative group and 6/5–6/36 (20/17–20/120) in the seropositive group, although both groups included those with extremely poor vision who could decipher only large objects (measured by finger counting) or who could not perceive light. Visual acuity differed statistically among groups and was more likely to be worse in the RVFV–seropositive group (visual impairment defined as ≥20/80: 12% of seronegative vs. 25% of seropositive participants; p = 0.047, χ^2 3.94). Among the 18 participants with retinal disease, 14 (78%) had visual impairment, and among the 7 seropositive participants with retinal disease, 5 (71%) had visual impairment. No distinctive lesion was associated with RVFV seropositivity, though the eye diseases differed among the groups. Seropositive participants with eye disease had of optic atrophy (3), retinal hemorrhage (2), and retinal scarring (3). One person had retinal hemorrhage and scarring. By contrast, seronegative participants with eye disease had uveitis (1), vasculitis (1), maculopathy (3), peripapillitis (1), retinal scarring (1), optic scarring (2), retinal atrophy (1), and retinal degeneration (1).

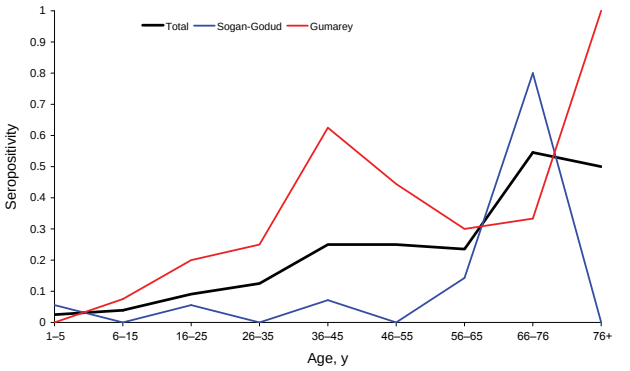

Figure 4. Rift Valley fever virus immunoglobulin G seropositivity by decade of age and village of residence; Gumarey had a higher rate than Sogan-Godud in almost all age groups.

Discussion

This study highlights the variability in RVFV seroprevalence in high-risk settings. In northeastern Kenya, older age, rural village location, male gender, disposal of an aborted fetus, and eye disease were associated with RVFV seropositivity. RVFV seropositivity was relatively high in our sample population in Masalani town, Kenya, particularly in the village area (Gumarey), where seropositivity rates were nearly 4 times higher than in the town area (Sogan-Godud); these areas were separated by only 500 m. Clues to the reasons for this discrepancy in seroprevalence were identified in our study. Those from Gumarey were more likely to have mosquito and animal exposures than those from Sogan-Godud. These risk factors, coupled with the most important predictors of rural seropositivity, male gender, and disposal of an aborted animal fetus, yield evidence for disparate risks for RVFV infection in different communities.

As identified in our prior work, RVFV seroprevalence can vary significantly across Kenya (*16*). Our current study shows that large seroprevalence discrepancies can also occur over short distances. Spatial risk assessments of RVF in animals in Senegal have been predicted by using measurements of seasonal rainfall, land surface temperature, distance to perennial water bodies, and time of year (*18*). Designing such risk maps with human risk factor data may enable improved surveillance systems and better prediction of the spatial distribution of RVFV. This information, gathered with satellite imagery (*19*) and large-scale cluster analysis (*20*), can be used not only to predict large outbreaks

but also to identify local hot spots of RVFV transmission to optimize RVF control in resource-limited settings.

For each year of life, the odds of being RVFV seropositive increased by 5%. Male participants were nearly 3 times more likely to be seropositive than female participants, a risk that was noted in the 1997 RVF outbreak investigation (*13*). The difference in seropositivity among genders is not explained on the basis of reported animal or nonanimal exposures, which were comparable and not statistically different between genders. The increased seropositivity among male participants may have a biological basis, given that outcome of infection and resultant immune response to other viruses have been linked to gender differences (*21*).

Disposal of an aborted animal fetus was associated with nearly 3 times increased odds of RVFV seropositivity. This finding may indicate the importance of RVFV transmission by aerosolization of blood and amniotic fluid during animal birthing. It is unknown whether aerosol or vector-borne transmission is the dominant form of transmission during interepidemic or epidemic periods. Our analysis indicates that disposal of an aborted animal fetus was a common associated risk factor at both the composite and sublocation level. Planned repeat sampling of our cohort since the most recent outbreak of 2006–2007 may enable the determination of the primary mode of epidemic transmission.

We found evidence of interepidemic human transmission of RVFV, which has not been previously shown. Our validation of seropositive young children, born after the documented outbreak in 1997–1998, indicate that low-level interepidemic transmission to humans is continuing in the Masalani area and likely in other areas of Kenya (*16*). The natural reservoir for RVFV and the mechanism by which humans become infected during interepidemic periods are unknown. Wild animals have been shown to be infected with RVFV, but further studies must determine whether these animals play a role in RVFV maintenance between outbreaks (*22*).

We demonstrated statistically significant differences between the seropositivity rates of those with and without eye disease. Those with chronic retinal disease were 5 times more likely to be RVFV seropositive. We did observe a difference in visual acuity between RVFV seropositive and seronegative persons in our sample tested 8 years after the 1997–1998 outbreak, and perhaps greater changes may have been present during acute RVF disease. Although there were no ocular findings that were pathognomonic for prior RVFV infection, the detected retinal disease supports evidence from previous studies on the oculopathogenesis of RVFV (*23*).

No specific nonocular examination finding was associated with RVFV seropositivity, but several reported symptoms were statistically more common among those who were RVFV seropositive. Most of these symptoms were severe neurologic manifestations of disease, such as neck stiffness, confusion, and coma. RVFV can cause encephalitis (*1*), and this type of inflammation may explain the higher prevalence of these reported symptoms among seropositive participants. Myalgia and backache may be present in most of the nonsevere RVF cases and are not specific to RVFV infection. Poor vision, which was noted to be more common among RVFV seropositive participants in our sample, may be an indicator for RVF retinitis, a common sequela of RVFV infection (*23*,*24*).

RVFV IgG ELISA and PRNT antibodies are believed to last decades after infection and therefore provide a reliable index of prior RVFV exposure. In contrast, though less well studied, it appears that IgM is lost in 50% of patients after 45 days and is absent in 100% by 4 months after infection (*25*). We did not perform IgM testing in our study, although it might have yielded useful additional information about acute RVFV infection. We also recognize that seropositive results may be false positive due to cross-immunoreactivity with viruses in the same family, although discrepancies between the neutralization test and the ELISA were only 4.9% in this population. The use of confirmatory PRNT testing of ELISA-positive samples can greatly improve viral specificity (*26*).

Our study was limited by its cross-sectional design; therefore, we are unable to conclude whether the identified risk factors specifically caused RVFV exposure. The validity of the associations in this study relies on accurate recall of exposures by the study participants. Although we asked about timing of symptoms and exposures, language differences during questioning limited our accurate collection of these data. Our study may have limited generalizability; we tested risk factors from a small population in Masalani, and risks may vary in other parts of Kenya or in other countries. Data on animal exposures were collected in a binary fashion, so no information about magnitude or duration of contact is known, which may have an effect on risk estimations. We also had no quantitative exposure data for the RVFV vectors in our study area.

This study highlights the large-scale variability in exposure and RVFV seropositivity among Kenyan villages and emphasizes the effect of age, gender, location, and animal husbandry in RVFV transmission. This information is useful for local public health agencies so that they can target protective interventions according to risk factors in different populations. Further studies are needed to examine the epidemiologic, biological, and genetic basis for the increased risk among persons of male gender and to quantify the potential public health impact of modifying the rural environment. RVFV transmission is known to be ongoing in livestock in areas where RVFV is endemic during interepidemic periods; we have shown that this extends

to humans, confirming past observations (*27*). Ongoing efforts to predict hot spots of infection on both small and large scales is useful only when at-risk communities are able to use the information to target mosquito or vaccine control efforts and prevent outbreaks. As RVF expands its geographic range and becomes recognized as a disease of global importance for human and animal health, more research is needed to define the most accessible modes of transmission control.

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Aquatic Invertebrates as Unlikely Vectors of Buruli Ulcer Disease

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Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans* and associated with exposure to aquatic habitats. To assess possible transmission of *M. ulcerans* by aquatic biting insects, we conducted a field examination of biting water bugs (Hemiptera: Naucoridae, Belostomatidae, Nepidae) in 15 disease-endemic and 12 non–disease-endemic areas of Ghana, Africa. From collections of 22,832 invertebrates, we compared composition, abundance, and associated *M. ulcerans* positivity among sites. Biting hemipterans were rare and represented a small percentage (usually <2%) of invertebrate communities. No significant differences were found in hemipteran abundance or pathogen positivity between disease-endemic and non–disease-endemic sites, and between abundance of biting hemipterans and *M. ulcerans* positivity. Therefore, although infection through insect bites is possible, little field evidence supports the assumption that biting hemipterans are primary vectors of *M. ulcerans*.

Mycobacterium ulcerans infection is an emerging skin disease often called Buruli ulcer (BU). Infection results in illness and lasting negative socioeconomic effects in rural areas of the tropics and subtropics (*1*). The pathologic changes, clinical signs and symptoms, and treatment have been reviewed elsewhere (*2*–*5*). In this article we evaluate field evidence for the potential of aquatic invertebrates to be vectors of *M. ulcerans*.

The exact mode of BU transmission remains unknown; however, past epidemiologic studies have associated BU with human activity near, or within, slow-flowing or standing water bodies that have been created or disturbed by humans (*2*–*4*). Although several water-related risk factors have been recognized, none has been consistently reported, making it difficult to identify specific water-related risk activities (*6*–*8*). Most studies suggest that infection occurs through inoculation of *M. ulcerans* into skin lesions or insect bites (*2*,*4*,*9*–*11*). Portaels et al. (*11*) were the first to propose that aquatic insects might serve as vectors of *M. ulcerans*. This hypothesis maintains that *M. ulcerans* is found in biofilms of aquatic habitats and concentrated by grazing or filter-feeding invertebrates that are then consumed by predators known to bite humans (*11*). Initial evidence for this hypothesis used PCR detection of the insertion sequence IS*2404* to document *M. ulcerans'* association with biting water bugs (Hemiptera), filtered concentrates of water, detritus, and aquatic plants (*4*,*12*–*14*). These studies were important for understanding the possible environmental reservoirs of *M. ulcerans*. However, IS*2404* is now understood to be not specific for *M. ulcerans* because this insertion sequence has been found in a number of other aquatic mycobacterial species, including *M. marinum* (*15*–*17*). When more discriminatory methods based on detection of variable number tandem repeats were used, many IS*2404*-positive environmental samples were reported to lack *M. ulcerans* (*18*). In light of these recent findings, the relative frequency or abundance of *M. ulcerans* among aquatic invertebrates or other environmental reservoirs, remains tenuous, and thus, the role of aquatic insect vectors is uncertain.

A series of laboratory experiments provided initial evidence for biting hemipteran vectors of *M. ulcerans* (*19*–*23*). Marsollier et al. (*9*,*24*) demonstrated that a South American isolate of *M. ulcerans* could survive and multiply within the salivary glands of aquatic bugs indigenous to France (Naucoridae: *Naucoris cimicoides*). Furthermore, *N. cimicoides* could transmit *M. ulcerans* by feeding on inoculated prey

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and then biting mice, which then exhibited BU (*9*). Most recently, exposure to hemipteran insect saliva was reported to infer protection against lesion development in laboratory mouse models (*21*). That study also reported correlations between aquatic insect salivary gland antibodies in humans categorized as exposed or patient, when the former group had exhibited BU. However, 3 limitations of that study have been noted (*25*): 1) the antibodies against salivary proteins might only be biomarkers of protection; 2) possible geographically related polymorphisms in the salivary proteins among hemipteran taxa could limit the generalizability of protection among distant communities; and 3) the overall relevance of biting aquatic insects infected with *M. ulcerans* in the natural environment is unknown.

A confounding factor in these experimental studies is that they used 1 South American isolate of *M. ulcerans.* Recent data support 2 major lineages of *M. ulcerans:* the ancestral strains that closely resemble *M. marinum* in chromosomal content, and the classic strains that have undergone substantial genome reduction (*26*). The latter strains account for all severe disease and include the African, Malaysian, and Australian isolates. The aforementioned laboratory studies have been elegantly performed, but the use of a French species of Naucoridae and a South American isolate of *M. ulcerans* makes it difficult to assess the importance of insect transmission in Africa. Thus, although provocative experimental data support a potential role for aquatic hemipterans as vectors of *M. ulcerans* in laboratory settings, no supporting evidence has been obtained from studies conducted in the natural setting. Results from field studies that identify the relative abundance and exposure potential of biting aquatic hemipterans can provide insight into the importance of biting insects in BU transmission.

This study had 3 objectives: 1) to describe the aquatic invertebrate samples collected during a large-scale, 2-year standardized field-sampling program of 27 bodies of water in Ghana, West Africa; 2) to investigate *M. ulcerans* positivity among the same aquatic invertebrates from those water bodies, directly linking aquatic invertebrate communities with pathogen positivity; and 3) to discuss the role of human-biting hemipterans as primary vectors of *M. ulcerans*. Data on the detection of *M. ulcerans* within aquatic samples based on the use of variable number tandem repeats analysis are presented in another article (*18*). In the current article, we associate presumptive *M. ulcerans* positivity rates with relative abundance and percentage composition of the same aquatic communities.

Methods

Study Sites

In June 2004 and August 2005, we sampled 27 water bodies associated with human communities in southern Ghana (Figure 1). The water bodies were located within or very near (<100–200 m) each community of housing structures and were routinely used for daily domestic purposes and reflect habitats of routine human exposure. These water bodies were chosen after discussions with community members who directed us to the main water source for drinking water, recreation, domestic washing, irrigation, or bathing for that community. Six of these sites were sampled in both years, providing information on annual variation: Afuaman, Amasaman, Abbeypanya, Afienya, Odumse, and Weija. Human BU case data for the years 2003–2005 were provided by the Ghana Ministry of Health and used to classify communities into 2 site types: 15 BU–endemic (BU+) and 12 BU–nonendemic (BU–). A site was classified as a BU+ type if at least 1 case of BU had been reported during the 3-year period.

Aquatic Invertebrate Sampling and Processing

Within each water body, two 10–20-m transects were measured parallel to the shoreline and positioned through the dominant macrophyte community. Along each transect, we randomly placed two 1-m^2 polyvinyl chloride quadrats and collected invertebrates by sweeping within the quadrat with a 500-µm mesh dip net. The quadrats floated on top of the water and delineated 1 m^2 of area to be sampled by using an aquatic dip net designed to capture the aquatic life stages of invertebrates. Three sweeps of the dip net were performed from the water surface to the bottom substrate for comprehensive sampling of specimens in the water column. All

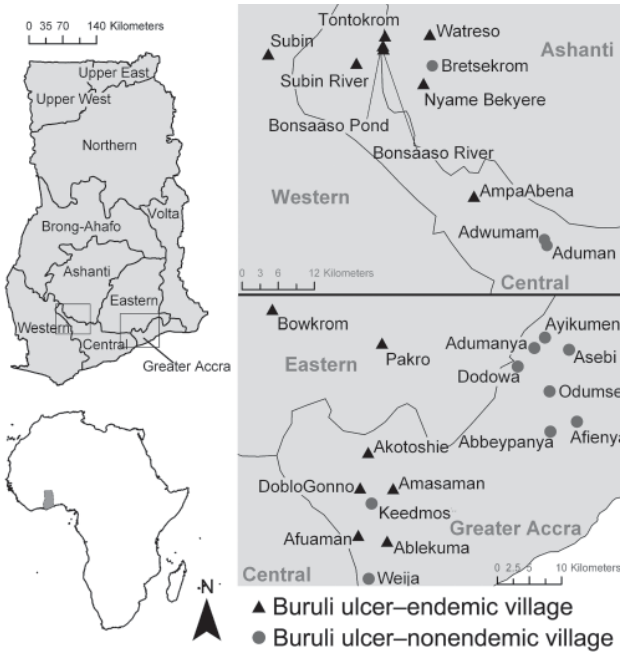

Figure 1. Regional site map of water bodies sampled in Ghana for aquatic invertebrates during 2004, 2005, or both. Small maps on left show location of Ghana in Africa and location of regions sampled within Ghana (boxes).

contents were washed through a 500-μm sieve and preserved in 100% ethanol for laboratory identification and PCR. The 2 quadrats were combined into 1 composite sample.

M. ulcerans Detection in Invertebrate Samples

Samples were analyzed in a 2-step procedure so that an initial screening reduced sample numbers. Small invertebrates were analyzed in pools of 3–15, whereas larger specimens were tested individually. DNA was extracted by using a protocol adapted from Lamour and Finley (*27*). Samples were ground and vortexed in 400 μL of lysis solution (100 mmol/L Tris, pH 8.0), 50 mmol/L EDTA, 500 mmol/L NaCl, 1.33% sodium dodecyl sulfate, and 0.2 mg/mL RNase A) and 1 g of 1.0-mm glass beads (Sigma-Aldrich, St. Louis, MO, USA), then centrifuged. After 150 μL of 5 mol/L potassium acetate was added, each sample was incubated overnight at –20° C. After a 30-min centrifugation, supernatants were transferred to new tubes containing 0.66 mol/L guanidine hydrochloride in a 63.3% ethanol solution. The samples were added to a spin filter (MO BIO Laboratories Inc., Carlsbad, CA, USA) in a 2-mL microcentrifuge tube (MO BIO Laboratories Inc.). The flow-through was discarded and the filter was rinsed first with 500 μL of wash solution (10 mmol/L Tris, pH 8, 1 mmol/L EDTA, 50 mmol/L NaCl, 67% ethanol) and then with 500 μL of 95% ethanol. The spin filters were dried by centrifugation and transferred to new 2-mL microcentrifuge tubes, immersed in 200 μL elution solution (10 mmol/L Tris, pH 8), and incubated at room temperature for 15 min. The DNA was eluted and stored at –20°C.

Presumptive identification of *M. ulcerans* in invertebrates was based on detection of the enoyl reduction domain (ER) in *mlsA* that encodes the lactone core of the mycolactone toxin, the major virulence determinant of *M. ulcerans.* All samples were screened for the presence of the ER gene, which has been evaluated for *M. ulcerans* specificity in a companion study that used a multitiered PCR approach (*18*). Amplification of the ER gene was achieved using a 50-μL reaction mixture containing 1 μL each of forward and reverse primer (*15*,*18*), 10 μL 5× Go Taq reaction buffer (Promega, Madison, WI, USA), 1 μL 10 mmol/L PCR nucleotide mix (Promega), 31.7 μL double-distilled water, 1.6 units Go Taq polymerase enzyme (Promega), and 5 μL DNA template. Cycling conditions began with an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 45 seconds, 72°C for 1 min, and a final 10-min extension at 72°C. The amplified DNA was subjected to gel electrophoresis by using a 1.5% agarose gel, and band sizes were compared by using a 1-kb DNA ladder (Invitrogen, Carlsbad, CA, USA). PCR products of appropriate size were cloned into the pCR2.1 Topo vector (Invitrogen) and sequenced by using an ABI 3100 automated genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Using all invertebrate data, we initially evaluated differences between site types (i.e., BU+ vs BU–) by comparing total abundance and percentage composition. Only those taxa that represented >3% of total invertebrates collected from all sites were used for subsequent statistical analyses because some taxa were so rare that any comparisons would limit meaningful conclusions. However, because we were interested in evaluating Hemiptera known to bite humans, the families Belostomatidae, Naucoridae, and Nepidae also were included, although each represented <2% of total collections.

To compare abundance differences between site types, *t* tests were used after data were log + 1 transformed to meet the assumptions of normality and equal variances. For percentage composition differences, data were arc-sine square root transformed, but they still did not demonstrate a normal distribution, so the nonparametric Wilcoxon/Kruskal-Wallis rank sum test was used. Because multiple tests were performed, it was necessary to calculate a Bonferroni adjusted α (and corresponding p value) of 0.006 to assist in interpreting statistically significant differences. However, to evaluate the biological meaning of these multiple tests, Cohen *d* effect size (and 95% confidence intervals) was calculated with Hedges adjustment (*28*). To compare overall ER positivity proportions between BU+ and BU– sites, a *t* test was used after data were arc-sine square root transformed. Lastly, we evaluated correlations between total biting hemipterans (and each individual family) and ER positivity using Spearman rank correlations with a Bonferroni adjusted α = 0.008. This nonparametric test was used after attempts to transform the data for normality and homogeneity of variances failed.

Results

Invertebrate Abundance and Composition

Of 22,832 invertebrates collected, ≈50% came from each group of BU+ and BU– site types (online Technical Appendix, available from www.cdc.gov/EID/content/14/8/1247-Techapp.pdf). A total of 85 taxa were represented among all sites: 80 taxa were collected from BU+ sites compared with 71 from BU– sites. The abundance of specific taxa was not consistent between site types, indicating that the invertebrate communities were highly variable. This variability was confirmed in statistical analyses comparing the most abundant taxa (>3%) with substantial effect size variation within and among taxa (online Technical Appendix). The invertebrates found in greatest abundance were 2 families of Diptera (i.e., Chironomidae and Culicidae), 1 family of Ephemeroptera (Baetidae), and several Crustacea. More than 300 individuals of some families of Hemiptera, Coleoptera, and

Odonata were encountered (online Technical Appendix). The biting Hemiptera were usually rare. For instance, 55 Naucoridae in total were collected, which was about 0.2% of all invertebrates sampled (online Technical Appendix).

Insects made up the greatest percentage of the invertebrates collected from BU+ sites but were nearly equivalent to the Crustacea in BU– sites. In BU– sites, Anura made up a relatively higher percentage, but most (1,231 of 1,303 individuals) were from a single site (Figure 2; online Technical Appendix). The Crustacea were most often represented by copepods, ostracods, and shrimp (Atyidae); fewer shrimp were collected from BU+ sites. Most shrimp were from BU– sites Adumanya (197) and Keedmos (120). Further, in BU– sites the large copepod abundance occurred primarily at Odumse, where 1,723 were collected from a total 1,884 (online Technical Appendix). Insects were reduced by 40% in BU– sites compared with BU+ sites (Figures 2, 3). When individual insect orders were compared, the Ephemeroptera (mayflies) and Diptera (true flies) made up the greatest percentages of insects in both BU+ and BU– site types (Figure 3; online Technical Appendix).

When the abundance and percent composition of dominant taxa were statistically compared between BU+ and BU– site types, there were no significant differences for any taxa (online Technical Appendix). However, the effect size varied greatly, reflecting a need to collect from more sites in future studies. On average, the Chironomidae (midges) made up the greatest percentage of the invertebrate communities, representing 9%–20% of the total, while the Baetidae (mayflies) ranged from 6% to 15% and the Culicidae (mosquitoes) from 2% to 5%. The biting Hemiptera made up a very small percentage of the dominant invertebrate communities, with Naucoridae ≤0.5%, Belostomatidae ≤2%, and Nepidae ≤0.3% (online Technical Appendix).

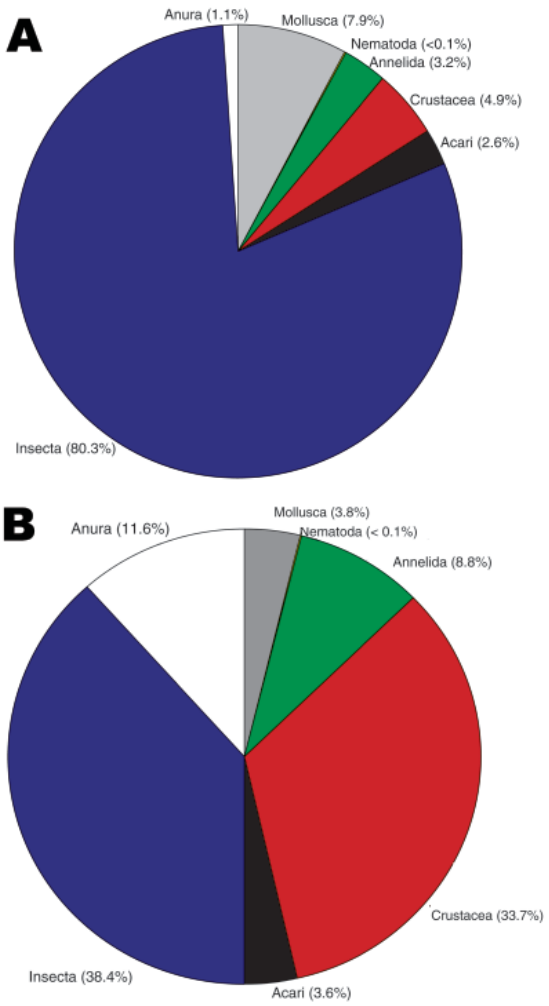

Figure 2. Higher level classification (e.g., class, phylum) taxa percentage composition between A) Buruli ulcer–endemic (n = 15) and B) Buruli ulcer–nonendemic (n = 12) site types, Ghana.

Presumptive Identification of *M. ulcerans* from Invertebrates

Presumptive identification of *M. ulcerans* from a total of 1,032 invertebrate sample pools tested found no significant difference between BU+ and BU– site types (online Technical Appendix). Furthermore, there was no detectable pattern of invertebrate taxa ER positivity among sites, indicating that no single taxon was more often likely to be positive at a particular site. The number of ER positive taxa that were detected at any site ranged from 0 to 15 and 0 to 6 in BU+ and BU– sites, respectively (Figure 4). Clearly, not all BU+ or BU– sites had ER positive invertebrates. There were 6/15 BU+ sites without a single taxon positive compared with only 3/12 BU– sites (Figure 4).

Taxon-specific ER positivity was highly variable, and percentage positivity ranged from 0% to 100% among taxa (online Technical Appendix). There were 26 taxa positive from BU+ site types compared with only 18 from BU– sites. Only 2 taxa were positive in BU– and not in BU+ sites, and for those taxa, <5 samples were tested from the BU+ type. When only those taxa with >5 samples tested were compared, no observable pattern in ER positivity was apparent among sites or taxa. The most abundant taxa did not always have the greatest ER positivity. For instance, positivity of Chironomidae (19.5% of all invertebrates) was only about 7%, even though positivity of Caenidae (<2% of all invertebrates) ranged from 6% to 17% (online Technical Appendix). For taxa with >5samples tested from either BU+ or BU– sites, the ER positivity was ≥20% for 5 taxa and from 10% to 20% for 12 taxa (online Technical Appendix). The biting Hemiptera had neither the highest nor consistently higher ER positivity compared with more abundant taxa (online Technical Appendix). Fifteen taxa with >5 samples

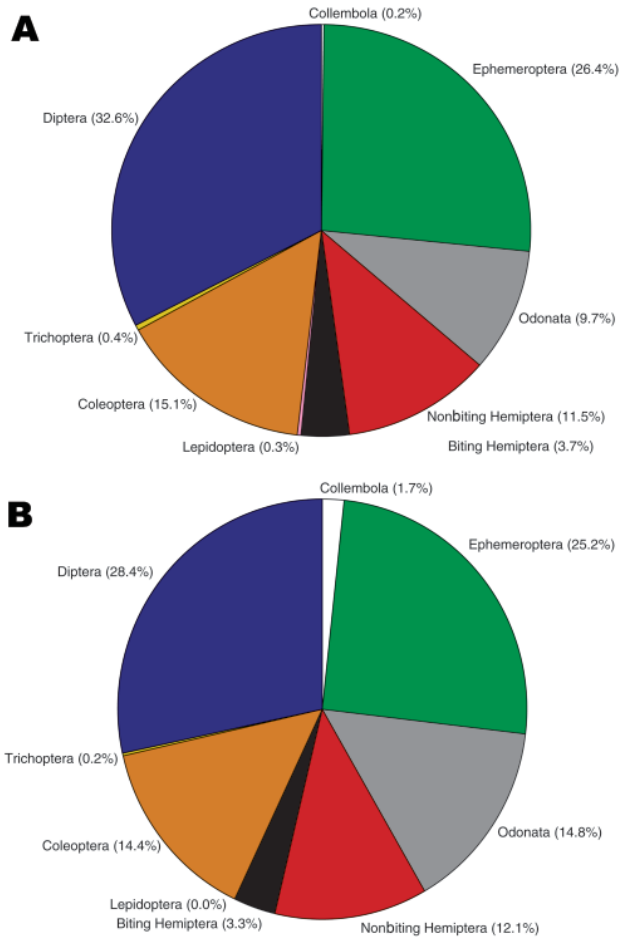

Figure 3. Insect order percentage composition between Buruli ulcer–endemic (n = 15) and Buruli ulcer–nonendemic site types (n = 12), Ghana.

tested had 0 positivity. These taxa represented all invertebrate functional feeding groups (e.g., predators, shredders, scrapers, collector-gatherers, and filterers).

Biting Hemiptera Correlations

No significant correlation was found between mean ER positivity and total biting Hemiptera (r = 0.25; p = 0.218) or any individual family: Belostomatidae (r = 0.31; p = 0.118), Naucoridae (r = -0.03; p = 0.850), and Nepidae (r = 0.37; p = 0.060). These results confirmed that biting Hemiptera were not significantly associated with the pathogen in the environment.

Discussion

The role of aquatic invertebrates in the transmission of BU has been proposed several times (*3*,*4*,*29*). However, to date, no large-scale field studies have assessed aquatic invertebrate communities from multiple locations or evaluated associated *M. ulcerans* positivity rates for specific invertebrate communities. Understanding the relative abundance and composition of the invertebrate taxa is a useful initial approach for assessing exposure risk of populations that use waterbodies for domestic needs. If biting water bugs are primary vectors of *M. ulcerans*, then the minimum (but not only) supporting evidence should confirm at least 1 of the following characteristics: 1) biting water bugs should be relatively more abundant at sites with BU cases compared with those without BU, indicating increased exposure potential to the vector in disease-endemic communities; 2) biting water bugs should have relatively higher *M. ulcerans* positivity rates within disease-endemic sites compared with disease-nonendemic sites; 3) *M. ulcerans* positivity rates should be higher in biting water bugs than in other invertebrates in the same sites, demonstrating increased potential pathogen exposure in the vector compared with background exposure; or 4) a correlation should exist between *M. ulcerans* positivity and vector abundance. This study addressed each of these characteristics and did not find strong confirming evidence that biting water bugs were any more important in the transmission of *M. ulcerans* than passive contact exposure to the environment. This finding is consistent with reports that few infected persons remember being bitten by water bugs (*30*). Although our results do not prove that infection could never occur from biting water bugs, they suggest that such an event would be rare.

In a companion study, Williamson et al. (*18*) reported *M. ulcerans* ER positivity from a broad spectrum of environmental samples, including animals, water filtrate, and biofilm on glass slides. They found that *M. ulcerans* DNA was detectable, not only at sites with or without a history of BU cases, but also in the environment, independent of invertebrates; positive results were detected for all sample types. Although *M. ulcerans* has been detected on the exoskeleston of experimentally infected Naucoridae (*9*), the possibility that invertebrates could serve as substrates for *M. ulcerans* in a natural environment has not been addressed, but it is certainly possible and may explain the wide range of taxa that were found positive in this study.

The invertebrate communities in this study demonstrated high intersite variation (online Technical Appendix), a finding similar to those of other studies of lentic invertebrate habitats (*31*,*32*). This variation suggests that additional collection sites should be included for a more comprehensive evaluation of invertebrate communities; an expanded study is under way. Hydrologic and physical/chemical attributes regulate the structure and abundance of invertebrate communities (*31*), while biotic factors such as macrophytes and fish can also influence communities (*33*). Few basic ecologic studies have been conducted on non–disease-related aquatic invertebrates in West Africa. The

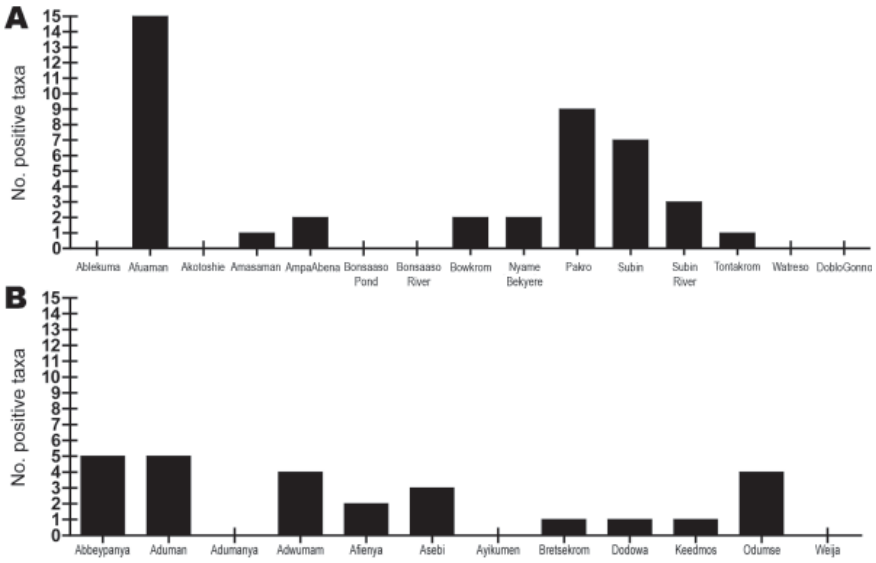

Figure 4. Number of enoyl-reduction-domain–positive taxa detected for each A) Buruli ulcer–endemic site (n = 15) and B) Buruli ulcer–nonendemic site (n = 12), Ghana.

most comprehensive articles on ecology have come from studies of small, fast-flowing streams or large lakes (*34*,*35*), which are different habitats than those in this study.

Season may also play a role in invertebrate abundance patterns; however, in many tropical and subtropical regions, most invertebrate taxa show minimal seasonally based abundance patterns (*36*–*38*). Most tropical species have multivoltine (multiple generations) and asynchronous (overlapping) life cycles throughout the year (*39*). For instance, all life stages of tropical naucorids have been reported through both wet and dry seasons over 2 years (*38*), and the same has been documented for other aquatic invertebrates in Kenya (*36*) and Lake Tanganyika (*37*). Therefore, although season might have had a small effect on the abundance variation of biting hemipterans and other invertebrates, this influence was unlikely to have limited our potential for detecting differences between BU+ and BU– sites.

If season affects biting Hemiptera populations, and these insects are important vectors, then human BU case data should reflect seasonal patterns, but this is not generally reported (*4*). In a recent study, no seasonal pattern was shown in monthly BU cases for 2003, 2004, and 2005 (*40*). In the current study, sampling each site throughout the year was not logistically feasible. In other ongoing studies, we have sampled an additional 55 sites, including 22 sites from 2004 to 2005 that have been sampled at least twice and 6 sites sampled 3 times over 3 years. The abundance of biting Hemiptera and other invertebrates from these additional sites are similar to what is reported here. Therefore, although season may have influenced our invertebrate community abundances, little evidence suggests that BU+ and BU– sites would be differentially affected, that Ghanaian invertebrate communities should respond differently to season compared with communities in other tropical and subtropical regions, or that any seasonal pattern in BU cases is related to seasonal population changes of biting hemipterans.

Various researchers have proposed that biting water bugs could be vectors for *M. ulcerans*, and laboratory studies have provided evidence for this possibility. However, no complementary field studies had tested these laboratory results. Results from this field study do not support the hypothesis that biting aquatic insects are primary vectors of *M. ulcerans*. The results do not rule out the possibility of biting Hemiptera or other invertebrates as vectors or possible reservoirs for *M. ulcerans*, but rather, they suggest caution in describing their role in transmission. These field data on biting hemipteran abundance and *M. ulcerans* positivity suggest a need to reevaluate future research directions for understanding BU transmission.

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Cutaneous Infrared Thermometry for Detecting Febrile Patients

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We assessed the accuracy of cutaneous infrared thermometry, which measures temperature on the forehead, for detecting patients with fever in patients admitted to an emergency department. Although negative predictive value was excellent (0.99), positive predictive value was low (0.10). Therefore, we question mass detection of febrile patients by using this method.

Recent efforts to control spread of epidemic infectious diseases have prompted health officials to develop rapid screening processes to detect febrile patients. Such screening may take place at hospital entry, mainly in the emergency department, or at airports to detect travelers with increased body temperatures (*1*–*3*). Infrared thermal imaging devices have been proposed as a noncontact and noninvasive method for detecting fever (*4*–*6*). However, few studies have assessed their capacity for accurate detection of febrile patients in clinical settings. Therefore, we undertook a prospective study in an emergency department to assess diagnostic accuracy of infrared thermal imaging.

The Study

The study was performed in an emergency department of a large academic hospital (1,800 beds) and was reviewed and approved by our institutional review board (Comité de Protection des Personnes se Prêtant à la Recherche Biomédicale Pitié-Salpêtrière, Paris, France). Patients admitted to the emergency department were assessed by a trained triage nurse, and several variables were routinely measured, including tympanic temperature by using an infrared tympanic thermometer (Pro 4000; Welch Allyn, Skaneateles Falls, NY, USA), systolic and diastolic arterial blood pressure, and heart rate.

Tympanic temperature was measured twice (once in the left ear and once in the right ear). This temperature was used as a reference because it is routinely used in our emergency department and is an appropriate estimate of central core temperature (*7*–*9*). Cutaneous temperature was measured on the forehead by using an infrared thermometer (Raynger MX; Raytek, Berlin, Germany) (Figure 1). Rationale for an infrared thermometer device instead of a larger thermal scanner was that we wanted to test a method (i.e., measurement of forehead cutaneous temperature by using a simple infrared thermometer) and not a specific device. The forehead region was chosen because it is more reliable than the region behind the eyes (*5*,*10*). The latter region may not be appropriate for mass screening because one cannot accurately measure temperature through eyeglasses, which are worn by many persons. Outdoor and indoor temperatures were also recorded.

The main objective of our study was to assess diagnostic accuracy of infrared thermometry for detecting patients with fever, defined as a tympanic temperature ≥38.0°C. The second objective was to compare measurements of

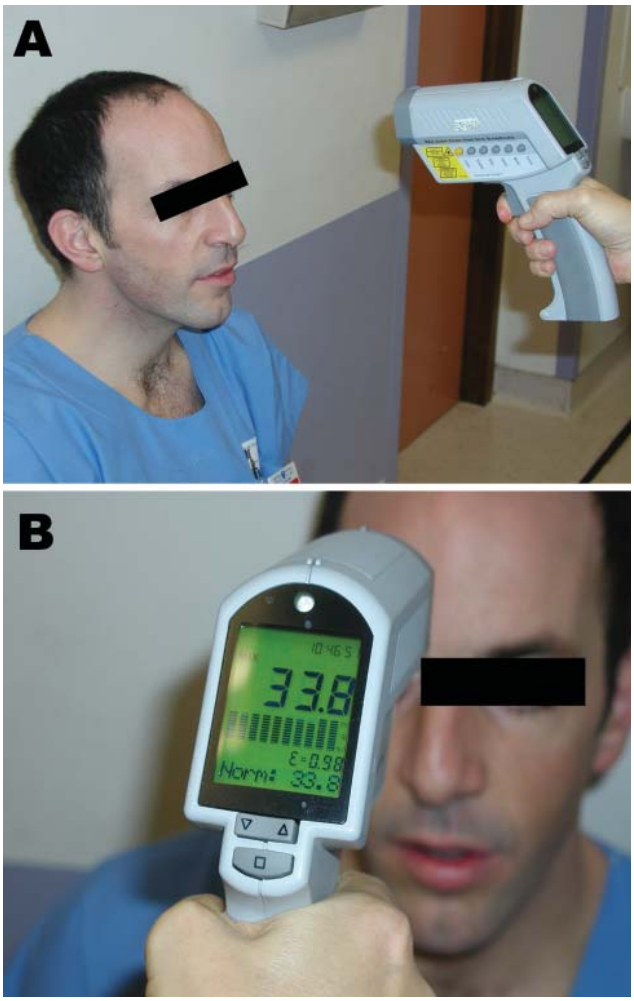

Figure 1. Measurement of cutaneous temperature with an infrared thermometer. A) The device is placed 20 cm from the forehead. B) As soon as the examiner pulls the trigger, the temperature measured is shown on the display. Used with permission.

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cutaneous temperature and tympanic temperature, with the latter being used as a reference point. Data are expressed as mean ± standard deviation (SD) or percentages and their 95% confidence intervals (CIs). Comparison of 2 means was performed by using the Student *t* test, and comparison of 2 proportions was performed by using the Fisher exact method. Bias, precision (in absolute values and percentages), and number of outliers (defined as a difference >1°C) were also recorded. Correlation between 2 variables was assessed by using the least square method. The Bland and Altman method was used to compare 2 sets of measurements, and the limit of agreement was defined as ± 2 SDs of the differences (*11*). We determined the receiver operating characteristic (ROC) curves and calculated the area under the ROC curve and its 95% CI. The ROC curve was used to determine the best threshold for the definition of hyperthermia for cutaneous temperature to predict a tympanic temperature >38°C. We performed multivariate regression analysis to assess variables associated with the difference between tympanic and infrared measurements. All statistical tests were 2-sided, and a p value <0.05 was required to reject the null hypothesis. Statistical analysis was performed by using Number Cruncher Statistical Systems 2001 software (Statistical Solutions Ltd., Cork, Ireland).

A total of 2,026 patients were enrolled in the study: 1,146 (57%) men and 880 (43%) women 46 ± 19 years of age (range 6–103 years); 219 (11%) were >75 years of age, and 62 (3%) had a tympanic temperature >38°C. Mean tympanic temperature was 36.7°C ± 0.6°C (range 33.7°C–40.2°C), and mean cutaneous temperature was 36.7°C ± 1.7°C (range 32.0°C–42.6°C). Mean systolic arterial blood pressure was 130 ± 19 mm Hg, mean diastolic blood pressure was 79 ± 13 mm Hg, and mean heart rate was 86 ± 17 beats/min. Mean indoor temperature was 24.8°C ± 1.1°C (range 20°C–28°C), and mean outdoor temperature was 10.8°C ± 6.8°C (range 0°C–32°C). Reproducibility of infrared measurements was assessed in 256 patients. Bias was 0.04°C ± 0.35°C, precision was 0.22°C ± 0.27°C (i.e., 0.6 ± 0.7%), and percentage of outliers >1°C was 2.3%.

Diagnostic performance of cutaneous temperature measurement is shown in Table 1. For the threshold of the definition of tympanic hyperthermia definition used (37.5°C, 38°C, or 38.5°C), sensitivity of cutaneous temperature was lower than that expected and positive predictive value was low. We attempted to determine the best threshold (definition of hyperthermia) by using cutaneous temperature to predict a tympanic temperature ≥38°C (Figure 2, panel A). Area under the ROC curve was 0.873 (95% CI 0.807–0.917, p<0.001). The best threshold for cutaneous hyperthermia definition was 38.0°C, a condition already assessed in Table 1. Figure 2, panels B and C shows the correlation between cutaneous and tympanic temperature measurements (Bland and Altman diagrams). Correlation between cutaneous and tympanic measurements was poor, and the infrared thermometer underestimated body temperature at low values and overestimated it at high values. Multiple regression analysis showed that 3 variables (tympanic temperature, outdoor temperature, and age) were significantly (p<0.001) and independently correlated with the magnitude of the difference between cutaneous and tympanic measurements (Table 2).

Conclusions

Infrared thermometry does not reliably detect febrile patients because its sensitivity was lower than that expected and the positive predictive value was low, which indicated a high proportion of false-positive results. Ng et al. (*5*) studied 502 patients, concluded that an infrared thermal imager can appropriately identify febrile patients, and reported a high area under the ROC curve value (0.972), which is similar to the area we found in the present study (0.925). However, such global assessment is of limited value because of low incidence of fever in the population. Rather than looking at positive predictive value or accuracy, one should determine negative predictive value. This determination might be of greater consequence if one considers an air traveler population or a population entering a hospital.

Ng et al. (*5*) identified outdoor temperature as a confounding variable in cutaneous temperature measurement. Our study identified age as a variable that interferes with cutaneous measurement, but the role of gender is less obvious. Older persons showed impaired defense (stability) of

Table 1. Assessment of diagnostic performance of cutaneous temperature in predicting increased tympanic temperature*

Characteristic	Predicted tympanic temperature, °C†		
	≥37.5	≥38.0	≥38.5
Cutaneous temperature threshold, °C‡	37.5	38.0	38.5
Sensitivity	0.76 (0.69–0.82)	0.82 (0.71–0.90)	0.82 (0.67–0.91)
Specificity	0.65 (0.63–0.67)	0.77 (0.76–0.79)	0.90 (0.88–0.91)
Positive predictive value	0.16 (0.14–0.19)	0.10 (0.08–0.13)	0.13 (0.09–0.18)
Negative predictive value	0.97 (0.96–0.98)	0.99 (0.99–1.00)	1.00 (0.99–1.00)
Accuracy	0.66 (0.64–0.68)	0.78 (0.76–0.79)	0.90 (0.89–0.91)

*Values in parenthesis are 95% confidence intervals.
†Definition of hyperthermia.
‡Corresponds to the best threshold for a definition of cutaneous hyperthermia determined by using receiver operating characteristic curve.

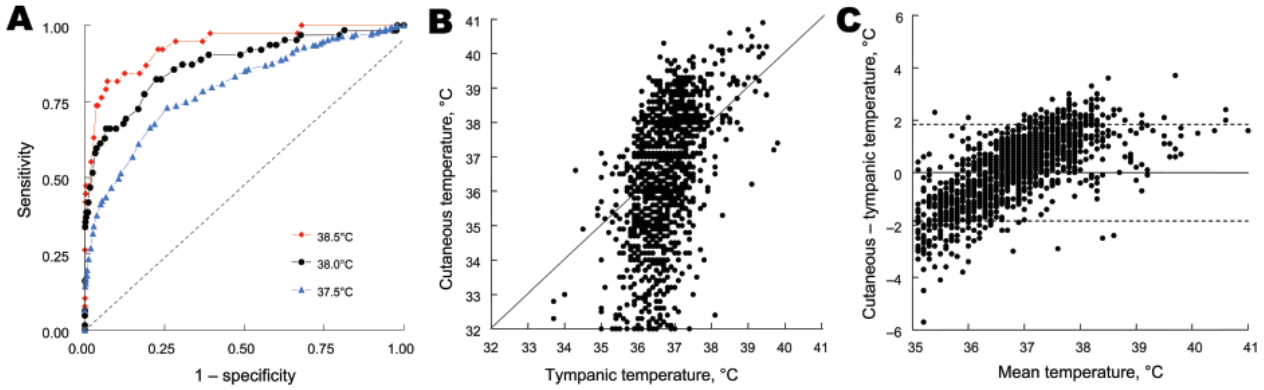

Figure 2. A) Comparison of receiver operating characteristic (ROC) curves showing relationship between sensitivity (true positive) and 1 – specificity (true negative) in determining value of cutaneous temperature for predicting various thresholds of hyperthermia definitions (37.5°C, 38.0°C, and 38.5°C) of tympanic temperature. Areas under ROC curves (95% confidence interval) were 0.935 (0.876–0.966), 0.873 (0.807–0.917), and 0.792 (0.749–0.829), respectively, and all were significantly (p<0.001) different from the identity line (dashed diagonal line). B) Correlation and C) Bland and Altman diagrams comparing cutaneous and tympanic temperature measurements (n = 2,026 patients). Values on the y-axis in panel C represent differences between cutaneous and tympanic temperatures. The solid horizontal line in panel C represents the null difference between cutaneous and tympanic temperatures, and the 2 dashed horizontal lines represent ± 2 standard deviations.

core temperatures during cold and heat stresses, and their cutaneous vascular reactivity was reduced (*12*,*13*).

Use of a simple infrared thermometry, rather than sophisticated imaging, should not be considered a limitation because this method concerns the relationship between cutaneous and central core temperatures. We can extrapolate our results to any devices that estimate cutaneous temperature and the software used to average it. Our study attempted to detect febrile patients, not infected patients. For mass detection of infection, focusing on fever means that nonfebrile patients are not detected. This last point is useful because fever is not a constant phenomenon during an infectious disease, antipyretic drugs may have been taken by patients, and a hypothermic rather than hyperthermic reaction may occur during an infectious process.

In conclusion, we observed that cutaneous temperature measurement by using infrared thermometry does not provide a reliable basis for screening outpatients who are febrile because the gradient between cutaneous and core temperatures is markedly influenced by patient's age and environmental characteristics. Mass detection of febrile patients by using this technique cannot be envisaged without accepting a high rate of false-positive results.

Table 2. Variables correlated with magnitude of the difference between cutaneous and tympanic temperature measurements*

Variables	Coefficient of regression (95% confidence interval)
Tympanic temperature, °C	0.27 (0.17 to 0.37)
Age, y	–0.012 (–0.015 to –0.009)
Outdoor temperature, °C	0.04 (0.03 to 0.05)

*p<0.001 for all comparisons.

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Increased Amoxicillin–Clavulanic Acid Resistance in *Escherichia coli* Blood Isolates, Spain

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To determine the evolution and trends of amoxicillin–clavulanic acid resistance among *Escherichia coli* isolates in Spain, we tested 9,090 blood isolates from 42 Spanish hospitals and compared resistance with trends in outpatient consumption. These isolates were collected by Spanish hospitals that participated in the European Antimicrobial Resistance Surveillance System network from April 2003 through December 2006.

In addition to being an essential component of the gut flora, *Escherichia coli* is an etiologic agent for both hospital- and community-acquired infections in humans (*1*–*3*). As with other bacterial pathogens, this bacterium can develop resistance and multidrug resistance to several antimicrobial families; consequently, antimicrobial treatment of invasive *E. coli* infections can be challenging (*1*).

Amoxicillin–clavulanic acid (AMC) is one of the most consumed antimicrobial agents in many countries (*4*–*6*), principally for respiratory and urinary tract infections. However, little is known about its impact on antimicrobial drug resistance, particularly in *E. coli*. *E. coli* is one of the indicator organisms of the European Antimicrobial Resistance Surveillance System (EARSS) (*7*), an international network of surveillance systems that attempt to collect reliable and comparable antimicrobial resistance data on invasive pathogens (*1*).

The Study

The 42 participating Spanish hospitals were selected according to EARSS criteria (*1*,*7*). The total catchment population was ≈9 million people, or ≈22.5% of the Spanish population. The first blood *E. coli* isolates obtained from each patient between 2003 and 2006 were included. Each laboratory identified the strains and tested their susceptibilities according to standard microbiologic procedures; all used commercial microdilution systems. Susceptibility data were interpreted according to Clinical Laboratory Standards Institute criteria (*8*). For epidemiologic purposes, intermediate susceptibility to AMC was considered as resistance. Multidrug resistance was defined as resistance to ≥3 of the following antimicrobial agents: ciprofloxacin, gentamicin, cotrimoxazole, and cefotaxime. To assess the comparability of susceptibility test results, an external quality assurance exercise (UK National External Quality Assessment Scheme) was performed yearly.

Hospital-acquired infections were defined as infections acquired at least 48 hours after hospital admission. Community-acquired infections were those in which *E. coli*–positive cultures were identified at or within 48 hours of hospital admission.

Outpatient consumption of penicillin/β-lactamase inhibitors (World Health Organization code J01CR02) for the period 2002–2006 was assessed from the Especialidades Consumo de Medicamentos database, which showed retail pharmacy sales of all medicines acquired with National Health System prescriptions and covered nearly 100% of the Spanish population (*5*). The information was tabulated, and the number of units was converted into defined daily doses (DDD) of active drug ingredients according to WHO methodology (*9*). The number of DDD per 1,000 inhabitants per day (DIDs) was calculated for each active drug ingredient.

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Differences in the antimicrobial resistance prevalence between different groups were assessed by Fisher exact test. Association was determined by calculation of the odds ratio (OR) with 95% confidence intervals (CI). The significance of the antimicrobial resistance trends was calculated by χ^2 test for trend. The null hypothesis was rejected for values of $p<0.05$. Statistical analyses were performed by using GraphPad Prism version 3.02 software (GraphPad Software, Inc., San Diego, CA, USA).

Participating hospitals reported data on 9,090 cases of *E. coli* bacteremia during the study period, corresponding to the same number of patients; 4,526 (49.8%) were male patients and 4,564 (50.2%) were female patients. A total of 1,531 cases (16.8%) were diagnosed in 2003; 2,526 (27.8%) in 2004; 2,438 (26.8%) in 2005; and 2,597 (28.6%) in 2006. Of the total number of isolates, 328 (3.6%) were obtained from children ≤14 years of age; 2,857 (31.4%) were obtained from patients ≥15 and ≤64 years of age; and 5,909 (65%) were obtained from patients >64 years of age. There were 3,384 (37.9%) isolates implicated in hospital-acquired infections and 5,540 (62.1%) in community-acquired infections; information was missing for 166 cases.

Of the 9,090 *E. coli* isolates tested, 1,136 (12.5%) were nonsusceptible to AMC, 5.1% were resistant, and 7.4% were intermediate. The prevalence of amoxicillin/clavulanic acid nonsusceptibility in relation to gender, age, infection origin, and resistance to other antibimicrobial drugs is detailed in the Table.

Multidrug resistance was present in 198 (17.4%) of the nonsusceptible AMC isolates. The most prevalent phenotypes included multidrug resistance to ciprofloxacin, cotrimoxazole, and gentamicin, which was detected in 73 nonsusceptible AMC isolates (36.9% of multiresistant isolates and 6.4% of isolates overall), and resistance to ciprofloxacin, cotrimoxazole, and cefotaxime was detected in 55 isolates (27.8% of multiresistant isolates; 4.9% of isolates overall). Multidrug resistance was more prevalent in nosocomial (23.5%) than in community-acquired isolates (15.1%; OR 1.99, 95% CI 1.46–2.72; $p<0.0001$).

Among nonsusceptible AMC isolates, susceptibility to other antimicrobial drugs, including ciprofloxacin, gentamicin, cefotaxime, and cotrimoxazole, was more frequent in community- (28.7%) than in hospital-acquired isolates (13.3%; OR 1.68, 95% CI 1.27–2.22; $p = 0.0003$). This suggests that more therapeutic options were available for community-acquired isolates.

The overall rate of invasive *E. coli* nonsusceptibility to AMC increased from 9.3% (2003) to 15.4% (2006) (χ^2 test for trend 36.51; $p<0.0001$) (Figure 1); this increase was observed in 64.3% of the participant hospitals. This increase was also detected in both intermediate and resistant isolates, with annual distributions of 5.6% and 3.8%, respectively, in 2003; 6.8% and 4.8% in 2004; 7.5% and 5.4% in 2005; and 9.4% and 6% in 2006.

AMC nonsusceptibility according to age groups increased over the study period as follows: children ≤14 years of age (10.6% in 2003, 14.6% in 2004, 14.3% in 2005, and 16.3% in 2006); patients ≥15 and ≤64 years 9.6% in 2003, 11.2% in 2004, 11.7% in 2005, and 13.3% in 2006); patients >64 years (8.8% in 2002, 11.3% in 2004, 15.9% in 2005, and 16.3% in 2006). The prevalence of AMC nonsusceptibility in community-acquired infections increased from 8.9% (2003) to 15.6% (2006) (χ^2 test for trend 29.43; $p<0.0001$). AMC nonsusceptibility in nosocomial infections increased from 9.2% (2003) to 15.2% (2006) (χ^2 test for trend 11.94; $p = 0.0006$).

In the final 2 years of the study period (2005–2006), the proportion of AMC-nonsusceptible isolates increased from 12.9% to 15.9%. This increase was due to community-acquired *E. coli* isolates only; the nonsusceptible proportion

Table. Association of AMC nonsusceptibility of *Escherichia coli* from blood with gender, age, infection origin, and resistance to other antimicrobial agents, Spain, 2003–2006*

Variable	AMC nonsusceptibility, %	Odds ratio	95% Confidence interval	p value
Gender				
Male	13.6	1.23	1.08–1.39	0.001
Female	11.4			
Age				
≤14 y	14.2	1.12	0.83–1.52	0.51
>14 y	12.8			
Infection origin				
Nosocomial	13.8	1.18	1.04–1.34	0.012
Community	12.0			
Antimicrobial susceptibility				
Ciprofloxacin susceptible	9.0	2.93	2.59–3.32	<0.0001
Ciprofloxacin resistant	22.5			
Gentamicin susceptible	11.5	2.64	2.22–3.14	<0.0001
Gentamicin resistant	25.6			
ESBL-negative	11.5	3.40	2.84–4.08	<0.0001
ESBL-positive	30.7			

*AMC, amoxicillin–clavulanic acid; ESBL, extended-spectrum β-lactamase.

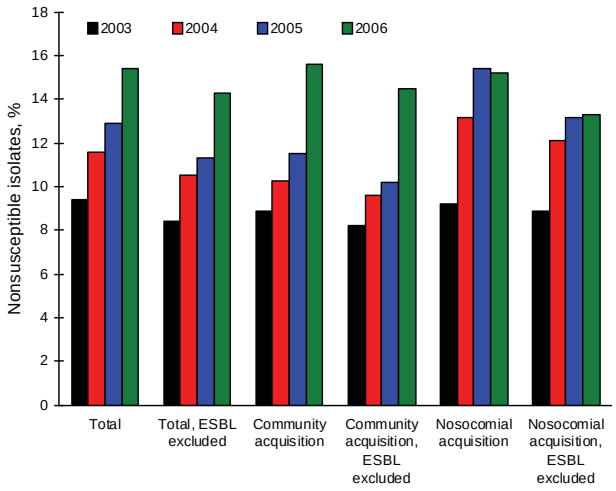

Figure 1. Evolution of amoxicillin-clavulanic acid nonsusceptibility of *Escherichia coli* from blood isolates, Spain, 2003–2006. ESBL, extended-spectrum β-lactamase.

varied from 11.5% (2005) to 15.6% (2006) (OR 1.42, 95% CI 1.16–1.74; p = 0.0009) in community-acquired isolates compared with 15.4% (2005) to 15.2% (2006) in hospital-acquired isolates (Figure 1). Community-acquired infection probably included healthcare-associated infections, a recently described epidemiologic category distinct from both community-acquired and nosocomial status.

In this study, the number of blood isolates of *E. coli* producing extended-spectrum β-lactamase (ESBL) was 614 (6.7%); 188 of them (30.6%) were nonsusceptible to AMC. When ESBL-producing *E. coli* isolates were excluded from analysis, AMC nonsusceptibility increased from 8.4% (2003) to 14.3% (2006) (χ^2 test for trend 34.39; p<0.0001) in total isolates; from 8.2% (2003) to 14.5% (2006) (χ^2 test or trend 25.23; p<0.0001) in community-acquired isolates; and from 8.9% (2003) to 13.3% (2006) (χ^2 test for trend 6.35; p = 0.012) in hospital-acquired isolates (Figure 1). The proportion of isolates highly susceptible to AMC (MIC ≤4 mg/L) steadily decreased over the study period as follows: 70.2% (2003), 70% (2004), 64.8% (2005), and 57.4% (2006) (χ^2 test for trend 99.36; p<0.0001).

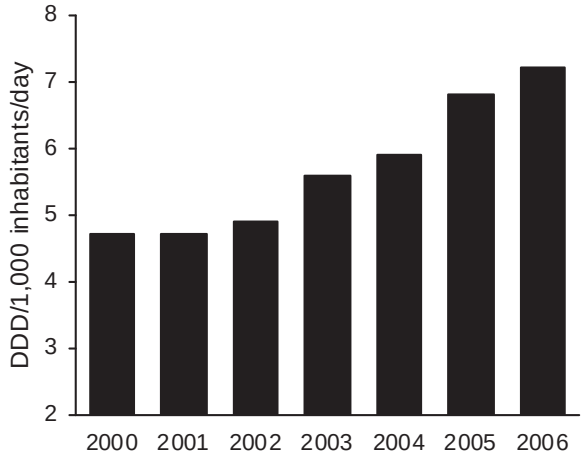

Figure 2. Evolution of consumptions of outpatient penicillin/β-lactamase inhibitors (World Health Organization code J01CR02), Spain, 2000–2006. DDD, defined daily dose.

Community consumption of penicillin/β-lactamase inhibitors, predominantly AMC, increased 34.7% from 2000 to 2006 (Figure 2), whereas total antimicrobial drug consumption remained relatively constant (19.6 DIDs in 2000 compared with 19.1 DIDs in 2006). After AMC, the most used β-lactam antimicrobial agents in the community in Spain were amoxicillin, cefuroxime, and cefixime; their consumption did not vary or slightly decreased from 2002 through 2005 (*6*).

Conclusions

The increased AMC resistance of *E. coli* isolates from blood observed in this study is of serious concern from clinical and epidemiologic standpoints because AMC is the first-choice antimicrobial treatment for many invasive *E. coli* infections. Increased AMC resistance coincided with growing AMC consumption at the community level. In urinary infections, previous treatment with AMC is a risk factor for the development of AMC resistance (*10*). AMC resistance mechanisms (β-lactamase overproduction, AmpC cephalosporinase hyperproduction, and inhibitor-resistant penicillinases) (*11*) might be favored by strong AMC consumption.

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Endemic Circulation of European Bat Lyssavirus Type 1 in Serotine Bats, Spain

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To determine the presence of European bat lyssavirus type 1 in southern Spain, we studied 19 colonies of serotine bats (*Eptesicus isabellinus),* its main reservoir, during 1998–2003. Viral genome and antibodies were detected in healthy bats, which suggests subclinical infection. The different temporal patterns of circulation found in each colony indicate independent endemic circulation.

The serotine bat (*Eptesicus serotinus*) is considered the main reservoir for European bat lyssavirus type 1 (EBLV1). Recently, southern Iberian populations of this species have been classified as *E. isabellinus* (*1*), a bat species previously known to exist only in North Africa.

In 1989, 5 EBLV1-infected serotine bats were found dead during a survey of natural colonies in Huelva (Andalusia). The prevalence of EBLV1 antibodies was up to 20% among completely healthy bats that were recaptured 1 year later, providing the first direct evidence of the survival of serotine bats after EBLV1 infection (*2*). Furthermore, high seroprevalence and presence of viral RNA in the oral cavity and bloodstream of different lyssavirus species have been reported in healthy bats captured in natural colonies in which the numbers of deaths have not increased (*3*–*5*), showing direct evidence of subclinical or asymptomatic disease after viral infection. Even viral RNA and antigens have been detected in the brains of healthy captive bats (*6*). However, reports of experimental lyssavirus transmission have drawn discrepant conclusions (*7*–*9*).

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The Study

During 1998–2003, a total of 1,030 *E. isabellinus* from 19 colonies located in the provinces of Huelva, Seville, and Granada (Andalusia), in southern Spain, were sampled. We focused on this region because all EBLV1 cases reported in Spain were from southern Spain. Distances between colonies varied from 0.5 to 317 km.

Bats were captured mainly in so-called maternity colonies made up of adult females and young of both sexes. Each animal was banded and assessed for sex, age, size, and weight. A total of 150 (14.5%) were recaptured. The frequency of repeated captures reached 22.7% in individual bats from 3 colonies that were sampled in all 6 years of the study. All recaptured bats were always found in the same colony in which they were first marked.

Viral RNA was detected in 34 (2.8%) of 1,226 oropharyngeal swab specimens from 33 bats of 8 colonies (1 bat was resampled after a 1-week interval). One bat that tested positive was recaptured and tested negative in a followup sampling effort. Positive results were identified as EBLV1 by direct sequencing as previously described (*4*,*10*). Samples for reverse transcription–PCR were stored in a buffer that was designed for RNA preservation but was not suitable for keeping the virus viable for isolation on cell culture.

A total of 626 plasma samples were tested for EBLV1-specific antibodies by using a modification of the rapid fluorescent focus inhibition test (*11*), but 77 (12.3%) of them were found to be toxic to cells. EBLV1 antibodies were found in 51 (9.3%) of the remaining 549 from 13 colonies. Only 2 of 22 samples showing viral RNA in the oral cavity were antibody positive (Table). Thirteen antibody-positive bats were recaptured in a healthy condition in the following campaigns.

The temporal pattern of circulation was completely different in each colony (online Appendix Table, available from www.cdc.gov/EID/content/14/8/12653-appT.htm). Only 1 colony showed continuous circulation from 1998 through 2002.

Brains were obtained from 20 bats of the same colony that had been studied previously (*4*). Differences in body condition between noninfected bats, bats with positive oropharyngeal swabs only, and bats with positive oropharyngeal and brain specimens were tested by an analysis of variance; index of body condition was the dependent variable. Individual body condition was expressed as the residuals from an analysis of covariance with an optimized design, including body mass as dependent variable; forearm length as a covariate; and sex, age, and year as fixed factors. We found a significant negative association (Figure 1) between RNA presence and body condition ($F = 11.78$; degrees of freedom = 2, 281; $p<0.001$, n = 292). Post hoc tests indicated that only bats whose brains tested

Table. European bat lyssavirus type 1 in *Eptesicus isabellinus* bats, Spain, 1999–2003*

Type of testing	RT-PCR	RFFIT
Total captures	1,226	626
No. colonies	19	13
Total no. (%) positive	34 (2.8)	51 (9.3)
1998		
No. captures	164	151
No. (%) positive	4 (2.4)	10 (6.6)
1999		
No. (%) captures	161	90
No. positive	4 (2.5)	5 (5.6)
2000		
No. captures	204	128
No. (%) positive	16 (7.8)	12 (9.4)
2001		
No. captures	209	96
No. positive	0	4 (4.2%)
2002		
No. captures	287	100
No. (%) positive	10 (3.4)	5 (5.0)
2003		
No. captures	201	54
No. positive	0	0

*Results obtained during active surveillance. RT-PCR, reverse transcription–PCR, RFFIT, rapid fluorescent focus inhibition test.

positive had a significantly worse body condition (Tukey honestly significant differences [HSD] test –2.33, p<0.001, and HSD –2.27, p<0.001, respectively). Differences in body condition between oropharyngeal swab–negative and oropharyngeal swab–positive bats were not significant (Tukey HSD 0.57, p = 0.99).

Brain, cerebellum, and spinal cord from 1 bat carcass with natural EBLV1 infection were studied by histopathologic and histochemical techniques. The bat was captured while flying but died during manipulation, which is an extremely rare event. The brain and oropharyngeal swab were positive for EBLV1 by reverse transcription–PCR, and the brain smear was also positive by immunofluorescence. Histopathologic studies showed moderate neuronal degeneration characterized by neuronal hyperchromatosis, chromatolysis, and satellitosis (Figure 2, panel A). The Sellers stain results were negative, proving the absence of Negri bodies (Figure 2, panel B). The occasional presence of basophilic intracytoplasmic structures in neurons located in the cerebral cortex that underwent necrobiosis was interpreted as a neurophagic process that was taking place in the glial cells because these inclusions were shown to be positive by the Feulgen reaction (Figure 2, panel C), whereas the Negri bodies have a strongly acidophilic nature. Moderate gliosis without perivascular infiltration was observed (Figure 2, panel 2D).

Conclusions

Despite the fact that serotine bats (*E. serotinus* and *E. isabellinus*) are the most frequently involved species in cases of bat lyssavirus exposure in humans in Europe, most published surveys of EBLV1 infection in natural bat colonies have focused on other bat species (*5*,*12*). Data from serotine bats have been obtained by either serologic or direct detection techniques but not from both (*2*,*4*), as in this report.

Our recapture data show an absence of switching among colonies, even between colonies located only a few kilometers apart. This result indicates highly philopatric behavior among female bats, as has been confirmed in a recent mtDNA-based study of these colonies (J. Juste, unpub. data). This could be the cause of the different temporal distribution of the positive results observed in each colony, which suggests a pattern of independent endemic viral circulation different from the model, based on periodic epidemic waves of fast viral spreading proposed for the mouse-eared bat (*Myotis myotis*) (*12*).

The analysis of body condition index as a measure of physiologic condition gives additional evidence for mild or subclinical infection in the previously described long-term survival of EBLV1 RNA or antibody-positive bats (*2*,*4*,*5*,*12*). Similar body condition values between bats with oropharyngeal swabs that were positive for the virus and those that were negative could be interpreted as a recent virus infection for which no symptoms have

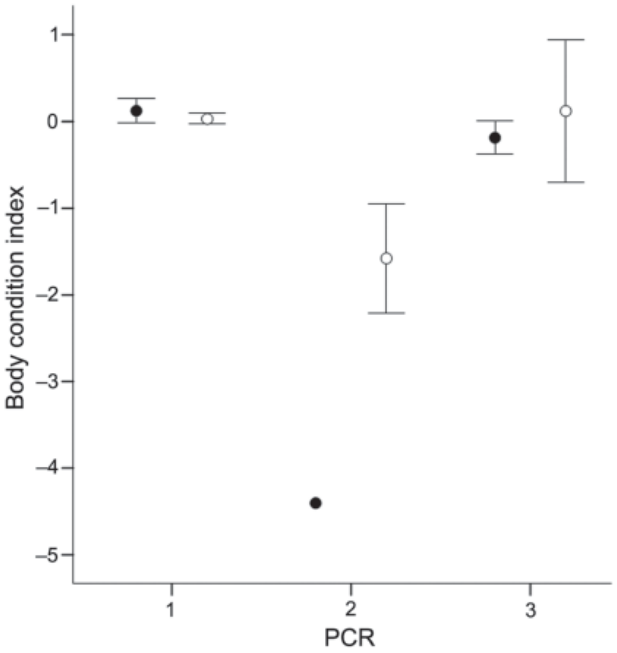

Figure 1. Relationship between body condition index (mean ± standard error) and diagnosis of European bat lyssavirus type 1 by reverse transcription PCR. Males and females are represented as filled and open circles, respectively. 1, only negative in oropharyngeal swab and brain specimens (● n = 49; ○ n = 225); 2, positive in oropharyngeal swab and brain specimens (● n = 1; ○ n = 4); 3, positive in oropharyngeal swab but negative in brain specimen (● n = 3; ○ n = 4).

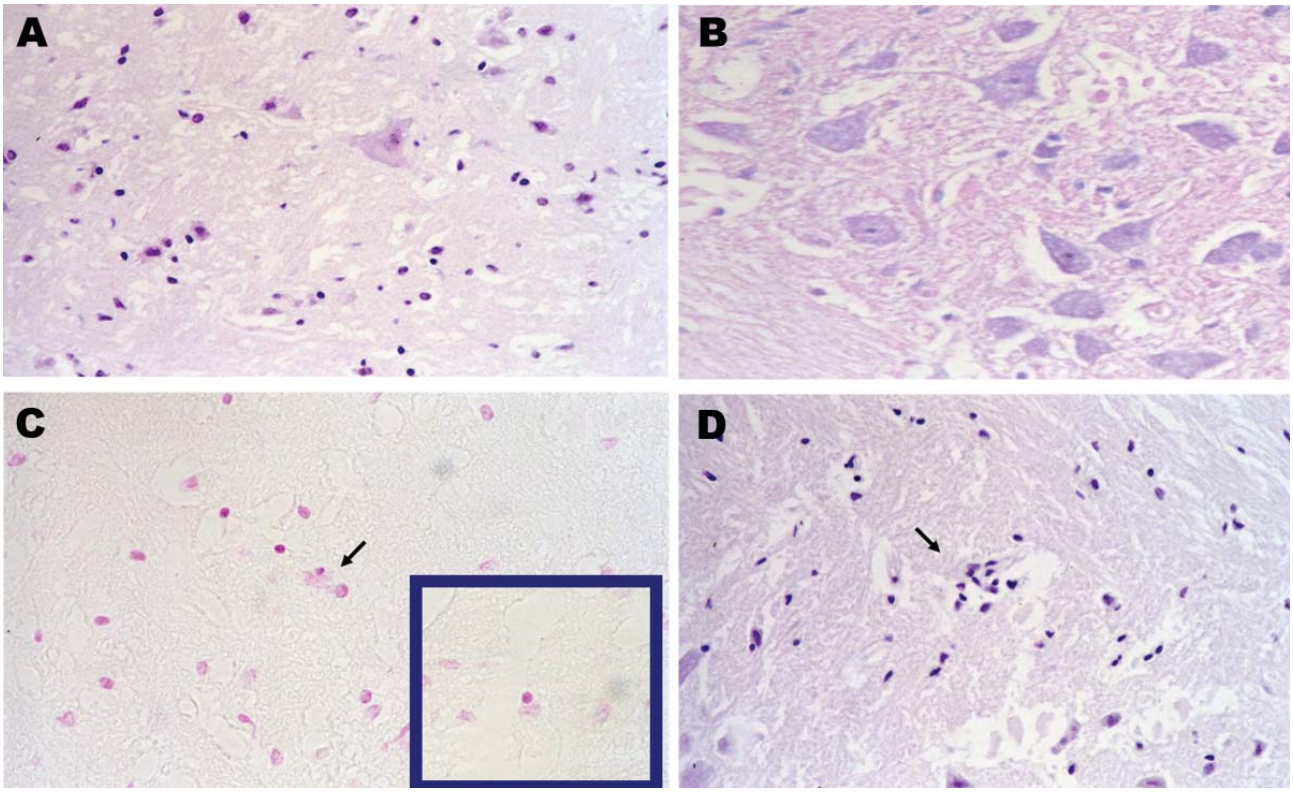

Figure 2. Pathologic images obtained from a carcass of *Eptesicus isabellinus*. The bat was captured while flying but died during handling. Brain specimen was positive for lyssavirus antigens by immunofluorescence and for European bat lyssavirus 1 RNA by reverse transcription–PCR. A) Neural degeneration in brain by hematoxylin and eosin stain (H&E); magnification ×400. B) Negative Seller stain in spinal cord indicating the absence of Negri bodies; magnification ×400. C) Positive Feulgen reaction in brain, glial cell neurophagia; magnification ×200. D) Focal proliferation of glial cells by H&E; magnification ×100.

developed. However, 1 lyssavirus-positive bat showed no viral RNA 3 years later. One bat even showed RNA in the oral cavity in consecutive samples separated by 1 week. Our findings suggest that asymptomatic EBLV1 RNA carriage may be common in serotine bats (*2*–*5*,*9*,*13*,*14*). In only a small subset of them does symptomatic neurologic infection progress to more severe body condition. However, most of these bats were captured while they were flying, and only 1 of those involved in an episode of human exposure had lost the ability to fly. This bat had the poorest body condition. The absence of Negri bodies and the moderate percentage of nerve cells affected found in the bat, captured while flying, suggest a subclinical process, as has been proposed for EBLV1 in *Rousettus aegyptiacus* (*6*,*14*). We cannot predict whether severe encephalitis was about to develop in these bats. The lack of pathogenicity could have arisen through long-standing coevolution between bats and viruses, as the phylogenetic data suggest (*15*). Nevertheless, additional work is necessary to establish whether the presence of EBLV1 RNA in the oral cavity is associated with the excretion of live virus.

In a similar study of *M. daubentonii* in the United Kingdom, only EBLV2 antibodies, but not viral RNA in the oropharyngeal cavity, were reported (*3*). Another study of the experimental transmission of Aravan, Khujand, and Irkut viruses to *E. fuscus* found oral excretion only in bats whose conditions were progressing to neurologic infection and death (*8*). These facts suggest differences between models of pathogenesis of lyssavirus infections in bats.

In summary, our findings indicate that natural maternity colonies of *E. isabellinus* behave as close communities in which EBLV1 independently circulates, which suggests an endemic pattern associated with asymptomatic or mild disease. Further studies are needed to 1) investigate the circumstances under which neurologic manifestations develop in bats (which lead to the abnormal behavior usually associated with human exposures) and 2) to establish the epidemiologic implications for public health of asymptomatic oral RNA carriage. The anthropophilic behavior of this bat species makes active surveillance highly recommended to predict risk factors that allow taking political decisions relating to public health.

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Highly Pathogenic Avian Influenza Virus (H5N1) in Experimentally Infected Adult Mute Swans

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Adult, healthy mute swans were experimentally infected with highly pathogenic avian influenza virus A/*Cygnus cygnus*/Germany/R65/2006 subtype H5N1. Immunologically naive birds died, whereas animals with preexisting, naturally acquired avian influenza virus–specific antibodies became infected asymptomatically and shed virus. Adult mute swans are highly susceptible, excrete virus, and can be clinically protected by preexposure immunity.

Since 2002, highly pathogenic avian influenza (HPAI) subtype H5N1 viruses have spread from endemically infected areas of Southeast Asia to Europe and Africa and infected poultry and wild birds. Especially in Europe, swans proved to be the most frequently affected wild bird species (*1*). Recently, Brown et al. (*2*) inoculated juvenile mute swans and confirmed that they are the most likely swan species to transmit HPAI (H5N1). Nevertheless, it remains unclear if an age-related susceptibility exists as it does for ducks (*3*). In addition, a more detailed knowledge regarding the role of preinfection with low-pathogenicity avian influenza virus is required. Our study was designed to answer these questions by experimental infection of adult mute swans (*Cygnus olor*).

The Study

All experiments with HPAI virus A/*Cygnus cygnus*/Germany/R65/2006 subtype H5N1 (*4*) were conducted under Biosaftey Level (BSL) 3+ conditions (trial approval LVL M-V/TSD/7221.3-1.1-003/07). The immunologically naive mute swans, 1–4 years of age, were divided into 2 groups. The high-dose group was inoculated oculo-oronasally with 10^6 50% egg infectious dose $(EID)_{50}$/animal (n = 4); 1 additional naive contact swan was included in this group. The low-dose group received 10^4 EID_{50}/animal (n = 5); 2 additional swans were in contact with this group. Two additional animals had preexposure avian influenza virus–specific antibody titers and were included in the high-dose group; 1 was also dedicated as contact animal.

In most birds the clinical signs were inconspicuous after HPAI virus (H5N1) inoculation. However, 3 swans exhibited severe neurologic disorders, including opisthotonus, torticollis, and ataxia. In addition, 3 animals died suddenly without any clinical signs developing. The incubation period for the high-dose group was at least 4 days. All swans of this group died or had to be humanely killed 5–9 days postinoculation (DPI) (Figure 1, panels A, B). The minimum

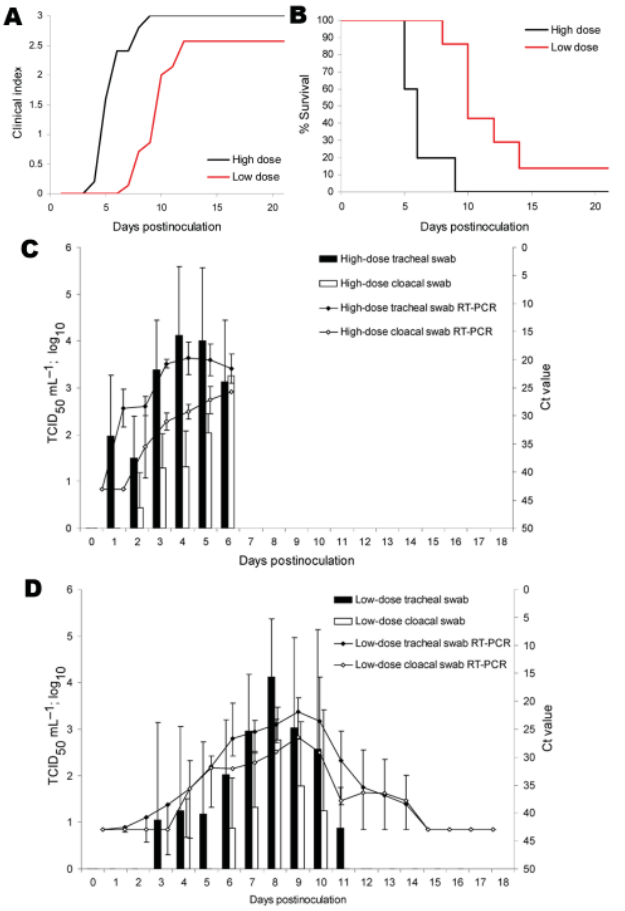

Figure 1. Clinical indices, mortality, and viral shedding of naive mute swans after inoculation with A/*Cygnus cygnus*/Germany/R65/2006 highly pathogenic influenza virus subtype H5N1. A) All animals were observed daily for up to 21 days for clinical signs and classified as healthy (0), ill (1), severely ill (2), or dead (3). A clinical index was calculated that represents the mean value of all naive swans per group for this period. B) Percentage survival of swans expressed as mean value of all naive swans per group. C and D) Mean values of the shedding of infectious virus of both groups (high dose = 10^6 50% egg infectious dose [EID_{50}]/animal, and low dose = 10^4 EID_{50}/animal) of naive mute swans are shown. Mean cycle threshold (Ct) values of real-time reverse transcription–PCR (RT-PCR) analyses of tracheal and cloacal swabs are depicted for both groups. Standard deviations are shown as error bars. $TCID_{50}$, 50% tissue culture infectious dose.

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incubation period of the low-dose group was 7 days (Figure 1, panel A). Only 1 animal of the low-dose group survived until the end of the trial (21 DPI), and all other swans of the group succumbed between 8 and 14 DPI.

Oropharyngeal and cloacal swab samples were collected daily in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and antimicrobial drugs. All individual swabs were tested by real-time reverse transcription–PCR (*5*) specific for subtype H5N1, and the genomic load was semiquantified by the cycle threshold (Ct) value. Infectivity titers of swab samples were calculated as the 50% tissue culture infectious dose/mL on Madin-Darby canine kidney (MDCK) cells (collection of cell lines in veterinary medicine, Friedrich-Loeffler-Institut, Südufer Insel Riems, RIE83).

Viral RNA as well as replicating virus could be detected from 1 until 6 DPI in oropharyngeal swabs of the high-dose group (Figure 1, panel C). The Ct values ranged from 17 to 33. The swans of the low-dose group excreted infectious virus from 3 until 11 DPI in oropharyngeal swabs (Figure 1, panel D), and real-time reverse transcription–PCR detected viral RNA in this group from 3 until 14 DPI. Virus excretion from cloacal swabs was demonstrated from 2 to 6 DPI in the high-dose group (Figure 1, panel C) and 4–10 DPI in the low-dose group (Figure 1, panel D). Viral RNA detection from cloacal swabs were positive 3 days longer than titration in cell culture (low-dose group; Figure 1, panel D). Maximum duration of viral shedding per individual swan was 6 days in both groups for cloacal and tracheal swab samples. Virus excretion of the contact animals was delayed, but the quantities of excreted virus were similar to those of the inoculated animals. One adult male swan of the low-dose group was the only surviving naive swan; his excretion pattern was delayed and shortened compared to other swans, which received the same virus dosage.

Sera were collected at 0, 7, 14, and 21 DPI from surviving swans as well as on the day of euthanasia. Serum samples were heat inactivated at 56°C for 30 min and subsequently nucleoprotein antibody ELISA, serum neutralization test (SNT), and hemagglutination-inhibition (HI) test were performed. Results of serologic tests from the day of euthanasia or the last day of serum collection before death are shown in Table 1. The comparison of the 3 serologic

Table 1. Distribution of viral genomic load and influenza A antigen in tissues of naive mute swans after challenge infection with highly pathogenic avian influenza virus (H5N1), related to assumed tropism and serologic data*

	Viral RNA load in tissue, Ct value†											
	High-dose group					Low-dose group						
Test	2	3	4‡	5	6	8‡	9‡	10	11	12§	13	14
Nasal concha	++	**+**	+	**++**	+	**+++**	/	**+++**	++	/	(+)	/
Trachea	/	**+**	/	(+)	(+)	(+)	/	**++**	(+)	/	(+)	/
Lung	(+)	**++**	++	+	(+)	**++**	/	**+++**	(+)	/	+	/
Brain	**++**	**++**	**(+)**	**+++**	**+**	**++**	**+**	**++**	**++**	/	**(+)**	**+**
Pancreas	**++**	**++**	+	**++**	**(+)**	**+++**	/	**++**	**+**	/	++	/
Adrenal gland	**++**	**+++**	(+)	**++**	(+)	**+++**	(+)	**+++**	(+)	/	+	/
Myocardium	/	**+**	(+)	+	/	+	/	**++**	(+)	/	/	(+)
Liver	+	**+++**	(+)	**+**	(+)	**+++**	/	**+++**	(+)	/	+	/
Kidney	(+)	**++**	(+)	(+)	/	++	(+)	**++**	(+)	/	+	(+)
Spleen	(+)	**+++**	(+)	+	(+)	++	(+)	**+++**	(+)	/	(+)	/
Bursa fabricii	/	**+**	+	(+)	+	**++**	/	++	+	/	+	(+)
Ovary/testis	**++**	**++**	(+)	**+++**	(+)	**+++**	/	**+++**	/	/	/	/
Proventriculus	(+)	**++**	(+)	(+)	(+)	++	(+)	**++**	+	/	(+)	+
Cecal tonsil	(+)	**++**	(+)	+	(+)	++	/	**++**	+	(+)	(+)	(+)
Tropism¶	N, EP	N, EP, EN	N	N, EP	N, EP	N, EP, EN	N, EP	N, EP, EN	N, EP	/	N	N
Serologic data												
ELISA	Pos	Neg	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Pos	Pos	Pos
SNT	1	ND	1	5.3	ND	ND	5.7	ND	5.3	10.3	6.7	8.7
HI	2	ND	2	2	ND	ND	7	ND	2	9	8	7
DPI serology	5	0	7	6	0	7	12	7	7	21	10	14
Died or euthanized, DPI	5	5	9	6	6	10	12	10	8	21	10	14

*Viral RNA detected by real-time reverse transcription–PCR (RT-PCR) in swans after challenge infection with highly pathogenic avian influenza virus strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1). N, neurotropism; EP, epitheliotropism; EN, endotheliotropism; ELISA, Pourquier AI A Blocking ELISA against nucleoprotein; Pos, positive; Neg, negative; SNT, serum neutralization test; [ND100 log2] modified from a previously described procedure (*6*); ND, not done; HI, hemagglutination-inhibition [log2] using homologous influenza virus (H5N1) as antigen according to standardized methods (*7*); DPI, days postinoculation.

†Real-time RT-PCR results are presented as cycle of threshold (Ct) values: /, >40; (+), ≥30–40; +, ≥25–<30; ++, ≥20–<25; +++, <20. **Boldface** indicates marked positive staining by immunohistochemical analysis.

‡Contact animals.

§Animal survived until the end of the study.

¶Tropism as assessed by immunohistochemical analysis.

Table 2. Viral shedding among mute swans with preexisting antibodies*

Individual serologically positive	Viral excretion, no. days shedding replication competent virus (DPI)		Genome detection, no. days with positive PCR results (DPI)		Peak titer log_{10}/mL swab (DPI)		Minimum Ct value (DPI)	
	OS	CS	OS	CS	OS	CS	OS	CS
No. 1 (contact)	1 (3)	0	7 (3-9)	6 (3-8)	1.75 (3)	0	32.12 (4)	31.65 (4)
No. 7	4 (2–5)	1 (4)	8 (1–8)	6 (3–7, 9)	2.63 (4)	1.75 (4)	26.44 (5)	29.49 (4)

*H5-specific real-time reverse transcription–PCR (RT-PCR) results of swab samples from swans. DPI, days postinoculation; Ct value, cycle threshold of the real-time RT-PCR; OS, oropharyngeal swab; CS, cloacal swab.

tests showed that the nucleoprotein-specific ELISA was the most sensitive assay, showing positive results as early as 5 DPI. All but 4 swan sera were positive in the nucleoprotein antibody ELISA after HPAI virus (H5N1) inoculation. Six of 8 ELISA-positive serum samples also exhibited neutralizing antibodies, whereas only 4 of 8 ELISA-positive serum samples exhibited positive HI titers against the challenge virus antigen. However, even postinfection antibody titers >400 SNT or 128 HI, respectively, did not protect swans from dying (Table 1). The 1 surviving swan developed antibody titers >1,000 SNT or 500 HI at the end of the experiment.

Two mute swans showed positive or questionable results in a nucleoprotein-specific ELISA before inoculation. The neutralizing activity of sera of both animals against HPAI virus (H5N1) was low, with titers of 10 and 3, respectively; no specific HI titers were detected. After HPAI virus (H5N1) inoculation, both swans survived without any clinical signs. When these swans were compared with the inoculated naive swans, viral shedding was delayed and had a shorter duration with reduced viral loads (Table 2).

Gross pathology showed widespread hemorrhages as predominant lesions in both infected groups. Ecchymoses were especially present within the myocardium, submeningeally in the brain, in the peritracheal connective tissue, and within the lungs. Petechiae were seen in the pancreas, liver, and subcutis and on serosal surfaces. Only 2 swans exhibited multifocal to coalescent foci of coagulative necrosis in the pancreas. Table 1 summarizes semiquantified viral RNA loads in comparison to immunohistochemical detection of avian influenza virus nucleoprotein in different tissues. Immunostaining for avian influenza virus antigen was positive in 11 of 14 animals and confined to 3 loca-

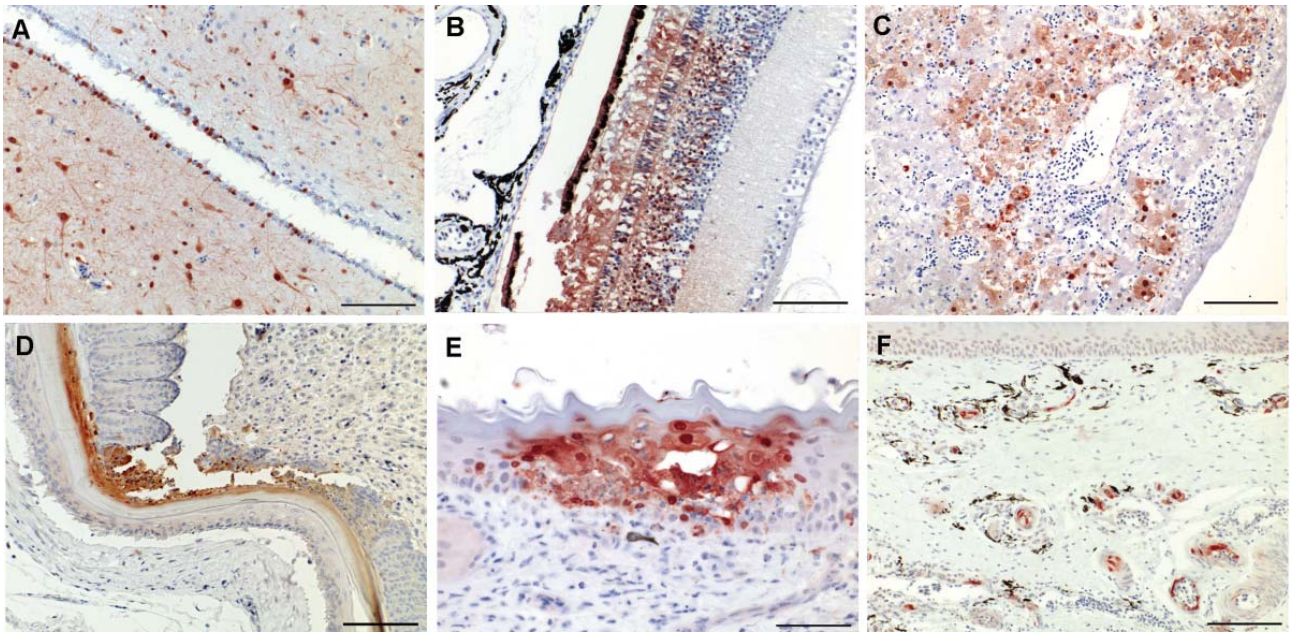

Figure 2. Immunohistochemical analysis for nucleoprotein of avian influenza virus. Tissue sections were stained by using the avidin-biotin-peroxidase complex method, 3-amino-9-ethylcarbazole (red), and hematoxylin (blue). A) Brain, cerebrum: numerous glial cells, neurons and ependymal cells stain positive for influenza virus antigen (scale bar = 200 μm). B) Eye, retina: cells of the pigmented epithelial layer, photoreceptor cells, and cells of the outer and inner nuclear layers are positive for the nucleoprotein of influenza virus (scale bar = 100 μm). C) Liver: subadjacent to the capsule there is hepatocyte degeneration and necrosis around a congested central vein (scale bar = 100 μm). D) Skin: keratinized layer of the feather follicular epithelium shows focal necrosis with intense nuclear and cytoplasmic immunostaining (scale bar =100 μm). E) Nasal cavity: focal intraepithelial necrosis of the mucocutaneous membrane associated with influenza virus infection (scale bar = 50 μm). F) Nasal concha: numerous submucosal arterioles and venules display strong endothelial staining, which partially extends into the media of the vessels (scale bar = 100 μm).

tions: neuronal, epithelial, and endothelial (Figure 2, Table 1). In all animals, a strong neuronal infection could be observed with viral antigen in the cytoplasm and nuclei of neurons, glial cells, and ependymal cells in the brain, spinal cord, and eye (Table 1, Figure 2, panels A, B). Peripheral nerves, e.g., innervating the adrenal glands, the ovary, or area located adjacent to the cecal tonsil also stained positive. Some swans showed immunoreactivity in epithelial cells, e.g., of the pancreas, adrenal glands, ovaries, liver (Figure 2, panel C), feather follicles, and nasal cavity (Figure 2, panels D, E). Endothelia of different organs stained strongly positive only in 3 swans (Figure 2, panel F); in addition, these animals exhibited very high loads of viral genome in all tissue samples (Table 1).

Conclusions

We demonstrated that few adult mute swans might have the ability to survive infection with HPAI virus (H5N1). Surviviors would most likely be older swans in good health infected with a low dosage (e.g., $<10^4$ EID_{50}/animal). However, because of viral shedding for several days without showing severe clinical symptoms, adult mute swans could play a key role in the spread of HPAI virus (H5N1), a conclusion that contradicts those of other investigators (*8–10*). Gross and histologic lesions in infected swans were independent of dosage, age, or sex of infected swans. Two parallel courses of pathogenesis with predominantly endothelial (n = 3) or epithelial/neuronal (n = 9) infections were distinguishable. Both forms have been described (*11*). However, normally only 1 dominant type was observed, depending on the experimental conditions (e.g., species, age of the animals, virus strain). At least 2 swans with the endotheliotropic course of infection were negative for avian influenza virus–specific antibodies. Thus, failure to mount an early antibody response might be responsible for or promote infection of the vasculature. In contrast, preexisting avian influenza virus–specific antibodies can be an efficient modulator of the outcome of an infection with HPAI virus (H5N1).

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Infection with Panton-Valentine Leukocidin–Positive Methicillin-Resistant *Staphylococcus aureus* t034

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Panton-Valentine leukocidin (PVL)–positive methicillin-resistant *Staphylococcus aureus* (MRSA), sequence type 398 is believed to be of animal origin. We report 2 cases of infection due to PVL–positive MRSA, *spa* type t034, in patients in Sweden who had had no animal contact.

The problem of methicillin-resistant *Staphylococcus aureus* (MRSA) is increasing worldwide. MRSA is no longer restricted to hospital settings but is found in homes, places of work, and kindergartens. Cases of animal MRSA infection and carriage are increasingly reported and cause substantial occupational health problems for farmers and veterinary staff (*1*–*3*).

MRSA carriage by domestic animals has been recently reported (*3*–*5*). In the Netherlands in 2005, MRSA of the lineage sequence type (ST) 398, *spa* type t108, was detected and found to be transmittable between animals and humans, e.g., pigs and pig farmers, as well as between humans (*2*). This zoonotic potential has warranted alertness to ask about animal contact when screening patients for MRSA.

Additional *spa* types of the clonal lineage ST398 have been identified among humans and animals. Witte et al. reported on *spa* types t011, t034, and t1197, isolated from colonized and infected humans and companion animals (e.g., dog, pig, horse) in Germany and Austria (*5*). Recently, farmers and veterinarians from 7 countries were found to carry MRSA strains of these *spa* types (*1*). Even more alarming are recent reports of these strains causing serious infection in humans (*4*–*6*). A common trait reported for the strains in the clonal lineage ST398 is the indigestibility of their whole cellular DNA when subjected to *Sma*I-macrorestriction analysis and their consequent inability to be typed by pulsed-field gel electrophoresis (PFGE) (*5*,*7*).

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The Cases

We report 2 patients infected with Panton-Valentine leukocidin (PVL)–positive MRSA t034. Each patient had a medical history typical of that reported for community-acquired MRSA of other lineages, which in most cases are PVL positive (*8*).

The first patient, a previously healthy 36-year-old male physiotherapist, sought medical care in March 2006 for a small abscess in his axilla. Culture of the abscess grew MRSA. Presence of m*ecA* gene was confirmed by PCR (*9*). During the next 2 months, furunculous developed twice, caused by the same strain. His youngest child, adopted from China, had been found to be MRSA positive (throat, perineum, and a small wound) a month earlier during routine screening for adopted children. During subsequent screening of the family, the older sister, adopted from South Korea, was also found positive (throat). Both parents were negative for MRSA at that time, which suggests that the father was newly infected when his abscess developed and that he had not acquired the strain abroad. Also, *spa* typing indicated that the children carried different strains from that of the father and from each other (t286, t1434) (*10*). Subsequent screening of family members for MRSA on several occasions found only the father to be repeatedly positive.

The second patient, a 43-year-old male clerk, also previously healthy, sought medical attention during the summer of 2007 for a MRSA-infected elbow wound. Follow-up examination determined that he carried MRSA also in the perineum and in a chronic external otitis eczema. He was later hospitalized for a larger abscess that required surgical drainage. His family members reported no symptoms and were thus not screened for MRSA.

The patients lived in geographically distinct areas in the western part of Sweden and had no connection to each other. No animal contact (e.g., pets, farming) was reported by the 2 patients, their family members, or other close contacts.

Both patient strains carried PVL, confirmed by identification of the *lukS-lukF* genes (*11*), and were resistant to digestion with restriction endonuclease *Sma*I when typing by PFGE was attempted. Both belonged to the t034 *spa* type. They produced β-hemolysin according to phenotypic detection methods that used rabbit blood agar with hot–cold analysis, which further indicated their animal origin (*12*). Their drug-susceptibility profiles differed; 1 was resistant to doxycycline and the other was resistant to ciprofloxacin, erythromycin, and clindamycin.

Conclusions

These strains carry PVL, a toxin partly responsible for the increased virulence of several of the MRSA clones in the community (*8*). Despite several recent publications concerning ST398 MRSA, few have reported PVL in this lineage, which is believed to be of animal origin (*2*,*4*,*6*,*13*). Most previous reports have described asymptomatic carriage in persons exposed to occupational hazards (e.g., veterinary personal and pig farmers) (*1*,*2*,*7*). However, severe clinical infections have been described (*4–6*). In our patients these strains caused repeated infections that needed medical attention, even hospitalization. Since neither patient had even a remote connection to animals and we found no common source of infection, these strains may already be more common in our region than we had thought. These case reports suggest that strains of this lineage may impose a threat in the community, even to patients with no obvious animal contact.

Dr Welinder-Olsson is a molecular microbiologist in the Bacteriological Laboratory at Sahlgrenska University Hospital. Her primary research interests are identification, epidemiology, and molecular subtyping techniques concerning *S. aureus*, especially MRSA.

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Identification of Residual Blood Proteins in Ticks by Mass Spectrometry Proteomics

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Mass spectrometry–based proteomics of individual ticks demonstrated persistence of mammalian host blood components, including α- and β-globin chains, histones, and mitochondrial enzymes, in *Ixodes scapularis* and *Amblyomma americanum* ticks for months after molting. Residual host proteins may identify sources of infection for ticks.

Without transovarial or venereal transmission, a vector-borne pathogen's persistence in nature depends on successful passage between ≥1 species of vertebrate reservoirs. For Lyme borreliosis in eastern North America, black-legged tick (*Ixodes scapularis*) larvae acquire *Borrelia burgdorferi* from a reservoir host during their first blood meal. Infection persists through subsequent molts, and when a tick feeds for the second time as a nymph it may transmit infection to another competent reservoir or to a human. Reservoir hosts for *B. burgdorferi* are commonly white-footed mice but also include chipmunks, voles, shrews, and ground-foraging birds.

When a tick-borne agent has multiple reservoir hosts, assigning relative contributions of each species to maintenance of the pathogen in the environment may be difficult. One approach is to capture animals, sample blood or tissue for evidence of infection, and examine embedded ticks for the microorganism (*1*,*2*). However, this approach is labor- and resource-intensive, and sample sizes are limited. Greater statistical power could be attained with fewer resources if questing ticks were examined not only for infection but also for the source of the last blood meal because the tick would likely have acquired the infection from that vertebrate. If the tick were engorged, this would be straightforward with the PCR, as demonstrated in mosquitoes (*3*,*4*). However, host-seeking nymphal hard ticks are flat because their last blood meals were months earlier. Use of PCR to identify DNA of vertebrate mitochondria in ticks has been reported (*5*,*6*), but results lacked full sensitivity (*7*,*8*).

An alternative approach is to detect residual proteins from the blood meal. Uptake and retention of host immunoglobulin into the hemolymph of different species of ticks have been documented (*9*), and Vennestrøm and Jensen found vertebrate actin in *I. ricinus* nymphs weeks after the molt (*10*). Given these observations, we hypothesized that sufficient host proteins remained in flat ticks for identification of blood meal by using proteins instead of DNA.

The Study

We used mass spectrometry (MS)–based proteomics, as described by Breci et al. and Koller et al. (*11*,*12*). Individual ticks or pools were pulverized after freezing in liquid nitrogen. Total proteins were precipitated in 95% ethanol at –20°C and recovered by centrifugation. Proteins of individual ticks were reduced with 100 mmol/L dithiothreitol, alkylated with 50 mmol/L iodoacetamide, digested with trypsin at a final concentration of 0.01 μg/μL, and filtered through a C18 cartridge before being subjected to liquid chromatography (LC) with a 5%–50% acetonitrile gradient in 0.1% formic acid, followed by tandem MS (LC-MS/MS; LTQ ThermoElectron, San Jose, CA, USA). Proteins of pooled ticks were separated by 1-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and gel slices were digested in situ with trypsin before using LC-MS/MS. Upwards of ≈7,000 spectra were submitted to a protein identification algorithm for each pooled sample. Sizes of sequenced peptides ranged from 5 aa to 34 aa. The SEQUEST search algorithm (http://fields.scripps.edu/sequest) with data-filtering criteria was used to identify sequence matches of output against databases of rabbit, sheep, deer, goat, mouse (*Mus musculus*), and tick proteins.

We studied *I. scapularis* and the lone-star tick, *Amblyomma americanum*, which is a vector of human monocytic ehrlichiosis in the United States. Ticks were provided by the tick-rearing facility of the Department of Entomology and Plant Pathology of Oklahoma State University (Stillwater, OK, USA). We examined pools of 15 *A. americanum* nymphs that had fed on sheep or rabbits as larvae and were 3 months postmolt. Predominant vertebrate peptides in all pools were α- and β-globin chains of hemoglobin and immunoglobulins. Sequences of these proteins corresponded to the source of the blood for the ticks. Other mammalian proteins detected in pools from ticks fed on sheep or rabbits were histone H3, histone H2, mitochondrial malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, mitochondrial ATP synthase, interferon regulatory factor, α tubulin, β tubulin, and transferrin.

We then studied individual ticks that had fed as larvae on mice (*I. scapularis*), rabbits (*A. americanum* and

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I. scapularis), or sheep (*A. americanum*) and examined flat nymphs of *A. americanum* at 7 months postmolt and *I. scapularis* at 3–11 months postmolt. We also examined *I. scapularis* adults (2 males and 1 female) that had fed as larvae and nymphs on mice and were 3–5 months postmolt. Concentrations of extracted proteins from individual ticks were 50–70 μg/tick. The Figure shows representative LC-MS/MS spectra of an *A. americanum* nymph that had fed on a sheep as a larva. Panel A shows the tandem mass spectrum for the singly charged peptide AAVTGFWGK, corresponding to residues 8–16 of sheep hemoglobin β-subunit (P02075). Panel B shows the tandem mass spectrum for doubly charged VKVDEVGAEALGR, corresponding to residues 17–29 of the same protein. These 2 peptides cover 15.2% of the protein sequence and differ from the orthologous sequence of rabbit (P02099) at 8 of 22 positions.

The Table summarizes results of all individual tick analyses. There was no correlation between number of proteins detected and postmolt period. Although some proteins, such as immunoglobulin and histone H3, were detected in both species, other proteins distinguished between *A. americanum* and *I. scapularis*. Globin chains were more commonly found in *A. americanum* than in *I. scapularis* nymphs. Cytochrome c-type heme lyase, which binds heme moieties and is transported from the cytoplasm to mitochondria of eukaryotes, was present in all samples of *I. scapularis* but not in *A. americanum* (2-sided $p<0.001$, by likelihood ratio). Peptides detected included those specific for the host animal for the blood meal.

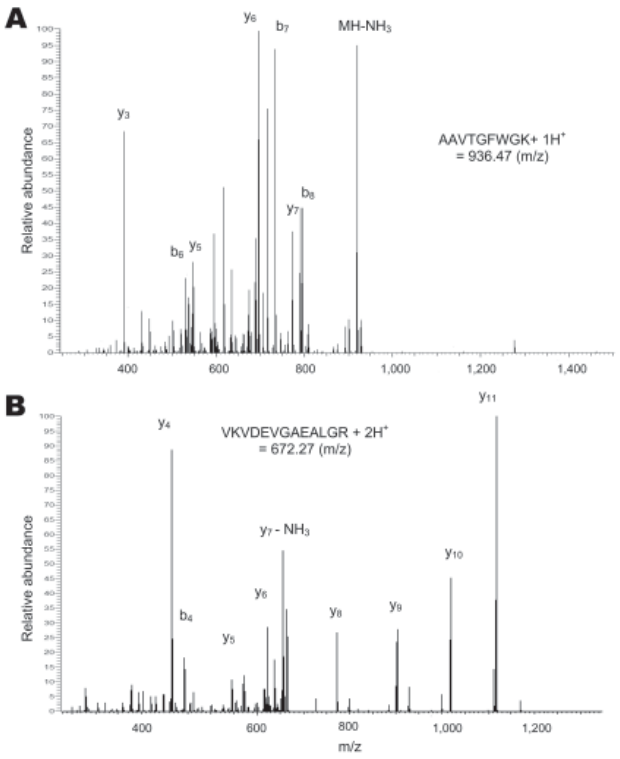

Figure. Tandem mass spectra of 2 peptides from sheep hemoglobin β-subunit identified in a nymph of an *Amblyomma americanum* tick. A) Singly protonated AAVTGFWGK. B) Doubly protonated VKVDEVGAEALGR. The peaks are labeled in the conventional manner: b ions include the N-terminus of the peptide and y ions include the C-terminus, with subscripts indicating the number of amino acid residues in the fragment.

Conclusions

Digestion of a blood meal in ticks differs from what generally occurs in hematophagous insects. In ticks, digestion takes place gradually within cells of the intestinal tract after endocytosis, rather than by intraluminal enzymatic breakdown of blood cells and plasma components, as in insects (*13*). The combination of slow assimilation and uptake of some host proteins into hemolymph may explain persistence of host blood proteins for months after feeding and molting. Immunoglobulins in hemolymph have been demonstrated, but demonstration of several other proteins, including globin chains, histones, and mitochondrial enzymes, indicates that host protein persistence is not limited to 1 type of molecule.

The success of our study depended on access to a large database of protein sequences for sheep, rabbits, and laboratory mice. For most host species for these ticks in nature, such as the white-footed mouse (*Peromyscus leucopus*),

Table. Vertebrate proteins detected by mass spectrometry in extracts of *Amyblomma americanum* or *Ixodes scapularis* flat ticks

	No. ticks in which protein was detected/no. examined				
	A. americanum nymphs		*I. scapularis* nymphs		*I. scapularis* adults
Protein in tryptic digest	Fed on rabbit	Fed on sheep	Fed on rabbit	Fed on mouse	Fed on mouse
Immunoglobulin	3/4	3/4	3/5	4/10	0/3
Globin (α or β)	1/4	3/4	0/5	0/10	3/3
Histone H3	1/4	3/4	3/5	5/10	3/3
Histone H2	3/4	3/4	4/5	6/10	3/3
Tubulin	1/4	2/4	5/5	9/10	3/3
Keratin	0/4	3/4	5/5	7/10	1/3
Actin	1/4	3/4	5/5	10/10	0/3
Cytochrome c-type heme lyase	0/4	0/4	5/5	10/10	3/3
≥1 of above	4/4	4/4	5/5	10/10	3/3

protein databases currently are much less extensive. Thus, a priority before application of this approach is MS-based sequencing of highly prevalent proteins from the blood of *P. leucopus* and other host species. Although LC-MS/MS of individual ticks is feasible and highly sensitive, its cost confines it to exploratory studies. High-throughput analysis of hundreds or thousands of specimens will likely require species-specific assays that use antibodies or aptamers for detection and identification of selected proteins.

Understanding contributions of different vertebrate hosts to pathogen maintenance is a prerequisite for effective monitoring, modeling, and disease prevention efforts that focus on natural reservoirs. Unfortunately, this level of understanding has not been broadly achieved. A major impediment to success in this area for most tick-borne zoonoses has been the absence of reliable and reproducible methods for identification of the vertebrate source of the infection for the tick vector by characterizing residual blood components. Our study shows a way to achieve this goal. Similar data on uninfected ticks would establish the denominator for prevalence studies and indicate the relative competence of different host species.

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Isolation and Molecular Characterization of Banna Virus from Mosquitoes, Vietnam

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We isolated and characterized a Banna virus from mosquitoes in Vietnam; 5 strains were isolated from field-caught mosquitoes at various locations; Banna virus was previously isolated from encephalitis patients in Yunnan, China, in 1987. Together, these findings suggest widespread distribution of this virus throughout Southeast Asia.

Banna virus (BAV) is a 12-segment, double-stranded RNA virus that is classified in the family *Reoviridae* and the genus *Seadornavirus* (*1*). BAV was first isolated from encephalitis patients in southern China, Yunnan Province, Xishuangbanna Prefecture, in 1987 (*2*). In addition, antigenetically BAV-consistent viruses were isolated from pigs, cattle, and humans in Yunnan Province and from the sera of febrile patients in Xinjiang Province (*3*). Therefore, BAV is suspected to be a pathogen of vertebrates and an encephalitis pathogen for humans. BAV was also isolated from mosquitoes in China (*3*–*5*) and Indonesia (*6*), prompting an alert about a pathogenic arbovirus in these countries. Until now, no mammalian cell line has been reported to propagate BAV, and experimental infection with BAV has not resulted in clinical encephalitis in mice (*7*). However, another species of *Seadornavirus*, the Liao Ning virus, has been propagated in various mammalian cell lines. Although this virus has caused hemorrhaging in mice, it has not been associated with the development of encephalitis (*7*).

The pathogenicity and precise distribution of *Seadornavirus* spp., including BAV, are not well understood, and the scope and impact of BAV infection in Asia require further investigation. We report the circulation of BAV in *Culex* spp. mosquitoes in Vietnam and demonstrate that 2 new phylogenetically distinct types of BAVs are co-circulating. These findings suggest that this virus is widely distributed throughout Southeast Asia.

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The Study

Mosquito samples were collected in Vietnam in 2002. Each species was separated and pooled, and each mosquito pool was then homogenized and centrifuged. The supernatant was filtrated through a 0.22-μm filter and then inoculated onto a monolayer C6/36 mosquito cell culture and subsequently incubated at 28°C for 7 days. Virus amplification in C6/36 cells was repeated twice, and the culture fluids were stored at –80°C for further analysis. RNA was extracted from the second cell culture fluid by TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed by using Superscript III Reverse Transcriptase (Invitrogen) and random hexamers, after RNA denaturation at a temperature of 95°C for 5 minutes in the presence of 15% dimethyl sulfoxide. PCR was conducted by using TaKaRa LA Taq DNA polymerase (Takara Bio, Inc., Otsu, Japan) with BAV targeting primers (for segment 5, 5′-CAGCTGCAGTGGTTATTGGA-3′ and 5′ACCGTGCATCTTAACCCTTG-3′; for segment 8, 5′-TTGCAGTCGCTGAGCTTTTA-3′ and 5′-CGCATTTGATCGTATGCTTG-3′). These targeting primers were designed from sequences of BAV strains from China and Java (GenBank accession nos. AF134519–AF134527,AY549307–AY549309,AF052024–AF052035, AY568287–AY568290,NC_004211,NC_004217-NC_004221, NC_004200-NC_004204, NC_004198, and AF052008–AF052013). The amplified cDNAs were sequenced in the 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The genomic sequences of all 12 genome segments for the 5 strains were determined (GenBank accession nos. EU265673–EU265727, EU312980).

The sequences were translated to protein sequences by using the Transeq program of the EMBOSS package (*8*), version 5.00. The protein sequences were aligned with the T-COFFEE program version 5.05 (*9*) with Chinese and Javanese BAVs. Nucleotide sequences were then aligned, on the basis of protein alignment, with the Tranalign program of the EMBOSS package. Maximum likelihood phylogenetic trees were constructed from the protein-guided alignments of nucleotide sequences with DNAML software of the PHYLIP package (*10*), version 3.67.

Five BAV strains were isolated in Ha Tay and Quang Bing Provinces (Figure 1) in Vietnam from *Culex annulus* and *Cx. tritaeniorhunchus* (Table 1). Phylogenetic analysis showed diversity within the phylogenetic clustering of each segment (Table 2). Segments 7 and 9 formed 5 independent clusters, segment 12 formed 3 clusters, and the remaining segments formed 4 clusters. Phylogenetic trees for some representative segments (segments 2, 6, 9, and

Figure 1. Mosquito collection sites in Vietnam.

12) are shown in Figure 2. Javanese strains formed a single cluster for segments 1, 2, 6, 8, 10, and 12. For segments 7, 9, and 11, however, they diverged into 2 clusters, I and II (Figure 2, panel C). The Vietnamese strains were distributed within 2 independent clusters for all 12 segments**.** Segments 11 and 12 of the 2 Vietnamese strains 02VN178b and 02VN018b were included in cluster III with Chinese BAVs. Segments 6 and 9 of isolate 02VN178b belonged to cluster V, although segments 1, 2, 3, 4, and 8 belonged to cluster IV. Moreover, segments 5 and 7 of isolate 02VN178b and segments 8 and 11 of isolate 02VN009b showed a mixed electrogram pattern of clusters V and IV or III.

Conclusions

We report isolation of BAV in Vietnam. The nucleotide sequences of the Vietnamese isolates' genomic RNA segments diverged into 2 phylogenetically distant clusters. Our data indicate that 2 BAV populations exist in the country and that both evolved independently. Two strains were clearly different from Chinese and Javanese BAVs (02VN078b and 02VN180b). Two other strains (02VN018b and 02VN178b) were phylogenetically close to Chinese BAVs when 2 shorter segments, segments 11 and 12, were analyzed but not when the remaining 10, longer segments were considered. This finding implies that a recent reassortment event has occurred and that segments of Chinese or Vietnamese strains were replaced. In addition, although some Vietnamese strains were phylogenetically distant, they could produce a viable progeny by reassortment of segments 5, 6, 7, 8, 9, and 11. This finding is clearly illustrated by the diversity in the phylogenetic clustering pattern and the mixed electrogram pattern of these segments (Table 2).

Analysis of segment 12 showed that cluster III (Table 2) contained 2 Vietnamese strains (02VN178b and 02VN018b) and the Chinese strains AF052030 and YN-6 (isolated in Yunnan Province) and BJ95-75 (isolated in Beijing). This finding suggests the possibility of wide circulation of BAVs containing segment 12 type III not only in Southeast Asia but also in East Asia.

A regionwide genotype shift of Japanese encephalitis virus (JEV), which is also carried by *Culex* spp. mosquitoes, from genotype III to genotype I, was witnessed in East Asia during the 1990s (*11*,*12*). These results and phylogenetic analysis of old JEV strains in Japan and Southeast Asia (*11*) strongly suggested that JEV could be transferred frequently from Southeast Asia to East Asia. Similarly, BAVs with segment 12 belonging to cluster type III were also distributed in both Southeast and East Asia. Therefore, we can speculate that BAVs are actively circulating within the Asian continent. To achieve a better understanding of the distribution dynamics of BAVs in Asia, more isolates are needed.

In Vietnam, a seasonal increase of viral encephalitis and meningitis cases is reported during the rainy season every year. Among these cases, 50%–70% are diagnosed as Japanese encephalitis, while the rest of them remain idiopathic (P.T. Nga et al., unpub. data). A clear association between BAV and human diseases, as well as the preva-

Table 1. Mosquito samples collected in Vietnam, 2002

Sample	Location	Month collected	Origin	No. mosquitoes
02VN009b	Ha Tay	Jan	*Culex annulus*	25
02VN018b	Quang Binh	Mar	*Cx. annulus*	170
02VN078b	Ha Tay	May	*Cx. tritaeniorhunchus*	100
02VN178b	Quang Binh	Aug	*Cx. tritaeniorhunchus*	102
02VN180b	Quang Binh	Aug	*Cx. tritaeniorhunchus*	83

Table 2. Phylogenetic clustering of Banna virus genomic RNA segments*

Strain	Isolation		Segments											
	Location	Year	1	2	3	4	5	6	7	8	9	10	11	12
Length (bp)			3747	3048	2400	2038	1716	1671	1136	1119	1101	977	867	862
Aligned area			990–3227	211–2541	108–2183	570–1748	110–760	106–1386	145–981	129–913	597–872	86–751	75–609	44–664
JKT-6423	Java, Indonesia	1980	I	I	I	I	I	I	I	I	I	I	I	I
JKT-6969	Java, Indonesia	1981	I	I	NA	NA	NA	I	II	I	II	I	II	I
JKT-7043	Java, Indonesia	1981	I	I	NA	NA	NA	I	II	I	II	I	II	I
Chinese	Yunnan, China	1987	III	III	III	III	III	III	III	III	III	III	III	III
YN-6	Yunnan, China	2000	NA	NA	NA	NA	NA	NA	NA	NA	III	NA	NA	III
BJ95-75	Beijing, China	1995	NA	NA	NA	NA	NA	NA	NA	NA	III	NA	NA	III
02VN018b	Quang Binh, Vietnam	2002	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	III	III
02VN178b	Quang Binh, Vietnam	2002	IV	IV	IV	IV	IV/V	V	IV/V	IV	V	IV	III	III
02VN009b	Ha Tay, Vietnam	2002	V	V	V	V	V	V	V	IV/V	V	V	V/ III	V
02VN078b	Ha Tay, Vietnam	2002	V	V	V	V	V	V	V	V	V	V	V	V
02VN180b	Quang Binh, Vietnam	2002	V	V	V	V	V	V	V	V	V	V	V	V

*Length, length of each segment in the reference sequence of Banna virus (BAV) from RefSeq (NC_004198, NC_004200–NC_004204, NC_004211, NC_004217–NC_004221); aligned area, the fragment used to make the alignment and phylogenetic tree. The numbers are set following the sequences of strain JHT-6423, which is the reference strain in National Center for Biotechnology Information Reference Sequences. I, the cluster including the Javanese JKT-6423 strain; II, the cluster specific to the JKT-6969 and JKT-7043 strains; III, the cluster including Chinese BAVs; IV, the cluster including the Vietnamese 02VN018b strain; V, the cluster including the Vietnamese 02VN180b strain. NA, not available.

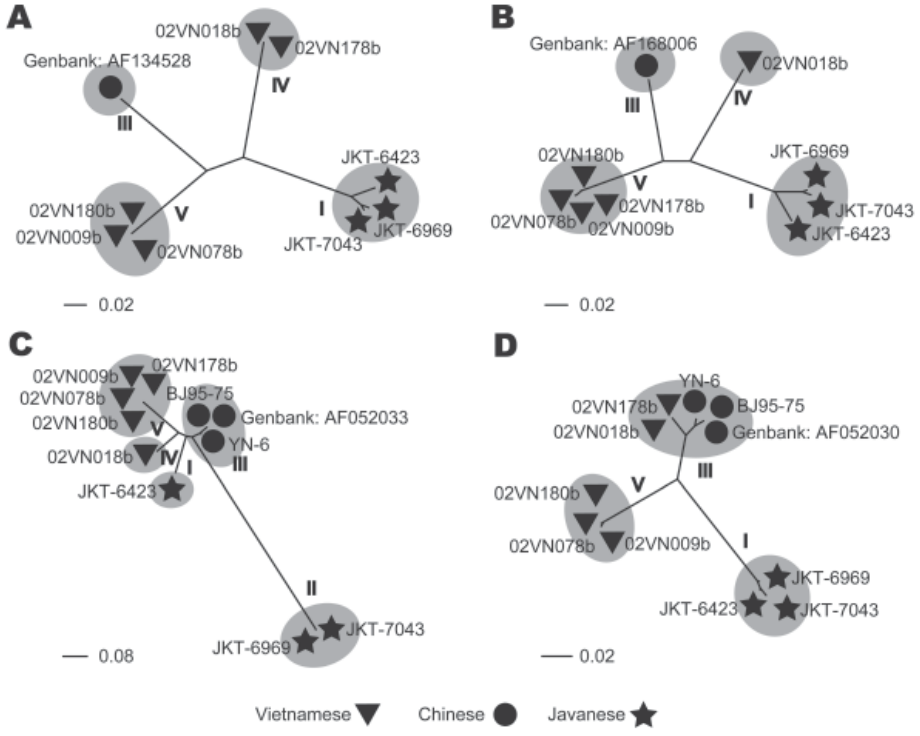

Figure 2. Phylogenetic trees of representative segments of Banna virus genomic RNA. A) Segment 2, encoding VP2, inner-layer coat protein. B) Segment 6, VP6, nonstructural protein, function is unknown. C) Segment 9, VP9, outer-layer attachment protein. D) Segment 12, VP12, dsRNA-binding protein. Clusters were numbered according to the clustering type classification presented in Table 2.

lence of BAV infection in humans, has not yet been established in Vietnam. This topic deserves further study. We also believe that BAV is a good candidate for the differential diagnosis of viral encephalitis and meningitis cases of unknown origin in tropical and subtropical Asia, where *Culex* mosquitoes are abundant.

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Oseltamivir Prescribing in Pharmacy-Benefits Database, United States, 2004–2005[1]

Justin R. Ortiz,*[2] Laurie Kamimoto,* Ronald E. Aubert,† Jianying Yao,† David K. Shay,* Joseph S. Bresee,* and Robert S. Epstein†

We reviewed information from a US pharmacy benefits manager database from 2004 through 2005 during periods with little influenza activity. We calculated rates of oseltamivir prescriptions to enrollees. Prescription rates increased significantly from 27.3/100,000 in 2004 to 134/100,000 in 2005 ($p<0.05$), which suggested that personal stockpiling of oseltamivir occurred.

From 2003 through 2006, avian influenza virus (H5N1) spread from Southeast Asia to Africa, Europe, and the Middle East (*1*), and media coverage about the risk for human infection and the potential for an influenza pandemic increased. Two classes of medications are available to treat influenza: neuraminidase inhibitors (NIs), which include oseltamivir and zanamivir, and adamantanes, which include amantadine and rimantadine (*2*). NIs are recommended by the World Health Organization for treatment of avian influenza virus (H5N1) infection because isolates have shown adamantane resistance (*3*). During the fall of 2005, NIs were in limited supply (*4*).

In 2005, concern was expressed in the medical literature about possible personal stockpiling of NIs for use during an influenza pandemic (*5*). We undertook this study to look for evidence of oseltamivir stockpiling, to understand the magnitude of the practice, and to discern who was receiving and prescribing these drugs. We collaborated with a pharmacy benefits management company to examine antiviral prescriptions and oseltamivir prescription filling in the United States during calendar weeks 36–44 in 2004 and 2005. These weeks were chosen because they had little influenza activity in either year and because reports of oseltamivir stockpiling occurred during this period in 2005 (*6*–*9*).

The Study

We used a database from Medco Health Solutions, Inc. (Franklin Lakes, NJ, USA), a pharmacy benefits management company serving >50 million US members. We examined filled prescriptions for oseltamivir by members from January 2002 through May 2006. Available member data included demographic information, medication dispensed, prescriber identification, and pharmacy dispensing history. Member-level historic pharmacy dispensing data were used to assign members into chronic disease classifications (*10*). Prescribers were cross-referenced with an American Medical Association member database to determine specialty and years since medical school graduation. We were able to cross-reference 64% of prescribing physicians by specialty and years since medical school graduation. The Centers for Disease Control and Prevention (Atlanta, GA, USA) determined that institutional review board approval was not needed for this study because we received aggregated data that was anonymous and not identified.

To assess media coverage, we queried the LexisNexis US News database (www.lexisnexis.com) for total weekly news reports from August 1, 2003, through August 30, 2006, referring to avian influenza and oseltamivir. Weekly virologic data from the World Health Organization and National Respiratory and Enteric Virus Surveillance System collaborating laboratories were used to assess US influenza activity during 2004 and 2005 (*6*,*7*).

Oseltamivir prescription rates were calculated per 100,000 enrolled members and per 1,000 prescribing physicians. Binomial distributions were used to estimate variances for rates. Relative rate ratios (RRs) and 95% confidence intervals (CIs) were calculated for 2004 and 2005 data. P values <0.05 were considered statistically significant. Analyses were performed with SAS version 9.0 statistical software (SAS Institute, Cary, NC, USA).

Weekly rates of filled prescriptions for oseltamivir and percentage of samples positive for influenza from October 1, 2002, through June 1, 2006, were temporally associated before the 2005–06 influenza season (Figure 1). During the fall of 2005, prescriptions for oseltamivir increased without an associated increase in the percentage of samples testing positive for influenza. In contrast, during the same period there was a temporal relationship between weekly oseltamivir prescription rates and media reports of avian influenza and oseltamivir (Figure 1).

The proportion of oseltamivir prescriptions to total anti-influenza prescriptions increased from 37.0% in 2004 to 76.9% in 2005 (Table 1). The 2005 oseltamivir prescription rate of 133/100,000 during weeks 36–44 was ≈5× the 2004 rate of 27.3/100,000 (RR 4.88, 95% CI 4.79–4.97) (Table 1).

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[1]This study was presented, in part, at the Infectious Diseases Society of America annual meeting in Toronto, Ontario, Canada, October 14, 2006, and in poster form at the Options for the Control of Influenza Conference, Toronto, June 17, 2007.

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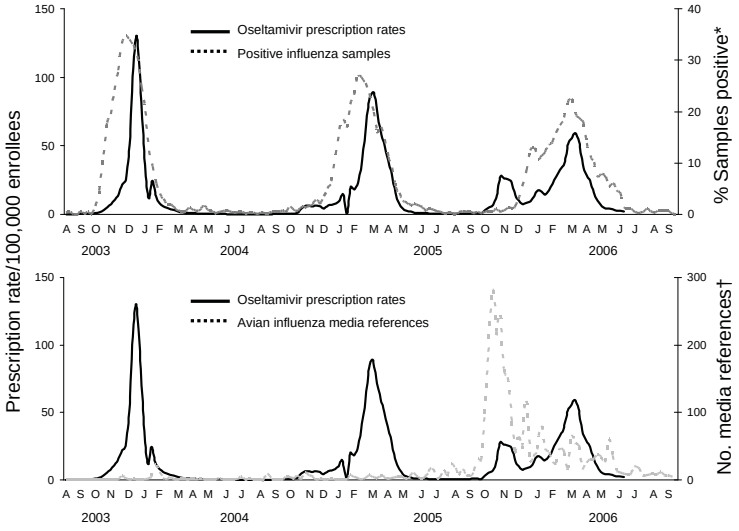

Figure 1. Weekly influenza activity, oseltamivir prescription rates for enrollees of all ages, and LexisNexis references to avian influenza and oseltamivir, United States, 2003–2006. *World Health Organization and National Respiratory and Enteric Virus Surveillance System collaborating laboratories in the United States. †LexisNexis US News database query for weekly news reports referring to "(avian or bird or H5N1) and (flu or influenza) and (Tamiflu or oseltamivir)."

Women were more likely to receive oseltamivir prescriptions than men in 2004 (RR 1.19, 95% CI 1.16–1.24) and 2005 (RR 1.07, 95% CI 1.05–1.09). Prescription rates increased from 2004 to 2005 for all age groups (Table 2). The highest prescription rates in 2005 were for persons 50–64 years of age (211/100,000) and those ≥65 years of age (168/100,000). Members <18 years of age had a ≥7-fold increase in prescription rates from 2004 to 2005.

Among adults, prescription rates were consistently higher in 2005 than in 2004, irrespective of chronic disease classification (Table 2). In 2005, the highest oseltamivir prescription rate was 268.5/100,000 for enrollees with pulmonary disease, and the lowest rate was 89.5/100,000 for those without chronic disease. The greatest rate increase from 2004 to 2005 occurred among those without chronic disease (RR 6.36, 95% CI 6.10–6.62). Among enrollees of all ages during weeks 36–44 in 2005, approximately one third of oseltamivir prescriptions were filled by members without chronic disease.

In 2004 and 2005, general internists had the highest average prescribing rate for oseltamivir (45.3/1,000 and 191.1/1,000, respectively) (Figure 2). Pediatricians had the lowest rates in both years (2.9/1,000 and 32.7/1,000).

For all prescribers in 2004 and 2005, oseltamivir prescription rates increased with years since the prescriber's medical school graduation. The lowest prescription rate in 2005 (1.7/1,000) was observed in prescribers who graduated from medical school in the previous 5 years, followed by prescribers with 5–10 years (4.9/1,000) and 11–19 years (6.6/1,000) since graduation. The highest rate (10.4/1,000) was observed in prescribers with ≥20 years since graduation.

Conclusions

Rates of filled oseltamivir prescriptions during calendar weeks 36–44 increased from 2004 to 2005. These weeks in 2005 were noteworthy for increased media references to oseltamivir and avian influenza, although there was little influenza activity. Low levels of influenza-like illness and respiratory syncytial virus activity also were documented during this period (*6*,*11*).

Among Medco enrollees, the highest prescription rates were for groups with the greatest risk for influenza-associated complications: persons ≥50 years of age and adults with chronic diseases (*2*). However, oseltamivir prescription rates increased from 2004 to 2005 for each age and

Table 1. Anti-influenza drug prescription rates/100,000 enrollees and proportions of all anti-influenza drug prescriptions, United States, weeks 36–44, 2004 and 2005*

	Anti-influenza prescription rates/100,000 enrollees of all ages, weeks 36–44				% Total anti-influenza prescriptions	
Medication	2004	2005	Rate ratio (2005/2004)	95% Confidence interval	2004	2005
Neuraminidase inhibitors						
Oseltamivir	27.3	133	4.88	4.79–4.97	36.99	76.89
Zanamivir	0.35	1.39	4.00	3.38–4.75	0.47	0.80
Adamantanes						
Amantadine	41.7	36.3	0.87	0.85–0.89	56.53	20.93
Rimantadine	4.43	2.37	0.54	0.50–0.58	6.00	1.37
All anti-influenza drugs	80.0	173.2	2.17	2.14–2.19	100	100

*Sums do not equal 100% because of rounding.

Table 2. Oseltamivir prescription rates/100,000 enrollees by age and chronic disease classification, United States, weeks 36–44, 2004 and 2005

Characteristic	2004	2005	Rate ratio (2005/2004)	95% Confidence interval
Age group, y				
<1	0.26	10.65	40.7	(5.6–294)
1–4	8.95	65.95	7.4	(6.3–8.6)
5–17	11.12	81.28	7.3	(6.8–7.9)
18–24	19.55	81.42	4.2	(3.8–4.5)
25–49	33.08	120.97	3.7	(3.5–3.8)
50–64	63.33	211.26	3.3	(3.2–3.4)
≥65	69.04	168.20	2.4	(2.4–2.5)
Chronic disease classification*				
Pulmonary	77.66	268.50	3.46	(3.33–3.60)
Immune deficient	67.75	240.16	3.54	(3.21–3.91)
Neurologic	54.57	207.43	3.80	(3.47–4.15)
Cardiac	54.11	196.92	3.64	(3.54–3.74)
Diabetes	50.85	143.85	2.83	(2.64–3.02)
Chronic disease absent	14.07	89.50	6.36	(6.10–6.62)

*Rates from chronic disease classification include only enrollees ≥18 y of age only. Except for the chronic disease absent category, all chronic disease classifications were not mutually exclusive. Chronic disease classification is derived from the chronic disease index, which has been validated (*10*).

chronic disease category. Although absolute prescription rates were lower in persons <50 years of age, persons without chronic medical diagnoses, and children, these groups had higher rate increases from 2004 to 2005, suggesting that they and their caretakers were influenced to stockpile oseltamivir in 2005.

Physician prescribing rates for all specialties increased from 2004 toh 2005. Prescription rates in 2004 were highest for general internists and family practitioners and likely reflect the primary care physician's gatekeeper role as the entry point for those seeking medical care. Given the nature of their specialty, infectious diseases physicians may be asked to prescribe oseltamivir for personal stockpiles more than the typical provider. Because physicians need not honor all prescription requests, these increases may not be fully explained by increased patient requests because physician attitudes regarding personal stockpiling likely affected whether requests were made or honored.

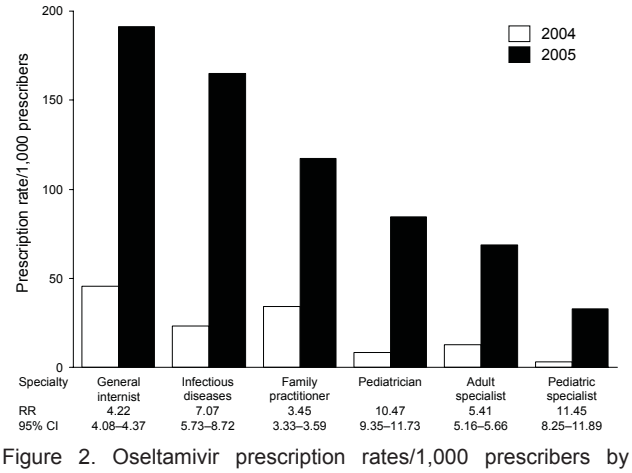

Figure 2. Oseltamivir prescription rates/1,000 prescribers by specialty, United States, weeks 36–44, 2004 and 2005. Infectious diseases classification includes pediatric and adult infectious diseases specialists. All classifications are mutually exclusive. RR, rate ratio; CI, confidence interval.

Our study is subject to limitations. Although we studied a large, national population, our study population may not be nationally representative. In addition, our analyses were limited to prescriptions of oseltamivir that were filled by a pharmacy, and we do not know whether prescriptions were written with the expressed purpose of personal stockpiling for use during a pandemic.

In summary, our findings suggest that increased media reports during the fall of 2005 about the influenza (H5N1) epizootic prompted concern about the possibility of an influenza pandemic. This heightened concern led to an increase in filled oseltamivir prescriptions for personal stockpiling among a national pharmacy benefits member population. Subsequently, as influenza virus began circulating in early 2006, oseltamivir prescriptions corresponded more closely with virus activity. Efforts by federal and state governments to procure sufficient supplies of NIs to treat every patient likely to become ill during the next pandemic may quell demand in personal stockpiles. Education campaigns about appropriate use of antiviral medications that target physicians and patients during seasonal epidemics and pandemics may reduce inappropriate requests for oseltamivir and other drugs.

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Cluster of Falciparum Malaria Cases in UK Airport

Alison J. Rodger,*† Graham S. Cooke,‡§ Rosalynn Ord,¶ Colin J. Sutherland,¶ and Geoffrey Pasvol*§

A cluster of 6 cases of *Plasmodium falciparum* malaria occurred in a UK airport among 30 travelers returning to the United States from East Africa. Molecular genotyping analysis indicated that all had been exposed to different parasites. The travelers' use of chemoprophylaxis was poor; their perception of risk was limited.

Although most malaria occurs in Africa and Asia, it is also found in travelers returning from malaria-endemic regions (*1*). In the United States in 2005, a total of 1,528 cases of malaria were diagnosed, an increase of 15% from the previous year (*2*). In the United Kingdom in 2006, a total of 1,758 malaria cases were reported (*3*). In each country, malaria prophylaxis was inadequate for 75%–80% of patients (*2*,*3*).

Most cases of malaria in travelers occur sporadically as single cases. We describe a cluster of *Plasmodium falciparum* malaria cases among a group of US citizens returning from East Africa.

The Cases

A group of 30 US citizens spent June and July 2005 in Kenya and Uganda as part of an educational program. Their return journey took them from Nairobi to London, where they spent a week before their flight to the United States. By the time they arrived at the airport departure lounge, 6 persons had become sick with high fevers, rigors, and some confusion. They were referred by airport staff to the Infectious Diseases Unit at Northwick Park Hospital, London.

All arrived at the unit within 3 hours of referral and were proven to have falciparum malaria on peripheral blood smears. Clinical features of persons with disease are shown in the Table. Of these 6 persons, all were 19–22 years of age, and 5 exhibited initial clinical features of severe malaria, e.g., impaired consciousness (disorientation as to time, place, or person; or prostration [inability to sit up and drink]). These 5 persons required intravenous quinine and intensive monitoring; the other person had less severe disease and was treated with a combination of oral atovaquone and proguanil. All improved by day 4 and returned to the United States shortly thereafter.

In light of these cases, all other members of the group who had returned to the United States were alerted to contact their healthcare providers if they became symptomatic. Another person became febrile while in the United States and was initially treated with conventional antimicrobial drugs; this person was later recalled to a hospital and treated appropriately for falciparum malaria after the diagnosis of his colleagues became known. Unconfirmed reports were received that 2 more patients had received treatment for malaria (species unidentified) within 6 months of return. The high attack rate in this group of students and the close temporal clustering of cases led us to examine the possibility that the group had experienced a mini-outbreak as a result of a point-source exposure.

A study-specific, self-administered questionnaire gathered information about demographics, onset and nature of symptoms, medical history, malaria protective measures, and chemoprophylaxis and adherence. The questionnaire was given to patients 1–6 in the UK hospital and to patient 7 by email. Clinical data for patients 1–6 were obtained during their UK hospital stay, and clinical data for patient 7 was obtained from the diagnosing and treating physician in the United States. Genetic diversity of parasite isolates was investigated by PCR amplification of polymorphic regions of the *P. falciparum* merozoite surface protein 1 (*Pfmsp1*) and *Pfmsp2* loci as previously described (*5*).

The group had traveled together through Kenya and Uganda, with almost identical itineraries. All had slept in shared accommodations under unimpregnated malaria nets. Despite weekly malaria education sessions, antimalarial drug use was not widespread in the absence of a clear predeparture recommendation. Only 1 of 7 patients had taken malaria prophylaxis, but this person did not continue using chemoprophylaxis for the full period at risk. None of the 7 patients recalled being bitten by mosquitoes. However, when returning to Nairobi on July 2, 2005, the group was delayed for several hours by the roadside in the rice-growing area of Mwea (northern Kenya). Many had little protective clothing with them and while waiting until dusk that evening, noticed a large number of mosquitoes of unknown species around them. This event occurred 20 days before first onset of symptoms. After visiting Uganda, the group returned to Nairobi on July 11, arrived in London on July 13, and went to London's Heathrow Airport on July 20 for the flight back to the United States.

Paired peripheral blood samples from pretreatment and day 1 posttreatment were provided by 6 of the 7 pa-

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Table. Clinical data for 6 US travelers with falciparum malaria returning from East Africa, 2005*

Patient no.	Malaria severity (*4*)	Temp, °C	BP, mm Hg	Parasitemia, %	HGB†	Platelets‡	Creatinine§	Bilirubin¶	ALT#	Base excess**
1	Severe	38.2	97/52	3.5	9.3	33	89	71	103	–2.4
2	Severe	40.1	132/73	3.5	16.0	38	108	40	101	3.1
3	Mild	38.0	110/75	1.0	13.3	48	70	20	34	ND
4	Severe	36.4	99/63	3.5	16.5	193	111	22	34	–2.3
5	Severe	39.4	105/72	1.5	16.8	121	103	20	38	-0.8
6	Severe	37.8	79/53	1.0	13.1	54	102	9	41	–4.8

*Date of symptom onset for each patient was 2005 Jul 20. Temp, body temperature; BP, blood pressure; HGB, hemoglobin; ALT, alanine aminotransferase; ND, not done.
†Reference range 11–15 g/dL.
‡Reference range 120–308 × 10^9/L.
§Reference range <100 μm/L. To convert to mg/dL, divide by 88.4.
¶Reference range <19 μm/L. To convert to mg/dL, divide by 17.1.
#Reference range 19–50 U/L.
**In mmol/L. To convert to mEq/L, divide by 1.0.

tients; for the other patient, only a pretreatment sample was available for DNA analysis. No 2 patients had identical alleles at both *Pfmsp1* and *Pfmsp2* loci (Figure). At least 12 different parasite genotypes were detected. Pretreatment and posttreatment samples were similar except for those of patient 2, for whom postquinine allelic patterns differed for *Pfmsp1* and *Pfmsp2* on day 1 and day 0.

Conclusions

Malaria-endemic areas, such as East Africa, are increasingly popular destinations for US travelers. Therefore, suboptimal adherence to malaria chemoprophylaxis and effective preventative measures against mosquito bites (such as use of bed nets) is cause for concern (*3*). Antimalarial drug use was poor among the case-patients, for whom emphasis was placed on personal protective measures rather than chemoprophylaxis. The high attack rate for this group is consistent with rates reported by other studies demonstrating the injudicious risk of traveling to a malaria-endemic area without taking effective antimalarial drugs (*6*,*7*).

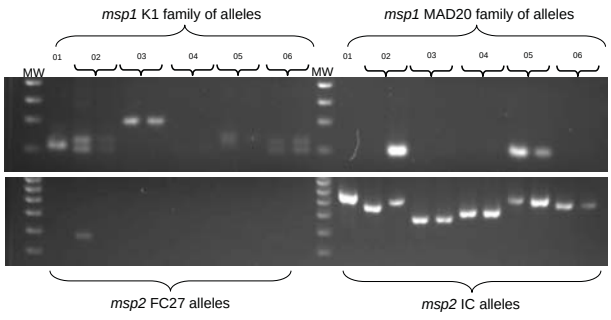

Figure. Molecular typing of malarial parasites from 6 US travelers with falciparum malaria returning from East Africa in 2005. *Plasmodium falciparum* merozoite surface protein 1 (Pfmsp1) (upper gel) and Pfmsp2 (lower gel) allelic variation among isolates was determined by nested PCR and agarose gel electrophoresis. DNA markers (MW) are in lanes 1 and 13 in each gel. Two family-specific primer sets were used for each of the 2 genes. No parasites of the Ro33 allelic family of msp1 were found (data not shown). Pretreatment (day 0) isolates are shown for all patients. Posttreatment (day 1) parasite isolates are also shown for patients 2–6.

The course of disease for patients 1–6 displayed remarkable synchrony; onset of symptoms occurred within 24 hours of each other and illness proceeded rapidly. These 6 patients received prompt diagnosis and treatment at a specialist unit and recovered fully. Had it had not been for alert airport staff, these 6 patients (5 with severe malaria) would have been on a 9-hour flight to the United States; consequences for the patients could have been fatal. These cases highlight the benefits of vigilant staff at airports, even for passengers boarding from non–disease-endemic areas. Prompt contact tracing enabled a missed diagnosis of malaria to be rectified at a US facility and highlights the advantage of actively seeking all those who might have shared the same exposure.

The clustering of the onset of symptoms for patients 1–6 suggested a common timing of infection, although other unproven factors, such as the effect of atmospheric pressure changes during air travel, may have led to the synchronous onset of the cases. Molecular evidence suggests that despite synchrony of symptoms, the genetically heterogeneous parasite variants were unlikely to have come from 1 infectious mosquito. We estimate that at least 12 distinct haploid parasite genotypes were circulating among the 6 patients. Our data also demonstrate, for patient 2, that circulating parasite genotypes can change profoundly immediately after treatment, as previously observed for quinine-treated persons (*8*). Caution is therefore required when interpreting genotyping data for treated persons (*9*).

The only reported intense exposure to mosquitoes was during the travel delay in Mwea. The main rice-growing season starts in June, which is also the time of *Anopheles* spp. peak abundance (*10*). However, the mosquitoes seen at Mwea were not positively identified as *Anopheles*, and although the students did not recall being bitten by mosquitoes on other occasions, we cannot rule out infection later in the journey, which would be more probable given the date of onset of symptoms in nonimmune persons.

This mini-outbreak demonstrates the need to encourage travelers to take malaria prophylaxis and to improve their knowledge about the risk of malaria in their area of proposed travel. It also underscores the value of alerting fellow travelers in the same party to the possibility of contracting malaria.

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Analysis of Collection of Hemolytic Uremic Syndrome–associated Enterohemorrhagic *Escherichia coli*

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Multilocus sequence typing of 169 non-O157 enterohemorrhagic *Escherichia coli* (EHEC) isolated from patients with hemolytic uremic syndrome (HUS) demonstrated 29 different sequence types (STs); 78.1% of these strains clustered in 5 STs. From all STs and serotypes identified, we established a reference panel of EHEC associated with HUS (HUSEC collection).

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a highly pathogenic subgroup of Shiga toxin–producing *E. coli* (STEC) that cause severe human diseases, including bloody diarrhea and hemolytic uremic syndrome (HUS) (*1*). The ability to cause severe human disease differentiates EHEC from other STEC found in the environment that are less pathogenic or nonpathogenic. *E. coli* O157:H7 is the most frequent EHEC implicated as a cause of HUS (*2*), but non–O157:H7 EHEC are variably present as the only pathogens in stools from HUS patients (*1*,*3*,*4*).

A recent phylogenetic analysis of *E. coli* isolated from humans and animals in different geographic areas by multilocus sequence typing (MLST), the current standard for phylogenetic analyses of bacteria, indicated extensive allelic variations and homolog recombinations in pathogenic lineages and demonstrated repeated and independent evolution of pathogenic strains (*5*,*6*). However, only a limited number of EHEC associated with HUS have been so investigated. Therefore, we performed a comprehensive MLST-based examination of the molecular phylogeny of EHEC isolated from HUS patients and established a collection of representative HUS-associated enterohemorrhagic *E. coli* (HUSEC) (www.ehec.org).

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The Study

From 1996 through 2006, 524 EHEC were isolated as the only pathogens from fecal samples of epidemiologically unrelated patients with HUS (1 strain per patient). The isolation was achieved by using previously described procedures (*7*). The isolates were confirmed as *E. coli* by API 20 E (bioMérieux, Marcy l'Etoile, France) and serotyped (*8*) by using antisera against *E. coli* O antigens 1–181 and H antigens 1–56. In all nonmotile isolates from serogroups O26, O103, O111, O145, and O157, *fliC* genes were genotyped (*9*,*10*). MLST was performed as described previously (*6*) with small modifications (*11*). Phylogenetic analyses were based on allelic data that used the BURST algorithm (*12*) to achieve a more robust interpretation of the clustering and to reduce the influences by the effects of the recombination, which are widespread in *E. coli* (*6*). In addition, the stringent definition of clonal complexes (CCs), with which strains sharing at least 6 identical alleles are grouped into the same CC, was applied. The minimum spanning tree was generated from the allelic profiles by using *Shigella dysenteriae* strain M1354 (ST243, by using data from http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli) as outgroup (online Appendix Figure, available from www.cdc.gov/EID/content/14/8/1287-appG.htm).

Among 524 EHEC isolated from HUS patients, 355 (67.7%) belonged to serotypes O157:H7/H$^-$ (249 were non–sorbitol-fermenting EHEC O157:H7/H$^-$ and 106 were sorbitol-fermenting EHEC O157:H$^-$), and 169 (32.3%) belonged to 34 non-O157 serotypes. Because the phylogeny of *E. coli* O157:H7/ H$^-$ has been extensively studied and is well established (*5*,*13*), we focused on MLST analysis of the 169 non-O157 strains, which represent all non-O157 EHEC serotypes from German HUS patients during the study period. We performed MLST analysis of only a subset of 10 O157 strains as controls.

MLST analysis of 169 non-O157 EHEC isolates distinguished 29 different sequence types (STs), which clustered into 10 CCs and 12 singletons (Table 1). The predominant ST was ST21, which consisted of 43 isolates (25.4% of non-O157 EHEC), followed by ST29 (30 isolates, 17.8%), ST32 (30 isolates, 17.8%), ST17 (15 isolates, 8.9%), and ST16 (14 isolates, 8.3%) (Table 1). These 5 STs included 78.1% of all HUS-associated non-O157 EHEC. The remaining 21.9% (n = 37) of the non-O157 EHEC strains belonged to 24 other STs that comprised only 1–3 strains; 14 of these STs were found only once (Table 1). Among the 10 CCs identified, CC29 was the most frequent. It comprised 89 strains of 5 STs, corresponding to 60.1% of 148 strains that could be assigned to a CC and to 52.7% of all non-O157 EHEC (Table 1).

After CC29, CC32 and CC20 were the most frequently identified CCs (Table 1).

The predominant serotypes identified among the 169 non–O157 HUS-associated EHEC were O26:H11/H– (n = 72; 42.6%), O145:H28/H– (n = 32; 18.9%), O111:H8/H– (n = 14; 8.3%), and O103:H2/H$^-$ (n = 14; 8.3%). The nonmotile strains within these serogroups shared the H antigen–encoding *fliC* gene with the motile strains that expressed the respective H antigen (Table 1). These 8 serotypes together constituted 132 (78.1%) of the non-O157 EHEC associated with HUS, whereas the other 37 strains (21.9%) belonged to 26 different serotypes, 17 of which contained only a single isolate (Table 1).

The most frequent serotypes including O26:H11/H–, O103:H2/H–, O111:H8/H–, and O145:H28/H– clustered into the 5 most prevalent STs (Table 1). However, not all isolates of the same serotype always belonged to the same ST (Table 1). One example is serotype O26:H11/H$^-$ (*fliC*$_{H11}$), which was the most common non–O157 EHEC associated with HUS and clustered into 4 STs as single-locus variants (Table 1). Each of four O rough (OR) strains (2 OR:H11, and 1 each OR:H2 and OR:H$^-$), none of which could be successfully serotyped, was matched by its ST to an O typeable strain, indicating a recent conversion from the smooth to the rough strain form.

The relationships among members of the different STs and CCs are demonstrated in the online Appendix Figure. Within the serogroup O111, 14 isolates belonging to serotypes O111:H8 and O111:H$^-$ (*fliC*$_{H8}$) were ST16 (CC29). In contrast, the EHEC O111:H10 isolate with ST43 (CC10) shared none of the 7 MLST loci with the O111:H8/H$^-$ strains, indicating that EHEC O111 causing HUS originate from 2 different clonal sources. Similar differences were observed between EHEC O145:H25 (ST342)/O145:H$^-$ (*fliC*$_{H25}$) (ST659) and O145:H28 (ST32). Whereas ST659 is a single-locus variant of ST342, both allelic profiles differ in all loci from ST32.

The combination of MLST analysis and serotyping enabled us to establish the HUSEC collection. This collection comprises 41 EHEC isolated from HUS patients in Germany, which includes all 36 EHEC serotypes (O157 and non-O157) isolated from HUS patients and all 31 STs identified

Table 1. Clonal complexes, sequence types, and serotypes of non-O157 EHEC from patients with hemolytic uremic syndrome*

CC	No. strains (%)†	ST	No. strains (%)†	Serotype‡ (no. strains)§
29	89 (52.7)	ST21	43 (25.4)	O26:H11/H$^-$ (*fliC*$_{H11}$) (41), OR:H11 (1), Ont:Hnt (1)
		ST29	30 (17.8)	O26:H11/H$^-$ (*fliC*$_{H11}$) (29), OR:H11 (1)
		ST27	1 (0.6)	O26:H11
		ST396	1 (0.6)	O26:H11
		ST16	14 (8.3)	O111:H8/H$^-$ (*fliC*$_{H8}$)
32	32 (18.9)	ST32	30 (17.8)	O145:H28/H$^-$ (*fliC*$_{H28}$)
		ST137	2 (1.2)	O145:H$^-$ (*fliC*$_{H28}$)
20	16 (9.5)	ST17	15 (8.9)	O103:H2/H$^-$ (*fliC*$_{H2}$) (14), OR:H2 (1)
		ST20	1 (0.6)	O119:H2
10	3 (1.8)	ST43	1 (0.6)	O111:H10
		ST330	2 (1.2)	Ont:H$^-$
11	3 (1.8)	ST335	3 (1.8)	O55:H7
40	1 (0.6)	ST40	1 (0.6)	O112:H$^-$
69	1 (0.6)	ST69	1 (0.6)	O73:H18
101	1 (0.6)	ST101	1 (0.6)	O55:Hnt
155	1 (0.6)	ST56	1 (0.6)	O113:H21
469	1 (0.6)	ST679	1 (0.6)	O163:H19
NA	2 (1.2)	ST25	2 (1.2)	O128:H2
NA	2 (1.2)	ST678	2 (1.2)	O104:H4
NA	2 (1.2)	ST655	2 (1.2)	O121:H19
NA	1 (0.6)	ST329	1 (0.6)	O136:Hnt
NA	3 (1.8)	ST342	2 (1.2)	O145:H25/H$^-$ (*fliC*$_{H25}$)
		ST659¶	1 (0.6)	O145:H$^-$ (*fliC*$_{H25}$)
NA	1 (0.6)	ST677	1 (0.6)	O174:H21
NA	1 (0.6)	ST39	1 (0.6)	O70:H8
NA	1 (0.6)	ST675	1 (0.6)	O76:H19
NA	3 (1.8)	ST442	3 (1.8)	O91:H21
NA	3 (1.8)	ST306	3 (1.8)	O98:H$^-$ (2), OR:H$^-$ (1)
NA	2 (0.6)	ST672	2 (1.2)	O104:H21 (1), Ont:H21 (1)

*CC, clonal complex; ST, sequence type; EHEC, enterohemorrhagic *Escherichia coli*; HUS, hemolytic uremic syndrome; NA, not assigned.
†% of strains of a CC and ST among all 169 non-O157 EHEC isolated from HUS patients.
‡H$^-$, nonmotile; OR, O rough (autoagglutinable strain); nt, not typeable by the *E. coli* O and H antisera used.
§Number of strains of the serotype that belonged to the respective ST; if no number is given, all strains of the serotype belonged to the respective ST.
¶ST659 is a single-locus variant of ST342.

within these serotypes (Table 2). The strains included in this HUSEC collection were reserotyped and characterized for their *stx* genotypes and the presence of the *eae* gene (Table 2). Phenotypic characteristics and additional properties such as putative virulence determinants are available at www.EHEC.org.

Conclusions

Most (81.1%) of the non-O157 EHEC clustered into 3 CCs and belonged to a limited number of serotypes. These strains were recovered independently from different regions in Germany over an 11-year period. For the remaining strains, epidemiologic support is not as strong, and the clonal analysis demonstrated that their chromosomal backgrounds are highly divergent from those of CC29, CC32, and CC20. In 14 STs, we have only 1 isolate. In these cases, excluding concurrent or recent infection by *E. coli* O157 serologically is even more important. This exclusion was not always possible because patients' serum for the investigation of immunoglobulin M anti-O157 lipopolysaccharide

Table 2. Strains of the HUSEC collection representing all serotypes of HUS-associated EHEC strains isolated in Germany, 1996–2006*

Strain	Original	Year of isolation	Serotype	ST (CC)	*eae*	stx_1	stx_2†
HUSEC001	05-946	2005	O111:H10	43 (10)	–	–	2
HUSEC002	5152/97	1997	Ont:H$^-$	330 (10)	–	–	2
HUSEC003	6334/96	1996	O157:H7	11 (11)	+	–	2
HUSEC004	3072/96	1996	O157:H$^-$	11 (11)	+	–	2
HUSEC005	2907/97	1997	O55:H7	335 (11)	+	–	2
HUSEC006	5376/99	1999	O157:H$^-$	587 (11)	+	–	2
HUSEC007	7382/96	1996	O103:H2	17 (20)	+	–	2
HUSEC008	2791/97	1997	O103:H$^-$	17 (20)	+	–	2
HUSEC009	6833/96	1996	OR:H2	17 (20)	+	–	2
HUSEC010	1805/00/A	2000	O119:H2	20 (20)	+	1	–
HUSEC011	2516/00	2000	O111:H8	16 (29)	+	1	2
HUSEC012	6037/96	1996	O111:H$^-$	16 (29)	+	1	2
HUSEC013	2245/98	1998	O26:H11	21 (29)	+	1	–
HUSEC014	5080/97	1997	O26:H$^-$	21 (29)	+	1	2
HUSEC015	126814/98	1998	OR:H11	21 (29)	+	1	2
HUSEC016	5028/97	1997	Ont:Hnt	21 (29)	+	1	–
HUSEC017	3319/99	1999	O26:H11	27 (29)	+	1	2
HUSEC018	1530/99	1999	O26:H11	29 (29)	+	–	2
HUSEC019	1588/98	1998	OR:H11	29 (29)	+	1	–
HUSEC020	3271/00	2000	O26:H11	396 (29)	+	–	2
HUSEC021	0488/99	1999	O145:H28	32 (32)	+	–	2
HUSEC022	4557/99	1999	O145:H$^-$	137 (32)	+	–	2
HUSEC023	1169/97/1	1997	O112:H$^-$	40 (40)	–	–	$2d_{act}$
HUSEC024	2996/96	1996	O73:H18	69 (69)	–	–	$2d_{act}$
HUSEC025	06-05009	2006	O55:Hnt	101 (101)	–	1	–
HUSEC026	99-09355	1999	O113:H21	56 (155)	–	–	$2d_{act}$
HUSEC027	03-07727	2003	O163:H19	679 (469)	–	–	$2d_{act}$
HUSEC028	03-06687	2003	O128:H2	25 (NA)	–	1c	2d
HUSEC029	4256/99	1999	O70:H8	39 (NA)	+	–	2
HUSEC030	05-03519	2005	O98:H$^-$	306 (NA)	–	1	–
HUSEC031	7792/96	1996	OR:H$^-$	306 (NA)	+	1	–
HUSEC032	2441/98	1998	O136:Hnt	329 (NA)	–	1c	2
HUSEC033	4392/97	1997	O145:H25	342 (NA)	+	–	2
HUSEC034	3332/99	1999	O91:H21	442 (NA)	–	1	$2+2d_{act}$
HUSEC035	1529/98	1998	O121:H19	655 (NA)	+	–	2
HUSEC036	2839/98	1998	O145:H$^-$	659 (NA)	+	1	2c
HUSEC037	02-03885	2002	O104:H21	672 (NA)	–	1	$2+2d_{act}$
HUSEC038	3356/97/B	1997	Ont:H21	672 (NA)	–	1	$2d_{act}$
HUSEC039	3651/96	1996	O76:H19	675 (NA)	–	1c	–
HUSEC040	220/00	2000	O174:H21	677 (NA)	–	–	2c
HUSEC041	01-09591	2001	O104:H4	678 (NA)	–	–	2

*HUSEC, hemolytic uremic syndrome–associated enterohemorrhagic *Escherichia coli;* EHEC, enterohemorrhagic *E. coli*. For each serotype, the multilocus sequence type (ST) and the corresponding clonal complex (CC) are given in accordance to the *E. coli* multilocus sequence typing website (http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli). Furthermore, the presence (+, present; – absent) of the intimin gene (*eae*), the Shiga toxin gene (*stx*), and its subtype(s) are specified. nt, not typeable by the O and H antisera used; H$^-$, nonmotile; OR, O rough (autoagglutinable strain); NA, not assigned.
†$2d_{act}$, $stx_{2d\text{-}activatable}$.

antibodies is frequently not available. However, at least some of these strains might represent emerging clones in the human population, such as O111:H10 (*10*), O113:H21 (*14*), and O121:H19 (*15*). Thus, strains of these serotypes included in our HUSEC collection can be used in future studies as a reference to compare EHEC isolated in other countries from HUS patients. This would allow timely discovery of the emergence of new non-O157 clones associated with HUS and the virulence traits that they contain (www.ehec.org).

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Virus Transfer from Personal Protective Equipment to Healthcare Employees' Skin and Clothing

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We evaluated a personal protective equipment removal protocol designed to minimize wearer contamination with pathogens. Following this protocol often resulted in virus transfer to hands and clothing. An altered protocol or other measures are needed to prevent healthcare worker contamination.

Caring for patients with communicable diseases places healthcare workers (HCWs) at risk. Infected HCWs may not only incur serious illness or death themselves but may spread infection to others. Methods to prevent HCW infections include vaccination (*1*), hand hygiene (*2*), and isolation of patients with communicable diseases (*3*).

A key aspect of patient isolation is proper use of personal protective equipment (PPE) to protect HCWs from pathogen exposure during patient care. PPE includes use of barriers (gowns, gloves, eye shields) and respiratory protection (masks, respirators) to protect mucous membranes, airways, skin, and clothing from contact with infectious agents (*3*). The importance of PPE was underscored in the recent outbreak of severe acute respiratory syndrome (SARS). HCWs accounted for ≈20% of cases (*4*); failure to properly use PPE was a risk factor for HCW infection (*5*).

This outbreak raised concern that HCWs could contaminate their skin or clothes with pathogens during PPE removal, resulting in accidental self-inoculation and virus spread to patients, other HCWs, or fomites. The Centers for Disease Control and Prevention (CDC) addressed this concern by designing a protocol to minimize contamination to the wearer during PPE removal (Figure 1) (*6*). However, the effectiveness of this protocol in preventing self-contamination has not been validated. To determine if removing PPE according to the CDC protocol prevents viral contamination of the wearer, a human challenge study was undertaken using a nonpathogenic virus.

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The Study

PPE (gowns, gloves, respirators, and goggles) donned by volunteers was contaminated with bacteriophage MS2, a nonenveloped, nonpathogenic RNA virus suspended in 0.01 mol/L phosphate-buffered saline and GloGerm (GloGerm, Moab, UT, USA), synthetic beads that fluoresce under UV light (for visual tracking of virus). Sites of contamination were as follows: front shoulder of gown, back shoulder of gown, right side of N95 respirator, upper right front of goggles, and palm of dominant hand. Each site was contaminated with a total of 10^4 PFU of MS2 in 5 drops of 5 μL each. Participants performed a healthcare task (measuring blood pressure on a mannequin) and then removed PPE according to CDC protocol. Hands, items of PPE, and scrubs worn underneath were sampled for virus. Hands were sampled by using the glove juice method (*7*). Each hand was placed inside a bag containing 75 mL stripping solution (0.4 g KH_2PO_4, 10.1 g Na_2HPO_4, 1.0 mL Triton-X/L) and massaged for 60 seconds to cover all hand surfaces with solution. PPE items were immersed in 1.5% beef extract, pH 7.5, and agitated on a shaker for 20 minutes. Eluent from hands and PPE was assayed by the most probable number (MPN) enrichment infectivity assay (*8*). To prevent cross-contamination, samples from only 1 volunteer were processed at a time, and individual eluent samples were processed separately in a biological safety cabinet, with decontamination in between.

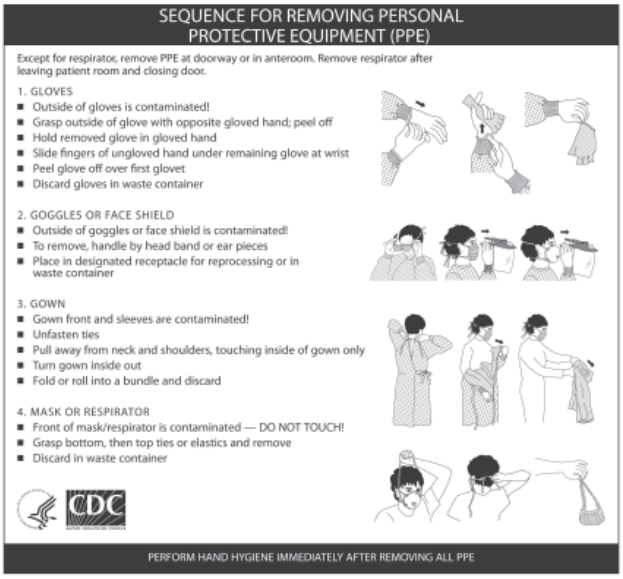

Figure 1. Centers for Disease Control and Prevention protocol for removing healthcare worker PPE.

When an a priori value of 25% was used for the 95% upper confidence limit when p (transfer) = 0, the sample size was N = 10. Protocols were approved by the University of North Carolina (UNC) Biomedical Institutional Review Board, and written informed consent was obtained. Enrolled participants met the following inclusion criteria: >18 years of age, nonpregnant, nonallergic to latex, no active skin disorders, and medical evaluation approval for N95 respirator fit testing and use (*9*). Experiments took place in a patient care room in the UNC Hospitals' General Clinical Research Center. The experimental protocol is shown in Figure 2. Participants were shown the poster distributed by CDC (Figure 1) and given an opportunity to read it and ask questions. The poster was placed in front of the participants for reference while they donned and removed PPE.

Ten study participants were enrolled in this study: 9 women and 1 man. Nine participants were right-handed, and 1 was left-handed. Transfer of virus to both hands, the initially uncontaminated glove on the nondominant hand, and the scrub shirt and pants worn underneath the PPE was observed in most volunteers (Table). Because of the difficulty of sampling large facial areas, visible fluorescent tracer was used as the criterion to determine whether the face would be sampled. No tracer was observed on the facial areas of any volunteer. The fluorescent tracer was not a consistent indicator of virus contamination; virus was recovered both from sites where tracer was visible and where it was not detected.

The amount of virus recovered was 1–3 $\log_{10}$ MPN for hands and 1–4 $\log_{10}$ MPN for scrubs. The mean amount of virus recovered from the right hand (the dominant hand of 90% of volunteers) was greater than that recovered from the left hand. While removal of gloves and gowns required 2 hands, mask and goggle removal was one-handed, which could have resulted in larger quantities of virus being transferred to the dominant hand during removal. In the single left-handed study participant, recovery of virus was greater from the left hand than the right (1.82 $\log_{10}$ vs. 0.98 $\log_{10}$ MPN). The mean amount of virus recovered from scrub shirts was significantly greater than that recovered from pants (p = 0.01), possibly because of contact with hands when the gown is pulled away from the shoulder during removal.

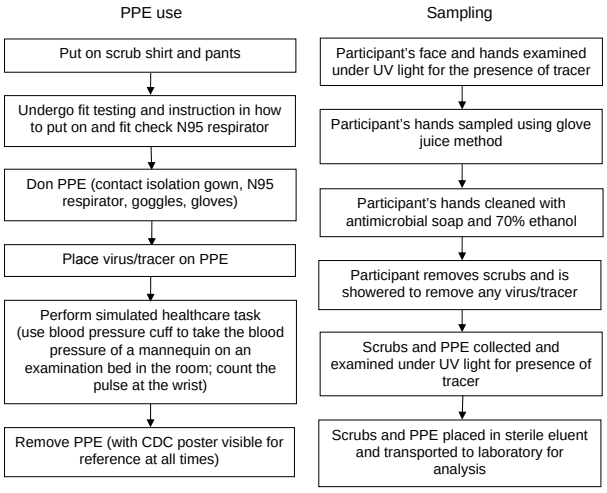

Figure 2. Protocol for human challenge experiments. PPE, personal protective equipment; CDC, Centers for Disease Control and Prevention.

Conclusions

PPE is vital for protecting HCWs from occupationally acquired infection during patient care, particularly from droplet- or airborne-transmitted diseases. However, removing PPE after patient care without contaminating skin or clothes is important. Although PPE is usually worn only for short periods, viruses such as influenza (*10*) and SARS coronavirus (*11*) can survive for hours on surfaces, and viral infection can be spread by surface-to-hand (*12*) and hand-to-hand contact (*13*).

Developing and validating an algorithm for removing PPE that prevents contamination of the skin and clothes of HCWs are key to interrupting nosocomial transmission of infectious agents. These experiments demonstrate that the current CDC algorithm is insufficient to protect HCWs from contamination during PPE removal. However, options that might prevent such contamination do exist, including double gloving, use of surgical protocols for PPE removal, and PPE impregnated with an antimicrobial agent.

A double-glove removal sequence would begin with removal of the outer glove, followed by removal of goggles or face shield, gown, and respirator/mask, and finishing with removal of the inner glove followed by hand

Table. Frequency and levels of viral contamination of selected sites, virus transfer study, 2007*

Site	% Volunteers who transferred virus to site (N = 10)	Mean viral titer recovered from site ($\log_{10}$ MPN)	% Contaminated sites with visible tracer (N = 10)
Nondominant glove	80	2.2	10
Right hand (skin)	90	2.4	20
Left hand (skin)	70	1.8	0
Scrub shirt	100	3.2	10
Scrub pants	75†	2.1	0
Face	0	–	–

*MPN, most probable number; –, not measured.
†N = 8.

hygiene; handling of PPE with ungloved hands is avoided. Borrowing PPE protocols from surgery, in which the ends of gown sleeves are tucked underneath gloves during wear, might also reduce contamination. When the HCW is finished, goggles and respirator are removed first, and gown and gloves are then removed together by peeling off both at the same time, again avoiding handling PPE with ungloved hands. Finally, the use of PPE impregnated with antimicrobial agents might also reduce or eliminate contamination of skin and clothes.

This study also indicates the need for continued emphasis on hand hygiene. A barrier to improving hand hygiene compliance rates is the belief that gloves make hand hygiene unnecessary (*14*). This is contradicted by our study and others showing that organisms can spread from gloves to hands after glove removal (*15*). Even if double gloving is incorporated into protocols for PPE use, it is not a substitute for proper hand hygiene. Before these or other candidate methods are introduced into clinical practice, their impact on the safety of HCWs should be validated by testing with methods such as we have described.

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Rickettsia felis in Fleas, Germany

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Among 310 fleas collected from dogs and cats in Germany, *Rickettsia felis* was detected in all specimens (34) of *Archaeopsylla erinacei* (hedgehog flea) and in 9% (24/226) of *Ctenocephalides felis felis* (cat flea). *R. helvetica* was detected in 1 *Ceratophyllus gallinae* (hen flea).

Rickettsia felis, the causative agent of the flea-borne spotted fever rickettsiosis, is pathogenic for humans (*1–4*). Since the first detection of *R. felis* from midgut epithelial cells of the cat flea, *Ctenocephalides felis felis*, in 1990 (*5*), interest in the role of this flea species as its main vector has increased. *R. felis* has been found in cat fleas on all continents (*6*). Because *R. felis* is not lethal for cat fleas and is transmitted transovarially by these fleas (*4*), *C. felis* could be a vector and a reservoir of this pathogen. For these reasons, the cat flea was considered the only flea species with a major role in the epidemiology of flea-borne spotted fever rickettsiosis. However, *R. felis* has been reported in other flea species (*4,6–8*), and flea-borne spotted fever rickettsiosis is now considered an emerging human infectious disease. We analyzed the presence of *R. felis* in different flea species collected from naturally infested cats and dogs in different locations in Germany.

The Study

A total of 310 fleas were collected from 49 dogs and 54 cats in 11 widely distributed locations in Germany (Berlin, Munich, Brandenburg, Leipzig, Chemnitz, Rostock/Laage, Bremen, Osnabrück, Münster, Freising, and Schongau) (Figure) in 2007. Specimens collected were recorded and kept at –20°C. Samples were shipped on dry ice to our laboratory, and species identification was performed by using light microscopy and following the determination key of Hopkins and Rothschild (*9*). Because of infestation variations (1–150 fleas per animal), 3 fleas per animal host were chosen randomly for species differentiation.

Fleas were homogenized individually in 80 µL of phosphate-buffered saline with a RETSCH Tissue Lyser Mixer Mill 300 (QIAGEN, Hilden, Germany) by using 5-mm steel beads. A 100-µL volume of ATL buffer and 20 µL of proteinase K (QIAGEN) were added, and homogenates were incubated at 56°C in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) until tissues were completely lysed. DNA was extracted from each flea by using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions (tissue protocol) and stored at –20°C until used.

PCR amplification of rickettsial DNA was performed by using oligonucleotide primer pairs Rp CS.877p/Rp CS.1258n (*10*) generated from the rickettsial citrate synthase (*gltA*) gene. Positive samples were analyzed for a 530-bp portion of the outer membrane protein A (*ompA*) gene with primer pair Rr 190.70p/Rr 190.602n (*10*) and for a 765-bp portion of the *ompB* gene with primer pair 120–1278/120–3599 (*11*). PCR amplification was accomplished in 50-µL volumes containing 5 µL DNA, 30 µL distilled water, 10 µL 5× *Taq* buffer (Roche, Mannheim, Germany), 3 µL 25 mmol/L $MgCl_2$ (Roche), 1 µL 10 mmol/L dNTP (Roche), 0.25 µL each primer (100 µmol/L), and 0.5 µL *Taq* polymerase (5U/mL; Roche). Conditions for the *gltA* and *ompA* PCRs were as described by Bertolotti et al. (*12*). Negative and positive controls were included in all PCRs.

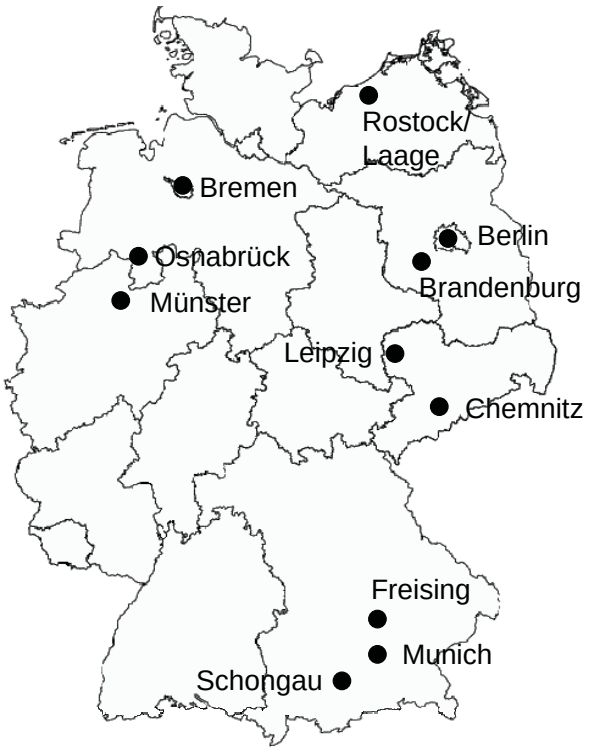

Figure. Locations of the 11 flea-collection study sites in Germany, 2007.

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All PCR products were separated by electrophoresis on 1.5% agarose gels at 100 V for 60 min and examined under UV light. Positive samples for both genes were purified by using the QIAquick PCR purification kit (QIAGEN) and sequenced by the MWG Biotech Company (Martinried, Germany). Sequences obtained were compared with those of characterized rickettsia in GenBank by using BLAST analysis (www.ncbi.nlm.nih.gov).

Five species of fleas were identified in the study. The most prevalent species was *C. felis* (93% of fleas in cats and 78% in dogs) (Table 1). *Archaeopsylla erinacei* (hedgehog flea), was the second most abundant species, with 26 specimens collected from dogs and 8 from cats. A few specimens of *Ctenocephalides canis* (dog flea), *Pulex irritans* (human flea), and *Ceratophyllus gallinae* (hen flea) were also identified (Table 1). Eight dogs had mixed populations of fleas; 5 had *C. felis* and *A. erinacei*, 2 had *C. felis* and *C. gallinae*, and 1 had *C. felis* and *C. canis*. Mixed populations of fleas were also detected in 3 cats; 2 were infested with *C. felis* and *A. erinacei*, and 1 with *P. irritans* and *C. felis*.

Thirty-six (25%) of 146 fleas collected from dogs and 24 (15%) of 164 fleas collected from cats were positive for the *gltA* gene. Positive fleas were found in 6 of 11 sampled locations. Proportions of infected fleas collected from dogs ranged from 25% (Berlin) to 56% (Münster), and proportions of infected fleas collected from cats ranged from 10% (Freising) to 100% (Münster) (Table 2).

Table 1. *Rickettsia felis* infection in fleas collected from dogs and cats, Germany, 2007*

Flea species	No. (%) *gltA*-positive fleas	
	Dogs	Cats
Ctenocephalides felis	114 (10)	152 (14)
Archeopsylla erinacei	26 (26)	8 (8)
Ctenocephalides canis	4 (0)	–
Ceratophyllus gallinae	2 (1)	3(1)
Pulex irritans	–	1 (0)
Total	146 (37)	164 (23)

**gltA*, citrate synthase.

Of 60 fleas positive for the *gltA* gene (for dogs and cats), only 2 were negative for the *ompA* and *ompB* genes. Sequencing analysis of the *gltA* gene for these 2 samples showed that 1 sequence (from *C. gallinae*) was 99% homologous with part of the *Rickettsia helvetica gltA* gene (AM418450.1) from an *Ixodes persulcatus* tick isolated in Russia; the other sequence (from *C. gallinae*) was 94% homologous with the *Rickettsia* sp. citrate synthase gene (U76908.1). Thus, we report *R. helvetica* in *C. gallinae* ticks.

Of the other 58 *gltA*-positive samples, 2 were positive for the *ompA* gene in the first round; 56 fleas were positive for the *ompB* gene. The 2 *ompA*-positive samples were

Table 2. Distribution of *Rickettsia felis* in fleas collected from dogs and cats, Germany, 2007*

Location	Animal	No. animals	No. fleas	Flea species	No. (%) *gltA*+	No. animals with/without infected fleas
Berlin	Dog	8	24	20 *Ctenocephalides felis*,† 4 *Archeopsylla erinacei*†	6 (25)	0/8
	Cat	6	18	17 *C. felis*,† 1 *A.erinacei*†	4 (22)	2/6
Brandenburg	Dog	4	12	12 *C. felis*	0	0/4
	Cat	1	3	*C. felis*	0	0/1
Bremmen	Dog	1	2	2 *C. felis*	0	0/1
	Cat	7	21	*C. felis*	0	0/7
Chemnitz	Dog	10	30	21 *C. felis*, 9 *A.erinacei*†	9 (30)	3/10
	Cat	8	24	*C. felis*	0	0/8
Freising	Dog	–	–	–	–	–
	Cat	7	21	20 *C. felis*,† 1 *A.erinacei*†	1 (5)	2/7
Leipzig	Dog	–	–	–	–	–
	Cat	2	6	*C. felis*	0	0/2
Munich	Dog	7	21	10 *C. felis*, 10 *A.erinacei*,† 1 *Ctenocephalides canis*	10 (50)	4/7
	Cat	8	27	21 *C. felis*, 6 *A. erinacei*†	8 (30)	3/8
Münster	Dog	3	9	6 *C. felis*,† 3 *A.erinacei*†	5 (56)	3/3
	Cat	1	3	*C. felis*†	3 (100)	1/1
Osnabrück	Dog	6	18	18 *C. felis*	0	0/6
	Cat	8	23	22 *C. felis*, 1 *Pulex irritans*	0	0/8
Rostock/Laage	Dog	5	15	12 *C. felis*, 3 *C. canis*	0	0/15
	Cat	1	3	*C. felis*	0	0/1
Schongau	Dog	5	15	12 *C. felis*,† 2 *Ceratophyllus gallinae*†	7 (47)	3/5
	Cat	5	15	13 *C. felis*,† 3 *C. gallinae*†	7 (47)	3/5

**gltA*, citrate synthase.
†Flea species positive for *gltA*.

sequenced, and sequences matched the *ompA* gene from *R. felis* (AJ563398.1; 99%–100% similarity). The 56 positive *ompB* samples were sequenced, and sequences matched with *ompB* gene from *R. felis* (CP000053.1; 98%–100% similarity). All hedgehog fleas (34 specimens) collected were infected with *R. felis*. Moreover, these 34 specimens were collected from 5 locations within a large area from Berlin (northeastern Germany) to Munich (southeastern Germany). Our findings indicate that *A. erinacei* may play a major role in the transmission of *R. felis* in Germany. Recent studies reported *R. felis* in 1 hedgehog flea in Portugal (*13*) and in 4 hedgehog fleas in Algeria (*8*).

Conclusions

Our study confirms that *C. felis* remains the most common flea species infesting cats and dogs in Germany. Nevertheless, only 24 of 266 cat fleas collected were infected with *R. felis*. Infected cat fleas were found only in 4 of 11 studied sites, in contrast with a recent study in France, where *R. felis*–infected *C. felis* were present in all locations studied (*14*). In the 4 positive sites in Germany, 3 had positive *A. erinacei* specimens and 1 had positive *C. gallinae* (Table 2). In the other sites where no positive fleas where found, only *C. felis* was present either alone or in association with *P. irritans* and *C. canis* (Table 2).

Although *C. felis* seems to be the main vector of *R. felis*, our findings indicate that *A. erinacei* may be a vector for human flea-borne rickettsiosis in Germany. Because hedgehogs may act as a reservoir of pathogens (*15*), further studies will be conducted to investigate the role of hedgehogs and hedgehog fleas in maintenance and transmission of *R. felis* in Germany.

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Macrolide-Resistant *Shigella sonnei*

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Shigella sonnei UCN59, isolated during an outbreak of *S. sonnei* in January 2007, was resistant to azithromycin (MIC 64 mg/L). The isolate contained a plasmid-borne *mph*(A) gene encoding a macrolide 2′-phosphotransferase that inactivates macrolides. Emergence of the *mph*(A) gene in *S. sonnei* may limit usefulness of azithromycin for treatment of shigellosis.

Shigellosis remains a common gastrointestinal disease in developing and industrialized countries. It occurs mostly in children <5 years of age; *Shigella sonnei* is the most frequently isolated species (*1*). Ampicillin and trimethoprim-sulfamethoxazole alleviate the dysenteric syndrome of shigellosis and reduce the infectious period. However, current resistance patterns limit the use of these drugs (*2*). Although fluoroquinolones are an effective alternative for adults, they are not approved for shigellosis treatment in children <18 years of age because of their potential toxicity (*2*,*3*). Azithromycin, a macrolide, represents an attractive treatment option for several reasons. It has in vitro activity against most *Shigella* spp. isolates (*4*), can be given once a day, and attains high intracellular concentrations (*5*). Despite MICs from 2 to 8 mg/L for *Shigella* spp., sufficient concentrations of azithromycin in the colon may inhibit *Shigella* spp. growth (*6*). Azithromycin is recommended by the American Academy of Pediatrics for treatment of shigellosis in children, by the World Health Organization as a second-line treatment in adults, and, since June 2004, by the Agence Française de Sécurité Sanitaire des Produits de Santé (*2*,*7*; www.agmed.sante.gouv.fr/htm/10/filcoprs/mp040601.pdf). In 1996, 2002, 2003, and 2007, outbreaks of shigellosis caused by *S. sonnei* resistant to ampicillin and trimethoprim-sulfamethoxazole occurred in children in northern Paris. The outbreaks occurred in religious schools, similar to cyclic outbreaks in US Jewish schools related to secondary transmission (*8*,*9*).We report an outbreak of shigellosis in and around Paris, France, in which azithromycin failure was related to emergence of plasmid-mediated resistance to macrolides.

The Study

On January 24, 2007, *S. sonnei* strain UCN59 was isolated from a 4-year-old girl admitted to Robert Debré Hospital, Paris, for bloody diarrhea and fever. The strain was resistant to ampicillin, trimethoprim, sulfonamides, and cotrimoxazole but susceptible to quinolones, third-generation cephalosporins, and doxycycline according to the disk-diffusion technique. MICs of macrolides were markedly increased for *S. sonnei* UCN59 compared with those for a susceptible control *S. sonnei* UCN62 (Table). From January to April 23, 2007, a total of 50 cases of laboratory-confirmed shigellosis were identified. Isolates included, in addition to UCN59, 31 *S. sonnei* that had an azithromycin MIC ≥64 mg/L from 31 children <15 years of age, who had each been prescribed azithromycin for diarrhea. All patients lived in the Paris area and attended 8 religious schools.

Typing by pulsed-field gel electrophoresis (PFGE) and repetitive sequence–based PCR (rep-PCR) by using the automated DiversiLab system (bioMérieux, La-Balmes-Grottes, France) (*10*) was performed on the 32 azithromycin-resistant and on 11 azithromycin-susceptible (MIC <16 mg/L) sporadic or outbreak isolates obtained during 1996–2007 in the Paris area. Five different PFGE patterns were obtained by using the enzyme *Bln*I. All the 2007 outbreak isolates, including the 32 azithromycin-resistant isolates and 2 azithromycin-susceptible isolates, were clustered into a single profile, profile 1 (Figure 1). The presence of azithromycin-susceptible isolates with profile 1 was detected among the 1996 and 2002–2006 outbreak isolates (data not shown), showing the persistence of this clonal type over 10 years in this area of Paris. Other isolates displayed PFGE types 2 to 5. Low diversity of PFGE profiles was consistent with isolation of strains in the same area and for most of them from the same community. Four different patterns with <97% similarity were distinguished by rep-PCR (Figure 2). Again, all the 2007 azithromycin-resistant isolates were clustered; however, they could be distinguished from the 2007 azithromycin-susceptible isolates. In contrast to PFGE findings, rep-PCR showed that isolates representative of the 1996, 2002, and 2003 outbreaks were genetically related.

The *mph*(A) gene, which encodes a macrolide 2′-phosphotransferase that inactivates macrolide antimicrobial drugs, was amplified from *S. sonnei* UCN59 DNA by PCR (*11*). PCR was negative for the *erm*(A), *erm*(TR), *erm*(B), *erm*(C), and *erm*(X) methylase genes; the *ere*(A), *ere*(B) genes encoding esterases; the *mph*(B) gene encoding a phosphotransferase; and the efflux genes *mef*(A) and *msr*(A). Sequence of the genes that encode ribosomal structures composing the target of macrolides, *rrl*, *rplD,* and

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Table. Macrolide susceptibility of outbreak and control *Shigella* isolates and *Escherichia coli* constructs

Strain	MIC, mg/L			
	Erythromycin	Clarithromycin	Azithromycin	Telithromycin
Shigella sonnei UCN 62	64	32	2	8
S. sonnei UCN 59	1,024	1,024	64	64
Escherichia coli K12 AG100A	2	2	2	1
E. coli K12 AG100A/pUV21	512	512	8	32
E. coli K12 AG100A/pUC18Ω*mph*(A)	>1,024	512	128	512

rplV genes in *S. sonnei* UCN59, did not display any mutation in the critical bases of resistance to macrolides.

The genes conferring resistance to ampicillin and erythromycin were transferred en bloc by conjugation from *S. sonnei* UCN59 to a macrolide-susceptible mutant *Escherichia coli* AG100A at a frequency of $\approx 10^{-3}$ per donor cell-forming unit after the mating period. A single plasmid was extracted from a transconjugant *E. coli* AG100A/pUV21. After restriction analysis, its size was estimated at ≈90 kb. PCR experiments showed that this plasmid belonged to incompatibility group I (*12*). MICs of macrolides for *E. coli* AG100A/pUV21 confirmed that this plasmid conferred cross-resistance to macrolides (Table).

*Eco*RI-restricted fragments of plasmid pUV21 were transferred to a nylon membrane and hybridized to an *mph*(A) probe. The *mph*(A) gene was borne by an ≈20-kb *Eco*RI fragment, confirming that resistance to azithromycin was plasmid mediated.

After plasmid digestion with *Pst*I enzyme, a DNA fragment that conferred resistance to erythromycin was cloned in plasmid pUC18 and introduced by transformation into *E. coli* AG100A to generate *E. coli* K12 AG100A/pUC18Ω*mph*(A). Sequence of the inserted DNA was determined. The fragment contained 4 open reading frames (ORFs) in the same orientation: *mph*(A), *mrx* that putatively encodes a membrane protein, *mphR*(A) that regulates the expression of *mph*(A), and an ORF of unknown function. This series of ORFs was flanked by a copy of IS*26* at the 5′ end and a copy of IS*6100* at the 3′ end. BLAST analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed that the nucleotide sequence was nearly identical to that of fragments of plasmid pU302L from *Salmonella enterica* serotype Typhimurium (C.Y. Chen et al., unpub. data, GenBank accession no. NC_006816), of *Shigella flexneri* transposon TnSF1 (J.H. Chen and J.Y. Chen, unpub. data, GenBank accession no. AF188331), and of plasmids pRSB101 and pSRB107 (*13*,*14*).

Conclusions

Few data are available on azithromycin resistance in *Shigella* spp. A recent report from Bangladesh mentioned that 16% of *Shigella* isolates were resistant to azithromycin and that 62% had intermediate resistance according to the Clinical Laboratory Standards Institute breakpoints recommended for streptococci (≥1 mg/L, resistant; <0.25 mg/L, susceptible) (*15*). However, the MIC_{90} (MIC at which 90% are susceptible) of 8 mg/L displayed by the microorganisms was within the normal range of MICs for this microorganism; no isolate had an azithromycin MIC >24 mg/L, which suggests that none had acquired resistance to azithromycin. Surveillance for resistance to azithromycin in *Shigella* spp. requires specific breakpoints for this species (*3*).

The *mph*(A) gene has been detected in the sequence of transposon TnSF1 isolated from *S. flexneri* (J.H. Chen and J.Y. Chen JY, unpub. data, GenBank accession no. AF188331). The *mph*(A) gene was first reported in an *E. coli* isolate from Japan (*10*). Since then, the gene has been found in *Aeromonas hydrophila*, *Pseudomonas*

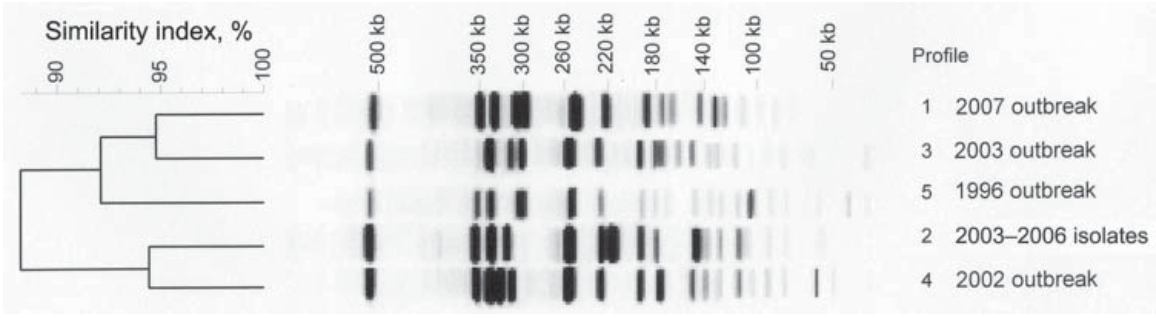

Figure 1. Pulsed-field gel electrophoresis–generated dendrogram for 43 *Shigella sonnei* isolates obtained from sporadic or outbreak cases during 1996–2007 in the Paris area. Profile 1) representative isolates from the 2007 outbreak, including 32 isolates with azithromycin MIC >256 mg/L by Etest and 2 isolates with azithromycin MIC <16 mg/L. Profile 2) 6 representative isolates from sporadic cases (2003–2006) with azithromycin MIC <16 mg/L. Profile 3) representative isolate Shi 03-3580 from 2003 outbreak with azithromycin MIC <16 mg/L by Etest. Profile 4) representative isolate Shi 02-9633 from 2002 outbreak with azithromycin MIC <16 mg/L by Etest. Profile 5) representative isolate Shi 96 1420 from 1996 outbreak with azithromycin MIC <16 mg/L by Etest.

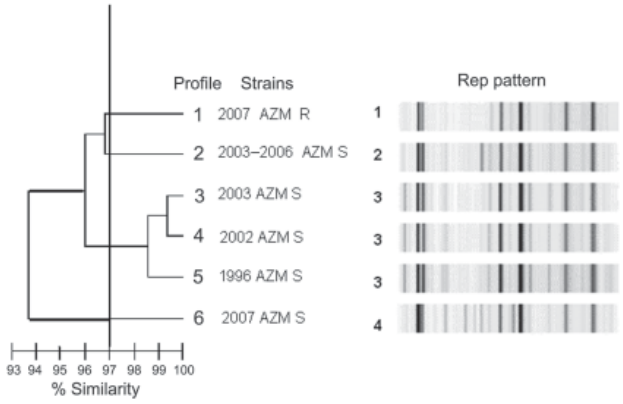

Figure 2. Repetitive sequence–based, PCR–generated dendrogram for 43 *Shigella sonnei* isolates obtained from sporadic or outbreak cases during 1996–2007 in the Paris area. Isolates with ≥97% similarity were considered to be closely genetically related. Profile 1) representative of the 32 isolates of the 2007outbreak with azithromycin (MIC >256 mg/L by Etest) . Profile 2) 1 of 6 isolates from sporadic cases (2003–2006) with azithromycin MIC <16 mg/L. Profile 3) representative isolate Shi 03-3580 from 2003 outbreak with azithromycin MIC <16 mg/L by Etest. Profile 4) representative isolate Shi 02-9633 from 2002 outbreak with azithromycin MIC <16 mg/L by Etest. Profile 5) representative isolate Shi 96 1420 from 1996 outbreak with azithromycin MIC <16 mg/L by Etest. Profile 6) isolate from 2007 with azithromycin MIC <16 mg/L by Etest; another AZM S 2007 isolate had an identical profile. AZM, azithromycin; R, resistant; S, sensitive; Rep, repetitive sequence-based PCR.

spp., *Stenotrophomonas* spp., and a variety of enterobacteria (listed at http://faculty.washington.edu/marilynr/ermweb4.pdf).

Azithromycin was used to treat shigellosis in France only after the release of the French recommendations in 2004. Subsequent rapid emergence of azithromycin-resistant isolates may be a limitation for the use of macrolides in shigellosis. Because use of azithromycin is proposed for treatment of shigellosis, susceptibility of the isolates to azithromycin should be routinely tested.

Dr Boumghar-Bourtchai is pursuing a PhD degree at the University of Caen; she completed this work as part of her PhD program. Her main research interest is emergence of unusual mechanisms of resistance to macrolides in various bacteria, such as *S. sonnei, Turicella otitidis,* and other gram-positive organisms.

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Genotyping *Rickettsia prowazekii* Isolates

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We developed a typing method that can differentiate 8 strains of *Rickettsia prowazekii* into 7 genotypes. This method can be used to type and trace the origin of *R. prowazekii* isolated from samples collected during epidemics after a bioterrorism attack.

Rickettsia prowazekii is the causative agent of epidemic typhus and also a potential bioterrorism agent. The disease may occur in epidemics when social, economic, or political systems are disrupted and expose a large population such as refugees to louse infestation due to lack of hygiene. Recent outbreaks of typhus have occurred in Burundi, Algeria, Peru, and Russia (*1*,*2*). *R. prowazekii* is transmitted by the human body louse, *Pediculus humanus corporis,* in the human cycle. Sylvatic typhus associated with *R. prowazekii* has been documented in the eastern United States. However, it is not clear whether *R. prowazekii* transmission to humans from flying squirrels results from the bite of fleas or lice or contaminated arthropod fecal material (*3*,*4*). Reemergence of epidemic typhus and the potential use of *R. prowazekii* in bioterrorist attacks requires a molecular method that can type isolates and trace the origin or epidemiology of the disease.

The Study

Our objective was to identify a minimal gene set in which PCR amplification and sequencing would allow the efficient differentiation of *R. prowazekii* strains for diagnostic purposes. Using BLAST analysis (www.ncbi.plm.nih.gov/blast/b12seq/wblast2.cgi) to identify target DNA sequences for genotyping, we compared the genomic sequences of Madrid E strain (E strain, NC_000963) (*5*) with those of Nuevo Leon strain, a new tick isolate of *R. prowazekii* (*6*), which was sequenced recently (unpub. data). We identified 6 loci with insertion or deletion in 1 of 2 strains. PCR primers were designed from the target sequences and used to amplify DNA from 8 strains of *R. prowazekii*, including human isolates Addis Ababa, Breinl, Cairo, and E strain; a guinea pig isolate of Evir strain (*7*); a tick isolate (ZRS) from Ethiopia (*8*); and 2 flying squirrel isolates (GvV-250 from Virginia and GvF-16 from Florida) (Table 1) (*4*). Rickettsial genomic DNA was extracted from the *R. prowazekii*–infected L929 cells or infected yolk sacs of embryonated chicken eggs by using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

For designing the primers (Table 1), we used Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi); primers were synthesized. Two microliters of the DNA preparation were amplified in a 50-μL RED taq ReadyMIX PCR (Sigma-Aldrich). The following conditions were used for amplification: an initial 5 min of denaturation at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 53 °C, and extension for 1 min at 72°C. Amplification was completed by holding the reaction mixture for 2 min at 72°C. PCR products were directly sequenced with PCR primers for both strands. PCR amplification and DNA sequencing were performed twice for each gene of each *R. prowazekii* strain. A PCR reaction without template DNA was included as a negative control in each PCR.

DNA sequences were aligned by using DNASTAR Lasergene software, version 6.0 (DNASTAR, Inc., Madison, WI, USA). The sequences amplified by 6 pairs of primers from each strain were joined together to form a concatenated sequence for each strain. A multiple alignment of the concatenated sequences was constructed by using ClustalW (www.ebi.ac.uk/clustalw) and was analyzed by using the neighbor-joining method in PAUP 4.0 Beta (Sinauer Associate, Inc., Sunderland, MA, USA). Bootstrap was estimated for neighbor-joining trees by 1,000 resamplings. The sequences reported here were assigned consecutive GenBank accession numbers from EU192931 to EU192949.

Conclusions

We amplified the 6 loci from all 8 *R. prowazekii* strains and compared the corresponding sequences of each strain to identify the variations among strains. Three loci were intergenic spacers (*rp272/rp273*, *rp308/rp309*, and *rp691/rp692*), and 2 loci were pseudogenes (*rp181* and *rp195*) in all *R. prowazekii* strains. We also sequenced *rp028*, the methyltransferase gene, because we wanted to know if this gene was inactivated in any virulent strain of *R. prowazekii*. Pseudogene *rp028* was inactivated in a virulent E strain but not in its virulent revertant Evir strain (*9*). Coincident with inactivation of the methyltransferase gene, E strain is deficient in methylation of surface proteins (*10*,*11*).

Our result shows that a single nucleotide insertion at position 732 in *rp028* occurred only in E strain among the

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Table 1. Primers for 6 loci of *Rickettsia prowazekii* genomic DNA sequences

Target sequence	Sequences of forward primer/reverse primer (5′ → 3′)	PCR product size, bp	Reference
rp028	TTGATATAGGTTGCGGAGTCGGTGTTA/TCATTGATGGCTTGTAGTTTTTCTGCT	682	(*9*)
rp181	ATTATGCAAATAATGCAG/GCATCGGATAAGTTAGTTCA	390	This study
rp195	TTTATTGGGGATTTACCTTT/CAAGTGTTAGATAGCTTGCT	384	This study
rp272/rp273	TCTTGCGATACAGTAAGCAC/TATTCGCTCCTTACCAGTTA	612	This study
rp308/rp309	TTAACAGAAGTAATAATAATTG/AGCAATAGAATTTGATAAGCA	369	This study
rp691/rp692	AGAAATTTGTATTGCATTTTTATG/GCTCTAGAAGCTATTGCTGA	447	This study

tested *R. prowazekii* strains (Table 2). However, single nucleotide polymorphism (SNP) existed in *rp028* among strains of *R. prowazekii* and was very useful in the differentiation of *R. prowazekii* strains (Table 2). Apparently none of these nucleotide substitutions caused attenuation of E strain because the E strain and Evir strain were identical at these sites.

DNA sequence comparison and phylogenetic analysis of the concatenated sequences indicated that the *R. prowazekii* strains were grouped together by geographic location and source of isolation (Table 2, Figure). Two flying squirrel isolates from the United States were differentiated by a single nucleotide substitution at position 480 in *rp028*. E strain and its revertant Evir strain differed by a single nucleotide insertion in E strain at position 732 in *rp028*, which we reported previously (*9*). Breinl and Cairo strains were closely related but were differentiated by several deletion/insertion mutations in *rp18*1 and the spacer between *rp272* and *rp273*. The cattle tick isolate ZRS and the human isolate Addis Ababa, both from Ethiopia, were identical in all 6 loci. ZRS strain and Addis Ababa strain were phylogenetically more closely related to E/Evir strains than other strains (Figure). There was only a single nucleotide difference between ZRS/Addis Ababa strains and Evir strain (Table 2).

Genotyping of *R. prowazekii* has been explored recently. Zhu et al., using intergenic spacers *rpmE*/tRNAf-Met and *serS/virB*4, differentiated 5 strains and PCR amplicons from 10 body lice of *R. prowazekii* into 4 genotypes (*12*). Ge et al. showed that *R. prowazekii* Breinl strain and E strain were different in the *rp084* gene, which was deleted from the Breinl strain (*13*). However, using the *rpmE*/*tRNAfMet* intergenic spacer, we were able to classify the 8 strains of *R. prowazekii* tested into only 2 genotypes. Genotype 1 contains Breinl strain and genotype 2 includes all other strains. All 8 strains were identical in the *serS/virB*4 spacer. With the exception of *R. prowazekii* Breinl strain, *rp084* was not deleted from any strains of *R. prowazekii* tested in our study. Conversely, using our methods, the 8 strains of *R. prowazekii* can be differentiated into 7 genotypes. ZRS and Addis Ababa strains are the only isolates that cannot be differentiated with our method. Because all *R. prowazekii* ZRS and Addis Ababa strains originated from Ethiopia, it is reasonable to believe that they might be genetically identical. Ge et al. recently showed that 5 *R. prowazekii* strains, including Breinl, Cairo, E, GvV257,

Table 2. Genotypes of *Rickettsia prowazekii* strains determined by nucleotide mutation in multiple loci

	*rp028**				*rp181*	*rp195*		*rp272–rp273*	*rp308–rp309*	*rp691–rp692*		
Strain	268†	286	480	732	713–714	140	1529	52–53	306	1306–1307	1415	GT
GvV-250	T	G	C	–	–	TACTTCAAG CTCATTTCG	C	AA	GTCATTA TCGTAT	TT	G	1
GvF-16	T	G	T	–	–	TACTTCAAG CTCATTTCG	C	AA	GTCATTA TCGTAT	TT	G	2
Breinl	T	A	C	–	–	TACTTCAAG CTCATTTCG	G	–	GTCATTA TCGTAT	–	–	3
Cairo	T	A	C	–	G	TACTTCAAG CTCATTTCG	G	A	GTCATTA TCGTAT	–	–	4
ZRS	G	A	C	–	G	–	G	AA	–	TT	–	5
Addis Ababa	G	A	C	–	G	–	G	AA	–	TT	–	5
Madrid E	G	A	C	A	GG	–	G	AA	–	TT	–	6
Evir	G	A	C	–	GG	–	G	AA	–	TT	–	7

*Gene names or intergenic spacers between genes.
†Positions of nucleotides with mutation, which were counted from the first nucleotide of the coding sequence or the first nucleotide after the stop codon in the case of intergenic spacers; –, deletion of nucleotides, in which the number of nucleotides deleted equals the nucleotides in the same column for the corresponding strains that do not have the deletion. For example, in *rp181,* the GvV-250 strain has 1 deleted nucleotide when compared with the Cairo strain, but it has deleted 2 nucleotides when compared with the E strain.

Figure. Phylogenic tree of *Rickettsia prowazekii* strains generated by using the concatenated sequences of 6 loci from each strain. *R. typhi* sequences were used to root the tree.

and GvF12 were different from each other by 1 to 4 SNPs in *ompB* and *sca4*, respectively (*14*). However, the differentiation of *R. prowazekii* based on SNPs between closely related strains may be complicated by PCR and sequence errors. Conversely, our method confers more confidence in the validation of the mutations because we differentiated all strains except for 2 flying squirrel strains by insertion and deletion mutations, which are rarely generated by PCR or sequence errors.

Our method provides a technique for typing and tracing the origin of new *R. prowazekii* isolates. This method will have a broad use in the biodefense against and the molecular epidemiology of *R. prowazekii* and in detection of laboratory cross-contamination of *R. prowazekii* strains.

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Environmental Contamination during Influenza A Virus (H5N1) Outbreaks, Cambodia, 2006

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To determine potential risk for bird-to-human transmission during influenza A virus (H5N1) outbreaks among backyard poultry in rural Cambodia, we collected environmental specimens. Viral RNA was detected in 27 (35%) of 77 specimens of mud, pond water, water plants, and soil swabs. Our results underscore the need for regular disinfection of poultry areas.

By June 19, 2007, the current epizootic of influenza A virus (H5N1) had caused 317 human cases in 12 countries, including 7 patients in Cambodia, all of whom died (*1*). Direct contact between infected bird secretions and human respiratory mucosa is thought to play an major role in poultry-to-human transmission (*2*). The role of indirect contact in virus transmission remains poorly understood. A few studies have suggested that some avian influenza viruses can be maintained in water fowl populations by waterborne transmission (*3*). Moreover, experimental studies have shown many types of avian influenza viruses could persist for a few months in cold waters or up to 8 days in feces at 22ºC (*4*). However, results obtained with various subtypes of influenza A virus may not apply to the current H5N1 subtype. Further, data are lacking regarding the survival of subtype H5N1 in natural settings and conditions. As an exploratory step, we have introduced environmental sampling during responses to influenza (H5N1) outbreaks. This report summarizes the results of the environmental investigations conducted in 3 villages with influenza virus (H5N1)–associated outbreaks in Kampong Cham and Prey Veng provinces, Cambodia, February–August 2006.

The Study

Cambodia is tropical and remains hot (24°–38ºC) all year with a rainy (May–October) and a dry (November–April) season. In response to notification of a confirmed case of influenza subtype H5N1 infection in humans or poultry, we surveyed all households located within a 1-km radius of the outbreak site. We gathered data on proportion of deaths in poulty flocks and on interaction with other species by conducting interviews. We also collected corresponding environmental specimens in some households and their surroundings, selected by proximity to the index household. We swabbed surfaces and collected materials by using 10-mL sterile flasks in the areas where poultry were reported to be free ranging. Swabs were placed in 1.5-mL virus transport medium; all environmental samples were transported at 4°C within 36 hours to Institut Pasteur in Cambodia for subtype H5N1 testing by real-time reverse transcription–PCR (rRT-PCR) after RNA extraction by using a viral RNA kit (QIAamp, QIAGEN, Valencia, CA, USA) and for virus isolation after inoculation onto MDCK cells. From each household's flock, we collected sick poultry and carcasses for subtype H5N1 virus testing, sampled 10 randomly selected ducks, and bled and swabbed cloacae and tracheas. Swab specimens were tested by hemagglutination after egg inoculation; positive samples were confirmed by rRT-PCR, and serum samples were tested by hemagglutination-inhibition assay with H5 antigens provided by the World Animal Health Reference Laboratory (Weybridge, UK). An influenza (H5N1)–associated household was defined as a household or a village poultry farm where 1) an influenza (H5N1)–infected patient resided, 2) influenza (H5N1) was identified in poultry, or 3) duck serum specimens were positive by hemagglutination-inhibition test for anti–influenza (H5N1) antibodies (*5*). Of note, none of the poultry owners who were interviewed reported having been vaccinated against "bird flu" (data not shown).

We collected a total of 167 environmental samples collected in 43 households; of 77 samples collected in 14 household areas, 27 (35%) were found positive for subtype H5N1 by rRT-PCR. Of these 14, the median positivity rate per household was 50% (range 9%–100%). Viral RNA was frequently detected in poultry feces (50%), soil swab specimens (50%), water plants in households' ponds (50%), swabs collected from feathers of recently dead poultry (50%), followed by results from mud collection (29%) (online Appendix Table 1, available from www.cdc.gov/EID/content/14/8/1303-appT1.htm). The subtype H5N1 genome was similarly identified in moist and dry surfaces (38% vs. 57%, p = 0.41). Viral loads were highest in contaminated mud (mean 94,000 copies). However, no viruses were subsequently isolated from the positive environmental specimens after 5 passages on MDCK cells.

All initially surveyed households owned chickens (5%), ducks (31%), or both (64%), although most poultry flocks were small (median 20, range 1–60 for chickens median 141, range 2–1,600 for ducks). All poultry were

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free ranging, and mixing between chickens and ducks was common. Deaths had occurred in the previous 3 months among 29 (67%) of the 43 households' flocks, although the flock mortality rate had a wide range (30%–100%). Of the 14 influenza (H5N1)–associated household areas, 4 had no evidence of influenza (H5N1) infection in poultry flocks, including household 12 in which no poultry died (online Appendix Table 2, available from www.cdc.gov/EID/content/14/8/1303-appT2.htm).

No association was found between positive environmental results and flock deaths or subtype H5N1–infected flocks. Of the 29 households at which poultry died, the median interval between the sampling date and death of the last bird was shorter among the 10 households for which environmental samples were positive (median days 0.5 vs. 16, p = 0.005) compared with 19 households with environmental samples with negative results. In addition, viral RNA was found to be detectable in the environment up to 12 days after the end of the flock outbreak. This RNA was present in soil beneath poultry cages with a viral load of 11,000 copies.

Conclusions

Our findings demonstrate that viral RNA was frequently present on various environmental surfaces or materials in the influenza (H5N1)–associated households and their surroundings. The presence of viral genome in water and feces supports R. Webster's finding (R. Webster, unpub. data) that the viruses could remain detectable in water and wet feces up to 4–6 days at 37°C (*6*). In addition, using regular techniques, we detected viral RNA in small volumes of unconcentrated water and in pond water plants, which suggests that levels of influenza A virus (H5N1) in these contaminated waters might have been relatively high (*6*). Notably, mud collection and dry soil swabbing have been efficient in detecting viral RNA in a contaminated environment. Nonetheless, the presence of RNA does not necessarily imply that the virus is alive or that transmission can occur; in addition, we were unable to isolate the virus by culture. This lack of culture growth may be related to a number of factors, including the fact that viruses could be short lived, whereas the decay of subtype H5N1 RNA may have been sufficiently slow to enable detection by rRT-PCR. Also, a live virus adsorbed on soil microparticles may have prevented viral binding onto MDCK cells, or these inoculated cell lines may have been damaged by bacteria or fungi present in the environmental specimens (*7*).

We used the interval between the last dead bird and the sample collection dates as a potential reflection of the survival of the virus in a natural setting. However, this interval may be subject to some limitations. First, we were not able to prove that infectious viruses were recovered after this interval. Second, these viruses could have been shed by duck survivors a long time after the end of the outbreak. Finally, interpretations were difficult because our analyses were limited by the modest number of flocks studied. Notably, however, an interval of 12 days was reported in 1 household, although none of the remaining birds was infected or had markers of influenza (H5N1) infection; this suggests that the virus was shed by the last dead birds infected and detected 12 days later.

Bird-to-human transmission is believed to occur largely through direct contact between infected bird secretions and human respiratory mucosa by inhalation of infectious droplets or transfer with contaminated hands to the upper respiratory tract through the nose, mouth,or conjunctival mucosa; subtype H5N1 has been understood to replicate primarily in the human respiratory tract (*7–9*). However, additional evidence suggests that influenza virus (H5N1) also replicates in the gastrointestinal tract, which indicates that ingestion of contaminated food (e.g., drinking duck blood) or water is not a negligible source of transmission (*6*,*10–12*). Most rural Cambodian households possess small ponds (≈10–20 m^2), which serve as water reservoirs for backyard animals and gardens. Ducks gather and deposit large amounts of feces in these ponds, while at the same time children commonly bath and play in them. Taken together, widespread dissemination of the virus in a subtype H5N1–infected household and high interaction between humans and poultry, the birds' environment may be particularly worrisome (*13*). On the other hand, current strains of subtype H5N1 may not yet easily be transmitted from poultry to humans; however, this transmission could increase as the virus continues to circulate and evolve (*3*,*14*). In addition to illustrating the need for good poultry-handling practices, our results underscore the importance of the following for preventing disease transmission: general basic hygiene, fencing domestic birds, and regular environmental disinfection of poultry places (*3*,*15*).

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Detection and Molecular Characterization of a Canine Norovirus

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We identified a novel calicivirus in a pup with enteritis. The isolate was related genetically (90.1% aa identity in the capsid protein) to a lion norovirus strain.

Caliciviridae are small, nonenveloped viruses, ≈35 nm in diameter with single-stranded, positive-polarity RNA genomes of 7.4–8.3 kb (*1*). Unlike calicivirus infections in cats, caliciviruses are not regarded as important pathogens in dogs and are not usually included in diagnostic algorithms for canine infectious diseases. Calicivirus-like particles have been occasionally identified by electron microscopy in specimens from dogs with diarrhea and, in some instances, glossitis, balanitis, or vesicular vaginitis. Most of these isolates were feline caliciviruses (*Vesivirus* genus) and were likely acquired from cats (*2*,*3*).

Thus far, only 2 documented reports have identified authentic canine caliciviruses in dogs. A calicivirus was isolated from the feces of a 4-year-old dog with bloody diarrhea and central nervous system disturbance in 1985 (Tennessee, USA). The virus replicated in experimentally infected dogs and elicited seroconversion, although disease was not reproduced. The virus was antigenically unrelated to feline calicivirus, and antibodies against the virus were identified in 76% of the canine serum specimens collected (*4*). However, the virus was not characterized molecularly, and its taxonomic status remains uncertain.

In 1990, another calicivirus was identified in Japan in a 2-month-old pup with intermittent fluid diarrhea (*5*). The virus, strain 48, was antigenically and genetically unrelated to feline calcivirus, was tentatively proposed as a "true" canine calicivirus (CaCV), and was included in the *Vesivirus* genus (*6*). Antibodies to CaCV 48 have been detected in 57% of dogs in Japan (*7*) and in 36.5% of dogs in South Korea (*8*). In this report, we describe the detection of a novel enteric CaCV.

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The Study

A gastroenteric disease with diarrhea and vomiting developed in a 60-day-old mixed-breed pup. Because of severe dehydration, the pup was hospitalized 3 days after onset of the gastroenteric symptoms. After 4 days of illness, the dog recovered completely. At the time of the dog's hospitalization, feces were collected and screened for common canine viral pathogens; test results were positive for canine parvovirus type-2a (CPV-2a). By using a broadly reactive primer pair, p289–p290, targeted to highly conserved motives "DYSKWDST" and "YGDD" of the RdRp region of the polymerase complex, we found, unexpectedly, that stool also tested positive for calicivirus (*9*). After the detection of calicivirus RNA in the dog's feces, the animal was kept under observation, and fecal samples were collected daily from 11 to 30 days posthospitalization (dph) to monitor virus shedding. CPV-2a DNA was detected until dph 10, whereas calicivirus RNA was detected until dph 22.

To determine the sequence and genomic organization of the novel calicivirus, a 3.4-kb region at the 3′ end of the genome was amplified by reverse transcription–PCR (RT-PCR) as described by Wang et al. (*10*), cloned into a vector, and sequenced. The sequence (3381 nt) from the 3′ end of open reading frame 1 (ORF1) to the poly-A tail of the CaCV (strain 170/07) was made available in GenBank (accession no. EU224456).

The 3.4-kb fragment of the calicivirus genome (the 3′ end of ORF1, the full-length ORF2, ORF3, and the noncoding region through the poly-A tail), is represented in Figure 1. The 3′ partial sequence of ORF1 spanned 824 nt and 273 aa at the COOH– terminus of the polymerase complex. Using BLAST (www.ncbi.nlm.nih.gov/BLAST) and FASTA (www.ebi.ac.uk/fasta33) analysis, we found the highest identity (84.6% nt and 96.7% aa) in a lion norovirus strain, Pistoia/387/06/ITA (*12*). A 14-nt overlap was present in the ORF1–ORF2 junction region, as with most described human and animal noroviruses. The ORF2 was 1737-nt long and predicted to encode for a capsid protein with a size of 578 aa. The highest sequence match was found to the lion GGIV.2 norovirus strain Pistoia/387/06/ITA (90.1% aa and 81.13% nt), while the identity was 69.4%–68.2% aa (75.5%–74.0% nt) to GIV.1 NoVs and <53.3% aa (<67.4% nt) to non–GGIV NoVs. There was a single nt overlap between ORF2 and ORF3; also, there was a 55-nt long nontranslated region between ORF3 and the poly-A tail. ORF3 was 783 nt in length and encoded for a 260-aa polypeptide.

A nucleotide identity plot of the canine norovirus genome 170/07 (from the 3′ end of ORF1 to the poly-A tail)

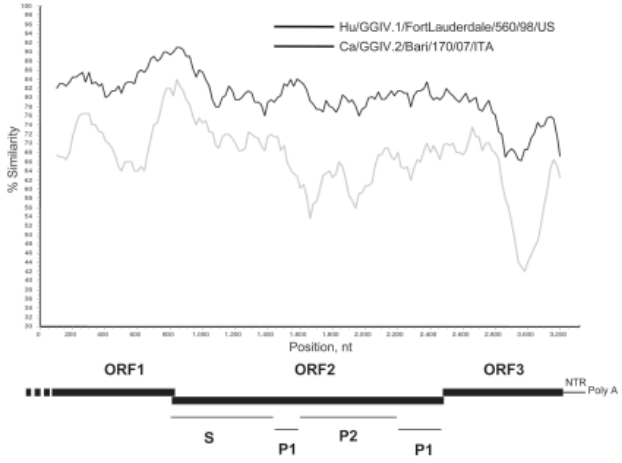

Figure 1. Genome organization of the canine norovirus GIV.2/Bari/170/07/ITA. The genome (from the 3′ end of open reading frame 1 [ORF1] to the poly-A tail) of the canine strain and of the human GGIV.1 norovirus, Fort Lauderdale/560/98/US (AF414426) was plotted against the genome of the strain lion/GGIV.2/Pistoia/387/06/ITA (EF450827). Sequences were analyzed with Simplot (*11*) by using a window size of 200 nt and step size of 20 nt with gap strip off and Hamming correction on. The conserved shell (S) domain and the variable regions (P1 and P2) are also indicated.

was compared with the lion GIV.2 NoV Pistoia/387/06/ITA and the human GGIV.1 norovirus, Fort Lauderdale/560/98/US (AF414426) (Figure 1). The 3′ genome of the canine norovirus 170/07 displayed high nucleotide conservation with the lion norovirus strain. A phylogenetic tree was constructed by using the capsid protein of selected human and animal noroviruses of the various norovirus genogroups (GGI to V) (*10*,*13*). In the tree (Figure 2), the canine calicivirus (strain 170/07) was grouped with the lion GGIV.2 norovirus strain Pistoia/387/06/ITA and with the GGIV.1 human NoVs Alphatron/98–2/98/NLD, Saint Cloud/624/98/US and FortLauderdale/560/98/US.

Conclusions

Noroviruses are regarded as the major cause of epidemic, nonbacterial gastroenteritis worldwide in humans of all age groups. The viruses are highly contagious and are transmitted by direct contact or by contaminated water and food (*15*). Because of the possibility of genetic recombination, a consistent and reliable classification of norovirus relies on the analysis of the complete capsid gene (*13*). Strains within the same genotype (or cluster) share >85% aa identity, while strains of different genotypes within the same genogroup share 55%–85% aa identity (*13*). Human noroviruses are classified into genogroups I, II, and IV. In addition, noroviruses detected in pigs, cows, and mice are classified in genogroups II, III, and V (*13*) (Table). Recently, a norovirus strain (Pistoia/387/06/ITA) was identified

Table. Distribution of norovirus genogroups and genotypes across the various animal species

Host	Norovirus genogroups and genotypes*				
	GGI	GGII	GGIII	GGIV	GGV
Human	1–8	1–10, 12–17		1	
Pig		11, 18, 19			
Cattle			1, 2		
Lion				2†	
Dog				2‡	
Mouse					1

*Norovirus classification follows the outlines of Wang et al. (*10*) and Zheng et al. (*13*).
†Martella et al. (*12*).
‡This study.

in a captive lion cub with severe hemorrhagic enteritis. By sequence analysis, the virus was found to resemble human GIV norovirus and was classified as a distinct genotype, GIV.2, whereas the human GIV noroviruses are genotype 1 (*12*). The canine norovirus strain 170/07 appeared to be most related genetically to the lion GGIV.2 norovirus (90.1% aa identity in the capsid protein); therefore, the virus may be considered as a variant of the genotype GGIV.2. Taken together, these findings suggest that noroviruses genetically similar to human GIV noroviruses are harbored in domestic and wild carnivores.

The pathogenic potential of this novel calicivirus in carnivores remains to be elucidated. In the pup, the norovirus strain was detected in conjunction with a CPV-2a strain that was likely responsible for the severity of the observed clinical signs, since CPV-2 is a major enteric pathogen of dogs. Accordingly, it is difficult to speculate on the pathogenic potential of the novel calicivirus for dogs and experimental infections in gnotobiotic dogs are required. Never-

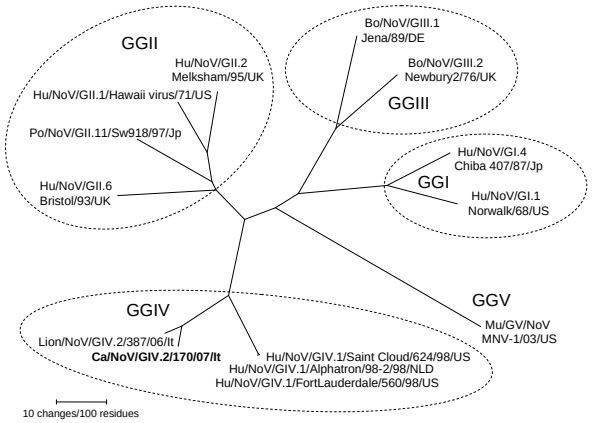

Figure 2. Phylogenetic tree constructed on the full-length amino acid sequence of the capsid protein. The tree was constructed by using a selection of norovirus (NoV) strains representative of the genogroups I to V. Phylogenetic analysis (neighbor-joining) with bootstrap analysis (1,000 replicates) and Kimura 2-parameter correction was conducted by using the MEGA software package version 3.0 (*14*). Strains designation follows the outlines of Wang et al. (*10*) and Zheng et al. (*13*). Bo, bovine; po, porcine; mu, murine; hu, human; ca canine.

theless, the norovirus 170/07 was detected in the subject pup for at least 22 days, which indicates active viral replication; the possibility of passive viral transit (mechanical passage) in the intestinal tract seems unlikely.

In conclusion, our study presents evidence for the existence of a novel calicivirus with enteric tropism that is related genetically to GGIV noroviruses and able to infect dogs. Future large-scale virologic and serologic investigations are needed to assess the ecology of this novel virus in wild and domestic carnivores. Also, because of the extensive social interactions between humans and pets, investigating the zoonotic potential of such animal noroviruses in humans is worthwhile.

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Dr Martella is associate professor at the Faculty of Veterinary Medicine of Bari, Italy. His research involves diagnosis, epidemiology, and characterization of enteric viruses, including rotaviruses and caliciviruses, in animals and humans.

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Henipavirus Infection in Fruit Bats (*Pteropus giganteus*), India

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We tested 41 bats for antibodies against Nipah and Hendra viruses to determine whether henipaviruses circulate in pteropid fruit bats (*Pteropus giganteus*) in northern India. Twenty bats were seropositive for Nipah virus, which suggests circulation in this species, thereby extending the known distribution of henipaviruses in Asia westward by >1,000 km.

Nipah virus (NiV) and Hendra virus (HeV) are zoonotic paramyxoviruses (genus *Henipavirus*) that have caused human deaths in Australia, Malaysia, Singapore, India, and Bangladesh (*1–4*). Known reservoirs for henipaviruses are *Pteropus* spp. fruit bats, which are distributed across the Indo-Pacific region from Madagascar eastward to the South Pacific islands (*5*). Evidence of henipavirus infection has been reported in *Pteropus* bats from Malaysia, Bangladesh, Australia, Thailand, Cambodia, Indonesia, and Madagascar, which supports the theory that these bats have co-evolved with henipaviruses (*6–8*).

The first known outbreak of NiV encephalitis in India occurred in 2001 in Siliguri, West Bengal (*1*). The fruit bat (*P. giganteus*) is present across the Indian subcontinent and, although it is suspected as the reservoir host for NiV in Bangladesh, its status as a reservoir for henipaviruses in India is unknown. Seven outbreaks of NiV encephalitis were recognized in Bangladesh from 2000 through 2008, and antibodies to NiV have been found in *P. giganteus* in several colonies there, including colonies adjacent to human case-patients (*3*,*5*,*9*). In the current study, we examined a population of *P. giganteus* bats in India, >1,000 km west of Siliguri, for antibodies to henipaviruses.

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The Study

We captured 41 *P. giganteus* bats from a colony in Haryana State in northern India from June 24 through June 30, 2003, by using mist nets. Blood was collected from the brachial or cephalic artery or from the vein by using a heparinized 3.0-mL syringe and a 22-gauge or 27-gauge needle and stored for 24 hours at 4°C to allow for plasma separation; the separated plasma was then stored at –20°C until use. Sex, age, body condition score, pregnancy status, lactation status, weight, and forearm length were recorded. Age was estimated by the presence of secondary sexual characteristics and dental wear. Body condition was assessed by digital palpation of the pectoral muscles and individuals were assigned a body condition score (BCS) of "poor," "fair," or "good." Unweaned juveniles were not assigned a BCS because of their physical immaturity. Pregnancy was determined by digital palpation, and a bat was considered "lactating" if milk could be expressed from either teat. All bats were released after sampling.

All 41 plasma samples were screened for antibodies to NiV and HeV by using virus-specific indirect ELISAs. Thirty-nine samples (2 samples had insufficient amounts of plasma remaining) were analyzed by using NiV and HeV serum neutralization tests (SNTs) under Biosafety Level 4 conditions (*10*). For the ELISA, coating antigen was derived from purified HeV- and NiV-infected Vero cells, and positive control serum specimens were obtained from experimentally infected horses (HeV) and pigs (NiV). Protein A/G conjugate was used to detect bound bat serum. A final serum dilution of 1:50 was used for the bat samples. A sample was considered reactive if the ratio of its average optical density at 450 nm (OD_{450}) of infected Vero cell antigen-coated wells (each sample was tested in duplicate) to uninfected Vero cell antigen-coated wells was >2.0 and the average OD_{450} value for the sample in the infected Vero cell antigen-coated wells was >0.2. Positive control serum samples were confirmed by both ELISA and SNT. SNT results were considered positive if virus neutralization occurred at ≥1:5 dilution (*11*). If neutralizing antibodies were present for both HeV and NiV, the higher titer was considered the positive test only if the difference between them was >4-fold (*11*). Samples that had positive titers to both viruses that differed by <4-fold were considered positive for an unspecified henipavirus.

The results of the serologic tests are presented in the Table, including comparisons of the results by gender, lactation status (females), and BCS. Twenty-six (63%) of 41 samples (95% confidence interval [CI] 47%–78%) were reactive in the NiV ELISA, 5 of which were also reactive in the HeV ELISA. No plasma samples reacted only in the HeV ELISA. Twenty (51%) of 39 samples (95% CI 35%–68%) had neutralizing antibodies to NiV, and 10 (26%) of 39 (95% CI 13%–42%) had neutralizing antibodies to

HeV. One (3%) of 39 samples (95% CI 0%–13%) had a neutralizing titer of 5 to NiV and HeV. This sample reacted in the NiV ELISA, but not in the HeV ELISA, although because it had equivalent neutralizing titers to both viruses, it was considered positive for an unspecified henipavirus. The ELISA showed 95% sensitivity and 75% specificity compared with the SNT.

Each of 2 unweaned pups matched their mother's serostatus, with 1 pup positive by SNT (pup 80, mother >640). Samples of the other mother–pup pair were seronegative. There were no significant differences in the NiV seroprevalence in male bats on SNT (8/12) compared to female bats (12/27) by using a Fisher exact test (FET; p = 0.300) or in lactating female bats (8/19) compared to nonlactating (5/8) female bats (FET; p = 0.420). We found significant differences in seroprevalence between bats with a poor and fair BCS (FET; p = 0.005), with bats in poor condition having a lower antibody prevalence than those with fair BCS. No difference in seroprevalence was found between the poor and good BCS groups or the fair and good groups.

Conclusions

Our study provides evidence that NiV, or a closely related henipavirus, circulates in Indian fruit bats (*P. giganteus*), thereby extending the range of the genus *Henipavirus* in Asia westward by >1,000 km. Our results are consistent with reports of NiV in *P. giganteus* bats in Bangladesh (*3*) and with *Pteropus* spp. being the primary reservoir of henipaviruses (*5*). Logistical limitations prevented us from attempting virus isolation and testing for viral RNA.

Previous studies have demonstrated that ELISAs, although less specific than SNTs, are useful screening tests for henipaviruses (*11*). Our results support this assertion, with the ELISA showing a high sensitivity. In our study, neutralizing antibodies to HeV and NiV were detected in 11 bats, 10 of which exhibited a >4-fold titer to NiV antibodies. Concurrent HeV and NiV titers are considered due to cross-neutralization rather than exposure to both viruses (*6*,*11*,*12*). Serologic studies provide information about the proportion of a population exposed to NiV, but not about the prevalence of bats that may be shedding virus or the virus itself. Further work in this area is required to fully characterize the henipavirus(es) involved and to confirm the status of *P. giganteus* as a reservoir.

Researchers have suggested that pregnancy plays a key role in henipavirus transmission among Australian *Pteropus* spp. and from bats to other species (*13*,*14*). In our study, we found no significant difference in seroprevalence between sexes, or between lactating and nonlactating females. Of the 2 lactating females carrying pups, 1 had a high titer of >640 and its pup had a titer of 80 against NiV, which suggests the passive transfer of antibodies; the other dam–pup pair was seronegative. Seroprevalence appeared to be significantly greater in bats with fair BCS when compared with those with poor BCS; however, no significant differences were found between good and poor or good and fair BCS groups. The findings that bats with fair BCS had a higher seroprevalence than poor BCS bats, but that there was no difference between good BCS bats and the other 2 groups, may be explained by the subjective classification of a bat's body condition. Those bats deemed to have fair body condition may have been more similar to those with robust bodies (good BCS) than those with thin, emaciated bodies (poor BCS). In fact, if one combines the good and fair categories, and compares the seroprevalence (18/28) with that of the poor group, the difference is still significant (p = 0.007); by contrast, combining the fair and poor categories (17/32) and comparing that seroprevalence to the good category results in no significant difference (p = 0.660). Having a lower seroprevalence in bats with the

Table. ELISA and SNT results and univariate associations between serostatus and other variables for wild-caught *Pteropus giganteus* bats in India*

Characteristic	ELISA		SNT	NiV SNT comparisons, p value†
	No. NiV reactive/ no. tested	No. HeV reactive/ no. tested	No. NiV positive/total (%) [median titer; range]	
Total	26/41	5/41	20/39‡ (51) [80; 5–640]	
Male	10/12	3/12	8/12 (67) [60; 20–640]	0.300
Female	16/29	2/29	12/27‡ (44) [80; 5–640]	
Lactating	12/20	2/20	8/19‡ (42) [80;20–640]	1.00
Nonlactating	4/9	0/9	4/8‡ (50) [80;5–80]	
Body condition score§				
Poor	5/9	0/9	1/9 (11) [640; NA]	P v F: 0.005; F v G: 0.315; P v G: 0.505
Fair	16/24	5/24	16/23 (70) [80; 5–640]	
Good	3/6	0/6	2/5 (40) [60; 40–80]	

*SNT, serum neutralization test; NiV, Nipah virus; HeV, Hendra virus; NA, not applicable; P, poor; F, fair; G, good.
†Fisher exact test p value significant at <0.05.
‡Two samples had insufficient plasma for SNT (both were ELISA negative); sample 1 was from a nonlactating adult female with a good body condition score (BCS) and the other was from a lactating adult with a fair BCS. A third sample, a nonlactating adult female with a good BCS had equivocal NiV/HeV SNT titers (*5*), which was attributed to an unspecified henipavirus and considered negative for NiV and HeV.
§Two pre-weaned pups (1 male, NiV SNT negative; 1 female, NiV SNT positive titer 80) were excluded from the BCS dataset because of their physical immaturity.

poorest BCS may be explained as an artifact of the nonrandom sampling (we sampled those bats that were first to be captured), by the limited sample, or it could suggest that NiV infection causes death in *P. giganteus* bats that are in poor physical condition. The latter explanation is less plausible because experimental infections of *Pteropus* spp. with henipaviruses produce only subclinical infection with no illness or death (*15*).

In northern India, as in Bangladesh, *P. giganteus* bats live in close association with the human population. Indeed, the colony examined in this study lives in a busy town above a major tourist attraction. Previous studies of NiV encephalitis outbreaks in Bangladesh have identified fresh date palm juice or fruit as plausible foodborne routes of transmission between bats and humans (*3*,*16*). The multiple outbreaks of NiV in Bangladesh, and the 2001 outbreak in West Bengal, show a continued risk for spillover infection between bats and humans in this region. Our findings suggest that the risk for NiV spillover to humans should be considered over a much wider area than previously regarded.

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Pediatric Pneumonia Death Caused by Community-acquired Methicillin-Resistant *Staphylococcus aureus*, Japan

To the Editor: Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), which carries genes for Panton-Valentine leukocidin (PVL), has become a major concern worldwide (*1*–*3*). CA-MRSA is mainly associated with skin and soft tissue infections in young, otherwise healthy, persons in the community (*3*) and also with life-threatening sepsis and community-acquired pneumonia (preceded by influenza) (*1*,*3*,*4*). The role of PVL in the pathogenesis of staphylococcal infections is controversial. Whereas Labandeira-Rey et al. (*5*) provided data that PVL, in combination with staphylococcal protein A, destroys respiratory tissue and bacteria-engulfing immune cells, Voyich et al. (*6*) and Bubeck Wardenburg et al. (*7*) showed that PVL was not essential for the pathogenesis of skin disease, sepsis, or pneumonia in a mouse model.

Several types of CA-MRSA clones exist, e.g., CA-MRSA belonging to multilocus sequence type (ST) 1 (called the USA400 clone) and ST8 (called the USA300 clone), which have been major clones in North America (recently, USA300 is becoming more prominent); CA-MRSA belonging to ST80, which has been a major clone in Europe; and CA-MRSA belonging to ST30, which is distributed worldwide, including Japan (*2*,*8*). MRSA carrying the PVL gene (a marker of CA-MRSA [ST30]) comprises 0.1% of MRSA isolated in hospitals in Japan (*9*).We describe a fatal case of pediatric pneumonia and septic shock from CA-MRSA in Japan.

A 16-month-old, previously healthy boy was admitted to the hospital for fever and shortness of breath on August 30, 2006. He had had cold-like symptoms for 14 days and fever for the 2 previous days. On examination, hordeolum of the right eyelid and cyanosis were observed; the patient's blood pressure was 106/ (undetectable) mm Hg, tachycardia 185 beats/min, tachypnea 72 breaths/min, and temperature 39.8°C. He had bilateral coarse breath sounds, and bronchovesicular breath sounds over the right lung. Chest radiography indicated lobar consolidation and pleural effusion on the right side. Laboratory analysis showed leukocytopenia, thrombocytopenia, elevated C-reactive protein level, and hypoxemia.

Intravenous administration of sulbactam/ampicillin and cefotaxime, and oxygen inhalation was started. Oxygen saturation did not improve, and laboratory values of disseminated intravascular coagulation (DIC) were observed: platelet count 121 K/mm^3, fibrinogen level 528 mg/dL, fibrin degradation products 37.7 μg/mL, prothrombin time 1.86 international normalized ratio, and D-dimer 37.7 μg/mL. The condition was considered septic shock, and consequently the boy was transferred to the pediatric intensive care unit, where he required intubation and mechanical ventilation.

Sulbactam/ampicillin was switched to meropenem, and cefotaxime was continued. On day 2 after admission, chest radiography showed bilateral consolidation. On day 3, blood culture yielded MRSA, and cefotaxime was changed to vancomycin. Meropenem therapy was continued to cover possible mixed bacterial infection. Immunoglobulin therapy and DIC syndrome treatment (nafamostat mesilate, ulinastatin, freeze-dried concentrated human antithrombin III) were also started. On day 4, computed tomographic examination detected pneumothorax and athelectasis. Because laboratory data confirmed the presence of only MRSA, meropenem was changed to flomoxef (which belongs to the oxacephem family of β-lactam antimicrobial agents) on the expectation that a possible synergistic effect of flomoxef and vancomycin might occur. No major changes occurred on days 5 and 6. On day 7, in addition to bilateral infiltrates on chest radiography, the oxygen index was 65 (partial pressure of arterial oxygen/fraction of inspired oxygen), and the patient was considered to have acute respiratory distress syndrome. A percutaneous cardiopulmonary support system (a portable heart-lung machine that provides temporary circulatory support) was used, but in spite of treatment, there was no improvement, and the child died on day 10 after admission (September 8). An autopsy was not performed.

Molecular characterization of MRSA isolated from the blood was performed as described previously (*8*,*9*). Isolated MRSA (strain NN32) was positive for PVL, belonging to ST30:*spa*19:staphylococcal cassette chromosome mec (SCC*mec*)IVa, and was resistant to only β-lactam antimicrobial agents (Table).

To date, all cases of PVL-positive CA-MRSA infections officially reported in Japan were caused by strains belonging to ST30 (*9*). All these strains can be classified into 2 types on the basis of *spa* type (Table), for example, ST30:*spa*19:SCC*mec*IVc. This type includes strain NN1, isolated from an 11-month-old patient with bullous impetigo (*8*); strain NN12, isolated from a 17-year-old patient with cutaneous abscess/osteomyelitis (*8*); strain NN31, isolated from an 18-year-old patient with pelvic abscesses (*9*); and strain EB00449, isolated from a 27-year-old patient with cutaneous abscesses (*9*). Another type is ST765 (single locus variant of ST30):*spa*43:SCC*mec*IVx. This type includes strain DB00319, isolated from a 61-year-old hospital inpatient (*9*).

Table. Characteristics of PVL-positive MRSA strains reported in Japan since 2000*

		PVL-positive MRSA				
		Present strain	Previous strains			
Type, gene, or resistance		NN32	NN1	NN12, NN31	EB 00449	DB 00319
Types	CC	30	30	30	30	30
	ST	30	30	30	30	765
	spa	19	19	19	19	43
	agr	3	3	3	3	3
	SCC*mec*	IVa	IVc	IVc	IVc	IVx*
	Coagulase	IV	IV	IV	IV	IV
Toxins						
Leukocidins	*lukS-PV, lukF-PV*†	+	+	+	+	+
	lukE-lukD, *lukM*	–	–	–	–	–
Hemolysins	*hla, hlg, hld*	+	+	+	+	+
	hlb	–	–	–	–	+
	hlg–v	–	–	–	–	–
Staphylococcal enterotoxins	*sea*	–	–	–	–	+
	tst, seb, sec, sed, see, seh, sej, sek, sep	–	–	–	–	–
	egc‡	+	+	+	+	+
	seu	+	+	+	+	+
Exfoliative toxins	*eta, etb, etd*	–	–	–	–	–
Others	*set*	+	+	+	+	+
	edin	–	–	–	–	–
Adhesins						
	icaA, sdrD, sdrE	–	–	–	–	–
	icaD, cna,§ *eno, fnbA, fnbB, ebpS, clfA, clfB, fib, sdrC, bbp*¶	+	+	+	+	+
Drug resistance and penicillinase plasmid	Aminoglycosides			GEN# KAN#*		GEN** KAN** STR**
	Macrolides					ERY**
	Lincosamides					CLI**
	Tetracycline			TET#		
	Penicillinase plasmid (kb)	+ (33)	+ (33)	+ (33)	+ (33)	+ (40)**

*PVL, Panton-Valentine leukocidin; MRSA, methicillin-resistant *Staphylococcus aureus*; SCC*mec* IVx, staphylococcal cassette chromosome *mec* type IV with unknown subtypes; +, positive; –, negative; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline.
†GenBank accession nos. for the PVL gene sequences from strains NN32 and NN1 are AB286959 and AB186917, respectively.
‡*egc*, enterotoxin gene cluster, including *seg*, *sei*, *sem*, *sen*, and *seo* genes.
§*cna*, gene encoding for collagen binding protein (adhesin).
¶*bbp*, gene encoding for bone sialoprotein binding protein (adhesin).
#Drug resistance encoded by a conjugative drug resistance plasmid (pGKT1) (*8*).
**Multidrug-resistant penicillinase plasmid encoding for resistance to GEN, KAN, STR, ERY, and CLI, in addition to penicillin resistance (*9*).

The molecular characteristics of strain NN32 were similar to those of strain NN1, except for SCC*mec*IV subtypes (Table). Moreover, pulsed-field gel electrophoresis patterns (data not shown) and the PVL gene sequences of the 2 strains (NN32 and NN1) were identical (Table).

This case of CA-MRSA ST30 infection in a child represents a progression from common cold–like symptoms (occurring outside the influenza season) to fatal pneumonia, despite intensive therapy, including the administration of sensitive antimicrobial agents. CA-MRSA ST30 contains several genes that mediate adhesion (e.g., *cna* and *bbp*) and toxin genes (PVL and *egc*, which encode for at least 5 superantigens, including staphylococcal enterotoxin G, I, M, N, and O). The gene cluster *egc* is associated with septic shock (*10*). Further studies are needed to clarify the pathogenesis of community-acquired pneumonia caused by CA-MRSA.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Rarity of Influenza A Virus in Spring Shorebirds, Southern Alaska

To the Editor: Knowledge of avian influenza (AI) virus and its host epidemiology and ecology is essential for effective monitoring and mitigation (*1*). Applicability of global and continental-scale models will be key for expanding this knowledge base. Research in the Delaware Bay area, eastern United States, suggests an ecologic and epidemiologic viewpoint of AI virus in wild birds in which shorebirds (family Scolopacidae) are predominant hosts in spring; however, research in Alberta, Canada, suggests that waterfowl are such in autumn (*2*,*3*). AI virus surveillance in Europe (*4*) suggests that the spring aspect of this scenario does not apply there. To increase knowledge of AI transport among shorebirds in spring in the North Pacific, we conducted AI virus surveillance during the springs of 2006 and 2007 at the Copper River Delta area of Alaska. Millions of birds congregate at this location in the spring, resulting in the highest spring shorebird concentrations in the New World (*5*). We also sampled gulls (Laridae), which are common and heretofore unsurveyed for AI in this ecosystem.

In 2006 and 2007, 1,050 shorebirds (Western Sandpiper, *Calidris mauri*, and Least Sandpiper, *C. minutilla*) and 770 Glaucous-winged Gulls (*Larus glaucescens*) were sampled during peak spring migration at Hartney Bay, Cordova, Alaska (60°28′N 146°8′W; Table). Fresh fecal samples were obtained from tidal flats within <1 to 90 min after identified flocks were dispersed, and samples were placed in sterile medium (brain heart infusion buffer with 10,000 U/mL penicillin G, 1 mg/mL gentamicin, and 20 μg/mL amphotericin B) and either kept cool (<1 week) before transport to Fairbanks (2006) or placed into liquid nitrogen within 2 h of collection

(2007). Samples were stored at –70° C; shipped frozen overnight to Athens, Georgia; and maintained frozen until analyzed.

Samples were screened by real-time reverse transcriptase–PCR (RT-PCR) for influenza A virus, and virus isolation was performed on samples that were positive. RNA was extracted by adding 250 μL of sample to 750 μL Trizol LS reagent (Invitrogen, Inc., Carlsbad, CA, USA). Samples were mixed and incubated at room temperature for 10 min. A total of 200 μL of chloroform was then added, incubation was continued for 5 min, and samples were centrifuged for 15 min at 12,000 × *g* at 4° C. Supernatant was removed, and 50 μL was extracted with the MagMax AI/ND viral RNA extraction kit (Ambion, Inc. Austin, TX, USA). RNA was tested for AI virus matrix (M) gene. A positive test result for this gene indicates the presence of any influenza viruses (*6*) when an internal positive control is used (*7*). Positive samples were processed for virus isolation in embryonated chicken eggs by standard methods (*8*). Real-time RT-PCR results were corroborated by processing 50 randomly selected negative samples for virus isolation with 3 egg passages.

Screening for AI virus was conducted on 1,820 samples (Table). Among these, 1 AI virus was identified (A/Glaucous-wingedGull/AK/4906A/2006; H16N?), reflecting an overall prevalence of 0.055% (0% in shorebirds and 0.13% in gulls).

Results of power analysis (*9*) suggested that our shorebird samples would detect infection rates >0.9% with 99% probability (95% probability of detecting rates 1%–2% or higher in each year). In gulls, probability of detecting infection rates >1% across both years of the study (≥6% in 2006 and ≥1%–2% in 2007) was 95%.

Virus prevalence in spring shorebirds in Alaska was substantially lower than prevalence in spring shorebirds in the Delaware Bay area (*3*) and more similar to prevalence in spring shorebirds in Europe (*4*). Our shorebird samples (1,050) were fewer than those in other studies (*3;* 4,266 samples from 4 species over 16 years, and *4;* 3,159 samples from 47 species over 8 years, with 35% from spring), representing 25% and 33% of those studies, respectively. Our study covered only 2 years, but it would detect AI virus infections in shorebirds at rates ≥1%–2% within each year with 95% probability and at rates ≥0.9% across years with 99% probability. Thus, the prevalence rate among Copper River Delta shorebirds in our study is lower than that found in the 16-year Delaware Bay study (*3*). In the Delaware Bay area, 4 shorebird species were sampled: 3 *Calidris* and 1 *Arenaria* (*3*). Precise statistics are unavailable, but the average 16-year prevalence rate was 14.2%, fluctuating annually from ≈2% to ≈38% (*3*).

Table. Species and sample sizes of wild bird hosts screened for avian influenza virus, Cordova, Alaska, May 2006 and May 2007

Species	Sample size		
	2006	2007	Total
Western sandpiper (*Calidris mauri*)	500	300	800
Least sandpiper (*C. minutilla*)	0	250	250
Glaucous-winged gull (*Larus glaucescens*)	100	670	770
Totals	600	1,220	1,820

In Europe AI viruses were absent among spring shorebirds (*4*). Differences in prevalence rates found among studies may be influenced by species sampled, sampling procedures, and seasonal timing (*4*). However, with >1,000 spring shorebirds sampled, results suggest that differences might exist between the world's major migration systems (*3*,*4*).

Our results corroborate other recent results (*10*) suggesting that AI prevalence rates among shorebirds at Delaware Bay are not typical within North America. Present evidence indicates (this study; *3*,*10*) that the role of shorebirds in AI virus ecology and epidemiology is heterogeneous within North America and within a genus (*Calidris*). These findings confirm that knowledge of how AI viruses cycle in wild bird hosts remains incomplete at continental and family-level taxonomic scales. Only further surveillance can fill these knowledge gaps.

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Isolation of *Brucella microti* from Soil

To the Editor: *Brucella microti* is a recently described *Brucella* species (*1*) that was isolated in 2000 from systemically infected common voles (*Microtus arvalis*) in South Moravia, Czech Republic. The organism is characterized by rapid growth on standard media and high metabolic activity, which is atypical for *Brucella* (*2*). The biochemical profile of *B. microti* is more similar to that of *Ochrobactrum* spp., of which most species are typical soil bacteria.

On the basis of the close phylogenetic relationship of *Brucella* spp. and *Ochrobactrum* spp. and the high metabolic activity of *B. microti*, we hypothesized that this *Brucella* species might also have a reservoir in soil. To test this hypothesis, we investigated 15 soil samples collected on December 11, 2007, from sites in the area where *B. microti* was isolated from common voles in 2000 (*2*). Ten of the samples were collected from the surface and at a depth of up to 5 cm near different mouse burrows 5 m apart. The remaining 5 samples were collected from an unaffected area without clinical cases of vole infection. The pH of soil samples ranged from 5.9 to 6.3. No frosts were recorded before the time of collection.

To specifically detect *B. microti* in soil samples, we have developed a PCR that targets a genomic island of 11 kb (H.C. Scholz et al., unpub. data) that is unique for *B. microti*. Briefly, primers Bmispec_f (5′-AGATACTGGAACATAGCCCG-3′) and Bmispec_r (5′-ATACTCAGGC AGGATACCGC-3′) were used to amplify a 510-bp fragment of the genomic island. PCR conditions were denaturation at 94°C for 5 min, followed by 29 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Total DNA was prepared from 0.5 g of each soil sample by using the MO BIO Ultra Clean Soil DNA Kit (Dianova, Hamburg, Germany). DNA was eluted with 50 μL of double-deionized water of which 2 μL was used in PCRs. Template DNA of *B. microti* CCM 4915^T was used as a positive control. Type strains of all recognized *Brucella* species, 1 strain of each biovar of all species, and type strains of 11 *Ochrobactrum* species were used as negative controls.

In this PCR, 5 of 15 soil samples and the positive control were positive for the 510-bp fragment; other *Brucella* spp. and *Ochrobactrum* spp. were negative. Of the 5 positive samples, 3 were collected from surface soil collected near mouse burrows. However, the remaining 2 positive samples were collected from the unaffected and supposedly negative-control area.

For direct cultivation of *Brucella* spp. from soil, 2 g each of 2 selected PCR-positive samples with the highest amplification rate (both from the affected area) were thoroughly homogenized in 5 mL of phosphate-buffered saline (PBS), pH 7.2, in 50-mL tubes. Of a serial dilution in PBS (10^0–10^{-4}), 100 μL was plated onto *Brucella* agar (Merck, Darmstadt, Germany) supplemented with 5% (vol/vol) sheep blood (Oxoid, Wesel, Germany) and *Brucella* selective supplement (Oxoid) and incubated at 37°C. Twenty suspicious colonies from the 10^0 dilution plate of 1 soil sample were subcultivated on *Brucella* selective agar. Two of the subcultivated bacteria (BMS 17 and BMS 20) reacted positively with monospecific anti-*Brucella* (M) serum. Both isolates were positive in the *B. microti*–specific PCR. Sequencing of the 510-bp fragments from both strains (GenBank accession nos. AM943814 and AM943815) and comparison with the known nucleotide sequence of *B. microti* showed 100% identity.

To confirm that strains BMS 17 and BMS 20 were *B. microti*, these strains were subjected to multilocus sequence analysis and multilocus variable number of tandem re-

peat analysis (MLVA) as described (*1*,*3*–*5*). Multilocus sequence typing profiles of these strains were identical to the type strain *B. microti* CCM 4915^T and strain CCM 4916. MLVA showed that these strains also clustered with *B. microti* strains CCM 4915^T and CCM 4916, with identical panel 1 and panel 2A genotypes but a different panel 2B genotype.

In summary, we successfully isolated *B. microti* from soil samples collected at the same site 7 years after primary isolation of this novel species from common voles. *B. microti* could still be isolated from the same soil samples 6 months after storage at 4°C. This finding indicates long-term survival of *B. microti* in soil; thus, soil might function as a reservoir of infection. Identification of *B. microti* as a potential soil bacterium is consistent with *Brucella* spp. whole genome sequencing data, in particular with the genome sequence of *B. suis*, which exhibits fundamental similarities with plant pathogens such as *Agrobacterium* spp. and *Rhizobium* spp. (*6*). Whether soil is the primary habitat of *B. microti* or other vectors, such as nematodes, remains to be investigated.

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Plasmodium falciparum in Ancient Egypt

To the Editor: Malaria is a disease caused by parasites of the genus *Plasmodium.* The infection is transmitted to humans through the bites of female flies of the genus *Anopheles.* Four species of *Plasmodium* are pathogenic to humans, and each leads to different clinical features: *P. falciparum* causes severe malaria with undulating high fever (malaria tropica); *P. malariae*, *P. vivax,* and *P. ovale* cause less severe clinical courses of disease with the manifestations of malaria quartana (*P. malariae*) and malaria tertiana (*P. vivax* and *P. ovale*). Literary evidence for malaria infection dates back to the early Greek period when Hippocrates described the typical undulating fever (*1*), highly suggestive of plasmodial infection. Although it is believed that malaria widely affected early pre-Hippocrates populations, until now only 1 study, which used molecular analysis, clearly identified *P. falciparum* in a Roman infant dating back to the 5th century AD (*2*). Two other studies used molecular analysis to identify more recent plasmodial DNA in ancient human remains, i.e., from 100–400 years ago (*3*,*4*). A substantial number of nonspecific amplifications in these previous studies raised concerns as to the specificity of current molecular markers for ancient malaria (*3*,*4*).

In this report, we describe the unambiguous identification of ancient DNA (aDNA) for *P. falciparum* in ancient Egyptian mummy tissues from ≈4,000 years ago. We analyzed 91 bone tissue samples from ancient Egyptian mummies and skeletons. The Egyptian material derived from the Predynastic to Early Dynastic site of Abydos (n = 7; 3500–2800 BC), a Middle Kingdom tomb in Thebes West (n = 42; 2050–1650 BC), and various tomb complexes in Thebes West, which were built and used between

the Middle and New Kingdom until the Late Period (n = 42; c. 2050–500 BC). All samples were first tested for *Plasmodium* spp. DNA by using the heminested PCR for the 18S rDNA primer targets usually used for malaria identification (*5*). Direct sequencing was performed on those with positive amplification products. Thereby, a high number of amplification products of various sizes (including the expected size) were detected. However, on sequencing, all amplicons provided nonspecific products. Consequently, in a second set, all material was tested for the *P. falciparum* chloroquine-resistance transporter gene (*pfcrt* gene) (*6*,*7*), which was also further characterized by direct sequencing.

In this second set of experiments, 2 of the 91 ancient Egyptian samples tested positive for the 134-bp fragment of the pfcrt region of *P. falciparum* (Figure). The specificity of the amplification was verified by sequencing, which showed 99% sequence concordance. The result was verified by parallel analysis in 2 independent laboratories; observations were fully concordant. The 2 positive samples originated from 2 different tomb complexes dating from the New Kingdom until Late Period (1500–500 BC). Each sample was obtained from adults who had osteopathologic evidence of chronic anemia. No positive results were found for the earlier samples from the Predynastic to Early Dynastic or Middle Kingdom periods.

Previously, immunologic tests have been used to investigate the presence and incidence of malaria in ancient Egyptian mummies (*8*,*9*). Because >40% of all samples and 92% of samples from persons with bone lesions suggestive of chronic anemia tested positive for the *P. falciparum* histidine-rich protein-2 antigen, doubts as to the specificity of those tests have been raised.

Our study unambiguously identified *P. falciparum* aDNA in Egyptian mummy samples, thereby proving a specific infection by falciparum malaria in ancient Egypt. With respect to the infection incidence, our molecular analysis suggests a more realistic frequency than had been previously suggested by paleoimmunologic methods. Consequently, the aDNA analysis is superior with respect to the reaction specificity, so that the latter should not further be used for that purpose.

This report adds another infectious disease to the spectrum of paleomicrobiology in ancient Egypt, thereby further explaining the previously postulated influence of infectious diseases on the low life expectancy for ancient Egyptian populations (*10*). Molecular detection of pathogen aDNA can be used not only to identify a certain disease, but it may also provide information on disease frequency, evolutionary origin, and pathways.

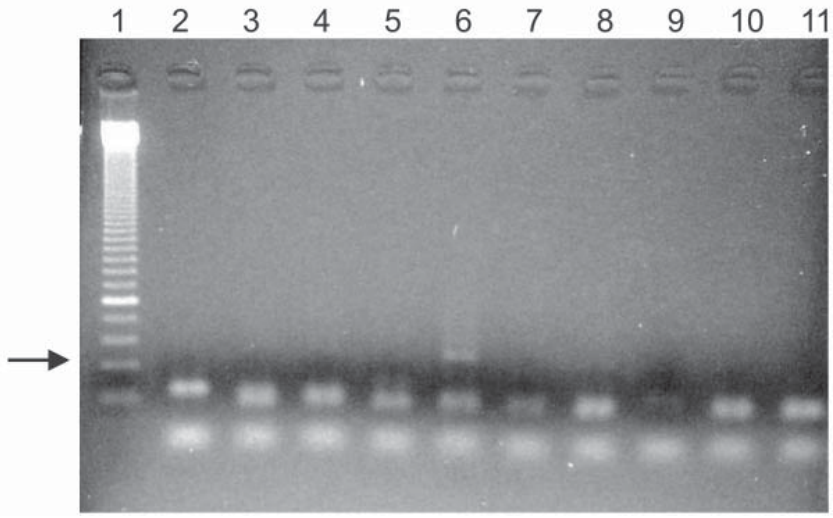

Figure. PCR amplification of a 134-bp fragment of ancient DNA of *Plasmodium falciparum* in Egyptian mummies. Lane 1, molecular marker; lanes 10 and 11, 2 negative controls. One (lane 6) of 8 samples shows a positive amplification product (arrow). Specificity of the product was verified by sequencing.

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Brucellosis in Infant after Familial Outbreak

To the Editor: Brucellosis is a known cause of small household outbreaks (*1*,*2*), usually attributed to exposure of all infected family members to the animal/animal product pathogen source. Although the means of disease transmission is well delineated (*3*), in certain cases the pathogen's entry into the human body cannot be clearly defined; this has led to suggestions of direct human-to-human transmission and also to the increasing recognition of airborne brucellosis, which is important in the context of the role of *Brucella* spp. as potential biological weapons (*4*). Another understudied transmission route is entry by direct contact through skin and mucosal abrasions. We report a case of infantile brucellosis in which airborne transmission in the context of familial brucellosis or indirect contact with the animal source through other family members was considered the only possible means of infant infection.

In 2006, a 2.5-month-old girl was admitted to the Pediatric Department of the University Hospital of Ioannina, in a region of northwestern Greece where animal and human infection from *Brucella melitensis* is still common (*5*,*6*). She had a 2-week history of poor feeding and a 5-day history of swelling of the right wrist. She was born after 38 weeks' gestation with a birthweight of 3,050 g and was fed formula milk exclusively. Results of the physical examination were normal except the finding of a tender swelling of the right wrist. The infant came from a family of shepherds, and her father and paternal grandfather had been treated for brucellosis 10 and 22 months ago, respectively. At that time, the whole family was screened for additional cases; screening was also often repeated at the patients' followup examinations. On admission, laboratory tests showed characteristic relative lymphocytosis (leukocytes 10.5 × 10^9/L, 65.3% lymphocytes) and an increase in inflammatory markers (C-reactive protein, 22 mg/L, and erythrocyte sedimentation rate, 47 mm/h). Results of a wrist x-ray were normal. Because brucellosis was suspected, serum agglutination test, ELISA, and blood PCR for *B. melitensis* were performed. Agglutination titer was 640; ELISA immunoglobulin M (IgM) antibodies and PCR results were positive. No organisms were grown in blood culture.

All family members were rescreened. The father and paternal grandfather had negative serum agglutination and ELISA IgM and positive IgG serologic results, indicating past infection. The mother and paternal grandmother were again negative. Veterinary investigation showed active disease in a few sheep of the family's herd. The infant was treated with a combination of oral trimethoprim-sulfamethoxazole and rifampin for 6 weeks. The course of the illness was uneventful, and she recovered completely. Followup PCR results were negative for *B. melitensis.* Six months later, only an ELISA IgG had positive results; IgM and IgA antibody and agglutination test results were negative. The patient remains without relapse 2 years after treatment.

Awareness of brucellosis is low in disease-endemic areas, including knowledge of its transmission potential and its medical consequences.. As a consequence, familial clusters of brucellosis are the norm. Recognition of a human case should prompt investigation of other family members so that early recognition and treatment for other household case-patients are possible. However, limitations in eradicating the initial animal disease source may lead to continuous exposure and appearance of new cases after a protracted period, or to infection of new household members.

Our case raises the need for awareness of the transmission dynamics of *Brucella* spp. because the disease emerged in a household member who did not have any direct contact with the animal source or any related products. The baby was not breastfed and had not digested raw milk. Her feeding bottle was specifically used for formulated milk and for feeding her only. Ingestion of breast milk from an infected mother (*7*) and vertical transmission transplacentally or during delivery are acknowledged means of transmission (*8*), but in this case the mother had never had brucellosis (she had been repeatedly screened during her husband's initial disease and followup). Neither previously infected household member had any clinical or laboratory sign of relapse or residual disease. The infant was never in contact with the infected animals and

never carried to the sites where they were housed or taken out for grazing. Thus, the only way for the infant patient to be infected would be environmental exposure. Since the infant had not been carried to the animal sites, the pathogen must have been transmitted to the household by the clothes or skin of the father or grandfather, who had been shepherding the infected animals. Subsequently, the baby became infected either by inhaling infected aerosolized particles or by direct transmission of the pathogen through minor skin abrasions (which were specifically looked for on admission but were not seen) or mucosal surfaces.

This case suggests that *B. melitensis* may even affect persons who are not directly exposed to infected animals, through direct contact with contaminated persons or the environment. In this context, brucellosis can be considered as not simply a household disease but as a disease of the house.

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Hepatitis E Virus Genotype 1, Cuba

To the Editor: Hepatitis E virus (HEV) causes acute viral hepatitis, which in rare cases leads to fulminant hepatitis with high death rates, especially among women in their third trimester of pregnancy (*1*). Sporadic infections and epidemics have been reported from all parts of the world, especially Asia, Africa, and Latin America. Although indigenous hepatitis E has rarely been observed in industrialized countries, higher than expected anti-HEV prevalence has been detected in these areas (*1*). In the Caribbean region, many countries including Cuba, Haiti, Guatemala, and Honduras have reported hepatitis E (*2*,*3*), but the viruses have not been characterized.

The transmission of HEV is primarily fecal–oral, through contaminated drinking water; limited zoonotic transmission has also been reported. Despite only 1 serotype, 4 major genotypes of HEV have been reported (*1*). Genotype 1 is mainly responsible for sporadic infections and large outbreaks in Asia and Africa. Genotype 2 was first found in Mexico and later on the African continent. Genotypes 3 and 4 have been reported from the United States, Europe, China, Japan, and Taiwan; this group also includes the related swine HEV (*1*).

We report the phylogenetic analysis of 11 HEV isolates from 2 outbreaks and sporadic cases in Havana, Cuba. The first outbreak occurred in 1999 in a factory; 20 persons were affected (12 women, 8 men; median age 45 years, range 22–53 years). The second outbreak was in 2005 in a suburb of Havana and involved 26 persons (15 women, 11 men; median age 24 years, range 17–45 years). We also analyzed HEV in 12 sporadic clinical cases obtained from the Cuban national surveillance program for viral hepatitis. Most patients reported asthenia, epigastric pain, nausea, and vomiting. None had any history of international travel, contact with persons traveling from disease-endemic areas, or consumption of exotic foods. Serologic screening showed all patients to be negative for immunoglobulin (Ig) M against hepatitis A and hepatitis C viruses. One patient had positive results for hepatitis B surface antigen but negative results for anti-hepatitis B core antigen IgM and hepatitis B virus DNA. All patients were positive for anti-HEV IgM (Genelab Diagnostics, Singapore) according to the manufacture's criteria. A total of 22 serum samples (outbreak 1, n = 9; outbreak 2, n = 7; sporadic cases, n = 6) were tested for HEV RNA; only 2 (both from sporadic cases) were positive. A total of 31 serum samples were also tested for anti-HEV IgG (Genelab Diagnostics), of which 22 were positive

(outbreak 1, n = 7/10; outbreak 2, n = 10/13; sporadic cases, n = 5/8).

A total of 44 stool samples were collected 2–4 weeks after onset of symptoms and stored at –70°C until use. Fecal samples were screened for HEV open reading frame (ORF) 2 by using reverse transcription (RT)–PCR (*4*). For genotyping, nested RT-PCR was then performed for the ORF1 RdRp region (*5*) on 18 samples that were positive for ORF2. Of the 12 PCR products obtained, 11 fragments were cloned into pGEMT Easy Vector (Promega, Madison, WI, USA). At least 3 positive clones for each sample were sequenced, and the consensus sequence was used for phylogenetic analysis. The GenBank accession numbers of ORF1 for the HEV outbreak cases from Cuba are CUB10-1999 (EU165504), CUB11-1999 (EU165502), CUB13-1999 (EU1655019), CUB19-1999 (EU165500), CUB24-1999 (EU165 499), CUB68-2005 (EU165496), and CUB71-2005 (EU165495). For the sporadic cases they are CUB9-2005 (EU165503), CUB1803-2003 (EU165494),CUB2-2005(EF493155), and CUB27-2005 (EU165498). Additionally, nested RT-PCR was conducted with ORF2-specific primer pairs (*6*) for 2 HEV isolates, 1 each from outbreak and sporadic cases, and sequences were obtained. The accession numbers for ORF2 are outbreak CUB10D-1999 (EU284749) and sporadic CUB2D-2005 (EU284748).

Phylogenetic analysis of ORF1 nucleotide sequences showed that HEV isolates from Cuba clustered in genotype 1 with high bootstrap values (Figure, panel A). The same genotype was detected in an outbreak of hepatitis E in UN peacekeepers deployed from Bangladesh to Haiti (*3*). Although the outbreak was adequately contained, anti-HEV immunoglobulin was subsequently detected in 3% of civilians in Haiti (*3*). Nucleotide identity between isolates from Cuba and other HEV strains from genotype 1 ranged from 91.7% to 99%. The strains from Cuba were closely related to the isolates from India and shared 97.8%–99% homology with Yam-67 (*7*). Absolute ORF1 nucleotide differences (p-distances; MEGA2 software, www.megasoftware.net) of isolates from Cuba ranged from 0% to 1.6%, demonstrating a high degree of relatedness. The ORF2 analysis supported our ORF1 findings because the CUB2D-2005 and CUB10D-1999 sequences also clustered with genotype 1 (Figure, panel B). Both strains from Cuba shared 96.1% nucleotide homology with a prototype strain from Burma (Bur82) and were related to the strains from India (Hyderabad and Yam-67), sharing 97.4%–99% homology. Absolute ORF2 nucleotide differences ranged from 0.8% to 1.9%. This value for ORF1 ranged from 0.05% to 0.08% for the same isolates from Cuba (CUB2-2005 and CUB10-1999).

HEV shows a global presence. The genotype distribution, although dominant in a given geographic area, is not limited to that area. For example, genotype 2, first identified on the American continent in Mexico (*8*), was later found in Namibia and Nigeria on the African continent (*9*,*10*). We report indigenous HEV genotype 1 strains in the Americas.

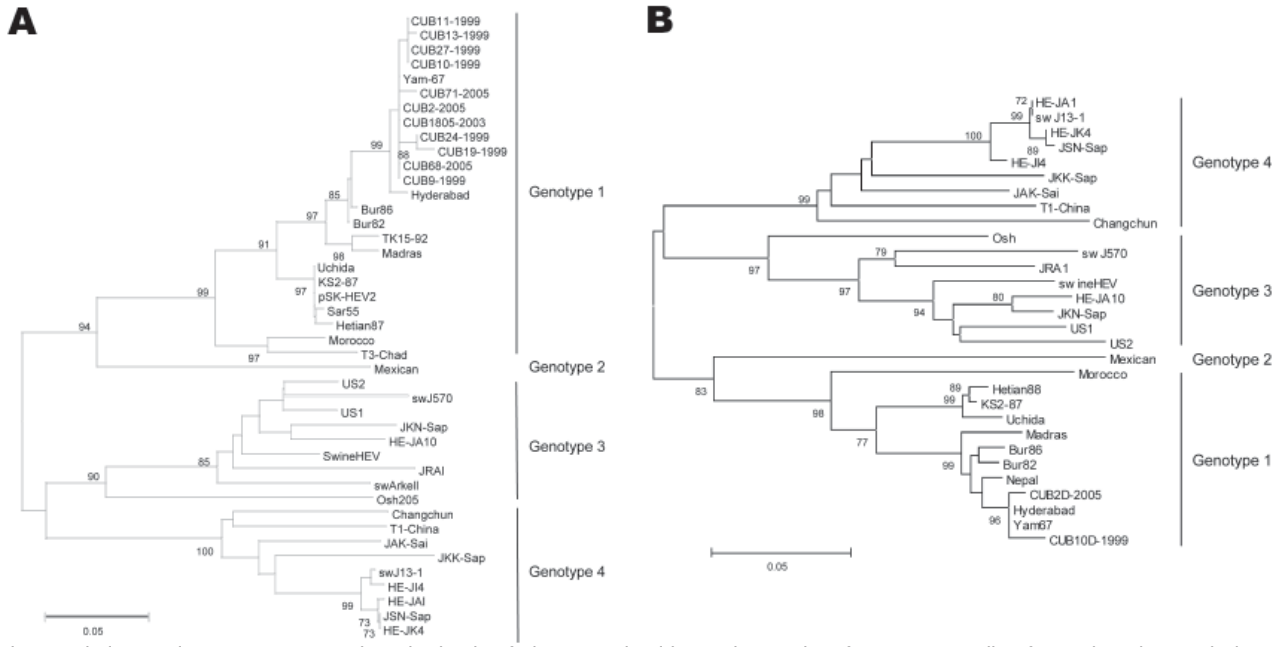

Figure. Phylogenetic trees constructed on the basis of A) 240 nucleotides, RdRp region, from open reading frame (ORF) 1, and B) 311 nucleotides from ORF2. Each tree was generated by using the neighbor-joining method; the distance matrix was calculated by using the Kimura 2-parameter method. The robustness of the trees was determined by bootstrap for 1,000 replicates. Values >70% are shown at the nodes. The major branches represent hepatitis E virus genotypes. Scale bar indicates 0.05 substitutions per nucleotide position.

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Ciprofloxacin Resistance in *Neisseria meningitidis*, France

To the Editor: Infections with *Neisseria meningitidis* may occur as outbreaks or epidemics. Consequently, chemoprophylaxis for contacts is generally recommended. Ciprofloxacin is frequently used in adults in a convenient 1-dose regimen (*1*). Resistance to this antimicrobial drug in *N. meningitidis* is rare (MIC>0.06 mg/L) and has been reported only in sporadic cases in Greece, France, Australia, Spain, Argentina and Hong Kong Special Administrative Region, People's Republic of China (*2–5*). However, recent reports have described ciprofloxacin-resistant (Cip-R) serogroup A meningococci from 2 outbreaks in Delhi, India, (*6*) and a cluster of 3 serogroup B meningococci in the United States (*7*). This information is of concern because of the high epidemic potential of serogroup A isolates, lack of vaccine against serogroup B meningococci, and possible horizontal Cip-R gene transfer to other meningococcal isolates.

Experimental work was conducted in the *Neisseria* Unit of the Institut Pasteur in Paris. We screened all clinical *N. meningitidis* isolates received at the French National Reference Center for Meningococci in Paris since 1999 for ciprofloxacin resistance. Of these isolates, 4,900 were from France and 246 were from African countries (Burkina Faso, Cameroon, Central African Republic, Côte d'Ivoire, Madagascar, Niger, Rwanda, Senegal, and Tunisia). Only 3 isolates tested were resistant to ciprofloxacin (MICs = 0.19 mg/L), and all were isolated from cases of invasive disease in France.

Two serogroup A, serotype 4, serosubtype P1.9, Cip-R isolates belonged to different sequence types (STs), ST-7 (Cip-R1) and ST-4789 (Cip-R2), although they belonged to the same clonal complex (ST-5/subgroup III). Cip-R1, which showed decreased susceptibility to penicillin, was isolated in 2004 from the blood of a 7-year-old girl. This isolate was most likely imported from Africa. Cip-R2 was isolated from the cerebrospinal fluid of a 77-year-old man who had arrived in France from India in 2006. The ST of this isolate (ST-4789) is the same as the ST of isolates from an outbreak in Bangladesh and similar to isolates from an outbreak in India (*6*; http://neisseria.org/nm/typing/mlstdb). Cip-R3 (serogroup W-135, nontypeable, subtype P1.5), which was isolated from blood and cerebrospinal fluid of an 82-year-old woman in 2006, belonged to a new ST (ST-6361). Current ciprofloxacin resistance has not been documented among invasive W-135 meningococcal isolates since 2 W-135 resistant meningococci were isolated from sputum samples of elderly patients in Spain (*3*).

To investigate the mechanism of resistance in the isolates, fragments of *gyrA* (847 bp) and *parC* (822 bp) genes were amplified by using primers gyrA-1F (5′-gttttcccagtcacgacgttgtaATGACCGACGCAACCATCCGCCAC-3′) and gyrA-1R (5′-ttgtgagcggataacaatttcCCAGCTTGGCTTTGTTGACCTGATAG-3′), and parC-1F (5′-gttttcccagtcacgacgttgtaATGAATACGCAAGCGCACGCCCCA-3′) and parC-1R (5′-ttgtgagcggataacaatttcGGAATTGGCGTTCGGCGGCAGCTC-3′), respectively (sequences in lower case letters are adaptors for universal forward and reverse sequences were added for sequencing after amplification). Primers used for amplification of the *parE* gene were as described (*8*).

Sequencing of fragments of *gyrA*, *parC*, and *parE* genes showed a mutation in the *gyrA* gene in the 3 Cip-R isolates resulting in a Thr91 → Ile substitution. Cip-R1 also showed additional alterations of Asn103 → Asp, Ile111 → Val, and Val120 → Ile, which were described for meningococcal isolates (*3*). Sequences of *parC* and *parE* genes were the same as in a ciprofloxacin-susceptible isolate tested. The association of the Cip-R phenotype with mutations in *gyrA* was confirmed by transformation into the susceptible isolate by using appropriate PCR products (*9*). In addition to the common Thr91 → Ile substitution, the 3 Cip-R isolates were distinguishable by additional *gyrA* alterations or phenotypic and genotypic characteristics. This finding suggests independent events and argues against clonal expansion of Cip-R meningococci.

Serogroup A meningococcal isolates in France are rare and mostly imported. Lack of detection of ciprofloxacin resistance among African isolates tested in this study may be caused by the relatively low number of these isolates (n = 246). Therefore, surveillance of antimicrobial drug susceptibility of meningococcal isolates should be enhanced by using molecular approaches that can also be used as nonculture techniques. This molecular approach will be useful in countries with limited access to classic microbiologic culture–based methods. Reports of invasive cases caused by W-135 Cip-R meningococci should alert physicians who use quinolones to treat respiratory infections in elderly persons. This age group is affected most often by invasive meningococcal pneumonia and 54.5% of such cases are caused by W-135 meningococci (*10*).

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Rare *Cryptosporidium hominis* Subtype Associated with Aquatic Center Use

To the Editor: Cryptosporidiosis is the most frequently reported gastrointestinal illness in outbreaks associated with treated (disinfected) recreational water venues in the United States (*1*). In 2003, an increased number of cryptosporidiosis cases occurred in the Tri-Cities area of the Lower Mainland region (near Vancouver), in British Columbia, Canada. Although all cases were associated with the use of a community aquatic

center, their onset dates were spread over a 3-month period, and the link between cases was unclear. The aim of this study was to determine if the cases in this disease cluster were related. Although suitable molecular markers had yet to be defined at the time of the outbreak, recent reports on the use of the gp60 gene for subtyping in molecular epidemiologic studies (*2*,*3*) have enabled us to reanalyze the isolates and report these results.

Fifteen laboratory-confirmed cases were identified from October 15 to December 5, 2003. This number was in excess of the anticipated incidence rate for this community, which averaged 5 reported cryptosporidiosis cases per year. During the period of investigation, an incident of fecal contamination at the aquatic center on October 10, 2003, was documented and remediation involved increasing the free chlorine concentration. Because the regional health authority was concerned about the increased number of cases, the facility closed voluntarily on December 5 for further remediation. However, recorded free chlorine concentrations did not exceed 2.0 ppm at any time during the investigative period (October 5–December 31).

The health authority released a public advisory encouraging those who used the facility to submit fecal specimens for laboratory testing. The health authority also sent letters to family physicians in the area, informing them of the disease cluster and requesting that unpreserved stool specimens be collected, in addition to the formalin-fixed specimens, for routine diagnostic testing. Nine fecal specimens were collected from clinically symptomatic case-patients with histories of exposure to the implicated aquatic center. Five specimens were selected by using the criteria that they were from patients with laboratory-confirmed cryptosporidiosis cases from 5 separate households. The specimens were then coded for anonymity before subsequent molecular analysis. Genomic DNA was extracted from purified *Cryptosporidium* oocysts by freeze-thawing, and the species was determined by PCR amplification and sequencing of the 18S rRNA gene as described previously (*4*). The gp60 gene was also amplified by PCR by using primers described by Ong and Isaac-Renton (*5*). DNA sequences of amplicons were determined by cycle sequencing and assembled as described previously (*4*,*5*). The gp60 allele and subtype were identified by multiple sequence alignment with GenBank reference sequences and phylogenetic analysis that used ClustalX version 1.8 (www.clustal.org) as well as manual quantification of microsatellite repeats.

The 18S rRNA and gp60 genes were amplified successfully from 4 specimens. On the basis of the 18S rRNA gene sequence, all case-patients were infected with *Cryptosporidium hominis*, a species associated primarily with human-to-human transmission. The gp60 sequences from all 4 case-patients were identical and were subtype IdA19, a rarely reported subtype of *C. hominis*. Globally, most reports of the gp60 Id allele, such as 9 reported cases from Australia, have identified the IdA15G1 subtype (*3*). Another subtype, IdA18, was isolated from 5 case-patients in a 1997 foodborne outbreak in Spokane, Washington (*6*). To date, the IdA19 subtype has been identified in only 1 sporadic case, in northern Ontario (*7*), and a subset of cases (seven 1dA19 and 2 mixed IdA19 and IbA10G2) in the 2001 waterborne outbreak in North Battleford, Saskatchewan (*5*,*8*). The IdA19 subtype is identical in sequence to the IdA18 subtype except for 1 extra TCA repeat in the microsatellite region. Neither subtype has been reported anywhere in the world except in Canada and the Pacific Northwest.

Because cases from all previous *C. hominis* outbreaks of cryptosporidiosis in British Columbia have been caused by the IbA10G2 subtype, the most prevalent subtype in sporadic and outbreak cases around the world (*2*,*5*,*9*,*10*), our results indicate the presence of a new subpopulation of *C. hominis* parasites that could cause future disease outbreaks. The identification of the same subtype in all 4 case-patients with cryptosporidiosis associated with the use of a community aquatic center was consistent with their exposure history and confirmed that all cases were linked epidemiologically. However, the association between the single northern Ontario sporadic case and the larger number of Saskatchewan and British Columbia outbreak cases is uncertain. The association with the IdA18 subtype in the Washington foodborne outbreak is also unknown. Further research is needed to determine the distribution and prevalence of gp60 subtypes in Canada as well as other parts of the world before we can more clearly understand the transmission of the IdA19 subtype.

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Two Imported Chikungunya Cases, Taiwan

To the Editor: Chikungunya is a reemerging infectious disease, endemic to Africa and Southeast Asia, caused by a mosquito-borne alphavirus in the family *Togaviridae*. Numerous chikungunya outbreaks have been reported in Africa and Southeast Asia since chikungunya virus (CHIKV) was first isolated in Tanzania in 1953 (*1*). Since 2005, several Indian Ocean islands and India have experienced massive CHIKV outbreaks caused by the East/Central/South African genotype (*2*,*3*), whereas all earlier isolates from India during 1963–1973 were of the Asian genotype (*4*). Other chikungunya outbreaks caused by the Asian genotype were frequently reported during 1960–2003 in many Southeast Asian countries, including India, Malaysia, Indonesia, Cambodia, Vietnam, Myanmar, Pakistan, the Philippines, and Thailand. Epidemics caused by reemerging CHIKV were reported in Indonesia and Malaysia during 2005–2007 (*1*,*5*).

We have previously reported on fever screening at airports in Taiwan as part of active surveillance for a panel of notifiable infectious diseases such as dengue, gastroenteritis caused by enteric bacteria, malaria, and yellow fever (*6*). The activity is carried out by using infrared thermal scanners to measure the body temperature of arriving passengers. Diagnostic testing algorithms for patients being screened for fever were based on evaluation by airport clinicians. The rationale behind this process is to minimize local outbreaks by reducing the number of imported cases. We report 2 imported chikungunya case-patients identified in Taiwan by fever screening at airports; 1 had returned from Singapore in 2006, infected with CHIKV East/Central/South African genotype, and the other had returned from Indonesia in 2007, infected with the Asian genotype.

To assess viremic fever patients with alphavirus infection, a multiplex 1-step SYBR Green I-based real-time reverse transcription–PCR (RT-PCR) was developed. A cocktail consisting of 3 sets of primers was mixed and used for RT-PCR screening. The alphavirus-specific primer set (AL-2: 5′-AAG CTY CGC GTC CTT TAC CAA AG-3′ and AL-3: 5′-GTG GTG TCA AAC CCT ATC CA-3′) targeted a consensus region of the nonstructural protein 1 (*nsp1*) genes to detect all alphaviruses. The CHIKV-specific primer set (F-CHIK: 5′-AAG CTY CGC GTC CTT TAC CAA AG-3′ and R-CHIK: 5′-CCA AAT TGT CCY GGT CTT CCT-3′) targeted a region of the envelope protein 1 (E1) gene of CHIKVs (*7*). The Ross River virus–specific primer set (RRV-1: 5′-GGG TAG AGA GAA GTT YGT GGT YAG-3′ and RRV-2: 5′-CGG TAT ATC TGG YGG TGT RTG C-3′) targeted a region of the envelope protein 2 (E2) gene of Ross River virus. Positive results were then confirmed by gene sequence analysis, virus isolation, and serologic tests. The nucleotide sequences of complete structural polyprotein genes were determined as previously described and submitted to GenBank (accession nos. EU192142 and EU192143) (*3*,*8*). A phylogenetic tree, based on a total of 23 CHIKV partial E1 gene sequences (255 bp), was drawn to trace the origin of 2 CHIKV isolates reported in this study (Figure).

The initial imported chikungunya case was detected at Taiwan Taoyuan International Airport on November, 20, 2006, in a 13-year-old Taiwanese boy who was returning from studying at an international educational training center in Singapore. The second imported case was also detected at Taiwan Taoyuan International Airport on June, 20, 2007, in a 5-year-old boy on his return from visiting relatives in East Kalimantan Province, Indonesia,

with his mother. Both case-patients had fever, fatigue, generalized arthralgia, and rash. Real-time RT-PCR screening showed a high level of alphavirus, but not flavivirus, viremia on day 2 (Singapore imported case) and day 3 (Indonesia imported case) acute-phase samples. Serodiagnosis with immunofluorescent antibody assay (immunoglobulin M + G + A titers ≥640), and ELISA showed positive seroconversions for both patients.

Analysis showed that these 2 imported cases were introduced from Singapore and Indonesia and that the patients were infected with CHIKV of East/Central/South African genotype and Asian genotype, respectively. Unlike dengue, chikungunya is not endemic to Singapore. However, a small chikungunya outbreak caused by an Indian strain of East/Central/South African genotype transmitted by *Aedes aegypti* was reported in January 2008; this occurrence suggests that imported CHIKV may not be detected because of limited transmission and because the signs and symptoms may be mistaken for those of dengue (*9*). In contrast, chikungunya has been endemic to Indonesia since 1973. Indonesia had epidemic outbreaks in 1980, 1983–1984, and yearly outbreaks after 1998. In following the ongoing chikungunya epidemic, we have identified 4 additional imported chikungunya cases from Indonesia since July 2007.

A recent chikungunya outbreak in Italy demonstrated that *Aedes albopictus* is a competent vector that can initiate local transmission of imported CHIKV (*10*). In Taiwan, *Ae. albopictus* is distributed throughout the island, and *Ae. aegypti* is distributed only in southern Taiwan. With increasing numbers of imported CHIKV infections, the risk for local transmission is similar to that of dengue, especially in southern Taiwan. Our results show that CHIKVs of both genotypes are spreading in Southeast Asia. The cocirculation of dengue and chikungunya would likely be increased in many Southeast Asian and African countries because of the rise in international travel and the wide distribution of the competent vectors, *Ae. albopictus* and *Ae. aegypti*.

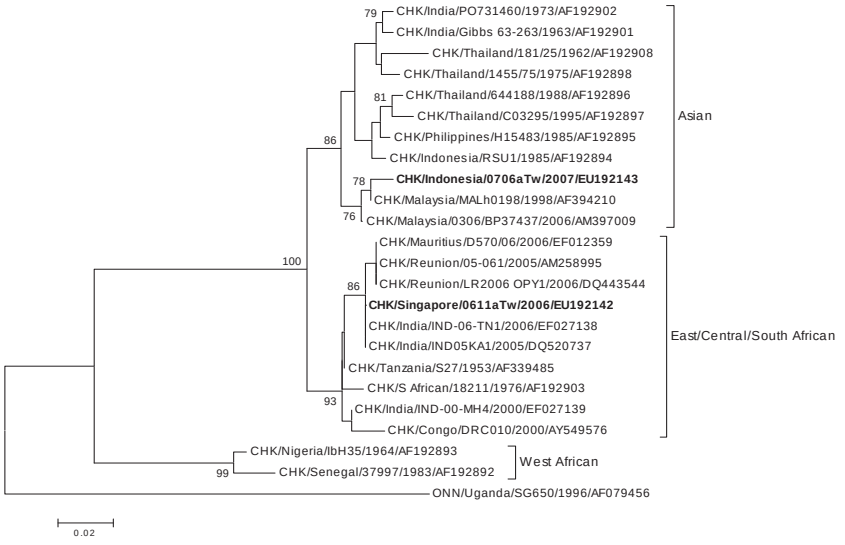

Figure. Phylogenetic relationships of chikungunya virus (CHIKV) isolates from 2 imported cases in Taiwan. The tree was constructed by the neighbor-joining method using partial nucleotide sequences of envelope protein 1 (E1) gene (255 bp) of 23 CHIKV strains. O'nyong-nyong (ONN) virus sequence was used as the outgroup virus. Bootstrap support values >75 are shown. The 2 imported CHIKV strains in Taiwan are designated by **boldface** type. Viruses were identified by using the nomenclature of virus/country/strain/year of isolation/GenBank accession no. Scale bar indicates substitutions per site.

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Chikungunya-related Fatality Rates, Mauritius, India, and Reunion Island

To the Editor: During the epidemic of chikungunya virus infection that occurred on Reunion Island in 2005–06, we reported an overmortality corresponding to the epidemic peak, which was estimated by comparing observed and expected deaths (*1*). The excess was similar to the number of deaths related to chikungunya infection reported by death certificates (*2*). The case-fatality rate (CFR) on Reunion Island was estimated to be 1/1,000 population.

According to Beesoon et al. (*3*), the fatality rate attributable to chikungunya infection was much higher on Mauritius: 743 deaths in excess of expected deaths led to a CFR of ≈4.5%, with 15,760 confirmed or suspected cases for 2005 and 2006 as reported in this letter. A similar CFR of 4.9% can be calculated for the city of Ahmedabad, India, during the 2006 chikungunya epidemic (*4*).

This 45- to 49-fold difference could be explained by a greater severity of chikungunya infection in Mauritius or Ahmedabad that could be due to a mutating strain, differences in the preexisting conditions of patients, differences in the management of patients, or by coincident deaths in excess from other causes.

However, the most probable explanation can be attributed to the surveillance systems of chikungunya cases. On Reunion Island, surveillance was highly sensitive and relied either on active case finding or on estimates of suspected cases. Results have been assessed by iterative external studies and serosurveys, and the CFR we found is likely consistent.

If we apply this rate to Mauritius, ≈60% of the population would have contracted chikungunya infection during this epidemic. If so, the risk of epidemic resurgence could be much lower than previously expected. This point raises the need to conduct seroprevalence studies in those territories, the only way to evaluate the herd immunity level of the population.

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Aquaculture and Florfenicol Resistance in *Salmonella enterica* Typhimurium DT104

To the Editor: In June 2006, the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organisation for Animal Health (OIE) convened an Expert Consultation to consider the risks to human health represented by the use of antimicrobial drugs in aquaculture. This would, therefore, appear to be an opportune time to reexamine some of the arguments that have been presented with respect to the assessment of these risks.

In their contributions to the debate regarding the risks associated with the use of antimicrobial agents in aquaculture, Angulo (*1*), Angulo and Griffin (*2*), Ribot et al. (*3*), and, more recently, Cabello (*4*) have argued that the available molecular evidence suggests that the *flo* gene that encodes chloramphenicol and florfenicol resistance in *Salmonella enterica* serovar Typhimurium DT104 (DT104) originally emerged in Japanese aquaculture and may have transferred horizontally from this host to DT104. This argument also appears in the report of the WHO/FAO/OIE consultation (ftp://ftp.fao.org/ag/agn/food/aquaculture_rep_13_16june2006.pdf). These authors (*1–4*) have based their argument on the assertions that florfenicol was first used in Japan and that *flo* gene–mediated resistance to this agent was first identified in bacteria isolated from Japanese fish farms.

In attempting to identify the date of the emergence of florfenicol resistance in Japanese aquaculture, Angulo and Griffin (*2*) state that florfenicol had been used in this country since the early 1980s. However, Schering Plough, the manufacturer of florfenicol, reports first marketing this

agent for aquacultural use in Japan in 1990 (D. Schofield, pers. comm.), and this date for the introduction of florfenicol is also provided by Kim et al. (*5*). It should, however, be noted that the *flo* gene encodes resistance to both florfenicol and chloramphenicol and, therefore, *flo*-containing bacteria could be selected for by the use of either agent. As a consequence, arguments about the chronology of the first use of florfenicol may have limited relevance.

Florfenicol resistance in Japanese aquaculture (*5*) was first reported in strains of *Pasteurella piscicida* (now renamed *Vibrio damsela)*. The sequence of the gene that encoded florfenicol resistance in these strains (*6*) was demonstrated to have a 97% nucleotide sequence similarity to that found in strains of DT104 resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (ACSSuT DT104) (*7*). The available data (*5*,*8*) allow a reasonably accurate estimate of the date when these florfenicol-resistant strains first emerged in Japanese aquaculture. The strains of florfenicol-resistant *P. piscicida* were first isolated in Japan in 1992 (*5*). However, a previous study (*8*) had demonstrated 100% susceptibility to florfenicol of *P. piscicida* strains isolated from 1989 through 1991. Because this study examined 175 *P. piscicida* strains isolated from fish farms distributed over a wide geographic area in Japan, the data it generated provide strong support for the conclusions that *flo*-mediated florfenicol resistance in *P. piscicida* first emerged in Japanese aquaculture in 1992 (*5*). However, the presence of the *floR* gene has been demonstrated in a strain of ACSSuT DT104 isolated in the United States in 1985 (*3*). Thus, the *floR* gene was present in DT104 strains isolated in the United States at least 7 years before the first bacteria containing this gene (*6*) were isolated from an aquaculture setting in Japan (*5*).

There are, however, data indicating that the *flo* gene was present in terrestrial bacteria associated with humans long before it was detected in multidrug -resistant DT104. A gene with a 95%–97% nucleotide identity with the *flo* gene of DT104 was detected in the Inc C plasmid R55 (*9*). This plasmid was originally identified in a chloramphenicol-resistant strain of *Klebsiella pneumonia*e isolated from a person in Paris in 1969 (*10*).

The earliest report of the isolation of a bacterium whose florfenicol resistance was encoded by a *flo* gene (*6*) and the earliest accession date for a *flo* gene sequence in GenBank (www.ncbi.nlm.nih.gov/Genbank) both related to *P. damsela* isolated from Japanese aquaculture. Publication and accession dates do not, however, constitute evidence of the date of the first isolation of a bacterium containing this gene. Analysis of the available chronological and molecular data presented here indicates that a variant of the *floR* gene in DT104 (*9*) was present in a terrestrial bacterium isolated in 1969 (*10*), 23 years before the first isolation, in 1992, of a bacterium associated with aquaculture that contained this gene (*5*). It further demonstrates that this gene was present in strains of DT104 isolated in 1985. Thus, these data provide no support for the arguments (*1–4*) that implicate aquacultural use of florfenicol or the subsequent occurrence of florfenicol-resistant *P. damsela* in the emergence of the *flo* gene in DT104.

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Imported Dengue Hemorrhagic Fever, Europe

To the Editor: Dengue infection is an endemic and epidemic urban disease (*1*), transmitted by infected *Aedes* mosquitoes. Its incidence is increasing in tropical and subtropical areas (*1*,*2*) because of 1) introduction of the virus into areas where it was not previously endemic, and 2) the spread of the 4 serotypes and the vector in disease-endemic areas (*2*,*3*). Infection with 1 serotype provides lifelong homologous immunity only for that serotype, and after a few months, the presence of nonneutralizing antibodies increases the risk for progression to dengue hemorrhagic fever (DHF) or dengue shock syndrome when the patient is infected by any of the other 3 serotypes (*3*,*4*). We report an imported case with severe clinical manifestations that fulfills DHF criteria (*5*).

A 33-year-old Spanish woman who had worked in Anantapur, India, for 180 days, returned to Spain on August 1, 2007; on August 3, she traveled to Dubrovnik, Croatia, on holiday. She also had visited Thailand 45 days before August 1 and Brazil 2 years ago. Two months previously, she experienced a 3-day episode of fever that spontaneously resolved but without laboratory evidence of dengue. On August 6, she exhibited a high fever, chills, headache, arthralgia, and myalgia, with hypotension and was admitted to the hospital. Three days later, a confluent maculopapular rash developed. Dubrovnik hospital laboratory values were hemoglobin (Hb) 143 g/L, packed cell volume (PCV) 41.6%, mean corpuscular volume (MCV) 84.6 fL, platelet count 97×10^9/L, leukocyte count 1.96×10^9/L, aspartate aminotransferase (AST) 45 U/L, alanine aminotransferase (ALT) 31 U/L, AP 73 U/L, and lactate dehydrogenase (LDH) 198 U/L. On the fifth day of illness, platelet count was 50×10^9/L. Because viral hemorrhagic fever was suspected, the patient was referred to a specialized hospital in Zagreb. Chest radiograph and abdominal ultrasound scan showed bilateral pleural and peritoneal effusions.

The patient was treated with fluid and plasma replacement, antipyretics, and ceftriaxone plus doxycycline to counteract bacterial and other possible tick-borne infections. She was placed under strict isolation measures while awaiting final diagnosis. The patient was transferred to Barcelona (Spain) University Hospital on August 14; on the basis of her clinical symptoms, hemorragic fever was suspected. She exhibited headache, arthralgia, and myalgia. The fever subsided 9 days after the onset of symptoms. Clinical examination showed a maculopapular rash involving the face, thorax, limbs, and palms and soles, with diffuse petechiae and bruising (Figure). Barcelona University Hospital laboratory values were Hb 105 g/L, PCV 32%, MCV 86, prothrombin time 12.4 s, AST 347 U/L, ALT 322 U/L, gamma-glutamyl transferase 114 U/L, alkaline phosphatase 194 U/L, LDH 544 U/L, bilirubin 0.5 mg/dL, and C-reactive protein level 6.93 mg/dL. Platelet count and renal function were within normal limits. Urine, blood, and stool cultures were all negative for bacterial infections.

Serologic tests on day 3 and day 11 after the onset of symptoms were not reactive for Crimea-Congo hemorrhagic fever (CCHF), chikungunya, yellow fever, Hantaan, Puumala, and Dobrava viruses; HIV 1 and 2; parvovirus B19; cytomegalovirus; Epstein-Barr virus; or rickettsial diseases. Immunoglobulin (Ig) M tests on day 3 for all 4 dengue virus serotypes were negative. Positive IgG were 1:320 (type 1) and 1:100 (type 3 and 4). A second sample on day 11 showed all 4 IgG serotypes >1:10,000, and IgM >1:10,000 for serotypes 1, 2, and 4. Results of real-time PCR for CCHF were negative but reverse transcription–PCR multiplex for dengue virus was positive for dengue type 1 virus. The patient recovered and was monitored for 2 months.

Since 1977, 15 cases of imported DHF have been reported in Europe (*6*,*7*). The 4 World Health Organization (WHO) criteria for DHF diagnosis are 1) fever related to the current process, 2) hemorrhagic manifesta-

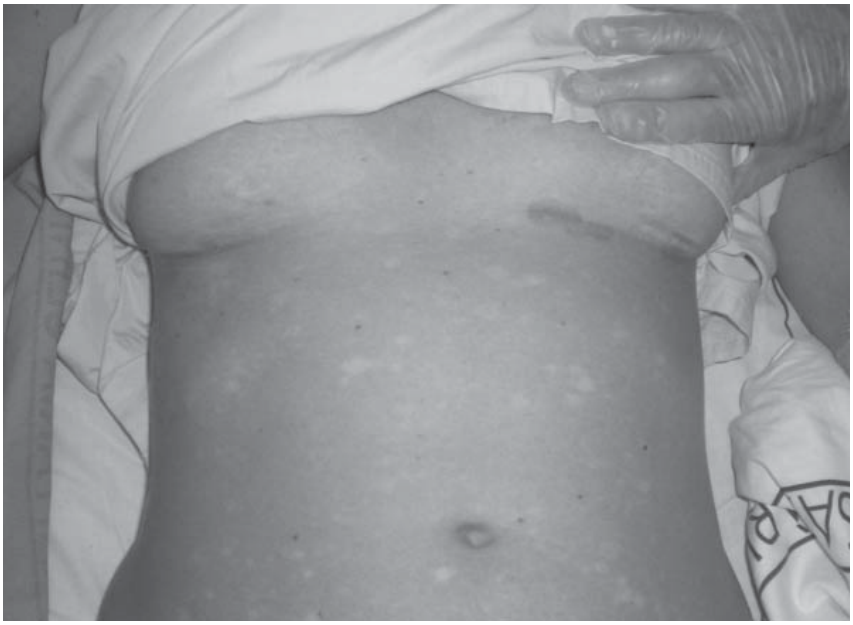

Figure. Maculopapular rash with diffuse petechiae, with areas of normal skin and bruising under the breast.

tions, 3) low levels of platelets (<100 × 10^9/L) and 4) increased capillary permeability (*5*). Our patient fulfilled all 4 criteria. Few cases of reported DHF fulfill criterion 3 due to the short duration of severe thrombocytopenia in mild clinical forms (*8*). Increased vascular permeability was shown in our patient by the peritoneal and bilateral pleural effusions.

The probability of diagnosing dengue fever in Europe increases with travel to dengue-endemic areas, in view of the increase of DHF numbers (2006–2007) and several outbreaks around the world, even during the nondengue season (*9*). Frequent travelers are more at risk for DHF. In a recent European publication, 17% of patients with imported dengue fever exhibited a secondary immune response, thus having a higher risk of developing DHF in the future (*6*). Serologic tests confirm dengue infection only if a 4-fold increase in titers in consecutive serum samples occurs, as in our case.

In dengue-endemic areas, despite the higher disease incidence, many cases still fail to meet WHO criteria (*9*). A comprehensive revision of dengue and DHF series (*8*) shows differences in applying WHO criteria for diagnosis, and sometimes the correlation was poor between criteria-fulfilling cases and severity of disease. Some reports (*6*,*8*) suggest that WHO criteria should be reviewed and perhaps new parameters should be established to define severe dengue disease.

Although our patient was not infected in Europe, lessons from the recently described chikungunya outbreak in Italy indicate the possibility of new arbovirus outbreaks in previously non–disease-endemic areas due to the increasingly established presence of vectors like *Ae. albopictus* (*10*).

Dengue virus infection should therefore be considered in the differential diagnosis of fever in returning travelers. DHF diagnosis, although unusual, could become more frequent in the future.

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Mycobacterium setense Infection in Humans

To the Editor: A 66-year-old man had a bone graft for treatment of an oroantral fistula in March 2007 in Marseille, France. The surgery consisted of a bilateral maxillary sinus filling with a parietal osseous graft to close the fistula (position 24–25). Painful edema of the hemiface and mild fever developed in the patient in July 2007. Computed tomography showed areas of hypodensity in the osseous graft in the left maxillary sinus consistent with osteolysis. Microscopic examination of a bone biopsy specimen after gram staining did not reveal any organisms but this specimen did grow *Enterobacter cloacae* and colonies of a gram-positive bacillus after a 2-day inoculation on 5% blood agar incubated at 37°C in an atmosphere of 5% CO_2. Tentative identification of this catalase-positive, oxidase-negative gram-positive rod (isolate 74023791) by an API Coryne

strip (API bioMérieux, La Balme-les Grottes, France) remained inconclusive. Isolate 74023791 exhibited acid fastness and was further identified by comparing its 16S rDNA (*1*) and heat shock protein 65 (hsp65) (*2*) sequences with those in the GenBank database and its RNA polymerase subunit B (*rpoB*) sequences (*3*) with those of *Mycobacterium* spp. in our local rpoB database.

Antimicrobial susceptibility testing using E-test (AB Biodisk, Bruz, France) indicated that isolate 74023791 was susceptible to ciprofloxacin with an MIC of 0.047 μg/mL and resistant to amoxicillin (MIC >256 μg/mL), ceftriaxone (MIC >256 μg/mL), erythromycin (MIC >256 μg/mL), clarithromycin (MIC >256 μg/mL), and rifampin (MIC >32 μg/mL). Disk testing and reference broth dilution method (*4*) showed that isolate 74023791 was susceptible to imipenem after a 3-day incubation. However, E-test showed heterogeneous resistance with colonies exhibiting an MIC >256 μg/mL. The same observations were made for *Mycobacterium setense* type strain CIP109395T (Collection de l'Institut Pasteur, Paris, France) (*5*). Daily treatment with 2 g imipenem and 1.5 g ciprofloxacin was prescribed for 1 month before the imipenem E-test and broth dilution results were available, and was followed by 1.5 g/day ciprofloxacin for 3 months, resulting in a favorable clinical and radiologic evaluation at 5-month follow-up.

Phylogenetic analyses indicated that isolate 74023791 belonged to the *M. fortuitum* group, along with *M. porcinum* and *M. conceptionense*, and was most closely related to M. *setense*, a recently described species of this group (*5*) (Figure). Isolate 74023791 shared 100% 16S rDNA (GenBank accession no. EU371507), 99.5% hsp65 (accession no. EU371508), and 99.0% *rpoB* (accession no. EU371509) sequence similarity with *M. setense* (accession nos. EF138818 and EU371504, EF138819 and EU371505, and EF414447 and EU371506, respectively). Because the 99% *rpoB* sequence similarity of the patient's isolate was above the 97% rpoB sequence similarity cut-off value used to identify rapidly growing mycobacteria (*3*), isolate 74023791 was therefore identified as *M. setense*.

M. setense, in association with an *E. cloacae* strain susceptible to antimicrobial drug therapy, was an agent of infection in our patient. It is noteworthy that *M. setense* and the closely-related species *M. conceptionense* were isolated from patients with posttraumatic osteitis (*5*,*6*); *M. porcinum* was isolated from 7/46 cases of osteomyelitis and additional cases of postsurgical infection, respectively (*7*); *M. fortuitum* osteomyelitis has also been reported (*8*). These data emphasize the role of *M. fortuitum* group organisms in posttraumatic and postsurgical osteitis.

In a later interview, the patient disclosed that he rinsed his mouth with well water during the weeks after receiving the bone graft. We initially suspected that the water was the source of *M. setense*, as previously suspected for *M. conceptionense* (*6*) and reported for *M. porcinum* (*7*). However, neither *M. setense* nor *M. setense* DNA were detected in the well water in October 2007.

Initially, *M. setense* was reported to be susceptible to imipenem by the disk diffusion method, which is not the reference method (*5*). In this report, the disk and reference broth dilution methods showed that both clinical and reference *M. setense* strains were initially susceptible to imipenem but the E-test disclosed that both strains exhibited heterogeneous resistance to imipenem; colonies exhibited an MIC >256 μg/mL. This result was unexpected because the E-test showed that related species *M. fortuitum* CIP104534T, *M. conceptionense*, CIP 108544T and *M. porcinum* CIP105392T were susceptible to imipenem (*6*). Likewise, the modified broth dilution method showed that the MIC for imipenem was ≤4 μg/ml for *M. fortuitum* (*9*). However, when a broth dilution method was used, the MIC for imipenem ranged from 0.5 μg/mL to 8 μg/mL in 42 *M. porcinum* strains (*7*). Together, these data challenge the susceptibility to imipenem in organisms of the *M. fortuitum* group.

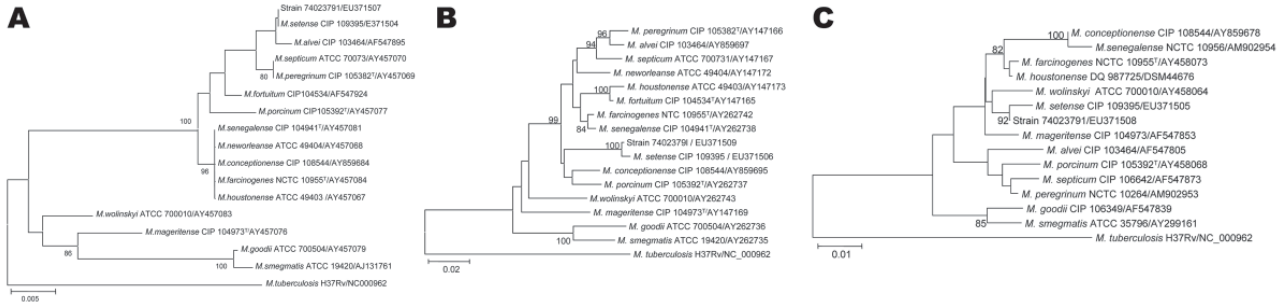

Figure. Phylogenetic position of isolate 74023791 and 16 rapidly growing *Mycobacterium* species based on A) 16S rDNA, B) partial RNA polymerase subunit B, and C) partial heat shock protein 65 sequences analyzed by using the neighbor-joining method and Kimura 2-parameter distance correction model. The support of each branch, as determined from 1,000 bootstrap samples, is indicated by the value at each node when ≥80% (as a percentage). *M. tuberculosis* was used as the outgroup species. Scale bars represent differences in nucleotide sequences.

M. setense is an emerging organism of the *M. fortuitum* group that must be added to the growing list of rapidly growing mycobacteria isolated from humans. The initial gram-positive rod appearance of *M. setense* may delay its accurate identification. Determination of antimicrobial drug susceptibility needs to be conducted by the reference broth dilution method. Further reports are warranted to characterize the role of *M. setense* in infection.

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Human Bocavirus in Tonsillar Lymphocytes

To the Editor: We read with great interest the recent report by Longtin and colleagues (*1*) describing human bocavirus (HBoV) infection among Canadian children with acute respiratory tract illnesses (ARI). The authors identified HBoV by PCR in nasopharyngeal aspirates from 13.8% of young children hospitalized with ARI, an infection rate well within the range reported by other studies on children (*2*). However, these authors also detected an unexpectedly high rate of HBoV for their control group (43%), >3 times the rate for ARI case-patients. In contrast, several similar studies did not detect HBoV in asymptomatic children. Kesebir et al. detected HBoV in 23 (5.2%) of 425 young children with ARI but in none of 96 children during routine well-child visits (*3*). Maggi et al. detected HBoV in 9 (4.5%) of 200 infants with ARI but in none of 30 healthy infants or 21 preadolescent healthy children without signs of ARI or history of asthma or wheezing (*4*). Finally, Allander et al. detected HBoV in 5 (19%) of 259 young children with acute expiratory wheezing but in none of 64 children who had not had respiratory symptoms during the preceding 4 weeks (*5*). In a recent study, we detected HBoV by PCR in 44 (12%) of 369 Thai children ≤4 years of age hospitalized with pneumonia but in only 2 (2%) of 85 asymptomatic age and temporally matched controls (*6*).

The inexplicably high rate of HBoV infection for patient controls reported by Longtin et al. (*1*) may reflect a unique feature of the children selected. The 100 controls were children without concomitant respiratory symptoms or fever at admission who were hospitalized during the study period for elective surgery, primarily of the ear, nose, and throat (71%). Most surgeries consisted of myringotomies, adenoidectomies, and tonsillectomies. The authors reported that these surgeries were more frequently performed on children found to be PCR positive for HBoV than on children negative for HBoV (84% vs. 61%). One possible explanation is that HBoV infection may directly induce inflammation of tonsillar tissues or facilitate bacterial superinfection prompting surgical intervention. Another possibility is that inflammation of mucosal lymphoid tissues enhances HBoV replication by recruitment of immune cells permissive for HBoV infection or by latent virus reactivation. Persistent infections and dependence on rapidly dividing cells are common features of the related parvoviruses, for example, human erythrovirus B19 (*7*). The presence of

low-level persistent HBoV infections may also help explain the exceptionally high rates of respiratory viral coinfections found with HBoV, as high as 90% in 1 study (average 42%) (*2*,*6*). Longtin et al. (*1*) found that 71% of the HBoV-positive patients were also co-infected with another respiratory virus. As we previously hypothesized (*6*), co-virus–induced cellular damage resulting in high levels of cellular division and differentiation may stimulate HBoV reactivation and replication, as has been shown for polyomavirus following induced kidney damage in newborn mice (*8*).

To assess whether HBoV is present in tonsillar lymphocytes and therefore possibly explain the high rate of PCR positives obtained by Longtin et al. (*1*) in their patient controls, we tested DNA extracts of lymphocytes separated by Ficoll-Paque from nasopharyngeal tonsils or adenoids (AL) and palatine/lingual tonsils (TL) from 164 patients (mostly children) undergoing routine adenoidectomies and tonsillectomies for HBoV DNA. Of these children, 21 had AL only, 57 had TL only, 18 had both AL and TL collected separately, and 68 had AL and TL combined in 1 container. Hypertropic tonsil and adenoid tissues were removed because of clinical complications, generally obstructive sleep apnea, otitis media, or chronic tonsillitis. Data were not available on whether the children had concurrent respiratory tract illness, although surgeries would likely be postponed if symptoms were apparent. The median age of 162 children for whom age data were available was 5 years (range 1–19.7 years). HBoV real-time PCRs were performed as previously described, targeting 2 unique regions of the HBoV genome (*9*). All extractions were performed in a separate laboratory from PCR activities, and negative-assay controls were included in all PCR runs to monitor for DNA contamination.

HBoV DNA was detected by both PCRs in lymphocytes from 53 (32.3%) children (median age 3.7 years, range 1–7.6 years). A single assay target (nonstructural protein gene NS1 or NP-1) was positive in specimens from 6 additional children (3.7%), but these specimens were classified as HBoV negative because they did not meet our strict criteria for a positive test result. Children PCR negative for HBoV were significantly older (median age 5.5 years, range 1.8–19.7 years; $p<0.001$). HBoV was more often detected in AL (56%) than TL specimens (16%; $p<0.001$); the age distributions for children from each group were similar. Among 12 HBoV-positive children from whom both AL and TL were available and collected in separate containers, 6 were positive for HBoV in both specimens, 4 were positive for AL alone, and 2 were positive for AL but only for a single PCR assay target. Moreover, HBoV was present at a substantially higher load in AL (median 3.1×10^5 copies/10^7 cells; range 2.8×10^1 to 1.2×10^9 copies/10^7 cells) than TL (median 1.6×10^3 copies/10^7 cells; range 0.1×10^0 to 5.3×10^7 copies/10^7 cells) as measured by quantitative PCR (*9*). We have no clear explanation for the relative predominance of HBoV DNA in AL. The adenoids are located at the back of the nasopharnyx in close proximity to inhaled pathogens and are covered with ciliated pseudostratified epithelium (respiratory epithelium) that may better support primary virus replication than the palantine and lingual tonsils, which are located in the lower pharynx and covered with nonkeratinized, stratified, squamous epithelium.

Our findings suggest that HBoV may establish latent or persistent infections of mucosal lymphocytes or contribute to tonsillar hyperplasia in young children. Further studies with appropriately matched controls will be necessary to fully understand the nature of HBoV infection and its role in human disease.

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Assessment of Reporting Bias for *Clostridium difficile* Hospitalizations, United States

To the Editor: Burckhardt et al. (*1*) recently reported on *Clostridium difficile*–associated disease (CDAD) in Saxony, Germany. In contrast to the observation by Wilcox and Fawley in the United Kingdom (*2*), the report from Germany argued against a reporting bias for gastroenteritides as a cause of the observed increase in the incidence of CDAD diagnoses from 2002 through 2006. To explore this issue further, I examined the potential influence of such reporting bias on the observed increase in the incidence of hospitalizations of patients with CDAD in the United States from 2000 through 2005.

In the 2000–2005 data from the National Inpatient Sample data from the Agency for Healthcare Research and Quality (*3*,*4*), I identified hospitalizations for gastrointestinal infections caused by *C. difficile*, *Salmonella*, rotavirus, and other unspecified infectious agents, using the corresponding diagnosis codes from the International Classification of Diseases, 9th Revision, Clinical Modification. I obtained censal and intercensal data on the numbers of the U.S. population from 2000 through 2005 from the U.S. Census Bureau (*5*). Based on these records, I calculated hospitalization incidence for each of the infectious causes.

Annual incidence of CDAD increased from 49.2 to 101.6 per 100,000 population within the period examined. Within the same time frame, the incidence of CDAD as the principal diagnosis also more than doubled, increasing from 11.6 to 25.8 hospitalizations per 100,000. Although the incidence of hospitalizations for *Salmonella* infections per 100,000 population remained stable, rotavirus infection showed a slight increase (from 10.8 to 14.5) as did other infectious gastroenteritides (from 38.9 to 49.9/100,000) (Figure). Thus, although a slight increase in the incidence was exhibited, a reporting bias for gastroenteric infections with organisms other than *C. difficile* does not appear to account fully for the observed doubling of the overall incidence of hospitalizations with CDAD in the United States from 2000 through 2005.

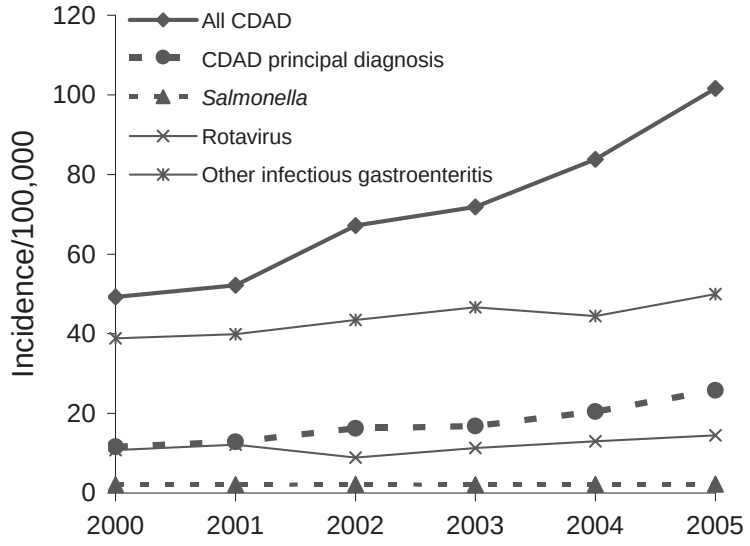

Figure. Annual incidence per 100,000 population of all hospitalizations for *Clostridium difficile*–associated disease (CDAD) compared with hospitalizations for a primary diagnosis of CDAD and with gastroenteritides caused by *Salmonella*, rotavirus, and other unspecified infectious agents, United States, 2000–2005.

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Improving Methods for Reporting Spatial Epidemiologic Data

To the Editor: A recent perspective in this journal (*1*) pointed out problems with the present, county-referenced system for reporting spatial epidemiologic data. Problems identified included coarse spatial resolution of county-referenced data and differences across the United States in size of counties, making data for the western part of the country coarser in resolution than data for the eastern part. Eisen and Eisen correctly pointed out that these problems complicate spatial analyses of epidemiologic data (*1*). However, the solutions that they propose, referencing epidemiologic data to ZIP codes or census tracts, partially solve only the first problem.

The problem of regional differences in spatial resolution of county-referenced data is, unfortunately, reflected in counties, ZIP codes, and census tracts, as shown in plots of nearest-neighbor distances among unit centroids as a function of longitude (Figure). Because all 3 regionalizations are based on human populations, the much greater population density in the eastern United States creates finer scale dispersion in the east. Thus, a shift to ZIP codes or census tracts does nothing to resolve the problem of regional differences in spatial resolution.

The problem of coarse spatial resolution is only partially addressed by the ZIP code or census tract solution. ZIP codes and census tracts cover fixed areas and can misrepresent the spatial precision of epidemiologic records. A traveling salesperson who covers the state of Wyoming each week would be represented identically as his or her next-door neighbor who is housebound, although spatial precision differs considerably between the 2 persons. Precision of the housebound neighbor could be better represented than county, ZIP code, or census tract. ZIP codes and census tracts change periodically, and ZIP codes do not have defined spatial extents per se (*2*). Thus, a better and more flexible solution is needed.

The biodiversity world has already addressed this challenge. The point-radius method for georeferencing locality descriptions (*3*) estimates a best guess for the exposure site (e.g., residence, workplace) but describes uncertainty in that georeference is a radius that expresses spatial uncertainty in the record (i.e., compare our traveling salesperson with his or her housebound neighbor) and in translation into geographic coordinates (including uncertainty in the locality descriptor, spatial footprint of the locality described, imprecision in the locality identified, and any other sources of imprecision). Point-radius georeferences are easily recorded and reported, are consistent and reproducible, and are more precise and considerably more stable than ZIP codes or census tracts.

As an example of how the point-radius method would be applied, the locality for our traveling salesperson would be assigned to his or her house, but the error radius would be 360 km (based on corner-to-corner distance across Wyoming). The housebound neighbor might have a similar set of coordinates (next door), but the error radius might be 0.1 km (breadth of the house plus the imprecision of the global positioning system unit). When a researcher uses these data, he or she might wish to analyze occurrence of this disease with a spatial precision of 1 km; e.g., applying a filter to exclude those data records too imprecise for this study, he or she would exclude the data record for the salesperson (because the salesperson may have contracted the disease in another sector of the state) but include that for the housebound neighbor. Alternatively, the researcher may include variable degrees of precision in the analysis according each to record a precision or certainty corresponding to its error radius, as in recent spatial analyses of Marburg virus transmission risk (*4*) and climate change effects on plague and tularemia transmission (*5*).

How specifically would this meth-

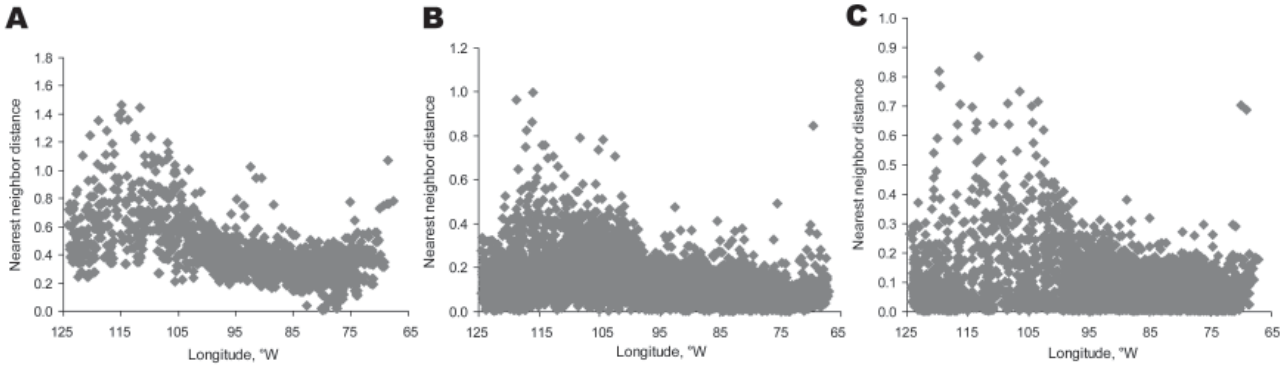

Figure. Longitudinal patterns in nearest-neighbor distances for A) counties, B) ZIP codes, and C) census tracts across the lower 48 United States, showing trends toward greater spacing among districts in the western United States compared with the eastern United States in all 3 regionalizations.

od be implemented in public health surveillance? If data are to be captured initially on paper, the data recorder would simply record the focal point of the person's activities (usually a residence) and an approximate description of the person's movements (e.g., broadly across the state, housebound, within 20 miles). These descriptions are easily georeferenced post hoc by using recently developed software tools (e.g., Biogeomancer, www.biogeomancer.org/). A more promising solution, if initial data capture is electronic, would be adaptation of some of these software solutions to the public health challenge. A flexible-resolution map with political boundaries, named places, and roads and streets could enable immediate digitization of the central point and the error radius even during direct consultation with the patient (when feasible).

The point-radius approach is novel to most epidemiologic applications but offers considerable advantages. When fine-resolution data are available, researchers will have this more precise information and can distinguish it from coarser resolution data; when actual data are coarser, this information is also expressed. Researchers will be able to filter epidemiologic occurrence information to retain those data that are sufficiently precise for particular applications, thus offering a considerable improvement over any of the 3 polygon-based approaches (ZIP codes, census tracts, and counties). Thus, the recent publication cited (*1*) got the question right but the answer wrong.

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In Response: In his comment, Peterson reiterates the need for improved methods for collecting and presenting spatial epidemiologic data for vector-borne diseases (*1*). He agrees with us that lack of reliable data on probable pathogen exposure site is an obstacle to the development of predictive spatial risk models (*2*). In that article we noted, "New methods are urgently needed to determine probable pathogen exposure sites that will yield reliable results while taking into account economic and time constraints of the public health system and attending physicians." Peterson suggests that the point-radius method is a viable solution to this problem. Unfortunately, its practical implementation for vector-borne diseases is neither reliable nor cost-effective.

With regard to practical implementation of the point-radius method in a public health setting, Peterson states, "If data are to be captured initially on paper, the data recorder would simply record the focal point of the person's activities (usually a residence) and an approximate description of the person's movements (e.g., broadly across the state, housebound, within 20 miles)" (*1*). We find a number of serious problems with this approach to determining probable sites of pathogen exposure, primarily that meaningful use of the point-radius method 1) will require not only recording detailed movements during the perceived window of opportunity for pathogen exposure but also weighting of risk by activity type and, for some vector-borne diseases, time of day; and 2) will require the public health community to allocate resources to in-depth interviews conducted by specially trained personnel.

Our first concern is that Peterson's scenario does not distinguish between a car trip to the mall at noon and spending an evening on the golf course. In reality, one activity presents minimal risk for exposure to mosquitoes infected with West Nile virus, whereas the other is a potential high-risk activity. Giving equal weight to the movements represented by these activities will assuredly produce an unreliable result for probable pathogen exposure site. Other issues are patient recall and reluctance to provide information on movement patterns and specific activities. Peterson's suggestion that the data recorder would simply record the focal point of the person's activities and an approximate description of the person's movements is therefore a grossly oversimplified solution to a complex public health problem.

With regard to the second concern, the average physician likely lacks the knowledge, time, and training in vector-borne disease epidemiology and ecology needed to accurately assess when and where risk for pathogen exposure occurred. To be of use, the method will require in-depth patient interviews by specially trained personnel from local or state health

departments. Even then, we doubt that the quality of data gleaned would justify the cost incurred.

We fail to see that the quality of information gathered by using the point-radius method would be an improvement over our suggestion. In our original article, we suggested using sets of standardized questions that are tailored to a given vector-borne disease. We also indicated that a critical minimal need includes a basic assessment of whether pathogen exposure likely occurred in 1) the peridomestic environment, 2) outside the peridomestic environment but within the county of residence, or 3) outside the county of residence (*2*).

The challenge of how to most effectively collect and present spatial epidemiologic data is neither conceptual nor technologic; rather, it is logistic and legal. Any new method must 1) weigh the public health utility of the method against the time and cost required for the public health system to implement it and 2) comply with existing patient privacy laws. The point-radius method clearly fails on the first count and also likely will present substantial problems in terms of patient privacy.

We agree that presenting data for case counts and disease incidence by ZIP code or census tract falls short of the desired level of spatial precision. However, this realistic compromise 1) is a marked improvement over the current practice to display only county-based spatial patterns for case counts or incidence; 2) incurs only minimal added time and cost for the public health community; and 3) can be implemented, especially for census tracts, with minimal concerns regarding patient privacy.

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etymologia

Brucella
[broo-sel′ə]

Genus of gram-negative, aerobic coccobacilli of the family Brucellaceae, named after Sir David Bruce (1855–1931), a Scottish physician who served abroad with the Royal Army Medical Corps. He investigated Malta fever, a mysterious undulating fever that affected many British soldiers. In 1887 Dr. Bruce established a causal relationship between the disease and an organism later designated *Brucella melitensis* (from Malta). *Brucella* spp. include animal parasites and pathogens, transmissible to humans through dairy products or contact with infected animal tissue.

Source: Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders/Elsevier; 2007; http://www.whonamedit.com

Wolbachia: A Bug's Life in Another Bug

Achim Hoerauf and Ramakrishna U. Rao, editors

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Wolbachia: A Bug's Life in Another Bug is timely and useful for understanding this bacterial genus. This book covers all aspects of *Wolbachia* organisms from basic science and history to medicine and veterinary science. The authors are the most renowned in their field.

These bacteria live intracellularly (similar to *Rickettsia* spp.) in arthropods or helminths and manipulate their hosts and progeny. They can select specific partners for their infected hosts and favor parthenogenesis or in ovo conversion from male to female. These bacteria were identified in 1924 in arthropods and are a paradigm of host–bacteria relationships. *Wolbachia* organisms became noteworthy in tropical medicine when they were found in nematode worms, specifically those causing filariasis. *Wolbachia* bacteria manipulate the fertility of most filarial worms; treating filariasis patients with antimicrobial drugs results in elimination of *Wolbachia* organisms and the microfilaremia. Unexpectedly, doxycycline was also found to kill the worm. Doxycycline could now be paradoxically recommended to treat certain cases of filariases.

I highly recommend reading this book because I believe that *Wolbachia* science will grow dramatically in the coming years as an example of the complex relationships between intracellular bacteria and hosts. Unfortunately, the most surprising discovery about these bacteria is too recent to be included in this book, namely, that they can integrate nearly their entire genome into the chromosome of their host, a unique example of massive lateral gene transfer. This characteristic may help investigators understand integration of mitochondrial genes into the eukaryotic nucleus.

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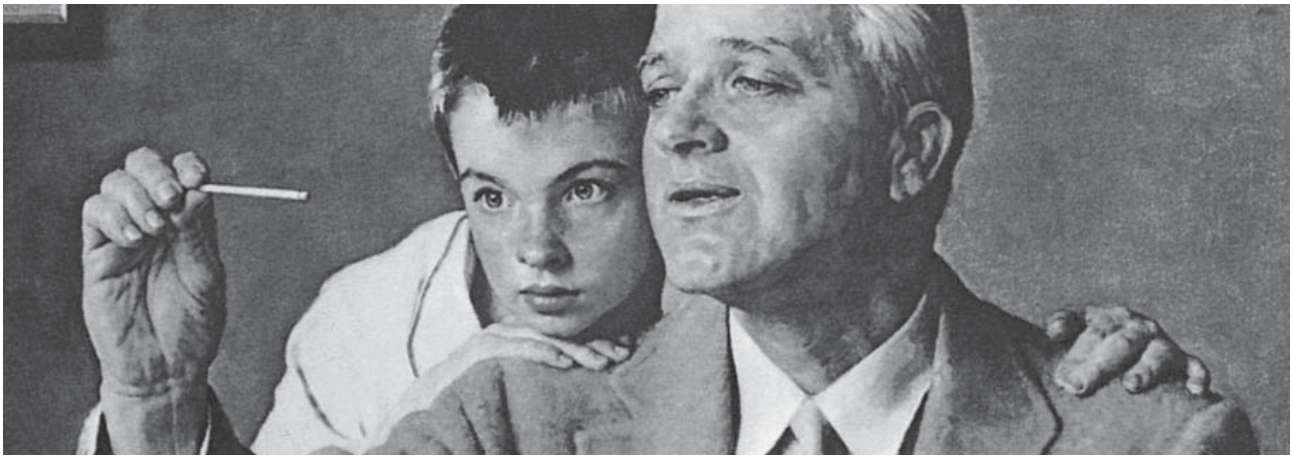

Norman Rockwell (1894–1978) Doctor and Boy Looking at Thermometer (1954) Display advertisement for the Upjohn Company. Oil on canvas (101.6 cm × 93.98 cm). Collection of Pfizer Inc. Licensed by Norman Rockwell Licensing, Niles, IL

"The Rainbow at the Edge of the Shadow of the Egg"

—John Updike

Polyxeni Potter*

"Rockwell is terrific. It's become too tedious to pretend he isn't," said art critic Peter Schjeldahl in 1999, summing up recent opinion about this well-loved American artist, whose work was dismissed by the art establishment during his lifetime (*1*). "Without thinking too much about it in specific terms," Rockwell said of himself, "I was showing the America I knew and observed to others who might not have noticed.... I guess I am a story teller" (*2*).

While hard at work as illustrator for The Saturday Evening Post, Encyclopaedia Britannica, Maxwell House Coffee, Parker Pens, Coca-Cola, Heritage Press, motion picture companies, and the U.S. government, Rockwell was keenly aware of the revolution brewing in the art scene of his day. He thought Picasso was "the greatest," and in The Connoisseur, painted when he was 68 years old, he showed a middle-aged man clad in a gray suit, fedora and umbrella in hand, pondering a credible representation of a Jackson Pollock canvas.

"I was born on February 3, 1894, in the back bedroom of a shabby brownstone front on 103rd Street and Amsterdam Avenue in New York City," wrote Rockwell in his autobiography. "I think I've always wanted to be an artist…. I drew, then I found I liked to draw, and finally, after I had got to know something about myself and the people and things around me, I found that I didn't want to do anything else but draw" (*3*).

He studied at the Chase Art School and then went on to the National Academy of Design and the Art Students League, where he worked under Thomas Fogarty, George Bridgman, and Frank Vincent Dumond. "In those days there wasn't the cleavage between fine arts and illustration that there is today," Rockwell wrote. "It was the end of what you might call the golden age of illustration." Frederick Remington had just died, and photographs were crowding the magazine and book publishing scene. Perhaps people had stopped demanding fine illustration, he thought; "You can't conduct a golden age with a heart of copper" (*3*).

Rockwell was scorned for becoming an illustrator, even by his fellow students. "You know, if I worked as hard as you do, I could be as good as Velásquez," one of them would joke. "Why don't you?" Rockwell would respond and return to some vexing problem at hand, how to draw an eye for instance so it did not look "like a fried egg."

"I can't say who has influenced me really. Or at least I can't say *how* the artists I have admired have influenced me…. Ever since I can remember, Rembrandt has been my

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favorite artist. Vermeer, Breughel, Velásquez, Canaletto; Dürer, Holbein, Ingres as draftsmen; Matisse, Klee—these are a few of the others I admire now. During my student days I studied closely the works of Edwin Austin Abbey, J.C. and Frank Leyendecker, Howard Pyle, Sargent, Whistler" (*3*).

"I had a secret ambition: a cover on the Saturday Evening Post. In those days the cover of the Post was the greatest show window in America for an illustrator…. 'Must be two million people look at that cover.'" After the Post, offers came from other magazines: Life, Judge, The People's Popular Monthly. "One of the most difficult problems in painting magazine covers is thinking up ideas which a majority of the readers will understand…. And it's darned hard to be universal, to find some situation which will strike the farmer, the housewife, the gossip…" (*3*).

During travels to Europe, especially Paris, Rockwell studied modern art and frequented the cafés in Montmartre, absorbing the bohemian scene and its preoccupation with one style over another, now cubism, now color. But it was no use. "I realized that I just didn't see things as the modern artists did." Throughout his career, he had periods of self-doubt and inactivity, but he always managed to "rise from the ashes." As his popularity increased, he became weary of the conventions and restrictions of commercial art so tied to its need to be understood and appreciated by the masses. "I don't do illustration any more. To tell the truth, I've priced myself out of the market. And then, frankly, I'm not too interested. I like to paint my own ideas, tell my own story" (*3*).

He resented being called old-fashioned. "I do ordinary people in everyday situations, and that's about all I can do…. But, as I say, every so often I get to hankering after immortality and, or so I think at those times anyway, that requires a picture tremendously conceived and tremendously executed." Along these lines, he painted big ideas: Freedom of Worship, Freedom of Speech, The Problem We All Live With.

"He went beyond the requirements," argued author, art critic, and fellow chronicler of American life John Updike. "He could have painted with less loving detail. He could have had fewer little anecdotal touches and facial expressions in his work. But he went always to fill the glass to the brim" (*4*). Updike came to understand the power of a detail noticed, when at age 12, he took art lessons. The teacher put an egg in the sun on a piece of white paper and asked him to draw it, then pointed out the detail missed: "the rainbow at the edge of the shadow of the egg" (*5*).

Doctor and Boy Looking at Thermometer, on this month's cover, shows how Rockwell used detail to capture authenticity. A fold in the skin, the angle of the neck, a crease in the clothes could deliver character. The doctor on the edge of the bed is a prototype. The patient, febrile face animated with curiosity, swollen eye glaring at the thermometer, trusts him. Bemused, the doctor holds up the instrument. The scene is homespun: the quilt, clean sheets, and picture on the wall are balanced by the doctor's bag and the friendliest suit and tie. In this home theater, the medical emergency is under control.

Modern art was not the only revolution in Rockwell's time. Much more was changing. The thermometer, in development since antiquity, became a practical tool in 1866, when an instrument that could read body temperature in 5 minutes was available for use. Rockwell's doctor was likely just as fascinated by it as his young patient. "See," he was probably saying, "If you hold it just right, you can see the mercury in there."

The mercury-and-glass thermometer has been replaced by less invasive, more refined versions. Now the race is on for a new, faster instrument to use in the field or hospital for mass screening. Cutaneous infrared thermometry is one such effort (*6*). The elements may all be there, but some fine critical detail is still missing.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Underreported Threat of Multidrug-Resistant Tuberculosis in Africa

Prevention and Control of Emerging and Reemerging Transmissible Diseases among the Homeless

Regulatory Systems for Prevention and Control of Rabies, Japan

Questions on Mediterranean Spotted Fever a Century after Its Discovery

Pediatric Parapneumonic Empyema, Spain

Transmissible Spongiform Encephalopathy Infectivity in Urine

Circulation of 3 Lineages of a Novel Saffold Cardiovirus in Humans

Forest Fragmentation as Cause of Bacterial Transmission among Nonhuman Primates, Humans, and Livestock, Uganda

Pigs as Source of MRSA CC398 Infections in Humans, Denmark

Mycobacterium haemophilum and Lymphadenitis in Immunocompetent Children, Israel

Obligations to Report Outbreaks of Food-borne Diseases under the International Health Regulations (2005)

Identification and Clinical Relevance of *Mycobacterium chimaera* sp. nov.

Bluetongue Virus Serotype 8 Reemergence in Germany, 2007–2008

Monkey Malaria in a European Traveler Returning from Malaysia

Highly Pathogenic Avian Influenza Virus (H5N1) Isolated from Whooper Swans, Japan

Uquitiba-like Hantavirus from 2 Unrelated Rodent Species, Uruguay

Genetic Variant of *Chlamydia trachomatis*, Sweden

Crack Cocaine Use and Infectious Tuberculosis

Ecoepidemiology of Cutaneous Leishmaniasis Outbreak, Israel

Human Case of Swine Influenza A (H1N1) Triple Reassortant Virus Infection, Wisconsin

Neurologic Disease Caused by *Brucella ceti* Infection in Stranded Dolphins, Costa Rica

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 5–15, 2008
IUMS 2008
Meetings of the Three Divisions of the International Union of Microbiological Societies
Istanbul, Turkey
http://www.iums2008.org/

October 30–November 1, 2008
Ninth International Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases Meeting (MEEGID IX)
University of California at Irvine
Irvine, CA, USA
http://www.th.ird.fr/site_meegid/menu.htm

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

The Public Health Image Library (PHIL)

The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health-related images, including high-resolution (print quality) photographs, illustrations, and videos.

PHIL collections illustrate current events and articles, supply visual content for health promotion brochures, document the effects of disease, and enhance instructional media.

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Systematic Literature Review of Role of Noroviruses in Sporadic Gastroenteritis

CME Questions

1. Norovirus (NoV) has been documented to cause what percentage of all-cause epidemic gastroenteritis worldwide?

A. 30%
B. 50%
C. 75%
D. 90%

2. Among 13 studies of community- or clinic-based mild-to-moderate diarrhea cases, what was the most likely pooled proportion attributed to NoV infection?

A. 5%
B. 12%
C. 40%
D. 65%

3. For severe diarrhea seen in emergency departments and/or resulting in hospitalizations, which of the following best describes the pooled proportion of cases attributable to NoV infection as reported in 23 studies?

A. 2%
B. 11%
C. 23%
D. 35%

4. Which of the following is considered the most common strain of NoV causing diarrhea among NoV cases?

A. GII.4 cluster
B. GII.1 cluster
C. GI.1 cluster
D. GIII.2 cluster

5. The worldwide morbidity and mortality from NoV among children in developing countries is best described by which of the following estimations?

A. 5 million hospitalizations
B. 400,000 deaths
C. 216,000 deaths
D. 2.5 million hospitalizations

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

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.

What are the goals of Emerging Infectious Diseases?

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.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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July 2008

Central Nervous System

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).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.