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June 2008

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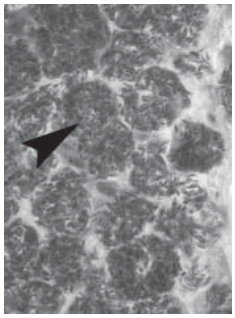

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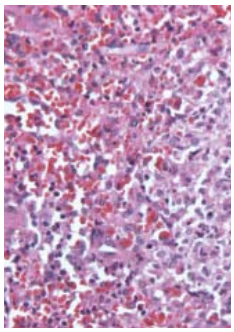

Books & Media

News & Notes

Major Issues and Challenges of Influenza Pandemic Preparedness in Developing Countries

Hitoshi Oshitani,* Taro Kamigaki,* and Akira Suzuki*

Better preparedness for an influenza pandemic mitigates its impact. Many countries have started developing and implementing national influenza pandemic preparedness plans. However, the level of preparedness varies among countries. Developing countries encounter unique and difficult issues and challenges in preparing for a pandemic. Deaths attributable to an influenza pandemic could be substantially higher in developing countries than in industrialized countries. Pharmaceutical interventions such as vaccines and antiviral agents are less likely to be available in developing countries. The public health and clinical infrastructure of developing countries are often inadequate to deal with a widespread health crisis such as an influenza pandemic. Such an event will inevitably have a global effect. Therefore, improving pandemic preparedness in every country, particularly developing ones, is urgently needed.

Avian influenza, caused by influenza A virus (H5N1), continues to cause outbreaks among poultry and wild birds worldwide. It has spread from Asia to other regions, including Europe, the Middle East, and Africa. The number of cases of human subtype H5N1 infection also continues to rise. These historically unprecedented outbreaks have raised serious global concerns about the imminent arrival of an influenza pandemic. The World Health Organization (WHO) urges countries to develop and implement national pandemic preparedness plans to mitigate the health and social effects of a pandemic (*1*). However, the level of preparedness varies among countries. In general, developing countries have limited financial and technical resources to strengthen pandemic preparedness. They also face some unique and difficult issues, which make preparing for a pandemic more challenging. These have not been addressed adequately during planning. Effective and feasible strategies are needed to mitigate the impact of the next influenza pandemic in developing countries.

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Major Issues

Potential Impact of Next Influenza Pandemic in Developing Countries

When an influenza pandemic emerges, all countries worldwide will inevitably be affected. However, the impact may vary both between and within countries. The estimated deaths for various countries during the Spanish flu pandemic from 1918 to 1920 shows that mortality rates in Europe and North America were significantly lower than those in Asia, Sub-Saharan Africa, and Latin America (*2*,*3*). A recent study that estimated the global impact of the Spanish flu pandemic indicated that a considerable difference in mortality rates was observed between high- and low-income countries (*4*). Why the pandemic caused such high mortality rates in developing countries is not entirely clear. Several factors may have been involved, including lack of access to adequate medical care, weak public health infrastructures, social factors such as housing conditions and population density, and host factors such as nutritional status and co-existing medical conditions. Another potential factor likely to influence mortality in a future pandemic is the high HIV/AIDS prevalence in some developing countries. Excess deaths attributed to pneumonia or influenza are significantly higher in HIV-positive persons during influenza seasons (*5*). HIV co-infection with a pandemic virus can be associated with more severe infections, which may further raise death rates in countries with high HIV/AIDS prevalence.

For these reasons, deaths associated with a future pandemic may be greater in developing countries than in industrialized countries. One study concluded that 96% of

the estimated 62 million deaths in a future pandemic would occur in developing countries (*4*). The impact of such high mortality rates obviously needs to be taken into account when creating pandemic preparedness plans for developing countries. However, no appropriate model that can estimate the impact of an influenza pandemic in developing countries exists. Models are based on data from industrialized countries (*6*), which may underestimate the actual impact of a pandemic in developing countries.

Availability of Vaccines and Antiviral Agents in Developing Countries

Several possible interventions can be implemented to control or mitigate the effects of an influenza pandemic, which include pharmaceutical interventions such as vaccines and antiviral agents, and nonpharmaceutical interventions such as quarantine, isolation, social distancing, and personal hygiene (*7*). Pharmaceutical interventions are needed for mitigating the impact of an influenza pandemic (*8*). Vaccines for subtype H5N1 viruses are currently being developed, and clinical trials are under way (*9*,*10*). However, worldwide vaccine production capacity is limited and is primarily in industrialized countries, where most seasonal influenza vaccine is produced (*11*). A recent WHO report estimated that the worldwide vaccine production capacity for current influenza vaccines is 350 million doses per year (*12*). That level of production is clearly insufficient to supply vaccines to all countries. Only a limited number of vaccine doses would be available, particularly in the early stages of the pandemic, and most of them would likely be supplied to industrialized countries. Many countries, especially developing countries, will be forced to confront the next pandemic with few or no available vaccines.

Antiviral agents are also considered effective for an influenza pandemic. They are particularly useful in the early stages of a pandemic when there is a shortage of vaccines (*13*). Two groups of antiviral agents for influenza are currently available, including M2 ion-channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). Neuraminidase inhibitors are preferred because some influenza viruses show high frequencies of resistance to M2 ion-channel inhibitors (*14*). Stockpiling of neuraminidase inhibitors is under way in many industrialized countries as part of national influenza pandemic preparedness (*15*). However, the stockpiles of antiviral agents available in developing countries are small and limited. WHO has global and regional stockpiles of antiviral agents, which are limited and are specifically used for early response and containment. The stockpile of antiviral agents is insufficient for a global pandemic.

The most critical limiting factor for stockpiling of neuraminidase inhibitors in developing countries is their high cost. One treatment course of oseltamivir (i.e., 10 tablets) costs US $15, even at a discount rate (*16*), which is far too expensive for developing countries. Some industrialized countries have set a target to stockpile oseltamivir to treat 25% of the general population. To purchase adequate oseltamivir for 25% of the total population, only 0.11% of the total annual health expenditure is required in high-income countries. In low-income countries, however, the expense would be 12.9% of the annual expenditure (Table 1). Therefore, it is not feasible for low-income countries to allocate scarce resources to stockpile sufficient quantities of oseltamivir for an unpredictable influenza pandemic.

Limitations of Pharmaceutical Interventions

The recent efforts to increase global availability of vaccines and antiviral agents can contribute to increasing the global availability of these pharmaceutical interventions. However, increased availability alone will not solve all the problems in many countries. Several other issues need to be addressed to implement pharmaceutical interventions. These pharmaceutical commodities, including syringes and needles for vaccines, should be delivered to healthcare facilities throughout the country. That is a difficult logistic challenge for many developing countries. Human resources are also required to implement these interventions. Yet, there are some uncertainties about the effectiveness of these pharmaceutical interventions. Even neuraminidase inhibitors may not be fully effective for a pandemic virus, whose pathogenesis in human hosts differs from that of seasonal influenza viruses. Another potential problem with the antiviral drugs is the risk that resistant strains will emerge. Vaccines may not be effective because of antigenic differences between a vaccine strain and a pandemic virus, or for other reasons. Full-scale implementation of pharmaceutical interventions that requires enormous financial and hu-

Table 1. Cost of purchasing oseltamivir to cover 25% of population with regard to total health expenditure in countries with different economic status*

Category of country	Average GNP, per capita†	Average annual health expenditure, per capita†	Cost of 1 treatment course of oseltamivir, % annual health expenditure
High income	30,168	3,376	0.11
Upper middle income	4,310	280	1.34
Lower middle income	1,364	77	4.87
Low income	753	29	12.93

*Data obtained from World Health Organization website (www.who.int/nha). GNP, gross national product.
†In US$.

man resources may not be the best use of limited resources in developing countries. The governments, international organizations such as WHO, and donors should consider various factors when providing support for pharmaceutical interventions in developing countries. Maintaining a balance between pharmaceutical and nonpharmaceutical interventions is necessary to achieve the best use of limited resources.

Lack of Medical and Public Health Infrastructure to Cope with an Influenza Pandemic

During an influenza pandemic, morbidity and mortality may be extremely high. Healthcare facilities would be quickly overwhelmed with increased numbers of patients. In the United States alone, an estimated 18–42 million outpatient visits and 314,000–734,000 hospitalizations could occur (*6*). The surge capacity in healthcare systems will likely be insufficient to cope with this rise in patient numbers, even in industrialized countries (*17*,*18*). Healthcare resources such as the number of physicians, nurses, and available hospital beds are limited in developing countries. In some countries, resources are insufficient to cope with patients even during normal circumstances. Hospitals and clinics in developing countries will be easily overwhelmed by the increasing number of patients during an influenza pandemic.

Using the method described by Wilson et al. (*19*), we estimated the number of required hospital admissions for countries of varying economic status. The percentages of available hospital beds occupied by influenza patients at incidence rates of 15% and 35% were calculated by using FluSurge software, version 2.0 (*20*). Demographic data were obtained from the US Census Bureau website (www.census.gov/ipc/www/idb) and information related to the number of available beds was obtained from a WHO database (WHOSIS, www.who.int/whosis/en). Results are shown in Table 2. The percentage of hospital beds required for patients with pandemic influenza is much higher in low-income countries than in high-income countries. With an incidence rate of 35%, up to 79.1% of hospital beds are required for patients with pandemic influenza in low-income countries. In countries like Bangladesh and Nepal, >100% of beds would be required for patients with pandemic influenza, even at the incidence rate of 15% (data not shown). This model is based on data from the United States, and the difference in disease severity among the countries was not considered. This model may underestimate the hospital bed requirements in developing countries, where a pandemic virus may cause more severe infections. Some hospitalized patients will require mechanical ventilation (*17*), but few mechanical ventilators, if any, are available in many hospitals in developing countries.

During an influenza pandemic, additional essential medical supplies such as gloves, masks, syringes, antipyretics, and antimicrobial agents will also be required. These supplies are insufficient in healthcare facilities in developing countries, even in nonemergency situations. Lack of these supplies may hamper provision of adequate medical care for patients with pandemic influenza. Basic personal protective equipment such as disposable gloves and surgical masks are needed for protecting healthcare workers. Antimicrobial agents are expected to be effective for secondary bacterial pneumonia, which can be a major cause of death for patients with pandemic influenza (*21*). Therefore, proper treatment with antimicrobial agents can be crucial for preventing deaths. However, in some developing countries, sufficient stocks of essential drugs, including antimicrobial agents, are often unavailable.

In countries with limited healthcare resources, providing routine medical care for other conditions may become difficult during a pandemic. For example, the treatment for tuberculosis or the antiretroviral treatment for AIDS patients may not be provided because of disruption in healthcare systems. Maintaining other public health programs, such as vaccination, may also be difficult when most of public health resources are spent for the response to a pandemic.

Future Directions

Improving Planning Process

To minimize the impact of an influenza pandemic, good preparedness plans need to be developed. With the increasing risk for a pandemic caused by the spread of influenza A virus (H5N1), most countries have started such planning. These national plans were recently reviewed from different perspectives (*15*,*22*–*24*). The level of planning in many developing countries is still inadequate to

Table 2. Hospital bed requirements during an influenza pandemic in countries with different economic status*

Category of country	Mean no. hospital beds/1,000 population (range)	Mean no. hospital beds required, as % of available hospital beds (range)	
		15% Incidence rate	35% Incidence rate
High income (N = 38 per capita)	50.7 (21–196)	8.9 (2.2–15.5)	20.8 (5.2–35.7)
Upper middle income (N = 28 per capita)	45.1 (9–99)	10.6 (3.9–30.1)	24.8 (9.0–70.3)
Lower middle income (N = 46 per capita)	30.0 (5–112)	15.5 (2.4–50.0)	36.2 (5.7–116)
Low income (N = 19 per capita)	26.2 (1.5–132)	33.9 (2.5–164)	79.1 (5.9–383)

*Only those countries with data on hospital beds and that were included in the World Bank country classification were included in the analyses. African countries are not incorporated in the analyses because they have no hospital bed data.

deal with such a major public health crisis. Some plans are based on the available plans of industrialized countries, or follow similar approaches to those of industrialized countries. As described above, the approaches used by industrialized countries may not be feasible or appropriate for developing countries. In addition, each country has specific issues, and therefore it should develop a plan based on its own requirements. This task can be difficult for most developing countries because they have little or no expertise with influenza and pandemic preparedness. For the few infectious disease experts working on infectious diseases in each country, numerous competing priorities exist, such as HIV/AIDS, malaria, tuberculosis, and vaccine-preventable diseases. Feasible, user-friendly tools are needed to assist these countries. WHO has developed several such tools, including a checklist for national preparedness (*25*). However, these tools describe the general approaches to pandemic preparedness and are not specifically designed for countries with limited resources. For developing countries more practical tools are needed, among them models to estimate the impact of a pandemic in developing countries, a list of feasible interventions to mitigate the impact of pandemic without available pharmaceutical interventions, and planning guidelines for hospitals with limited resources.

Increasing Availability of Antiviral Agents and Vaccines

If the next pandemic occurs in a few years, vaccines and antiviral agents, particularly neuraminidase inhibitors, may not be available as a main intervention in developing countries. Availability needs to be increased to fill the gaps between developed and industrialized countries. WHO recommends an increase in worldwide vaccine production to meet the demand during a pandemic (*12*). Several countries have initiated projects to improve influenza vaccine production with technical and financial support from WHO and donors. However, improved vaccine production capacity is not sustainable if only used for pandemic influenza vaccines. The use of seasonal influenza vaccines would also need to increase in these countries. However, the cost of the vaccines (US $3–$7 per dose) is a barrier in increasing their use (*12*). There is also little available evidence on the effectiveness and cost benefits of seasonal influenza vaccines in tropical developing countries. Further efforts should be made to reduce the cost and to collect additional scientific data to increase the use of seasonal influenza vaccines.

Some approaches have been proposed and tested to reduce the amount of antigens per vaccine dose for pandemic vaccine so that more vaccines, including adjuvant and whole virion vaccines, can be supplied (*10*). The world is expected to have an increased capacity to produce vaccines for pandemic influenza viruses by 2010 (*12*). In some countries, the vaccines for the subtype with a pandemic potential are being produced and stockpiled as a prepandemic vaccine, which can be a useful tool to mitigate the impact of a pandemic (*26*). However, both pandemic and prepandemic vaccines would not be available in developing countries unless an international mechanism exists to share such vaccines with them at a low cost.

Some actions have also been taken to reduce the cost of neuraminidase inhibitors such as oseltamivir. It is being produced in sublicensing companies in developing countries to increase its supply at a lower cost. However, oseltamivir may still not be affordable for many developing countries. In industrialized countries, M2 ion-channel inhibitors are not considered a first choice of treatment because of the high rate of resistance to these inhibitors. However, amantadine is much cheaper than neuraminidase inhibitors and is more widely available. Most subtype H5N1 isolates that belong to clade 1 are resistant to amantadine, but many clade 2 viruses are still susceptible to amantadine (*27*). M2 ion-channel inhibitors can be a valid option for a pandemic, especially in developing countries (*28*). The value of M2 ion-channel inhibitors as a treatment option for an influenza pandemic should be evaluated further.

Providing Better Medical Care

The health consequences of a pandemic, including deaths, can be substantially reduced by providing better medical care. Several issues need to be addressed to provide adequate medical care during a pandemic. First, essential medical supplies such as masks, gloves, and antimicrobial agents should be available in hospitals and clinics. The stockpiles of these basic supplies can be more cost-effective in developing countries than the stockpiles of more expensive antiviral agents. Guidelines on the types and quantity of essential items that are required in hospitals and clinics should be developed. Second, healthcare personnel should be trained for infection control measures. Even surgical masks are not commonly used in many developing countries, and hand hygiene practices are not always followed. Basic training on infection control should be provided to improve pandemic preparedness in healthcare settings. Third, healthcare and public health systems need to be maintained to minimize the impact of a pandemic. These systems should be maintained to deal not only with a pandemic but also with other health problems such as malaria, tuberculosis, and HIV.

Developing Feasible Mitigation Strategies

More feasible and effective strategies should be developed as soon as possible to mitigate the negative impact of an influenza pandemic in developing countries. Since the availability of pharmaceutical interventions in developing countries is less likely, nonpharmaceutical interventions such as social distancing and personal hygiene may

be the only available interventions. Public health measures such as school closure and household quarantine have been evaluated by using mathematical models for their effectiveness in mitigating the impact of a pandemic (*29*,*30*) and may have potential beneficial effects. However, the models suggest that substantial benefits of these measures require implementation with antiviral prophylaxis or vaccines (*29*,*30*). The evidence for effectiveness of public health measures is limited and is based primarily on experience in industrialized countries (*31*,*32*). For example, handwashing and hand hygiene have been highly publicized as a core management strategy for avian and pandemic influenza in developing countries (*33*). Although handwashing is effective in reducing the incidence of common diseases such as acute respiratory infections (*34*), data on its effectiveness specifically for community-acquired influenza infections are limited (*31*). Recommendations on nonpharmaceutical interventions have been based on available evidence (*35*). Accumulation of further scientific evidence for these measures, which can be implemented at a low cost, is urgently required.

Strengthening Core Capacities

Many health programs in developing countries depend on financial support from donors. Influenza had little donor interest before the current avian influenza outbreaks. More donor funds are available for avian and pandemic influenza. These funds are often earmarked for specific activities. However, a more general approach is required to improve pandemic preparedness in developing countries. Improving pandemic preparedness without establishing a proper national program for seasonal influenza is unrealistic. For example, increasing the availability of pandemic vaccines without increasing the use of vaccines for seasonal influenza is difficult. It is also difficult to implement infection control measures in hospitals and personal hygiene during a pandemic if they are not routinely implemented for seasonal influenza and other infections.

Lack of adequate infrastructure and technical expertise is a fundamental issue for developing countries, not only for influenza pandemic preparedness but also for any other infectious disease threats. Revised International Health Regulations (2005) were adopted at the World Health Assembly in 2005, under which each country is required to have core capacities for disease surveillance and response (*36*). Strengthening the core capacity in each country should be an essential step to improve preparedness for any public health emergency, including an influenza pandemic. Although some actions should be taken immediately to address urgent issues regarding a pandemic threat posed by influenza A (H5N1), a long-term vision is required to establish such core capacity in every country.

Strengthening International Collaboration

An influenza pandemic will spread to every corner of the world; hence, every country must be prepared for such a global event. All human cases of infection with influenza A virus (H5N1) have so far occurred in less industrialized countries, and thus the pandemic virus is likely to emerge from these countries. Epidemiologic models have indicated the possibility of rapid containment of the virus with a pandemic potential (*37*,*38*). WHO has stockpiles of oseltamivir specifically for the early containment of a potential pandemic. However, the window of opportunity is narrow, and early containment operations should be initiated as soon as the initial sign of a potential pandemic is detected. Timely sharing of the virus strains and relevant information is essential for such containment to be successful.

Sharing of the virus stains is also critical to develop pandemic vaccines. However, some countries do not share the virus strains with WHO reference laboratories. These countries argue that the virus strains from their countries would be used to develop pandemic vaccines that would only be available for rich countries (*39*). Developing countries have no incentives to share the virus strains if they do not benefit from the vaccines developed from these strains. The gaps in resources, including vaccine production capacity between the developing and industrialized countries, hinder the global effort to respond to a pandemic. Unequal distribution of resources, including antiviral stockpiles, could also be a major international issue when an influenza pandemic occurs. Countries with limited or no antiviral stockpiles and other resources may not be able to cope with the pandemic. A pandemic poses a serious threat to global health security if large gaps in capacity and available resources continue to persist. Large numbers of people may attempt to cross international borders to obtain better medical care, including antiviral treatment, or to escape a chaotic situation. Preparing for a pandemic by simply strengthening preparedness within a single country is not possible. A pandemic is a global issue, and pandemic preparedness should be considered from a global perspective.

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Managing Potential Laboratory Exposure to Ebola Virus by Using a Patient Biocontainment Care Unit[1]

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In 2004, a scientist from the US Army Medical Research Institute of Infectious Diseases (USAMRIID) was potentially exposed to a mouse-adapted variant of the Zaire species of Ebola virus. The circumstances surrounding the case are presented, in addition to an update on historical admissions to the medical containment suite at USAMRIID. Research facilities contemplating work with pathogens requiring Biosafety Level 4 laboratory precautions should be mindful of the occupational health issues highlighted in this article.

Recent interest and increased investment in biodefense research have resulted in construction of new research laboratories with Biosafety Level 4 (BSL-4) capability (*1*). In addition to ensuring biosafety, due consideration should be given to managing medical, public health, and public relations issues related to occupational exposures to highly hazardous infectious pathogens.

We present a potential exposure to Ebola virus that occurred in a BSL-4 laboratory at the US Army Medical Research Institute of Infectious Diseases (USAMRIID). Background and prior use of the medical containment suite (MCS) are reviewed briefly, followed by discussion of pertinent issues related to the event and recommendations for response.

Case Report

In 2004, a virologist at USAMRIID was working in a BSL-4 laboratory with mice that had been infected 2 days before with a mouse-adapted variant of the Zaire species of Ebola virus (ZEBOV) (*2*). The virulence and infectious dose of this variant of ZEBOV are unknown in humans; wild-type virus has a case-fatality rate of up to 90% (*3*).

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The person had been following standard procedure, holding the mice while injecting them intraperitoneally with an immune globulin preparation. While the person was injecting the fifth mouse with a hypodermic syringe that had been used on previous mice, the animal kicked the syringe, causing the needle to pierce the person's left-hand gloves, resulting in a small laceration. The virologist immediately squeezed the site to force the extravasation of blood. After decontamination of the blue suit in the chemical shower, the injured site was irrigated with 1 liter of sterile water and then scrubbed with povidone-iodine for 10 minutes.

In terms of exposure risk, the needle was presumed to be contaminated with virus-laden blood, although it was suspected that low levels of virus were present on the needle. The animals had not yet manifested signs of infection, and much contamination may have been removed mechanically when the needle pierced the gloves. The local decontamination of the site also reduced potential for infection.

USAMRIID medical, scientific, and executive staff concluded that the person with potential exposure warranted quarantine in the MCS. Contact plus airborne precautions (gown, gloves, N95 mask, eye protection) were used, with a plan to upgrade to BSL-4 precautions for signs or symptoms of illness. These extra precautions were instituted while the patient was asymptomatic for several reasons: 1) the timing of initial clinical manifestations with regard to potential for shedding virus were not known for this specific isolate in human infection; 2) there was interest in ensuring all infection control procedures were being followed appropriately in advance of clinical illness; and

[1]This article is derived from the lecture "10 Steps in Managing an Ebola Exposure," presented at the Interscience Conference on Antimicrobial Agents and Chemotherapy, December 15, 2005, Washington, DC, USA, and September 26, 2006, San Francisco, CA, USA.

3) there was interest in reducing any potential confounders, such as a caregiver transmitting a febrile respiratory infection to the patient, which might lead to unnecessary procedures or additional isolation. The person was monitored for routine vital signs; daily laboratory studies (coagulation studies, blood counts, chemistries, viral isolation, D-dimer) and regular physician assessments were performed.

Over the next several days, discussions were held with several internationally recognized filovirus experts regarding potential treatments or postexposure prophylaxis options. Local and state public health officials were also notified. The consensus opinion was that there was no safe, readily available source of immune plasma and little evidence existed to support its use. Emergency investigational new drug (IND) protocols were established for treatment with recombinant nematode protein (rNAPc2) and antisense oligomers, with the intention to consider implementation only if the patient demonstrated evidence of infection.

Ultimately, none of the 5 mice had confirmed viremia at the time of the incident. The patient did not become ill or seroconvert and was discharged after 21 days. The story received national and local media attention (*4*,*5*).

The MCS ("Slammer")

In planning for USAMRIID (established in 1969), the decision was made to include a maximum containment (now termed BSL-4) capability to care for 2 personnel who may have been exposed to a biologic agent. This MCS would also be available for managing occupational exposures at USAMRIID or other government agencies to diseases requiring containment (R. McKinney, pers. comm.).

The 1,288-square-foot MCS includes 2 patient rooms and a treatment room and is equipped for intensive care monitoring, ventilator use, and teleradiology capability. It has an independent ventilation system and a chemical shower for decontaminating caregivers' encapsulating suits (identical to those worn in the BSL-4 laboratories), and it is isolated from adjacent areas by doors fitted with airtight gaskets. This latter feature earned the facility the moniker "the slammer," which was popularized in the book The Hot Zone (*6*).

The MCS is staffed by USAMRIID personnel, with augmentation and specialized care provided by nearby medical center staff, who serve on a medical augmentation team (*7*). Passage of consumables and supplies in and out of the suite occurs through 3 conduits: a double-door autoclave, an ultraviolet light passbox, and a disinfectant dunk tank, which enables decontamination and transport of specimens to external areas within the facility for laboratory analysis. The MCS is maintained under negative pressure. Air undergoes HEPA filtration upon entry to and exit from the facility. The septic system links into USAMRIID's laboratory sewer system, which undergoes steam sterilization.

Prior MCS Admissions

Twenty-one patients have been considered candidates for admission to the MCS (Table 1) (*7*,*8*). Eighteen were USAMRIID investigators and 3 were from elsewhere. Four patients (3, 6, 7, and 18) would likely not be admitted today: 2 involved dengue virus (now a BSL-2 pathogen) and 1 each involved Japanese encephalitis B virus and Rift Valley fever virus (BSL-3 pathogens with licensed and investigational vaccines, respectively) (*1*).

Three patients (6, 7, and 15) were managed in an improvised manner. The MCS was unavailable for 1 patient because the unit was undergoing maintenance. Thus, the observation was conducted in another set of rooms without high-level containment features. Two other patients (16 and 19) were deemed low risk; consequently, isolation was permitted under more conventional conditions.

Of the remaining 14 admissions after potential exposure to BSL-4 viruses, 8 involved percutaneous injury and 6 involved potential aerosol exposure. Eight persons (5 evaluated for exposure to Lassa virus, 2 for Machupo virus, and 1 for Junin virus) received immune plasma (prepared from recovered patients in virus-endemic areas); 1 patient potentially exposed to Lassa virus also received intravenous ribavirin. No patient developed disease or seroconverted. From 1985 through 2003, no potential exposure in a USAMRIID BSL-4 laboratory was deemed a high enough risk to require quarantine.

Discussion

Laboratories remain a potential venue for exposures to BSL-4 viruses (*1*). Filoviruses, in particular, have been associated with laboratory-acquired infection, being first identified after exposure to African green monkeys in Marburg, Germany, in 1967 (*9*). Since then, laboratory-acquired Ebola virus infections have occurred in England (*10*) and Côte d'Ivoire (*11*). The death of a Russian researcher in 2004 (*12*) from laboratory-acquired Ebola virus infection and USAMRIID's recent experience demonstrate the seriousness of this issue.

Although USAMRIID periodically manages potential laboratory exposures (*13*–*16*), potential exposures in BSL-4 laboratories are rare. Nonetheless, as more facilities conduct research on viruses requiring BSL-4 containment, cases such as the one presented herein may become more commonplace.

The decision to place someone in quarantine is a difficult one. When this potential exposure occurred, several alternatives were considered: sending the patient home with periodic home or clinic assessments, admission to a medical center, and admission to the MCS. Some less serious potential exposures had been managed with twice-a-day vital sign assessments and exclusion from the laboratory. The current situation appeared to present a much higher risk to the person

Table 1. Admissions into the medical containment suite at the US Army Medical Research Institute of Infectious Diseases, 1972–2004*

Patient no.	Date of admission	Days in isolation	Virus†	Reason for admission	Therapy‡	Comments§
1	1972 Oct	18	Machupo	Cut finger	IP	
2	1975 Oct	42	Machupo	Cut finger	IP, IG	
3	1976 Oct	21	JEB	Fingerstick		
4	1977 Sep	14	Machupo	Vial leak		
5	1977 Sep	14	Machupo	Vial leak		
6	1978 May	11	Dengue	Not specified		Modified CC
7	1978 May	8	Dengue	Not specified		Modified CC
8	1978 Jun	17	Lassa	Dropped vial	LIG	
9	1978 Jun	17	Lassa	Dropped vial	LIG	
10	1978 Jul	8	Lassa	Field exposure		
11	1978 Nov	14	Lassa	Suit seam failed		
12	1979 May	20	Lassa	Fingerstick	IP	
13¶	1979 Jul	21	Lassa	Fingerstick	IP	
14	1979 Nov	20	Lassa	Fingerstick	IP, Rib	
15	1981 May	14	Ebola/Lassa	Field exposure		Modified CC
16	1982 Oct	14	Junin	Defective suit seal		Conventional
17	1982 Dec	21	Junin	Fingerstick	IP	
18	1983 Jan	3	Rift Valley fever	Waste exposure		
19	1983 Apr	14	Junin	Defective suit seal		Conventional
20	1985 May	4	Junin	Fingerstick		
21	2004 Feb	21	Ebola	Fingerstick		

*Modified from Cieslak et al. (*8*) with permission.
†JEB, Japanese encephalitis virus B; Ebola/Lassa, potential exposure to these viruses.
‡IP, immune plasma from previously infected survivors; IG, immune globulin; LIG, Lassa immune globulin; Rib, ribavirin.
§CC, containment care; modified CC, provided by converting a separate physical facility into a Biosafety Level 4–like suite; conventional, Biosafety Level 3 isolation was permitted for 2 lower risk exposures.
¶Not noted in previous reports (*7,8*).

than past potential exposures: the patient had a break in the skin caused by a potentially contaminated needle, a route of infection known to transmit efficiently and associated with enhanced risk for death (*17*). Therefore, the most reasonable approach was determined to be quarantine in the MCS, thus enabling closer monitoring than could be provided at home and ensuring the safety of caregivers and family members.

Quarantine in a hospital was considered. This option presents certain safety challenges in an unprepared facility, including safe handling, transport, and analysis of laboratory specimens within the hospital; safe disposal of waste; potential reluctance of hospital staff (unfamiliar with viral hemorrhagic fevers) to care for such an infected person; and lack of a specific area within the hospital configured for handling of this type of patient.

There are advantages and disadvantages to using the MCS as a stand-alone medical facility. It enables close monitoring separate from other patients (thus eliminating risk of nosocomial spread and cross-contamination); its personnel are already trained in managing a patient in containment; public access is limited; a proven system is in place for waste disposal; and an on-site containment laboratory (with the ability to culture virus or perform sophisticated diagnostic testing under containment conditions) reduces risk for infection of clinical laboratory personnel and contamination of laboratory equipment. Disadvantages of a stand-alone facility include lack of ready access to consultative physicians; critical care nursing; radiologic and other imaging studies; blood products, medications, and resuscitative procedures; and other services available at a large medical center. Activities undertaken to compensate for these deficiencies include staffing the MCS with intensive care and infectious disease physicians and having other consultants available, as needed. Moreover, ventilator and dialysis machines and blood product and laboratory support can be kept on stand-by status within or near the facility. However, it is easier to have an isolation unit located with or within a major medical center, as has been conducted elsewhere (*18*).

One might question whether a facility such as the MCS is the most appropriate place to isolate an infected patient. The US Centers for Disease Control and Prevention (CDC) advises that patients with viral hemorrhagic fevers can be managed safely in a conventional hospital with, at the most, airborne and contact precautions (*19–21*). Those patients with potential exposures (close or high-risk contacts of infected persons) who are not ill should be placed under surveillance with twice-a-day temperature checks, and a period of observation is appropriate (*19*). However, setting(s) for this observation period were not specified. Home observation works well for potential exposures deemed low risk; however, waiting for a patient at higher risk for highly hazardous or contagious diseases to manifest fever at home is not ideal.

The MCS was constructed with the premise that certain rare situations might call for extraordinary precautions to isolate victims of severe contagious diseases. These precautions reduce risk for a virus such as Ebola being introduced into the community by minimizing risk for nosocomial spread and optimizing known effective infection control practices. Although these precautions are useful for filoviruses, they may be more useful for other viral hemorrhagic fevers that are transmitted more readily by the aerosol route or are potentially adaptable to local animal reservoirs. The facility may provide some reassurance to the community (and thus serve to lessen public anxiety related to a filovirus exposure) and to laboratory researchers that there is a place for their care if they become infected. A patient with a filovirus infection in an unprepared medical facility would be handled as safely as possible, using CDC guidelines (if the disease were recognized). It is acknowledged that BSL-4–like infection control precautions may not be necessary for observation or illness. However, most clinical experience managing filovirus infections is from sub-Saharan Africa, where increased temperature and humidity may reduce stability of viruses in aerosol (*22*). Contrast that environment with a US hospital where air is cool, dry, and recycled within the facility, a setting potentially more conducive to airborne virus spread.

A laboratory-acquired case of Sabia virus provides an example of how a patient infected with a BSL-4 agent might be managed safely in a community hospital but also provides cause for caution and heightened vigilance, especially among facilities that might receive patients referred from containment laboratories (*23*). The Sabia virus–infected scientist did not report the initial potential exposure and only came to the attention of healthcare providers after 5 days of illness. He was evaluated initially at a tropical medicine clinic and was subsequently referred to an emergency department. Although the department was notified that the arriving patient might have been infected with an arenavirus, there was a 12-hour delay before heightened infection control measures (specific for managing a viral hemorrhagic fever patient) were instituted. A total of 142 persons were identified as potential case-contacts, including 61 workers in the hospital clinical laboratory. Although no secondary cases occurred, potential risk and anxiety of contacts, as well as costs of an investigation by 3 agencies (CDC, the Connecticut Department of Public Health, and Yale University) and a 6-week period of surveillance, argue in favor of 1) an aggressive program of reporting and evaluating any mishap or potential exposure occurring in a containment laboratory; 2) use of facilities familiar with and prepared in advance, when possible, for managing a similar patient; and 3) a preestablished method for surveillance and site for potential quarantine of high-risk exposures.

USAMRIID is not unique in foreseeing the need for a special isolation unit. Emory University and the University of Nebraska maintain special isolation wards for patients with potentially contagious, highly hazardous diseases, and recommendations on design and planning for biocontainment patient care units have recently been published (*18*).

In our recent case, the patient voluntarily entered the MCS. Had the patient refused to be quarantined, decision and authority on forcible quarantine would have rested with local or state health departments. Although there is some variability in local and state regulations, authority to enforce isolation and quarantine derives from the states' power to "safeguard the health, safety, and welfare of its citizens" (*24*).

There are no approved treatments or postexposure prophylaxis regimens for filovirus infections. Use of passive immunotherapy was considered; however, no studies support a definite benefit, and no readily available safe source of such products exists. On the basis of limited data indicating improved survival in rhesus macaques challenged with ZEBOV and treated with rNAPc2, an emergency IND protocol was obtained for using rNAPc2 (*25*). Another emergency IND protocol was obtained for use of antisense oligonucleotides on the basis of demonstrated safety with these compounds for other indications (*26*). Use of either protocol was without proven safety or efficacy in Ebola virus–infected humans. Both products were available for therapeutic use had the patient developed infection with clinical manifestations that warranted aggressive treatment.

Subsequent studies have demonstrated promise for treatment of ZEBOV infections with antisense oligonucleotides and small interfering RNAs (*27*,*28*). An effective vaccine would reduce inherent hazards in working with these viruses. There have been some recent developments with virus-vectored vaccines with and without naked DNA vaccine priming (*29–31*). A phase I human study of such a vaccine is ongoing (*32*).

Management Considerations

Given increasing interest in construction of additional laboratories for study of BSL-4 agents, potential exists for clinicians to manage an occupational exposure to these viruses. Our experience led us to formulate a stepwise approach that might help others plan for and manage similar incidents.

Step 1: Prepare

Occupational health clinics associated with containment laboratories should develop methods of assessing need for isolation and laboratory decontamination, exit, and notification procedures. Maintaining a close relationship with the biosafety office, thereby knowing the agents

in use, will make planning appropriate treatments in advance easier.

It should be determined in advance where an asymptomatic patient might be observed and where to isolate and treat an infected patient. Separate locations may be required, but moving an ill patient may be challenging. Thus, memoranda of understanding must be established in advance that articulate each facility's role. One should also have 24-hour recall rosters of key personnel that are used occasionally.

Step 2: Assess the Patient

A primary physician should be designated to develop the treatment/isolation plan in consultation with other experts. New diseases or medications need to be queried at the time of exposure evaluation if employees did not previously notify occupational health officials. This information must be gathered in a nonpunitive environment so that reporting of potential exposures is not discouraged. Details of the exposure incident should be obtained from the patient, the patient's supervisor, department chief, and laboratory co-workers.

Risks for exposure and disease should be estimated with available information as reported (*13*,*14*). Care for family members, including children, the elderly, or pets, may need to be addressed, in addition to issues such as powers of attorney, advanced directives, last wills and testaments, and similar legal matters.

Step 3: Gather Appropriate Consultants and Team

Designating another person to coordinate other activities surrounding a high-profile exposure (arranging conferences with external experts, handling media inquiries, issuing press releases, and interacting with external agencies) frees the primary physician to care for the patient. For any clinically important exposures, especially in the absence of licensed therapeutics, it is appropriate to seek advice of consultants (Table 2). These persons may vary, depending on the organization, the pathogen in question, and individual expertise.

Local and state public health agencies will need to be part of discussions if there is potential public health impact; these organizations will likely be fielding queries from the public and the press simultaneously. Local hospitals should be informed if there is potential for transferring the patient to those facilities. The Food and Drug Administration should be informed if establishment of an emergency use IND is contemplated. Any laboratory that might test clinical samples should also be informed in advance of specimens arriving.

Step 4: Determine the Appropriate Level of Infection Control Measures

Although specialized containment care procedures and facilities may play a limited role in certain extraordinary cases, such as those discussed here, CDC has published guidance for management of viral hemorrhagic fevers in more conventional settings (*19*–*21*). Standard, contact, and droplet precautions and a private room are recommended in initial outpatient or inpatient assessments in early stages of illness, and a face mask should be placed on patients with respiratory symptoms. A room capable of airborne isolation should be considered early to prevent later need for transfer. Precautions should be upgraded to airborne isolation if a prominent cough, vomiting, diarrhea, or hemorrhage

Table 2. Consultants to consider for establishing a team to manage a potential laboratory exposure

Consultant no.	Title and description
1	Designated primary physician
2	Scientific expert: This person knows the latest medical/scientific literature on the organism.
3	Director of safety: This person will assess the mechanism of injury and how to avoid a repeated occurrence.
4	Research institute or laboratory director's representative: This person may serve as the liaison to external political, media, or scientific agencies and will need to be aware of the progress of the patient or any investigation to convey accurate information externally and internally.
5	Patient's supervisor or department chief: This person will need to reassess the specific laboratory methods used (in conjunction with safety) and modify procedures as needed.
6	Representative from regulatory affairs: This person may serve as a liaison to regulatory agencies such as the Food and Drug Administration, especially if establishing an emergency investigational new drug protocol is contemplated.
7	Public affairs representative: This person needs accurate information to hold press briefings or to generate press releases.
8	Occupational health representative: This person should work in conjunction with safety experts to analyze the mechanism of exposure and ways to prevent a recurrence.
9	Scribe: This person will keep track of the key contacts and decisions, as well as the different courses of action considered.
10	Patient: In many cases, the patient may be the most well-informed person on the specific pathogen. His or her level of expertise and interest will determine whether to include the patient in group discussions. If the patient is already in isolation, a family representative may be considered to participate in group discussions with the patient's approval. Health Insurance Portability and Accountability Act* privacy regulations still apply.

*Public Law 104-191 Health Insurance Portability and Accountability Act of 1996, August 21, 1996 [cited 2007 Aug 27]. Available from www.hhs.gov/ocr/hipaa

develop in a patient, or if the patient undergoes procedures that may stimulate coughing or generation of aerosols.

Step 5: Provide Additional Communications

Because filovirus exposure has a particular cachet and media interest may be intense, it is preferable to inform the media proactively. Public affairs personnel will need to develop press releases and arrange interviews in conjunction with a medical or scientific expert. Lessons can be learned from the negative publicity received after the tularemia exposures at Boston University (*33*,*34*) and the death of the Russian researcher from infection with Ebola virus (*12*) after a delay in disseminating that information.

Regular communication with the laboratory's workforce should be maintained. Medical care personnel also need regular updates on modifications of procedures and ongoing reemphasis of infection control practices.

Step 6: Conduct Appropriate Isolation Logistics

A patient in quarantine results in logistical challenges (providing food and equipment and decontaminating personal, medical, and food waste) even before illness develops. CDC provides recommendations for specimen handling of viral hemorrhagic fever patients (*19–21*) that include 1) minimizing laboratory procedures, 2) alerting the laboratory of the nature of the specimens, 3) transporting specimens in decontaminated leak-proof plastic containers, 4) processing laboratory specimens in a class II biologic safety cabinet with BSL-3 practices, and 5) performing virus isolation or culture in a BSL-4 laboratory. If possible, CDC recommends pretreatment of serum specimens with heat (56°C) combined with polyethylene glycol *p*-tert-octylphenyl ether (Triton X-100) at a concentration of 10 μL/mL of serum to reduce viral titer; however, 100% inactivation may not occur (*21*). Automated analyzers should be cleaned and disinfected according to manufacture recommendations or with sodium hypochlorite at a concentration of 500 ppm (1:100 dilution) (*20*,*21*).

One should also limit the number of staff performing 24-hour monitoring and establish restricted room access and an entry-tracking log. If the patient becomes ill, some staff who entered the room may require illness surveillance, especially if there were any breaches in infection control practices. A visitation policy may need to be addressed. Because spending weeks in quarantine can be particularly stressful for the patient, it is useful to consider ways to keep the patient occupied, such as Internet connectivity, a television/video player, and a telephone.

Step 7: Decide on Treatment

Decisions on treatment/prophylaxis are difficult for viruses requiring BSL-4 precautions that lack any licensed therapy or prophylaxis. Therefore, having access to subject matter experts (as discussed in step 3) is essential. Collectively, difficult treatment decisions may be required that balance risk from investigational therapies against presumed risk for disease.

Step 8: Keep a Journal

Designation of a scribe early on should be considered to track major events, decision points, and options that were considered. Records of dates and times of important contacts should be included. Meeting minutes should be generated. Maintaining accurate logs may be useful to defend difficult decisions later and may help drive an after-action review.

Step 9: Learn from the Experience

It is useful to conduct a formal incident review that assesses how the event was managed. Results from any safety or epidemiologic investigations should be included. With appropriate review of procedures and training, additional potential exposures may be prevented.

Conclusions

There are few institutions in the United States currently capable of working with viruses that require BSL-4 containment, although the list is expected to expand in the near future. This article highlights medical issues and provides management considerations on the basis of USAMRIID's experience related to a recent potential exposure to a filovirus. The expectation is that as other facilities contemplate conducting research with BSL-4 pathogens, this report may enable them to improve their preparation for potential exposures in the future.

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Transmission of Human Papillomavirus in Heterosexual Couples

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- Specify the most common mode of transmission of HPV between couples
- Describe the role of anatomic sites in the transmission of HPV
- Identify behavioral factors associated with the transmission of HPV

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We examined the transmission of human papillomavirus (HPV) in 25 heterosexual, monogamous couples (25 men, 25 women), followed up over an average of 7.5 months. A total of 53 heterosexual transmission events were observed among 16 couples (14 male-to-female and 39 female-to-male). Sexual transmission involved 13 different oncogenic and nononcogenic HPV types; 8% were vaccine-covered types transmitted between partners. The overall rate of HPV transmission from the penis to the cervix was 4.9/100 person-months, which was substantially lower than that from the cervix to the penis (17.4/100 person-months). Transmission between the hands and genitals, as well as apparent self-inoculation events (primarily in men), were also observed. Couples who transmitted HPV were more sexually active and used condoms less frequently. These results have implications for HPV prevention and control strategies, including the targeting of prophylactic vaccines.

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Cervical cancer remains a major source of illness and death among women globally, and infection with oncogenic human papillomaviruses (HPVs) is its principal cause (*1*,*2*). Men are assumed to be the main reservoirs of genital HPV infection for women, although comparatively little is known about the natural history of HPV in men.

A limited number of cross-sectional and case-control studies have evaluated genotype-specific HPV concordance in male-female couples (*3*–*7*). There are, however, no empirical data on the heterosexual transmission of HPV. Our investigation evaluates the transmission of HPV in a cohort of male-female sexual partners.

Methods

Study Participants

The study was conducted at the University Health Services of the University of Hawaii at Manoa from February 2005 through November 2006. Promotional efforts includ-

ed flyers and email invitations, which were part of larger ongoing studies of HPV. The study was approved by the Committee on Human Studies of the University of Hawaii. All study participants provided written informed consent. Eligible participants were at least 18 years of age, English-speaking, not currently pregnant, and in a monogamous relationship with the index partner.

Specimen Collection

Couples attended concurrent study visits at 2-month intervals. Trained clinicians collected exfoliated cell samples for HPV DNA detection. For men, separate genital specimens from the penis glans/corona, penis shaft, scrotum, and inner foreskin (uncircumcised men) were collected by using textured paper and a saline-moistened swab (*8*,*9*). Anal canal specimens were collected by using a saline-moistened swab. A cytobrush was used to collect oral specimens (buccal cavity, tongue). Specimens from the dominant hand (palm, fingertips, under the fingernails) were collected by using a saline-moistened swab. Participants self-collected first-catch urine samples (30 mL) at the clinic. Using latex gloves, men collected semen specimens at home during masturbation within 24 hours of each visit.

For women, a cervical cytology (Papanicolaou [Pap]) smear was collected, and a swab and cytobrush were used to consecutively sample the ectocervix and endocervix, including the transformation zone. The same methods used for collection of anal, oral, hand, and urine specimens from men were used for collection of specimens from women.

HPV DNA Testing and Genotyping

DNA was extracted from specimens by using commercial reagents (QIAGEN, Valencia, CA, USA). The PCR used PGMY09/PGMY11 primers to amplify a 450-bp region of the L1 HPV genome (*10*). Amplification of the human β-globin gene was included as an internal control for sample sufficiency. HPV-positive specimens were subsequently genotyped by using commercial reagents (Roche Molecular Systems Inc., Branchburg, NJ, USA) originating from a prototype line blot assay (*11*). The assay detects 37 different HPV types, including oncogenic/probable oncogenic types (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, and IS39, a subtype of HPV 82), nononcogenic types (HPV 6, 11, 40, 42, 54, 61, 70, 72, 81, and CP6108, also known as candidate HPV 89), and types with undetermined risk status (HPV 55, 62, 64, 67, 69, 71, 83, and 84) (*12*,*13*). This PCR-based assay has demonstrated a high degree of sensitivity and reproducibility (*14*–*16*). HPV testing and genotyping procedures have been given in detail previously (*9*).

Statistical Analysis

The main objective of the statistical analysis was to evaluate HPV transmission between partners. Each type-specific HPV infection was assigned a status of transmitted or not transmitted by time period and anatomic site. For some statistics, the penis anatomic site was further divided into foreskin, glans, shaft, and urine, which is a proxy for urethral infection. HPV in female urine, which has demonstrated type-specific concordance with cervical measurements (*17*), was considered to be a proxy for cervical and other lower genital tract infections. Therefore, cervix and urine were combined as 1 anatomic site. When there were >1 possible source sites, sites were grouped and evaluated as 1 transmission event. When there were >2 destination sites, each was counted as a separate transmission event.

Partner transmission was defined as the presence of a specific HPV genotype at an anatomic site in 1 partner and its absence in all sites of the other partner at a given visit, along with the presence of this HPV type in the unaffected partner at the subsequent visit. Auto-inoculation was defined as the presence of a particular HPV type at an anatomic site in 1 partner and its absence in all sites of the other partner at a given visit, and the presence of this HPV type in a different anatomic site in the affected partner at the subsequent visit. An event was defined as self-inoculation only after possible transmission from the partner was ruled out, i.e., when the partner was negative for the HPV genotype at prior and concurrent visits.

The rate of HPV transmission was calculated as the number of HPV type-specific transmission events divided by the number of person-months of exposure × 100 and expressed as the rate per 100 person-months. Exact confidence intervals (CIs) for transmission rates were calculated by assuming a Poisson distribution for the number of events (*18*). Person-months of exposure for each HPV infection by anatomic site were computed based on the period of time between successive visits. When HPV was detected at a given visit and HPV type was absent at the successive visit, the exposure period was estimated at half of the visit interval. Comparisons between couples by transmission status were made by using the *t* test, χ^2 statistic, and linear rank statistic.

Results

Study Participants

Thirty-eight couples (38 men, 38 women) were enrolled. Six couples left the study, including 2 whose relationships ended. The present analysis focuses on 25 couples with at least 2 visits. Couples were followed up at ≈2-month intervals over an average of 7.5 months.

The mean age was 28 years (range 18–59 years) for men and 26 years (range 18–57 years) for women. Participants comprised Caucasians (52%), Asians (8%), Pacific

Islanders (6%), and persons of other single- or mixed-race backgrounds (34%). Couples were single/never married (48%), living together as married (32%), separated/divorced/widowed (12%), and married (8%). All participants indicated that the index partner was their only current sexual partner, and all reported vaginal intercourse with penile penetration during the follow-up period.

Five men were uncircumcised. At study entry, 1 woman had cervical low-grade squamous intraepithelial lesions, and 2 men had genital warts. Cervical atypia was diagnosed in 2 women during follow-up.

HPV Transmission

Overall, 957 (94%) of 1,015 specimens were adequate based on β-globin detection and were included in the present analyses. At study entry, 7 couples were HPV negative at all anatomic sites, 4 couples had 1 HPV-positive partner (3 were anal infections in the man), and 14 couples had both partners HPV positive. Of the 14 couples positive for HPV, 3 couples were positive for different HPV types, and 11 were positive for the same HPV type(s) at 1 or more anatomic sites (Table 1). All 11 HPV type-specific concordant infections involved the penis in the male partner and, with 2 exceptions, the cervix and/or urine in the female partner.

All transmission events occurred in couples with 1 or both partners positive for HPV at study entry. A total of 78 transmission events were observed in 16 couples, including 14 male-to-female, 39 female-to-male, 21 male auto-inoculation, and 4 female self-inoculation (online Appendix Table, available from www.cdc.gov/content/14/6/888-appT.htm). Overall, 15 different genotypes were transmitted, including oncogenic types and nononcogenic/undetermined risk types. Partner transmission involved 13 genotypes. Forty-one (53%) of the 78 transmission events involved multiple potential sources of infection.

Male-to-female transmission was observed in 7 couples. All infections transmitted from male to female partners originated in the penis with or without additional involvement of the scrotum. In particular, the penis shaft was a predominant source of infection either alone or with other genital sites. The cervix and anus were the most frequent targets of transmission from the men. Transmission of oncogenic HPV 16 comprised 1 of the 5 genital-to-cervix events. Male genital to female hand transmission was observed in 3 couples (D, E, and G).

Female-to-male transmission was observed in 12 couples. Transmission from the cervix and/or urine to the male genitals comprised most female-to-male events. Within penis subsites, the glans was targeted more frequently than the shaft. The anus was frequently an additional source site along with the cervix/urine. In addition, the anus was the sole source of transmission to the male genitals on 4 occasions, of which 3 targeted the partner's scrotum (couples A, B, and J). The penis and scrotum, respectively, were the most frequent targets of infection from women. There were 4 instances of transmission from the woman's hands to the man's genitals, including 1 case in which it was the sole source (couple I). Oncogenic HPV types 16 and 18 were transmitted in only 2 of the 39 female-to-male events.

Male self-inoculation was observed in 11 men, including 3 for whom no heterosexual transmission was observed during the entire period of follow-up. Sixteen events in-

Table 1. HPV status of couples at study entry*

		Man		Woman	
HPV status at baseline	No.	Site	HPV	Site	HPV
Both partners HPV neg	7	All	Neg	All	Neg
One partner HPV pos	4	Anus	18	All	Neg
		Anus	18	All	Neg
		Anus	42/51/52/84	All	Neg
		All	Neg	Urine	CP6108
Both partners HPV pos					
Different HPV type(s)	3	Anus	6	Anus	39
		Shaft	6	Cervix/urine	16
		Shaft, scrotum	6	Cervix/urine, anus	31
One or more of the same HPV type(s)†	11	Shaft	59	Cervix	59
		Shaft	59	Hand	59
		Shaft	84	Cervix	84
		Shaft	62	Urine	62
		Shaft	55	Anus	55
		Glans, shaft	56, 59	Cervix/urine, anus, hand	56, 59
		Shaft, scrotum	62	Urine, anus, hand	62
		Foreskin, glans, shaft, scrotum	CP6108	Urine, anus	CP6108
		Shaft, scrotum	42	Cervix/urine, anus	42
		Glans, shaft, scrotum	53	Cervix	53

*HPV, human papillomavirus; neg, negative; pos, positive.
†Only concordant types shown.

volved transmission between different genital sites, 2 involved anal-to-genital transmission, and 3 involved genital-to-hand transmission. Most genital-to-genital events involved transmission between penis subsites. In 3 instances, male self-inoculation immediately preceded transmission of HPV to female partners (couples A and B).

Female self-inoculation was observed in 4 females. All involved urine as a source site and 3 of these targeted the hands.

Heterosexual transmission of the same HPV genotype to >1 anatomic site (excluding penis subsites) was observed on 13 occasions. Eight of these cases involved transmission from the cervix/urine to the penis and scrotum.

Heterosexual transmission of multiple genotypes to the same anatomic site during the same period was observed in 5 instances (couples A, B, D, F, and G); the scrotum was the target site on 3 of these occasions. In 1 case, different genotypes in the cervix and urine were each transmitted to both the penis glans and shaft (couple K).

Transmission of some HPV types in preference to others was also observed. In couple L, after cervical infection with HPV 16 and 31 was detected at study entry, only HPV 31 was transmitted to the penis (glans, shaft) and scrotum. Alternatively, the female anus may have been the source of infection because HPV 31, not HPV 16, was present in the anus at baseline. In couple D, HPV 16, 59, and 62 were present in the penis shaft at baseline. Subsequently, only HPV 16 was transmitted to the woman's cervix/urine and anus by the second visit.

A number of instances indicated apparent reinfection after viral clearance. In 1 couple (A), the woman's anus was positive for HPV 39 infection at baseline and at visit 2. By visit 3, HPV 39 had been transmitted to the man's scrotum, and the woman was negative for HPV 39. HPV 39 had been transmitted (presumably by auto-inoculation) to the penis by visit 4, and the infection remained through visit 5. By visit 6, or 9 months after initial clearance in the woman, HPV 39 had been transmitted to the woman's anus.

Four cases of transmission to the female anus required >4 months of exposure to an infected partner. In 27 incident infections, no source of infection could be ascertained; 17 of these infections were in the male genitals.

The rates of HPV transmission by source site are shown in Table 2. Overall, the rate of transmission from the penis to the cervix/urine was 4.9 per 100 person-months of exposure (95% CI 1.6–10.0). By contrast, the overall rate of transmission from the cervix/urine to the penis was 17.4 per 100 person-months of exposure (95% CI 10.6–25.8). Transmission of oncogenic types to the male genitals was greater from the female urine alone than from the cervix alone. Transmission of HPV from the penis to the female anus was higher than that to the cervix; this was particularly true for transmission of oncogenic types to the anus (12.2 per 100 person-months of exposure, 95% CI 3.9–24.9). The highest rates of transmission were observed from the female anus to the male genitals (47.1 per 100 person-months of exposure, 95% CI 30.2–67.7), followed by cervix to the male genitals (27.8 per 100 person-months of exposure, 95% CI 19.0–38.3). In men, the rate of transmission by auto-inoculation was comparable to that of transmission from women. For example, the rate of transmission from the scrotum alone to the penis (6.0 per 100 person-months of exposure, 95% CI 1.2–14.5) was comparable to that of cervix only to penis (5.0 per 100 person-months of exposure, 95% CI 1.0–11.9).

Behavioral Factors

All couples with genital-to-genital transmission reported vaginal intercourse during the period corresponding to the transmission event. Among the 5 couples with penis-to-anus transmission, 4 reported anal intercourse during the corresponding time period.

Table 3 compares baseline characteristics of couples with and without HPV transmission. Male partners in transmitting couples had had more sexual partners over their lifetime. Transmitting couples had more frequent sexual intercourse with one another, were more likely to have contact between the male's mouth and the female's anus, were more likely to use birth control injections and have withdrawal before ejaculation, and had fewer periods of abstinence. Over half of nontransmitting couples reported use of condoms 100% of the time during sexual intercourse within the previous 4 months, compared with only 3% of transmitting couples.

Discussion

This study demonstrates that HPV is efficiently transmitted between sexual partners and that multiple transmission events can occur within a couple. The rates of genital transmission from women to men were substantially higher than from men to women. Greater rates of female-to-male transmission should imply higher HPV prevalence in men. Studies in men to date, including our own cohort, have reported male genital HPV prevalences at least as high as in women, with most reporting prevalences of at least 20% and up to 73% (*9*,*19*). The penis shaft was the primary source of transmission to the cervix; the cervix and urine were the primary sources of infection to male genitals.

Sexual transmission also involved the scrotum, the anus of women, and the hands of both sexes. The oral cavity and semen were not involved in transmission.

The anus of women was both a major source and target of heterosexual transmission. We observed consistency between penis-to-female anus transmission and reported anal intercourse during the corresponding period. We previously demonstrated high genotypic concordance between

concurrent cervical-anal infections in women, which indicates possible common sources of infection (*20*).

Transmission through nonpenetrative sexual contact was demonstrated between the female anus and the scrotum, as well as the female hand and male genitals. The male anus was not a major source or a target of HPV transmission. However, 3 of the 4 couples with baseline infection in only 1 partner involved anal infection in men.

Male self-transmission frequently involved the scrotum, likely facilitated by passive contact between proximate genital sites. The scrotum may be an important reservoir of infection for penile infections that can subsequently be transmitted to partners. Hands may also serve as reservoirs of infection in both men and women. Autoinoculation involving the hands may result from casual contact or masturbation.

To some extent, our study results suggest that HPV is relatively indiscriminate in its patterns of transmission. We observed the transmission of a given viral genotype to multiple anatomic sites in a partner and concurrent transmission of multiple genotypes to the same site.

Other observations suggest that HPV transmission is not entirely arbitrary and may reflect tissue or genotype differences or both. Rates of transmission of oncogenic types to the male genitals from the urine were higher than from the cervix. This may reflect differences in genotypes found in the vagina and vulva compared with those found in the cervix. All transmission events requiring extended periods of exposure involved the female anus target site.

A total of 15 genotypes were transmitted, including 13 which were transmitted through heterosexual means. HPV 16 and 18 and nononcogenic HPV 6 and 11, the 4 types

Table 2. Rate of HPV transmission by source and target site in male-female couples*

		No. transmission events		Duration of exposure, mo†		Transmission rate/100 person-months (95% CI)	
Source site	Target site	Overall	Oncogenic	Overall	Oncogenic	Overall	Oncogenic‡
Male to female							
Penis only	Cervix/urine	2	1	102	41	1.9 (0.2–5.4)	2.4 (0.06–9.0)
Penis only	Anus	3	3	102	41	2.9 (0.6–7.1)	7.3 (1.5–17.6)
Penis only	Hand	2	1	102	41	1.9 (0.2–5.4)	2.4 (0.06–9.0)
Any penis	Cervix/urine	5	1	102	41	4.9 (1.6–10.0)	2.4 (0.06–9.0)
Any penis	Anus	6	5	102	41	5.9 (2.2–11.4)	12.2 (3.9–24.9)
Any penis	Hand	3	2	102	41	2.9 (0.6–7.1)	4.9 (0.6–13.5)
Any scrotum	Cervix/urine	3	0	50	–	6.0 (1.2–14.5)	–
Any scrotum	Anus	3	2	50	27	6.0 (1.2–14.5)	7.5 (0.9–20.9)
Any genital	Cervix/urine	5	1	111	51	4.5 (1.5–9.3)	2.0 (0.1–7.2)
Any genital	Anus	6	5	111	51	5.4 (2.0–10.6)	9.8 (3.2–20.0)
Female to male							
Cervix only	Penis	3	0	61	–	5.0 (1.0–11.9)	–
Cervix only	Scrotum	1	0	61	–	1.6 (0.04–6.1)	–
Cervix only	Any genital	4	0	61	–	6.6 (1.8–14.5)	–
Urine only	Penis	2	1	55	36	3.7 (0.4–10.2)	2.8 (0.1–10.4)
Urine only	Scrotum	3	2	55	36	3.7 (.04–10.2)	5.6 (0.7–15.7)
Urine only	Any genital	5	3	55	36	9.2 (3.0–18.8)	8.4 (1.7–20.3)
Cervix/urine only	Penis	6	2	115	83	5.2 (1.9–10.1)	2.4 (0.3–6.7)
Cervix/urine only	Scrotum	5	3	115	83	4.3 (1.4–8.9)	3.6 (0.7–8.7)
Any cervix/urine	Penis	20	12	115	83	17.4 (10.6–25.8)	14.6 (7.5–23.9)
Any cervix/urine	Scrotum	12	8	115	83	10.4 (5.4–17.1)	9.7 (4.2–17.4)
Any cervix/urine	Any genital	32	20	115	83	27.8 (19.0–38.3)	24.2 (14.8–35.9)
Any cervix/urine	Anus	1	1	115	83	0.9 (0.02–3.2)	1.2 (0.03–4.50
Any cervix/urine	Hands	1	1	115	83	0.9 (0.02–3.2)	1.2 (0.03–4.5)
Anus only	Scrotum	3	1	51	36	5.9 (1.2–14.2)	2.7 (0.1–10.1)
Any anus	Any genital	24	16	51	36	47.1 (30.2–67.7)	44.0 (25.1–68.0)
Any hands	Any genital	4	2	426	373	28.2 (7.7–61.8)	16.1 (1.9–44.8)
Self-inoculation							
Male§							
Penis only	Scrotum	5	1	102	41	4.9 (1.6–10.0)	2.4 (0.1–9.0)
Scrotum only	Penis	3	2	50	27	6.0 (1.2–14.5)	7.5 (0.9–20.9)
Any genital	Any genital	15	6	111	51	13.6 (7.6–21.3)	11.7 (4.3–22.8)
Any genital	Hands	3	2	111	51	2.7 (0.6–6.5)	3.9 (0.5–10.9)
Anus only	Any genital	3	2	29	21	10.2 (2.1–24.6)	9.3 (1.1–26.0)
Female							
Cervix/urine only	Anus	1	1	115	83	0.9 (0.02–3.2)	1.2 (0.03–4.5)
Cervix/urine/anus	Hand	3	2	166	119	1.8 (0.4–4.4)	1.7 (0.2–4.7)

*HPV, human papillomavirus; CI, confidence interval.
†Based on the duration of infection in the source site. When HPV was detected at a given visit followed by the absence of that HPV type at the successive visit, the exposure period was estimated at half of the visit interval.
‡Includes probable oncogenic types.
§Includes penis-to-penis subsite inoculations.

Table 3. Characteristics of male-female couples by HPV transmission status*

Characteristic	Transmission (n = 16 couples)	No transmission (n = 9 couples)	p value
Mean age (SD)			
M	25.4 (8.8)	32.2 (14.4)	0.22
F	25.5 (10.2)	26.3 (7.3)	0.82
Length of relationship, mo, median (range)	10.7 (1.9–73.4)	13.1 (0.23–185)	0.22
Monthly frequency of sexual intercourse, mean (SD)	17.7 (10.0)	6.9 (7.8)	0.0002
Lifetime no. sexual partners, mean (SD)			
M	13.9 (10.4)	5.9 (5.1)	0.04
F	6.7 (6.1)	2.8 (2.2)	0.08
Circumcised man, no. (%)	13 (81)	7 (78)	0.84
Sexual practices (ever/never), no. (%)			
Vaginal intercourse	32 (100)	18 (100)	1.00
Anal intercourse	14 (44)	6 (33)	0.47
Oral-vaginal	29 (91)	13 (72)	0.09
Oral-penile	32 (100)	17 (94)	0.18
Oral (M)–anal (F)	6 (19)	0	0.05
Oral (F)–anal (M)	2 (6)	0	0.28
Finger/other object-vaginal	28 (88)	18 (100)	0.12
Finger/other object (M)–anal (F)	9 (28)	4 (22)	0.65
Finger/other object (F)–anal (M)	12 (38)	4 (22)	0.27
Contraception ever used with partner, no. (%)			
Birth control pill	21 (66)	8 (44)	0.15
Birth control shot	6 (19)	0	0.05
“Morning after” pill	15 (16)	0	0.08
Spermicides	3 (9)	2 (11)	0.84
Withdrawal	19 (59)	5 (28)	0.03
Vasectomy	2 (6)	4 (22)	0.10
Female condom	2 (6)	3 (17)	0.24
Abstinence	5 (16)	8 (44)	0.03
Condom	26 (81)	16 (89)	0.48
Frequency of condom use (prior 4 mo), no. (%)			
Always	1 (3)	10 (56)	
Never/some use†	31 (97)	8 (44)	<0.0001
Sexually transmitted infection history, no. (%)			
Chlamydia	4 (13)	0	0.12
Genital herpes	1 (3)	2 (11)	0.25
Cigarette smoking (ever)‡, no. (%)			
M	9 (56)	3 (33)	0.27
F	4 (25)	0	0.10

*HPV, human papillomavirus; SD, standard deviation.
†Some use defined as usage less than half the time, half the time, or more than half the time.
‡History of smoking daily for ≥6 months.

included in the current quadrivalent vaccine, comprised <10% of transmitted types. Notably, we observed greater transmission of HPV 16 to the cervix than such transmission by other types, which underscores the possibility of selective transmission of some HPV types.

Compared with couples not experiencing HPV transmission, transmitting couples were more sexually active and were more likely to use certain nonbarrier forms of contraception. Few HPV-transmitting couples reported always using condoms during recent sexual activity, compared with over half of nontransmitting couples.

A major limitation of our study was the potential for misclassification of HPV transmission events. Variable detection of HPV could be due to natural fluctuation in viral levels or variable sampling of sites could confound the observation of viral transmission. For example, instances of apparent reinfection of sites may alternatively represent possible reactivation of latent infections.

Another potential source of misclassification was the lack of a priori knowledge of the time required for HPV to be acquired from an infected partner. Viral transmission could have occurred more frequently than the 2-month visit intervals used in the study, and transmission events could have been missed.

Another limitation of the study was the inclusion of couples who had already had sexual contact with one another; initial viral transmission was likely to have occurred before study entry. Indeed, nearly half of the couples had

type-specific concordant infections at study entry, indicating previous transmission of HPV, which limited our ability to evaluate incident infections.

Because our study relied on self-reported sexual activity, it was subject to recall bias. Furthermore, although all persons reported monogamous relationships, some of the incident infections without a source, most of which involved the male genitals, could have been acquired through sexual activity with another partner. Despite these limitations, the present study included intensive follow-up of a well-characterized cohort, sampling of multiple genital and nongenital sites, and state-of-the-art HPV testing and genotyping methods.

The development of comprehensive HPV prevention and control strategies, which incorporate HPV vaccine usage and contraceptive practices, is impeded by lack of information on the risk and routes of sexual transmission between heterosexual partners and potential genotype-specific differences in transmission efficiency. The small size of the cohort and the diversity of genotypes precluded type-specific analysis of transmission.

This study contributes to a growing body of knowledge of HPV in men because we directly examined HPV transmission. However, study results are preliminary and need to be verified in larger cohorts. Future HPV transmission studies are critical to address major gaps in our knowledge of the natural history of this virus.

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Population-Attributable Risk Estimates for Risk Factors Associated with *Campylobacter* Infection, Australia

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In 2001–2002, a multicenter, prospective case-control study involving 1,714 participants ≥5 years of age was conducted in Australia to identify risk factors for *Campylobacter* infection. Adjusted population-attributable risks (PARs) were derived for each independent risk factor contained within the final multivariable logistic regression model. Estimated PARs were combined with adjusted (for the ≥5 years of age eligibility criterion) notifiable disease surveillance data to estimate annual Australian *Campylobacter* case numbers attributable to each risk factor. Simulated distributions of "credible values" were then generated to model the uncertainty associated with each case number estimate. Among foodborne risk factors, an estimated 50,500 (95% credible interval 10,000–105,500) cases of *Campylobacter* infection in persons ≥5 years of age could be directly attributed each year to consumption of chicken in Australia. Our statistical technique could be applied more widely to other communicable diseases that are subject to routine surveillance.

Foodborne gastroenteritis is a major public health concern in many countries, including Australia. A recent study estimated that 5.4 million cases (95% credible interval [CrI] 4.0–6.9 million), 15,000 hospitalizations (95% CrI 11,000–18,000), and 80 deaths (95% CrI 40–120) annually are caused by foodborne gastroenteritis in Australia (*1*). Norovirus, enteropathogenic *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. accounted for 88% of the estimated 1.5 million (95% CrI 1.0–1.9 million) cases of foodborne disease caused by known pathogens.

Among known foodborne pathogens, *Campylobacter* spp. are the most frequently reported enteric pathogens in Australia (*2*). The incidence of *Campylobacter* infection steadily increased from 1991 through 2001 but has been relatively stable since. In 2005, >15,000 cases were reported in Australia, a crude rate of 113.0/100,000 population. However, because of underreporting, ≈223,000 *Campylobacter* infections are estimated to occur annually; ≈75% of these are foodborne (*3*). Most of these infections are sporadic.

Case-control studies have identified a range of different risk factors for infection; consumption of chicken is the most frequently reported (*4*–*9*). Some of these studies report population-attributable fractions associated with independent risk factors, but no estimates of the total magnitude of infection caused by chicken or other risk factors have yet been reported. Using a multicentered, prospective case-control study, we aimed to develop a multivariable logistic regression model that identified independent foodborne and nonfoodborne risk factors for *Campylobacter* infection for this sample (*7*) and calculate population-attributable risk (PAR) proportions. These PARs were then combined with annual *Campylobacter* infection surveillance data to estimate the total number of infections (with associated CrIs) among persons ≥5 years of age attributable to specific risk factors that occur in the community each year in Australia.

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Methods

Study Design and Population

From September 2001 through August 2002, a multicenter, prospective case-control study was conducted across 5 of the 8 states and territories in Australia to identify risk factors for *Campylobacter* infection in persons ≥5 years of age. These jurisdictions were those with legislation that required physicians and laboratories to notify health departments about patients infected with *Campylobacter*. At the time of this study, the population of the 5 states combined was ≈12 million, and the total population of Australia was ≈19 million.

Case-Patients and Controls

A case-patient was defined as a person ≥5 years of age reported with a culture-positive stool result for *Campylobacter* infection and a recent history of acute diarrhea, who was not part of an outbreak investigation unless identified as the index patient. Controls were sourced from a national control bank and frequency matched to case-patients by age groups in each state. The age groups were selected on the basis of potential variation in risk factors due to different behavior at different ages. Age groups were children (5–9 years), adolescents (10–19 years), young adults (20–29 years), middle-aged adults (30–59 years), and elderly (≥60 years).

A total of 881 case-patients and 883 controls were recruited for this study. A telephone-administered questionnaire was used to collect detailed information on exposures in the 7 days before onset of illness for case-patients and in the 7 days before interview for controls. The questionnaire comprised several sections, each representing a separate exposure group that listed questions pertaining to potential risk factors related to that group. The following sections were included: meat, poultry and seafood consumption; egg and dairy product consumption; produce consumption; water consumption; food-handling practices; animal and pet exposures; host factors; dining locations outside the home; overseas travel; and demographic information. To measure the effects between illness and consumption of cooked meat products or undercooked meat products, additional information was sought on whether the meat appeared undercooked (pink on the inside) when eaten. A detailed description of the study design, sample, and exposure measurements has been published elsewhere (*7*).

Data Analysis

A 2-stage model-building strategy was undertaken, first by determining a parsimonious multivariable model for each exposure group, and second by deriving an omnibus parsimonious model that combines significant exposure variables from all the exposure group multivariable models. A more comprehensive description of the analytical model has been published elsewhere (*7*) and is included in the online Technical Appendix (available from www.cdc.gov/EID/content/14/6/895-Techapp.pdf).

We calculated PARs by using adjusted odds ratios (aORs) from the final multivariable logistic regression model for each variable that was significantly associated with an increased risk for infection, apart from host factors (*10*). Stata statistical software, release 7 (Stata Corp, College Station, TX, USA), was used for calculating 95% confidence intervals (CIs) around the PAR estimates. Using community incidence data derived from adjusted national surveillance data (*3*) coupled with PAR data from our case-control study, we used simulation techniques to estimate the total number of *Campylobacter* infections attributable to specific risk factors that occur in the community each year in Australia and to derive credible regions for these estimates by modeling the uncertainty in each variable component.

Simulation Methods

We assumed that 223,000 (95% CrI 94,000–363,000) cases of campylobacteriosis occur in Australia in a typical year (*3*). We then adjusted this figure by reviewing Australian notification data for the years 2001 through 2003 (*11*) and applying simulation techniques to estimate the proportion of cases that occur among persons ≥5 years of age. Similarly, we randomly generated simulated PAR values for each risk factor using aORs from the final model. The simulated campylobacteriosis case numbers and PAR-simulated values were multiplied together to produce distributions of the total number of *Campylobacter* infections attributable to each specific risk factor. Because some distributions are skewed, we present medians and 95% CrIs (defined to be the 2.5 and 97.5 percentiles) for the simulation results. Simulations were undertaken in SAS System for Windows, version 9.1 (SAS Institute Inc., Cary, NC, USA). A detailed description of the simulation technique used to derive these estimates is provided in the online Technical Appendix. The full description of the sample and the development of the final multivariable logistic regression model have been published elsewhere (*7*).

Results

Multivariable Analysis of Risk Factors

Table 1 reports results of univariable (crude) and multivariable logistic regression analyses for variables within each exposure group (adjusted for state, sex, and education), and the final multivariable model showing frequency and sample size, percentages, and crude odds ratios (ORs) and aORs, together with 95% CIs. The independent risk factors that were identified in the final model explained

only a limited proportion of illness (Nagelkerke $R^2 = 0.16$). Consumption of undercooked chicken (aOR 4.7, 95% CI 2.6–8.4), consumption of offal (aOR 2.0, 95% CI 1.0– 4.0), ownership of domestic dogs <6 months of age (aOR 2.1, 95% CI 1.1–4.2), and ownership of domestic chickens <6 months of age (aOR 12.4, 95% CI 2.6–59.3) were the only independent risk factors for infection after adjusting for all other variables in the model. Consumption of cooked chicken was positively but not statistically associated with illness and warranted further consideration (aOR 1.4, 95% CI 1.0–1.9, p = 0.06). Eating fresh fish, eating homemade foods containing raw eggs, eating organically grown fruit and/or vegetables, and eating homegrown fruit were independent factors associated with a statistically significant reduced risk for infection. Eating raw salads or vegetables, as measured by the vegetable index variable, was also associated with a reduced risk for infection. Drinking commercial bottled water, placing barbequed cooked meat back on the same plate used for raw meat, having liver disease, and having any immunosuppressive therapy in the 4-week

Table 1. Results of univariable (crude) and multivariable logistic regression analysis for variables within each exposure group and the final multivariable model, *Campylobacter* infection, Australia, 2001–2002*

Exposure group/variables†	Case-patients, n/N (%)	Controls, n/N (%)	Univariable analysis		Multivariable logistic regression analysis (exposure groups)		Final multivariable model‡	
			OR	95% CI	OR	95% CI	aOR	95% CI
Meat, poultry and seafood					Model 1			
No chicken	110/711 (15.5)	162/808 (20.0)	1.0		1.0		1.0	
Chicken, cooked	528/711 (74.3)	618/808 (76.5)	1.3	1.0–1.7	1.3	1.0–1.8	1.4	1.0– 1.9
Chicken, undercooked	73/711 (10.3)	28/808 (3.5)	3.8	2.3–6.3	4.4	2.6–7.5	4.7	2.6– 8.4
Offal	36/852 (4.2)	16/830 (1.9)	2.2	1.2–4.4	2.1	1.1–3.9	2.0	1.0– 4.0
Fresh fish	256/833 (30.7)	332/827 (40.1)	0.7	0.5–0.8	0.6	0.5–0.8	0.7	0.5– 0.9
Eggs and dairy products					Model 2			
Homemade foods containing raw eggs	40/837 (4.8)	70/822 (8.5)	0.5	0.4–0.8	0.5	0.3–0.7	0.5	0.3– 0.8
Produce					Model 3			
Organic fruit and vegetables	50/805 (6.2)	100/804 (12.4)	0.5	0.3–0.7	0.6	0.4–0.8	0.6	0.4–1.0
Homegrown fruit	84/845 (9.9)	169/828 (20.4)	0.4	0.3–0.6	0.5	0.4–0.7	0.4	0.3–0.6
Vegetable index§								
0 (no vegetables)	141/853 (16.5)	87/830 (10.5)	1.0		1.0		1.0	
1 (1–2)	339/853 (39.7)	305/830 (36.7)	0.7	0.5–0.9	0.7	0.5–1.0	0.7	0.5–1.0
2 (3–4)	352/853 (41.3)	382/830 (46.0)	0.6	0.4–0.8	0.6	0.4–0.9	0.6	0.4–0.9
3 (5–6)	21/853 (2.5)	56/830 (6.7)	0.2	90.1–0.4	0.3	0.1–0.5	0.2	0.1–0.5
Water consumption					Model 4			
Commercial bottled water	72/846 (8.5)	47/820 (5.7)	1.5	1.0–2.3	1.6	1.1–2.3	NS	
Food-handling practices					Model 5			
Barbequed cooked meat placed back on plate used for raw meat	21/511 (4.1)	9/471 (1.9)	2.2	1.0–5.5	2.3	1.0–5.4	NS	
Animal and pet exposure								
Domestic chickens					Model 6			
No domestic chicken	783/846 (92.6)	777/821 (94.6)	1.0		1.0		1.0	
Chicken <6 mo of age	18/846 (2.1)	5/821 (0.6)	3.6	1.3–9.7	5.2	1.5–17.8	12.4	2.6– 59.3
Chicken ≥6 mo of age	45/846 (5.3)	39/821 (4.8)	1.1	0.7–1.8	1.3	0.8–2.2	1.7	0.9– 3.0
Domestic dogs								
No dog	397/839 (47.3)	452/819 (55.2)	1.0		1.0		1.0	
Dog <6 mo of age	48/839 (5.7)	17/819 (2.1)	3.2	1.8–5.7	2.9	1.6–5.3	2.1	1.1– 4.2
Dog ≥6 mo of age	394/839 (47.0)	350/819 (42.7)	1.3	1.1–1.6	1.2	1.0–1.5	1.2	0.9–1.5
Host factors					Model 7			
Chronic gastrointestinal condition	101/873 (11.6)	50/831 (6.0)	2.0	1.4–3.0	2.0	1.4–2.9	2.3	1.5–3.4
Liver disease	14/875 (1.6)	2/830 (0.2)	6.7	1.5–61.2	5.1	1.1–23.0	NS	
Any immunosuppressive agent/therapy	35/881 (4.0)	12/833 (1.4)	2.8	1.4–6.0	2.8	1.4–5.5	NS	

*Each model adjusted for state, sex, and education. aOR, adjusted odds ratio; CI, confidence interval; NS, not significant.
†The exposure period for foods is 7 d before onset of illness for case-patients and 7 days before interview for controls.
‡After removal of nonsignificant interaction terms.
§The vegetable index was created to indirectly measure the range of raw produce consumed in the 7-day exposure period for patients and controls. The values of this index variable represented a count of the number of different types of salad/vegetable foods eaten during the exposure period.

exposure period were all removed from the final model during the sequential backward elimination procedure. None of the investigated 2-factor interactions was statistically significant. There was no reason to suspect the adequacy of the final multivariable model (Hosmer-Lemeshow goodness-of-fit test, p = 0.98). Additional statistical information, including β-coefficients, standard errors, statistical significance tests, and goodness-of-fit statistics for all multivariable models, is provided in Table 1A in the online Technical Appendix.

PAR Proportions

Among the food exposures, the proportion of study patients who reported eating undercooked chicken was 10.3%. The proportion of *Campylobacter* illness in the study population that could be attributed to the consumption of undercooked chicken was estimated to be 8.1% (95% CI 5.2%–11.1%) (Table 2). A further 21.2% (95% CI 0.0%–36.9%) of *Campylobacter* infections in the population could be attributed to cooked chicken. The overall PAR associated with consumption of chicken was 29.3%.

The proportion of campylobacteriosis patients ≥5 years of age that typically occurs each year in Australia was estimated from the simulations to be 191,000 (95% CrI 79,000–310,000). Applying the simulated PAR estimates to the number of cases of campylobacteriosis in Australia among persons ≥5 years of age, we estimated 15,000 (95% CrI 6,000–26,500) cases of *Campylobacter* infection could be attributed to eating undercooked chicken in a typical year. Similarly, an additional 35,500 (95% CrI 0–83,500) cases of infection could be attributed to apparently well-cooked chicken. Overall, an estimated 50,500 (95% CrI 10,000–105,500) cases of campylobacteriosis could be attributed to consumption of chicken each year in Australia.

The proportion of case-patients who reported eating offal was 4.2%. The proportion of illness in the study population that could attributed to the consumption of offal was estimated to be 2.1% (95% CI 0.0%–4.9%). This equates to ≈3,500 (95% CrI 50–8,500) cases of campylobacteriosis each year in Australia.

Among the nonfood exposures, ≈5,000 (95% CrI 500–11,500) cases of campylobacteriosis could be attributed to contact with dogs <6 months old each year in Australia. Similarly, an estimated 3,500 (95% CrI 1,000–7,000) cases of campylobacteriosis could be attributed to contact with domestic chickens <6 months old.

Discussion

The PAR proportions from this study indicate that chicken meat may be associated with >50,000 cases of *Campylobacter* infection each year in Australia. These figures provide a strong argument for government and industry to focus efforts on reducing contamination of chicken carcasses with *Campylobacter* through either improved on-farm control or interventions during processing. In addition, the figures justify the continued need for government to continue educating consumers and foodhandlers about the risks associated with the handling of raw chicken and the potential for cross-contamination in the kitchen.

Several case-control studies of sporadic *Campylobacter* infection have calculated PARs of independent foodborne risk factors (*4*,*5*,*9*,*12*,*13*). In these studies, the PAR percentage associated with chicken meat was 4.9%–31%, compared with 29.3% in our study. However, none of these studies extrapolated their PAR proportions to provide estimates of the total magnitude of infection in their study populations. The use of surveillance data coupled with an understanding of underreporting of illness from the com-

Table 2. PAR proportions with 95% CIs and community estimates with 95% CrIs for exposures associated with an increased risk for *Campylobacter* infection in persons ≥5 y of age, Australia, 2001–2002*†

Risk factor	No. case-patients	Proportion of case-patients (p_i)	aOR	PAR, %	95% CI	Estimated no. community case-patients	95% CrI
Food exposures							
Chicken consumption							
No chicken	110	0.155	Reference				
Chicken, cooked	528	0.743	1.4	21.2	0.0–36.9	35,500	0–83,500
Chicken, undercooked	73	0.103	4.7	8.1	5.2–11.1	15,000	6,000–26,500
Offal consumption							
No	816	0.958	Reference				
Yes	36	0.042	2.0	2.1	0.0–4.9	3,500	50–8,500
Nonfood exposures							
Dogs (domestic)							
No dog	397	0.473	Reference				
Dog <6 mo of age	48	0.057	2.1	2.9	0.3–4.8	5,000	500–11,500
Dog ≥6 mo of age	394	0.47	1.2	–			
Chickens (domestic)							
No domestic chickens	783	0.926	Reference				
Chickens <6 mo of age	18	0.021	12.4	1.9	0.9–2.9	3,500	1,000–7,000
Chickens ≥6 mo of age	45	0.053	1.7	–			

*PAR, population-attributable risk; CI, confidence interval; CrI, credible interval.
†Calculated from adjusted odds ratios (aOR) derived from the final multivariable logistic regression model.

munity to surveillance systems allows for this extra important step to quantify the extent of illness caused by specific risk factors (*3*,*14*,*15*).

A recent Australian study indicates that 75% (95% CrI 67%–83%) of cases of *Campylobacter* infection may be due to foodborne transmission (*1*). In our study, the foodborne risk factor with the highest attributable risk was cooked chicken, with an estimated median of 21.2% (95% CrI 0.0%–36.9%); followed by undercooked chicken, with an estimated median of 8.1% (95% CrI 5.2%–11.1%); and offal, with an estimated median of 2.1% (95% CrI 0.0%–4.9%). Although the aOR for cooked chicken is considerably lower than that for undercooked chicken, the high proportion of exposed case-patients (74.3% reported eating cooked chicken) explains the higher PAR. The combined significant foodborne attributable risk estimate found in the study, 31.4% (95% CrI 10.4%–46.8%), is <75%, which suggests that transmission of infection from foodborne vehicles other than chicken is likely to occur.

We interpret the risk associated with cooked chicken as most likely due to the consumption of undercooked chicken that was reported by patients as apparently well cooked or from poor handling during the preparation and cooking of raw chicken. Cross-contamination of cooked or ready-to-eat foods from handling raw chicken and poor food hygiene are considered to be alternative routes of transmission of *Campylobacter* infection (*12*,*16*–*21*). Although no other foods were significantly associated with illness in our study, food-based risk factors implicated from case-control studies conducted outside Australia include eating barbecued red meat or sausages, raw seafood, nonpoultry meat prepared at a restaurant, or pork and drinking unpasteurized milk (*4*,*6*,*8*,*9*,*22*). The Nagelkerke R^2 value of 16% for the final most parsimonious multivariable model also suggests that a considerable proportion of our case-patients had unexplained risk factors. The difficulty associated with recalling exposures is a major limitation of case-control studies designed to identify multiple potential risk factors. Bias caused by misclassification of reported exposures invariably dampens estimated effect sizes and may partly explain the failure to identify significant associations between some potential risk factors and illness. It is also likely that a proportion of unexplained cases were in persons infected by a variety of foods that had been subject to cross-contamination from raw chicken in the kitchen during preparation (*23*). Because eating chicken meat is a relatively common exposure among both patients and controls, our estimates of effect for cooked and undercooked chicken meat may be underestimates, as will be the derived PAR for chicken meat. However, it is reasonable to assume that at least some of the infections that occur in persons in Australia may be acquired from foods other than chicken or offal.

Two Australian case-control studies, including a study of risk factors among young children, have now identified household puppies and domestic chickens as risk factors for *Campylobacter* infection (*7*,*24*). Among persons ≥5 years of age, an estimated 8,500 cases of infection could be attributed to these 2 exposures in a typical year; the numbers could be expected to be considerably higher if sporadic infections among children <5 years of age were taken into account. These estimates indicate that a substantial portion of disease is caused by transmission of infection through these routes and provide a timely reminder that public health interventions to reduce this infection in the community should not be directed only at foodborne sources. Although variables associated with a reduced risk for infection did not contribute information to this article, several foods were independently associated with a reduced risk for infection, in particular raw fruit and vegetables. A more detailed discussion on factors associated with a reduced risk for infection in our study is published elsewhere (*7*).

The method used in this study provides an innovative approach to calculate estimates of the total magnitude of infection associated with a specific risk factor in a population, including an estimate of uncertainty. The required components for these calculations include 1) the PAR obtained from a case-control study in which estimates of effect can be generalized to the population under study and 2) an estimate of total community incidence. The method used to derive the incidence used in this study was from reportable disease data from an existing surveillance system and an estimate of underreporting to the surveillance system. Underreporting factors were derived from data on the proportion of case-patients in the community who visit a doctor (P_D), the proportion of case-patients seen by a doctor who have a stool sample taken (P_S), the proportion of correctly identified pathogens in stool samples submitted to laboratories (P_L), and the proportion of positive results that are reported to the surveillance system (P_R). The product of these proportions ($P_D \times Ps \times P_L \times P_R$) is the reported fraction (*3*). The extent and nature of underreporting will vary with different surveillance systems and for different pathogens. In the future, as more refined methods for calculating the degree of underreporting are developed, these estimates will become more accurate.

PAR estimates are useful for providing a measure of the proportion of illness that can be attributed to individual or multiple causal factors; however, in case-control studies, errors in the estimates of the proportion of cases exposed to a risk factor and/or errors in the estimate of ORs may lead to biased PAR estimates. For example, 1 requirement for estimating PAR is that the study patients be randomly selected from the population of interest and that exposure information be reported without bias. One could argue that the use of culture-confirmed cases in our study is not repre-

sentative of all *Campylobacter* case-patients in the population because patients with more severe symptoms are more likely to have stools collected and tested (*3*). Therefore, if the exposure information collected from our study patients was different from all case-patients in the general population, the proportion of case-patients exposed to a particular risk factor may be a biased estimate.

Recall and reporting bias are other concerns with case-control studies that may lead to biased estimates of the OR and subsequently the PAR. This is a particular concern for subjective exposures such as undercooked chicken, which are very difficult to measure accurately within a case-control design, so significant associations need to be interpreted with caution. Similarly, it may be difficult for a study participant who reportedly consumed cooked chicken meat to know if the meat was thoroughly cooked. Whether there are differential information biases between case-patients and controls in the reporting of undercooked chicken meat is not clear. In fact, consumption of undercooked chicken may well be systematically underreported by patients. Given the very high prevalence of chicken consumption in the Australian community (81% during the 7-day period before interview among our study controls), finding consumption of undercooked chicken as a risk factor for infection, despite the low reported frequency of exposure, is not surprising. Our PAR estimate for undercooked chicken meat was 8.1%, similar to that reported elsewhere (3%–11%) (*4*,*5*,*9*). No other types of undercooked meat that were measured in our study (e.g., pork, lamb, and beef) were significantly associated with *Campylobacter* infection.

For diseases with multiple risk factors, the PAR estimate for any single factor should be adjusted for possible confounding and interaction by these other factors (*25*,*26*). Multivariable adjustment methods that use logistic regression allow estimates of PAR to a single factor while simultaneously adjusting for other factors in the model. However, if all relevant factors are not included in the model or the model does not have correct parametric form, the adjusted estimates of PAR may be biased (*27*).

The use of simulation techniques provides a simple but robust approach to accommodate asymmetric component distributions and account for uncertainty in our final estimates of the magnitude of foodborne *Campylobacter* infection in the community. Rather than calculate a single point estimate for the number of cases attributable to each foodborne risk factor, a simulated distribution of credible values was generated to model the uncertainty for each component in our calculations. Generating 95% CrIs enabled us to confer a degree of confidence around our estimates.

Intercountry comparison of foodborne disease incidence is difficult without standardization of methods; however, the approach taken in this study may allow those countries that have the available data to conduct similar studies. Furthermore, this model could be adopted or applied more widely to other foodborne and nonfoodborne pathogens under surveillance and enable calculation of population estimates of the magnitude of infection associated with specific risk factors.

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Influenza A Virus (H3N8) in Dogs with Respiratory Disease, Florida

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In 2004, canine influenza virus subtype H3N8 emerged in greyhounds in the United States. Subsequent serologic evidence indicated virus circulation in dog breeds other than greyhounds, but the virus had not been isolated from affected animals. In 2005, we conducted virologic investigation of 7 nongreyhound dogs that died from respiratory disease in Florida and isolated influenza subtype H3N8 virus. Antigenic and genetic analysis of A/canine/Jacksonville/2005 (H3N8) and A/canine/Miami/2005 (H3N8) found similarity to earlier isolates from greyhounds, which indicates that canine influenza viruses are not restricted to greyhounds. The hemagglutinin contained 5 conserved amino acid differences that distinguish canine from equine lineages. The antigenic homogeneity of the canine viruses suggests that measurable antigenic drift has not yet occurred. Continued surveillance and antigenic analyses should monitor possible emergence of antigenic variants of canine influenza virus.

Influenza A viruses (family *Orthomyxoviridae)* are known to cause acute respiratory disease in humans, horses, pigs, and domestic poultry (*1*,*2*). Influenza A virus subtype H3N8 has recently emerged as a respiratory pathogen in dogs, associated with outbreaks of acute respiratory disease in racing greyhounds (*3*). The disease is caused by a novel virus closely related to contemporary equine influenza A virus subtype H3N8. These viruses share ≥96% nucleotide sequence identity, which suggests direct transmission of the entire virus from horses to dogs without reassortment with other strains (*3*).

Canine influenza virus (CIV) was first identified in racing greyhounds in Florida in January 2004 and was later associated with respiratory disease outbreaks in racing greyhounds in 9 states from 2004 through 2006 (*3*,*4*). Most affected greyhounds had clinical signs associated with virus infection of the upper respiratory tract—cough for 10–30 days, nasal discharge, low-grade fever—followed by recovery. However, some dogs died peracutely with extensive hemorrhage in the lungs, mediastinum, and pleural cavity. Histologic examination showed tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia associated with extensive erosion of epithelial cells and infiltration with neutrophils. The isolation of 4 closely related influenza A subtype H3N8 viruses from dogs that died in different geographic locations over a 25-month period, together with substantial serologic evidence of widespread infection among racing greyhounds in 9 states, suggested sustained CIV circulation in this population by dog-to-dog transmission (*3*,*4*).

The first evidence of CIV infection in dogs other than greyhounds came from serologic testing of dogs with acute respiratory disease in shelters, boarding kennels, and veterinary clinics in Florida and New York in 2004 and 2005 (*3*). Since August 2005, a national syndromic serosurvey for canine influenza has been conducted on >5,000 samples collected from nongreyhound dogs with compatible clinical signs (Cornell University College of Veterinary Medicine, http://diaglab.vet.cornell.edu/issues/civ-stat.asp). As of April 2008, seropositive dogs have been identified in 25 states and the District of Columbia.

In April and May 2005, an outbreak of respiratory disease occurred in dogs housed in a shelter facility in northeastern Florida (*3*). The outbreak involved at least 58 dogs, ranging in age from 3 months to 9 years, and included purebred dogs as well as mixed breeds; 6 were euthanized. In May 2005, a respiratory disease outbreak occurred among ≈40 pet dogs at a veterinary clinic in southeastern Florida; 1 died. We performed molecular analyses on 2 influenza

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A subtype H3N8 viruses isolated from these 7 nongreyhound dogs that died and genetically and antigenically compared them with influenza (H3N8) viruses from racing greyhounds.

Materials and Methods

Specimen Collection

Postmortem examinations were performed on the 6 mixed-breed shelter dogs that died in April and May 2005 and on the 1 pet Yorkshire terrier that died in the veterinary clinic in May 2005. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin; 5-μm sections were stained with hematoxylin and eosin for histopathologic diagnosis. Unfixed tissues for virologic and molecular analyses were stored at –80°C.

RNA Extraction

Frozen lung tissues from each dog were thawed and homogenized in lysis buffer containing β-mercaptoethanol by using a disposable tissue grinder (Kendall, Lifeline Medical Inc., Danbury, CT, USA). Total RNA was extracted by using a commercial kit (RNeasy Mini Kit, QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instructions and eluted in a final volume of 60 μL of buffer. Total RNA was also extracted from lung tissue collected from specific-pathogen–free dogs without respiratory disease.

Real-Time Reverse Transcription–PCR

A single-step quantitative real-time reverse transcription–PCR (RT-PCR) was performed on total RNA extracted from the canine tissue samples by using the QuantiTect Probe RT-PCR Kit containing ROX as a passive reference dye (QIAGEN). Briefly, 2 primer-probe sets were used for detection of influenza A sequences in each sample (Table 1). One primer-probe set was selective for canine influenza subtype H3 gene sequences. The other primer-probe set targeted a highly conserved region of the matrix (M) gene of type A influenza virus. The sequence of the M probe contained 3 locked nucleic acids. For each real-time RT-PCR, 5 μL of total RNA was added to a reaction mixture containing 12.5 μL of 2× QuantiTect Probe RT-PCR Master Mix, 0.25 μL of QuantiTech RT Mix (both QIAGEN), forward and reverse primers (0.4 μmol/L final concentration for each), probe (0.1 μmol/L final concentration), and RNase-free water in a final volume of 25 μL. Real-time PCR for eukaryotic 18S rRNA was performed by using commercially available assay reagents (VIC/TAMRA; TaqMan, Applied Biosystems, Foster City, CA, USA), according to manufacturer's instructions for detection of endogenous 18S rRNA, as an internal control for RNA extraction from the tissues.

Quantitative one-step real-time RT-PCR was performed on the reaction mixtures in an Mx3000P QPCR System (Stratagene, La Jolla, CA, USA). Cycling conditions were a reverse transcription step at 50°C for 30 min, an initial denaturation step at 95°C for 15 min to activate the HotStarTaq DNA Polymerase (QIAGEN), and amplification for 40 cycles. Each amplification cycle included denaturation at 94°C for 15 s, followed by annealing/extension at 60°C for 1 min. The FAM (emission wavelength 516 nm) and VIC (emission wavelength 555 nm) fluorescent signals were recorded at the end of each cycle. The threshold cycle (Ct) was determined by setting the threshold fluorescence at 1,000 for each individual experiment. The Mx3000P version 2.0 software program (Stratagene) was used for data acquisition and analysis. The results were normalized by calculating H3 Ct or M Ct to 18S rRNA Ct ratios for each sample. Samples were considered positive for influenza A virus when the normalized H3 or M Ct ratio was 3 U smaller than the normalized Ct ratio for lung tissues from dogs without respiratory disease. The positive control was amplified RNA that had been extracted from A/canine/Florida/242/2003 (H3N8) virus grown in MDCK cells (*3*).

Virus Isolation

Inoculation of Cell Culture

Frozen lung tissues from each of the 7 dogs were thawed and homogenized in 10 volumes of Dulbecco modified Eagle medium (DMEM) supplemented with 0.5% bovine serum albumin and gentamicin and ciprofloxacin. Solid debris was removed by centrifugation, and supernatants

Table 1. Primers and probes for identification of canine influenza virus (H3N8) in tissues by quantitative real-time reverse transcription–PCR*

Primer	Target	Sequence	Application
Ca-H3-F387	H3 (nt 387–406)	5′-tatgcatcgctccgatccat-3′	Forward primer for H3
Ca-H3-R487	H3 (nt 487–467)	5′-gctccacttcttccgttttga-3′	Reverse primer for H3
Ca-H3-P430	H3 (nt 430–459)	FAM-aattcacagcagagggattcacatggacag-BHQ1	TaqMan probe
FluA-M-F151	M (nt 151–174)	5′-catggartggctaaagacaagacc-3′	Forward primer for M
FluA-M-R276	M (nt 276–253)	5′-agggcattttggacaaakcgtcta-3′	Reverse primer for M
FluA-M-P218	M (nt 218–235)	FAM-acgcTcaccgTgcccAgt-BHQ1	TaqMan probe

*Conserved regions of the matrix (M) and hemagglutinin 3 (H3) genes of canine influenza virus were selected. The underlined r represents a mixture of a and g nucleotides at this position in the oligonucleotide; the underlined k represents mixtures of g or t; uppercase letters in the sequence for the M probe represent locked nucleic acids.

were inoculated onto MDCK cells cultured in DMEM supplemented with 1 μg/mL TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)–treated trypsin (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.35% bovine serum albumin, and antimicrobial drugs. Cells were grown in 25-cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were observed daily for morphologic changes and harvested 3 days after inoculation. The harvested cultures were clarified by centrifugation, and the supernatants were inoculated onto fresh MDCK cells as described for the initial inoculation; up to 3 additional passages were performed for samples that did not show evidence of influenza virus by hemagglutination or RT-PCR. Hemagglutination activity in the clarified supernatants was determined by using 0.5% turkey red blood cells as described (*5*,*6*). RT-PCR was performed as described below.

Inoculation of Embryonated Chicken Eggs

Frozen lung tissues were homogenized as described above for inoculation of MDCK cells by using phosphate-buffered saline instead of DMEM, and a volume of 0.2 mL was inoculated into the allantoic sac of 10-day old embryonated chicken eggs. After 48 h of incubation at 35°C, the eggs were chilled at 4°C overnight before the allantoic fluid was harvested. Hemagglutination activity in the clarified allantoic fluid supernatants was determined by using 0.5% turkey red blood cells as described (*5*,*6*). RT-PCR was performed as described below. Harvested allantoic fluid samples lacking evidence of influenza virus were reinoculated up to 2 more times in embryonated eggs and evaluated as described.

RT-PCR, Nucleotide Sequencing, and Phylogenetic Analyses

Virus RNA was extracted from MDCK culture supernatant or allantoic fluid by using the QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. The virus RNA was reverse transcribed to cDNA by using the QIAGEN OneStep RT-PCR Kit according to manufacturer's instructions. PCR amplification of the coding region of the 8 influenza virus genes in the cDNA was performed as described (*7*) by using universal gene-specific primer sets (*3*). The resulting DNA amplicons were used as templates for automated sequencing in the ABI PRISM 3100 automated DNA sequencer by using cycle sequencing dye terminator chemistry (Applied Biosystems). Nucleotide sequences were analyzed by using the Lasergene 6 Package (DNASTAR, Inc., Madison, WI, USA). The PHYLIP Version 3.5 software program was used to estimate phylogenies and calculate bootstrap values from the nucleotide sequences (*8*). Phylogenetic trees were compared with those generated by neighbor-joining analysis with the Tamura-Nei model implemented in the MEGA3 program (*9*) and confirmed by the PAUP 4.0 Beta program (Sinauer Associates, Inc., Sunderland, MA, USA). The complete genome sequences from the 2 new reported CIV isolates (A/canine/Miami/2005 and A/canine/Jacksonville/2005) were deposited in GenBank under accession nos. EU402407–EU402408 and EU534193–EU534204.

Hemagglutination Inhibition Assay

Convalescent-phase immune serum samples were obtained from horses and dogs naturally infected with influenza A virus (H3N8) in 2005. Antiserum from ferrets infected with A/canine/Florida/43/2004 (H3N8) was prepared as described (*3*). All serum samples were incubated with receptor-destroying enzyme (DENKA SEIKEN Co., Ltd., Tokyo, Japan) (1 part serum: 3 parts receptor-destroying enzyme) for 16 h at 37°C before heat inactivation for 30 min at 56°C. Influenza A/canine/Jacksonville/2005 virus (H3N8) was grown in MDCK cells for 72 h at 37°C in 5% CO_2. Virus culture supernatants were harvested, clarified by centrifugation, and stored at –80°C. All other canine and equine viruses used in the hemagglutination inhibition (HI) assay were grown in 10-day old embryonated chicken eggs from which allantoic fluid was collected after 72 h and stored at –80°C. The HI assay was performed as described (*6*). On the basis of assay results from serum of uninfected specific-pathogen–free dogs with HI titers $\leq$4, HI titers $\geq$32 were considered as evidence of previous exposure to CIV.

Results

Clinical Findings

Among the 58 affected shelter dogs, the most common clinical signs were low-grade fever, purulent nasal discharge, and cough for 10–21 days. Paired acute- and convalescent-phase serum samples were collected from 5 dogs and tested for CIV-specific antibodies by using the HI assay. All the dogs seroconverted to A/canine/Florida/43/2004 (H3N8) and had an increase in the geometric mean antibody titer (GMT) from 37 in the acute phase to 626 in the convalescent phase. Single serum samples were collected from another 18 dogs that had had clinical disease for at least 7 days, and 17 (94%) were seropositive for A/canine/Florida/43/2004 (H3N8); HI antibody titers ranged from 32 to 2,048 and GMT was 533. Pneumonia developed in at least 10 dogs, of which 6 were euthanized and submitted for postmortem examination. These 6 mixed-breed dogs were 3 males and 3 females ranging in age from 4 months to 3 years. The duration of clinical signs at the time of euthanasia was 2–10 days.

Among the ≈40 affected pet dogs at the veterinary clinic, the most common clinical signs were low-grade fever, purulent nasal discharge, and cough for 10–30 days. Paired acute- and convalescent-phase serum samples were

collected from 19 dogs and tested for CIV-specific antibodies by using the HI assay. Of these, 11 (58%) dogs seroconverted to A/canine/Florida/43/2004 (H3N8), and the GMT increased from 9 in the acute phase to 329 in the convalescent phase. Single serum samples were collected from another 9 dogs that had had clinical disease for at least 7 days, and 6 (67%) were seropositive for A/canine/Florida/43/2004 (H3N8); HI antibody titers ranged from 64 to 512 and GMT was 228. Pneumonia developed in 3 dogs; 1, a 9-year-old male Yorkshire terrier, died 3 days after onset of clinical signs and was submitted for postmortem examination.

Postmortem examinations showed that all 7 dogs had tracheitis and bronchitis with inflammatory changes that involved submucosal glands. Tracheitis and bronchitis were characterized by surface and glandular epithelial necrosis and hyperplasia with infiltration by lymphocytes, neutrophils, and macrophages. Suppurative bronchopneumonia was found in 2 of the shelter dogs and the pet dog; histologically identified bacteria in the lesions indicated a bacterial contribution to the pneumonic lesions. None of the dogs had intrathoracic or pulmonary hemorrhage.

Laboratory Diagnosis

Lung tissues from the 7 dogs were analyzed by quantitative real-time RT-PCR assays that detect the M gene of influenza A and the H3 gene of CIV. The M and H3 genes were amplified from the lungs of all 7 dogs, which confirmed the presence of CIV (Table 2). Lung tissue from the specific-pathogen–free dogs did not show evidence of amplification of influenza virus genes. MDCK cultures were inoculated with these lung homogenates to identify viable influenza virus. Pathologic cell damage was noted in the MDCK cell monolayer on the third passage of the lung homogenate from 1 of the shelter dogs that died after 3 days of pneumonia. Influenza A virus (later identified as subtype H3N8) was recovered from the supernatant, and the isolate was named A/canine/Jacksonville/2005. After 2 passages in embryonated chicken eggs, influenza A virus (later identified as subtype H3N8) was recovered from the lung homogenate of the pet dog that also died of pneumonia 3 days after onset. This virus was named A/canine/Miami/2005. These isolates provided virologic evidence of CIV infection in nongreyhound dogs.

Genetic Analyses of the Canine Influenza A (H3N8) Isolates

Sequence analyses of A/canine/Jacksonville/2005 and A/canine/Miami/2005 showed that their H3 gene nucleotide sequences were 98% identical to those of the A/canine/Florida/242/2003, A/canine/Florida/43/2004, A/canine/Texas/2004, and A/canine/Iowa/2005 isolates recovered from the lungs of racing greyhounds that died of pneumonia during influenza outbreaks in 2004 and 2005 (*3*,*4*). Phylogenetic comparisons of the H3 genes showed that the A/canine/Jacksonville/2005 and A/canine/Miami/2005 viruses from nongreyhound dogs clustered with the greyhound isolates and contemporary equine isolates, forming a distinct group from the older equine viruses isolated in the early 1990s (Figure, panel A). Furthermore, the A/canine/Jacksonville/2005, A/canine/Miami/2005, and A/canine/Iowa/2005 isolates were more closely related to A/canine/Texas/2004 than to either A/canine/Florida/43/2004 or A/canine/Florida/242/2003. The H3 genes from the 2005 isolates formed a subgroup that appeared to branch off from the earlier 2003 and 2004 canine viruses with nucleotide differences at 10 sites. Most nucleotide changes are silent, as can be appreciated by the shorter branch lengths in the phylogenetic tree constructed from deduced amino acid sequence data (Figure, panel B).

Amino Acid Sequence of the CIV H3 Hemagglutinin

To identify changes with possible functional significance with regard to antigenicity or receptor binding, we compared the deduced amino acid sequences of the H3 hemagglutinins from the 6 available CIV isolates. All 6

Table 2. Quantitative real-time reverse transcription–PCR and virus isolation from tissue specimens*

Dog	Location	Duration of clinical disease, d	Real-time reverse transcription–PCR					Virus isolation
			18S rRNA (Ct)	M (Ct)	M:rRNA	H3 (Ct)	H3:rRNA	
1079	Shelter	2	26.18	29.81	1.14	28.84	1.10	NVD
1078	Shelter	3	26.82	30.37	1.13	29.71	1.11	MDCK cells 3rd passage
1080	Shelter	6	24.17	38.87	1.61	38.23	1.58	NVD
319	Shelter	6	24.94	33.87	1.36	32.23	1.29	NVD
318	Shelter	9	23.54	33.89	1.44	32.97	1.40	NVD
320	Shelter	10	23.91	39.44	1.65	37.09	1.55	NVD
374	Veterinary clinic	3	22.89	24.05	1.05	22.65	0.99	Egg 2nd passage
A/canine/Florida/242/2003			29.37	28.15	0.96	27.36	0.93	NA
Normal dog lung			20.78	>40	>1.92	>40	>1.92	NA

*Ct, cycle threshold; NVD, no virus detected; NA, not applicable; M:rRNA and H3:rRNA ratios were calculated by dividing the matrix (M) or hemagglutinin 3 (H3) Ct by the 18S rRNA Ct.

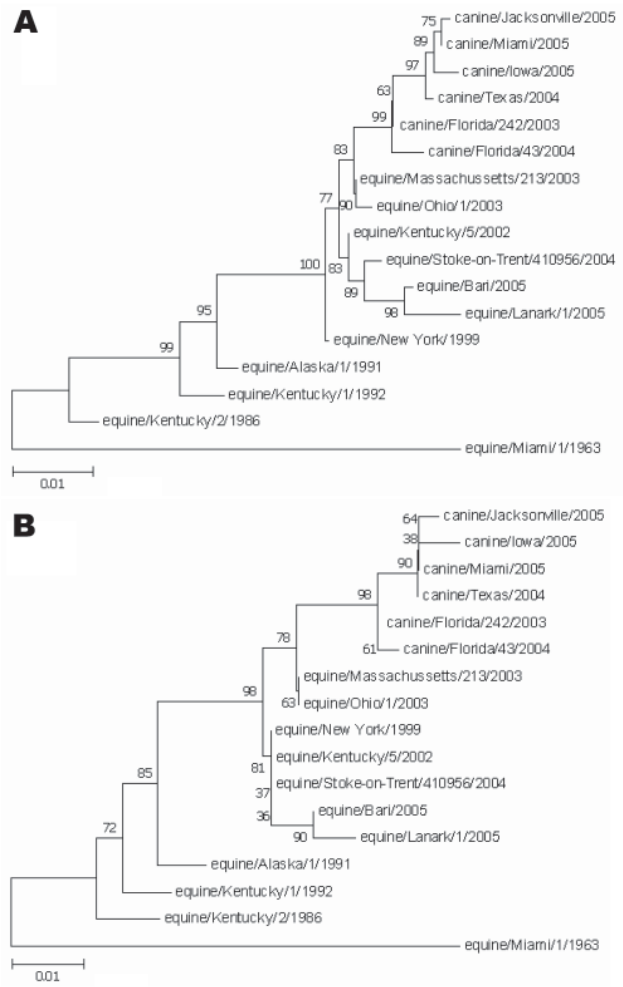

Figure. Phylogenetic relationships among the hemagglutinin 3 (H3) genes. A) Nucleotide tree of the canine influenza virus H3 genes with contemporary and older equine H3 genes. B) Amino acid tree of the canine influenza virus H3 protein with contemporary and older equine H3 proteins. Bootstrap analysis values ≥80% are shown. Scale bar indicates nucleotide or amino acid substitutions per site.

canine isolates had 5 conserved amino acid substitutions that differentiated them from contemporary equine influenza viruses (Table 3). These conserved substitutions (N54K, N83S, W222L, I328T, and N483T) can be considered as a signature of the circulating CIV H3 hemagglutinin. Phylogenetic comparisons of the mature H3 protein showed that the A/canine/Jacksonville/2005, A/canine/Miami/2005, and A/canine/Iowa/2005 viruses formed a subgroup with the A/canine/Texas/2004 isolate (Figure, panel B). Three amino acid changes (L118V, K261N, and G479E) differentiated this subgroup from the earlier A/canine/Florida/43/2004 and A/canine/Florida/242/2003 isolates (Table 3). Finally, A/canine/Jacksonville/2005 differed from A/canine/Miami/2005 at a single amino acid, S107P.

Antigenic Analyses of the Canine Influenza A (H3N8) Isolates

HI tests were performed by using an antigen panel of previously circulating and contemporary equine influenza viruses and the available CIVs as well as convalescent-phase immune serum from horses and dogs naturally infected with influenza virus (H3N8) in 2005 (Table 4). An antiserum from ferrets infected with A/canine/Florida/43/2004 was also included in the analyses. HI antibody titers in equine serum were 2-fold to 16-fold higher with contemporary equine viruses (1999–2003) than with older isolates (1963–1992). The heterologous titers of equine serum to canine viruses were generally similar to the homologous values for the contemporary equine viruses. The canine serum failed to substantially inhibit hemagglutination by the older equine influenza viruses (1963–1992), but the antibody titers to contemporary equine isolates (1999–2003) and the canine isolates were comparable. Similar results were observed for serum from ferrets infected with CIV. These patterns of inhibition demonstrated the antigenic similarity between the CIVs and contemporary equine influenza viruses and were consistent with the phylogenetic analyses. The antibody titers to the A/canine/Miami/2005 isolate in equine, canine, and ferret serum were similar to those for the 2003 and 2004 canine isolates, which indicates that the amino acid substitutions in the isolates did not result in measurable antigenic drift. The antibody titers to the A/canine/Jacksonville/2005 isolate were, in general, 2- to 4-fold lower than those to A/canine/Florida/43/2004 and other canine viruses, which suggests a potential antigenic difference.

Discussion

The genetic and phylogenetic analyses of influenza A subtype H3N8 viruses recovered from racing greyhounds affected by respiratory disease outbreaks and fatal pneumonia in 2003 and 2004 have been described (*3*). These greyhound influenza viruses were most homologous to contemporary equine influenza A subtype H3N8 viruses isolated from horses in 2002 and 2003. Although serologic evidence of influenza virus infection in nongreyhound dogs was reported (*3*), whether these infections were caused by the same virus that infected the greyhound dogs is not clear. The influenza subtype H3N8 viruses that we describe in this report came from nongreyhound dogs involved in fatal canine influenza outbreaks independent of any known outbreaks in greyhounds.

Because viral hemagglutinin is a critical determinant of the host specificity of influenza virus (*10*), we compared the nucleic acid and deduced amino acid sequences for the canine and equine H3 to identify residues that may be associated with efficient replication in different species or dog breeds. The 5 conserved amino acid substitutions in all

Table 3. Amino acid differences among the hemagglutinin proteins of canine and contemporary equine influenza viruses*

	Amino acid positions in mature HA																	
Virus	7†	29	**54**	78	79	**83**	92	107	118	159	218	**222**	261	**328**	479	**483**	492	541
A/equine/Kentucky/5/2002	G	I	N	V	F	N	S	S	L	N	G	W	K	I	G	N	R	K
A/equine/Massachusetts/213/2003	.	.	.	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/equine/Ohio/1/2003	.	.	.	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/equine/Bari/2005	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/canine/Florida/242/2003	D	.	**K**	A	.	**S**	.	.	.	S	.	**L**	.	**T**	.	**T**	.	.
A/canine/Florida/43/2004	.	M	**K**	A	.	**S**	N	.	.	S	.	**L**	.	**T**	.	**T**	.	R
A/canine/Texas/1/2004	.	M	**K**	A	.	**S**	.	.	**V**	S	.	**L**	**N**	**T**	**E**	**T**	.	.
A/canine/Iowa/2005	.	M	**K**	A	**L**	**S**	.	.	**V**	S	**E**	**L**	**N**	**T**	**E**	**T**	.	.
A/canine/Miami/2005	.	M	**K**	A	.	**S**	.	.	**V**	S	.	**L**	**N**	**T**	**E**	**T**	K	.
A/canine/Jacksonville/2005	.	M	**K**	A	.	**S**	.	P	**V**	S	.	**L**	**N**	**T**	**E**	**T**	K	.

*Dots represent the same amino acids as in A/equine/Kentucky/5/2002 hemagglutinin (HA). Conserved residues in the canine HA that differentiate them from the equine HA are in **boldface** within blue-shaded boxes. Residues that differentiate the A/canine/Texas/2004 clade from A/canine/Florida/242/2003 and A/canine/Florida/43/2004 are in **boldface** within gray-shaded boxes.
†Only variable positions are shown; numbering is for mature HAO.

the canine viruses differentiated them from contemporary equine viruses. The substitution of lysine for asparagine at position 54 maintains the positive charge of the residue and is of unknown functional significance. Position 83 located within antigenic site E of human H3 has been implicated in antigenic drift (*11*). The substitution of serine for asparagine at position 83 in canine H3 maintains the polar nature of the residue, but the functional significance in the evolution of canine influenza is not immediately apparent. The substitution of leucine for tryptophane at position 222 represents a nonconservative change adjacent to the sialic acid–binding pocket, which suggests a potential modulator function in adaptation of equine influenza virus to canine sialic acid receptors. This leucine substitution has also been reported in avian influenza subtype H4 infection of pigs (*12*) and subtype H9 infection of humans (*13*). The strictly conserved isoleucine at position 328 near the cleavage site of the equine H3 hemagglutinin has been replaced by threonine in all the canine isolates, which suggests the potential importance of threonine for recognition of the hemagglutinin cleavage site by canine proteases. The replacement of asparagine with threonine at position 483 results in the loss of a glycosylation site in the hemagglutinin 2 (HA2) subunit. This glycosylation site is conserved in all other hemagglutinin subtypes (*14*).

The phylogenetic tree of the canine and equine influenza H3 genes shows that the canine and equine lineages have diverged considerably. The hemagglutinin sequences from the 2004 and 2005 equine influenza isolates belong to the Florida sublineage and do not have the mutations found in the canine strains. The H3 genes from the 2 Florida 2005 canine isolates formed a clade with high bootstrap support that included A/canine/Texas/2004 and A/canine/Iowa/2005. Three amino acid substitutions in H3—L118V, K261N, and G479E—differentiated this group from the earlier isolates. Positions 118 and 261 are in the hemagglutinin 1 (HA1) subunit of canine H3; position 479 is in the HA2 subunit. The HA1 subunit of human H3 contains the antibody-binding sites where amino acid substitutions occur at high frequency, presumably the result of escape

Table 4. Hemagglutination inhibition antibody titers to older and contemporary equine viruses and canine influenza viruses*

	Serum sample no.						
	Equine†			Canine‡			Ferret§
Virus	65694	73147	84376	13	25	27	A/canine/Florida/43/2004
A/equine/Miami/63	40	40	160	<10	<10	10	16
A/equine/Kentucky/86	40	40	160	10	40	40	32
A/equine/Kentucky/92	40	20	80	<10	<10	10	32
A/equine/NewYork/99	320	40	320	40	160	40	128
A/equine/Kentucky/05/2002	320	40	320	40	160	160	256
A/equine/Massachussetts/213/2003	640	80	320	40	160	160	512
A/equine/Ohio/01/2003	640	80	320	80	320	160	512
A/canine/Florida/242/2003	160	40	320	40	160	160	512
A/canine/Texas/2004	160	40	320	40	160	160	512
A/canine/Florida/43/2004	160	40	320	40	160	80	512
A/canine/Miami/2005	320	40	320	40	160	80	256
A/canine/Jacksonville/2005	40	10	80	20	40	40	128

*The antibody titer is the reciprocal of the highest serum dilution that inhibited hemagglutination by the virus.
†Serum from horses infected with equine influenza virus in 2005.
‡Serum from dogs infected with canine influenza virus in 2005.
§Serum from ferrets infected with A/canine/Florida/43/2004 (H3N8).

from humoral immune responses (*11*,*15*,*16*). Although no evidence supports high variability at positions 118 and 261, neighboring positions 121 (antigenic site D) and 262 (antigenic site E) are sites of frequent positive selection in human H3 genes (*11*). The 2005 isolates from nongreyhound dogs differed from each other by substitution of proline for serine at position 107 in the HA1 subunit of A/canine/Jacksonville/2005. Serine is conserved at position 107 in all other equine and canine isolates except for A/equine/Jilin/1/89, which has a threonine substitution (*17*). A/canine/Jacksonville/2005 has potentially significant antigenic variation from the other canine isolates, which may be partially related to the proline substitution at position 107. However, no serologic evidence indicates that residue 107 modulates the antigenicity of human, porcine, or equine H3 hemagglutinins.

The HI results showed antigenic similarity between the canine and contemporary equine influenza viruses and were in agreement with the phylogenetic clustering of canine hemagglutinin sequences near the equine counterparts. The amino acid and antigenic differences between canine and equine hemagglutinin illustrate the ongoing process of divergent evolution of the canine viruses from the ancestral equine viruses. These differences also support the hypothesis that CIV is a separate virus lineage, which is efficiently maintained in the dog population by horizontal dog-to-dog transmission.

With the introduction and sustained transmission of influenza virus (H3N8) in dogs, the H3 hemagglutinin became the most broadly distributed subtype in mammalian species, including humans, swine, horses, and dogs. Continued virologic and serologic surveillance will be important for monitoring the evolution of CIV and its health effects in dogs as well as the possible transmission to other species, including humans.

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Tuberculosis from *Mycobacterium bovis* in Binational Communities, United States

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The epidemiology of tuberculosis (TB) in the United States is changing as the incidence of disease becomes more concentrated in foreign-born persons. *Mycobacterium bovis* appears to be contributing substantially to the TB incidence in some binational communities with ties to Mexico. We conducted a retrospective analysis of TB case surveillance data from the San Diego, California, region from 1994 through 2005 to estimate incidence trends, identify correlates of *M. bovis* disease, and evaluate risk factors for deaths during treatment. *M. bovis* accounted for 45% (62/138) of all culture-positive TB cases in children (<15 years of age) and 6% (203/3,153) of adult cases. *M. bovis* incidence increased significantly (p = 0.002) while *M. tuberculosis* incidence declined (p<0.001). Almost all *M. bovis* cases from 2001 through 2005 were in persons of Hispanic ethnicity. Persons with *M. bovis* were 2.55× (p = 0.01) as likely to die during treatment than those with *M. tuberculosis*.

The pattern of tuberculosis (TB) in the United States is changing as the incidence of TB disease becomes more concentrated in foreign-born persons. Of the annual total US TB cases, >54% are now concentrated in persons born outside of the United States (*1*); in communities with high immigration, the proportion can exceed 70% (*2*). TB prevention and treatment strategies, particularly those in communities on the border with Mexico, will need to be adapted to accommodate the changing epidemiology of TB (*3*).

San Diego, California, together with its sister city Tijuana-Tecate, Mexico, is the largest binational metropolitan region in the United States, accounting for 34% of the southern border population (*4*). In San Diego County, the Hispanic population has grown from 20% to 29% in the past 15 years (*5*). Of the total annual TB cases in San Diego, >70% occurred among foreign-born persons, of whom nearly half originated from Mexico (*2*). A review of culture-positive TB cases in San Diego County in the late 1990s indicated that 6.6% of all adult TB cases and 39% of all pediatric (<15 years of age) TB cases from this region were not caused by *Mycobacterium tuberculosis*, the most common TB pathogen in the United States, but were instead caused by *M. bovis*, a pathogen more often associated with TB in cattle (*6*). This finding represented the highest reported proportional incidence of TB from *M. bovis* among industrialized countries (*7*).

M. bovis is a pathogen in the complex of bacteria that includes *M. tuberculosis*, which causes TB in humans and animals. TB from *M. bovis* has been generally considered rare in the United States after its successful eradication from cattle in the mid-1900s (*8*), but wider use of laboratory tools for species-level diagnosis of TB pathogens has started to shed light on an unexpected regional presence of *M. bovis* in communities with large Hispanic populations. While *M. bovis* TB has been most often documented in Hispanic communities with close proximity to Mexico (*6*,*9*), a recent review of *M. bovis* cases in New York City indicates that the problem is not limited to US regions that border Mexico (*10*).

The clinical and pathologic characteristics of *M. bovis* TB is indistinguishable from *M. tuberculosis* TB in most cases, but there are relevant considerations for prevention and treatment strategies in communities where *M. bovis* contributes to TB incidence. First, *M. bovis* is thought to be spread to humans primarily through consumption of raw

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dairy products and inhalation of infectious droplets from cattle (*11*,*12*), with only minimal human-to-human transmission (*13*). Second, *M. bovis* is almost universally resistant to the key antituberculous drug pyrazinamide (PZA), which necessitates a 9-month treatment duration instead of the standard 6-month, short-course therapy, which is possible with PZA in the treatment regime. Third, higher mortality rates during treatment may be associated with *M. bovis* (*14*).

Multidrug-resistant (MDR) strains of *M. bovis* (*15*–*17*), the high proportional incidence of *M. bovis* (*9*,*18*) in pediatric TB cases, and frequent HIV co-infection (*19*) are important additional considerations in developing effective treatment and prevention strategies for *M. bovis*. To document the trends and the effect of *M. bovis* on TB epidemiology, we examined TB case surveillance data from 1994 through 2005 in San Diego County and identified risk factors related to *M. bovis* disease and deaths during treatment in the last 5 years.

Methods

Data Sources

This study used routine TB surveillance data from 1994 through 2005. The study protocol was approved by the Institutional Review Boards of San Diego State University and the University of California, San Diego. Demographic and clinical data were obtained from the Tuberculosis Information Management System (TIMS) database maintained by the San Diego County TB Control Program. Since the early 1990s, a TB isolate has been submitted to the county public health laboratory for every reported TB case. All TB isolates from patient specimens were initially identified as *M. tuberculosis* complex on the basis of the AccuProbe hybridization protection assay (GenProbe, San Diego, CA, USA). Specimens were further identified as either *M. bovis* or *M. tuberculosis* on the basis of culture morphologic findings, the results of the niacin strip test, the nitrate reduction test, and the specimens' susceptibility to PZA (*20*). Furthermore, all isolates identified as *M. bovis* from 2004 and 2005 were confirmed to have spoligotypes consistent with *M. bovis* (*21*). Population data for San Diego County were obtained from San Diego Association of Governments' estimates based on census and calculated data.

Study Design

We conducted a retrospective trend analysis of all culture-positive TB cases in the San Diego County TIMS database from 1994 through 2005 that were confirmed as either *M. bovis* or *M. tuberculosis*. We also conducted a detailed retrospective analysis of demographic and clinical variables associated with *M. bovis* case-patients and deaths during treatment from 2001 through 2005.

Demographic variables from the TIMS database used in the correlates and mortality analyses included sex, age, ethnicity, and country of birth. Clinical variables included: previous history of TB disease, presence or absence of pulmonary disease, presence or absence of multisite disease, presence or absence of acid-fast bacilli (AFB) in sputum smear, presence or absence of pulmonary lesions by chest radiograph, presence or absence of MDR TB, and HIV status.

Analysis

Trends

Trends in TB incidence were evaluated by using Poisson regression with time in years as the predictor variable, case number as the dependent variable, and population size as an additional exposure variable. Trend lines for *M. bovis* and *M. tuberculosis* were based on incidence predicted by Poisson regression fitted to the data. Trends in proportional incidence of *M. bovis* cases (relative to all TB cases) were assessed with a χ^2 test for trend.

Correlates of *M. bovis* Disease

Demographic and clinical variables shown previously to be associated with TB diagnoses (*6*) were compared between *M. bovis* and *M. tuberculosis*. Variables significant at the 5% level by χ^2 test in univariate analyses were entered into a multiple logistic regression model. The final model was derived by using the likelihood ratio method (*22*).

Analysis of Mortality Rates during Treatment

All deaths that occurred from the time that a TB case was reported until treatment was completed were documented with death certificates and recorded in TB case files. For the purposes of this study, causes of death in *M. bovis* and *M. tuberculosis* case files were transcribed from death certificates or California state death records and collated into 7 major causes of death based on the most common causes.

We investigated the apparently higher mortality rates during treatment among *M. bovis* cases relative to *M. tuberculosis* cases (*14*) by using a multiple logistic regression analysis with *M. bovis* as the exposure variable; death before treatment was completed as the outcome variable; and demographic and clinical variables as potential covariates. Univariate differences between causes of death in *M. bovis* and *M. tuberculosis* cases were analyzed with the Fisher exact test.

Results

Trends

Analysis of TB trends from 1994 through 2005 included 3,291 culture-positive cases of TB and excluded 806 cases (20%) that were based only on national and local clinical case definitions. Among all culture-positive cases, *M. bovis* was isolated in 8% (265/3,291) and *M. tuberculosis* in 92% (3,026/3,291). *M. bovis* accounted for 45% (62/138) of all culture-positive TB cases in children <15 years of age and 6% (203/3,153) of cases in adults (≥15 years of age). No cases of *M. bovis* occurred in children <12 months of age.

During the period under study, incident cases of *M. bovis* TB increased linearly (p = 0.002; Figure) at 4.1% per year from 17 cases (0.65/100,000 population) to 28 cases (0.93/100,000) per year. TB cases from *M. tuberculosis* declined in a nonlinear fashion (p<0.001) from 317 cases per year (12.1/100,000 population) to 221 cases (7.33/100,000). The annual proportion of TB cases attributed to *M. bovis* increased from 5% of all culture-positive cases in 1994 to 11% in 2005 (p<0.001, Table 1). The proportion of TB cases with culture-positive results remained relatively stable at ≈81% of annual reported TB cases.

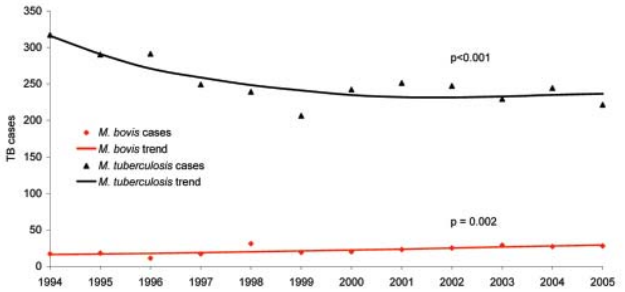

Figure. Trends in incidence of culture-positive tuberculosis (TB) cases from *Mycobacterium bovis* and *M. tuberculosis* in San Diego County, California, 1994–2005.

Correlates of *M. bovis* Disease

Of the 1,324 culture-positive TB cases reported from 2001 through 2005, *M. bovis* accounted for 10% (132/1,324), comprising 54% (29/54) of cases among children <15 years of age and 8% (103/1,270) among adults (≥15 years of age). Of the *M. bovis* TB cases, >96% were found in persons of Hispanic ethnicity, and 60% were among those of known Mexican origin. Univariate analysis indicated that sex, previous TB episode, and sputum AFB smear results were not significantly different between *M. bovis* and *M. tuberculosis* case-patients (Table 2). No MDR TB cases were identified among the *M. bovis* cases, whereas 1.5% of the *M. tuberculosis* cases had initial isolates that were MDR (defined as resistant to at least both isoniazid and rifampin). All of the *M. bovis* isolates were resistant to PZA, whereas 0.8% of the *M. tuberculosis* cases were PZA resistant. Of the 1,316 TB cases included in the multiple logistic regression model, factors associated with *M. bovis* disease included Hispanic ethnicity, multisite disease, being 5–14 years of age, and having extrapulmonary disease with a normal chest radiograph. HIV co-infection was not significantly more associated with *M. bovis* disease compared with *M. tuberculosis* (p = 0.08; Table 3).

Analysis of Mortality Rates during Treatment

Of 1,324 culture-positive TB case-patients, 1,119 were evaluated in the analysis of mortality rates during treatment. Fifteen percent (205/1,324) were excluded because of missing data on case survival, including patients who were lost to follow-up or moved during treatment. Of the 1,119 cases, 110 (19 *M. bovis* and 91 *M. tuberculosis*) patients died during TB treatment (n = 81) or before

Table 1. Proportional contribution of *Mycobacterium bovis* and *M. tuberculosis* to total culture-positive TB cases, San Diego County, California, 1994–2005*

Year	Total TB cases	Total no. (%) culture-positive TB cases†	No. (%)‡ *M. bovis* cases	No. (%)‡ *M. tuberculosis* cases
1994	420	334 (80)	17 (5)	317 (95)
1995	438	308 (70)	18 (6)	290 (94)
1996	384	302 (79)	11 (4)	291 (96)
1997	332	266 (80)	17 (6)	249 (94)
1998	342	270 (79)	31 (11)	239 (89)
1999	299	225 (75)	19 (8)	206 (92)
2000	295	262 (89)	20 (8)	242 (92)
2001	330	274 (83)	23 (8)	251 (92)
2002	317	272 (86)	25 (9)	247 (91)
2003	315	258 (82)	29 (11)	229 (89)
2004	320	271 (85)	27 (10)	244 (90)
2005	305	249 (82)	28 (11)	221 (89)

*TB, tuberculosis.
†Excludes 11 case-patients who had an isolate of *M. tuberculosis complex* resistant to pyrazinamide, but did not have species-level identification. Percent given is of all TB cases.
‡Percent given is of total culture-positive TB case-patients.

treatment was begun (n = 29). *M. bovis* patients who died during treatment were treated for a similar duration (mean 61 days, standard deviation [SD] 93.9) as *M. tuberculosis* patients (mean 60 days, SD 101.3).

No deaths during treatment were recorded in the pediatric *M. bovis* TB case-patients; 1 pediatric death was recorded among the *M. tuberculosis* TB treatment cases. In a multivariate analysis (n = 1,119), *M. bovis* patients were

Table 2. Univariate comparison of *Mycobacterium bovis* cases relative to *M. tuberculosis* cases, San Diego County, California, 2001–2005*

Characteristic	No. (%)† *M. bovis* cases, n = 132	No. (%)† *M. tuberculosis* cases, n = 1,192	Univariate p value
Sex			0.15
M	75 (56.8)	754 (63.2)	
F	57 (43.2)	438 (36.7)	
Age group, y			<0.001
0–4	15 (11.4)	10 (0.8)	
5–14	14 (10.6)	15 (1.3)	
15–24	21 (15.9)	156 (13.1)	
25–44	46 (34.8)	409 (34.3)	
45–64	20 (15.2)	356 (29.9)	
>64	16 (12.1)	246 (20.6)	
Race/ethnicity			<0.001‡
Hispanic	128 (96.9)	529 (44.3)	
White	3 (2.27)	151 (12.6)	
Asian	1 (0.75)	420 (35.2)	
Black	0	86 (7.2)	
Other	0	6 (1.0)	
Country of birth			<0.001
Mexico	79 (59.8)	382 (32.0)	
United States	53 (40.2)	302 (25.3)	
Philippines	0	248 (20.8)	
Other	0	260 (21.8)	
Previous TB			0.52
Yes	5 (3.8)	60 (5.0)	
No	127 (96.2)	1128 (94.6)	
Unknown§	0	4 (0.3)	
Sputum AFB smear result			0.16
Positive	40 (30.3)	590 (49.4)	
Negative	43 (32.5)	461 (38.6)	
Not done§	49 (37.1)	141 (11.8)	
Disease site			<0.001
Pulmonary	71 (53.7)	1031 (86.4)	
Extrapulmonary	61 (46.2)	161 (13.5)	
Clinical manifestations			<0.001
Single site disease	84 (63.6)	1036 (86.9)	
Multisite disease	48 (36.4)	156 (13.1)	
Chest radiograph lesions			<0.001
No lesions	55 (41.7)	123 (10.3)	
Pulmonary lesions consistent with TB	75 (56.8)	1063 (89.2)	
Unknown§	2 (1.5)	6 (0.5)	
HIV status			<0.001
Negative	48 (36.4)	611 (51.3)	
Positive	33 (25.0)	107 (9.0)	
Unknown	51 (38.6)	474 (39.8)	
Treatment outcome			0.02
Died before treatment completed	19 (14.3)	91 (7.6)	
Alive at end of treatment	102 (77.2)	913 (76.5)	
Unknown	11 (8.3)	188 (15.7)	

*TB, tuberculosis; AFB, acid-fast bacillus.
†Percentages do not always add up to 100% due to rounding.
‡Fisher exact p value.
§Not included in χ^2 calculation.

Table 3. Odds ratios from final logistic regression model of variables correlated with TB from *Mycobacterium bovis* versus *M. tuberculosis*, San Diego County, California, 2001–2005*

	M. bovis vs. *M. tuberculosis* (n = 130 vs. n = 1,186)	
Risk factors	OR (95% CI)	p value
Age group, y (ref ≥65 y)		0.002
0–4	2.43 (0.81–7.28)	0.11
5–14	4.38 (1.38–13.9)	0.01
15–24	1.06 (0.45–2.49)	0.90
25–44	0.68 (0.31–1.45)	0.32
45–64	0.50 (0.21–1.15)	0.10
Race/ethnicity (ref = white)		<0.001
Hispanic	7.97 (2.36–26.93)	<0.001
Asian	0.08 (0.01–0.76)	0.03
Black	0	0.99
Other	0	0.99
Extrapulmonary disease	4.51 (2.36–8.62)	<0.001
Normal chest radiograph results	3.16 (1.63–6.11)	<0.001
Multisite disease	4.31 (2.54–7.3)	<0.001
HIV status† (ref = negative)		0.13
Positive	1.75 (0.93–3.29)	0.08
Unknown	0.87 (0.48–1.58)	0.65

*n = 1,316. TB, tuberculosis; OR, odds ratio; CI, confidence interval; ref, referent.
†Variable not significant in multivariate analysis.

2.55× (p = 0.01) as likely to die before treatment completion than *M. tuberculosis* patients, after differences in age, race and ethnicity, country of birth, chest radiograph abnormalities, multisite disease, and HIV status were accounted for (Table 4). Univariate analyses of the causes of death in *M. bovis* and *M. tuberculosis* cases showed no significant differences (p>0.05) except for the category of "other noninfectious disease," which was overrepresented in the *M. tuberculosis* group (Table 5).

Discussion

From 1994 through 2005, incidence of *M. bovis* TB cases in San Diego County increased in absolute number, as a proportion of total TB cases, and relative to the population. In contrast, TB incidence caused by *M. tuberculosis* declined during the same period. *M. bovis* cases were concentrated in persons of Hispanic descent, especially those of Mexican origin, and among those <15 years of age, in whom *M. bovis* accounted for 45% of the culture-positive cases. Deaths during treatment were largely confined to adults and were twice as high in *M. bovis* TB case-patients when compared with *M. tuberculosis* patients.

Our findings indicate that the incidence of TB caused by *M. bovis* in southern California is substantially higher than the national rate of 1.5% estimated from TB surveillance data (*23*) but is similar to the proportional incidence (13%) among Mexican-born case-patients in New York, New York. It was previously hypothesized that TB attributed to *M. bovis* in San Diego is most likely being driven by recent infections in children and largely reactivated latent infections in adults, secondary to HIV co-infection (*6*). Our findings confirm the continued high incidence of *M. bovis* in children >12 months of age, but the role of HIV co-infection in *M. bovis* case-patients relative to *M. tuberculosis* cases is less clear.

Almost half of the culture-positive pediatric TB cases in this binational region of >3 million persons were caused by *M. bovis*, which has clinical implications. Since *M. bovis* is intrinsically resistant to PZA, a critical component of the standard 6-month, short-course treatment for *M. tuberculosis*, *M. bovis* treatment is usually extended to 9 months of isoniazid and rifampin (*14*). In southern California, and perhaps other Hispanic communities with close ties to Mexico, empiric extended TB treatment for children without culture-positive disease, particularly those with a history of consuming unpasteurized dairy products, should be considered.

Although one quarter of the *M. bovis* TB case-patients were co-infected with HIV, HIV was not significantly more associated with *M. bovis* TB compared with *M. tuberculosis* in our study or in the previous *M. bovis* study in this community (*6*). Confidence in this finding is somewhat limited because 40% of the TB case-patients did not have their HIV status reported. However, because most of the case-patients with an unknown HIV status were <15 or >55 years of age, the age groups at lowest risk for HIV, these missing data likely did not mask an association if one exists.

HIV co-infection plays a role in the epidemiology of adult *M. bovis* TB, but likely the growing Hispanic population with close ties to Mexico, and not HIV, is the major driving force behind the increasing number of *M. bovis* cases we observed. Given the long latency of this disease, the unclear role of airborne transmission in *M. bovis* TB (*13*),

Table 4. Factors associated with death before completion of TB treatment in TB patients, San Diego County, California, 2001–2005*

Risk factor	OR (95% CI)	p value
Mycobacterium bovis disease	2.55 (1.27–5.11)	0.01
HIV infection (ref = negative)		<0.001
Positive	4.50 (2.19–9.24)	<0.001
Unknown	2.09 (1.19–3.67)	0.01
Age group, y (ref = 0–4 y)		<0.001
5–14	Not defined	1.00
15–24	0.79 (0.06–9.88)	0.86
25–44	1.54 (0.17–13.78)	0.70
45–64	4.75 (0.55–41.12)	0.16
≥65	17.19 (2.04–145.01)	0.01
Country of birth (ref = United States)		<0.001
Mexico	0.52 (0.30–0.90)	0.02
Philippines	0.52 (0.28–0.97)	0.04
Other	0.25 (0.12–0.53)	<0.001
Chest radiograph lesions	2.21 (0.97–5.05)	0.06
Multisite disease	1.86 (1.10–3.15)	0.02

*n = 1,119. TB, tuberculosis; OR, odds ratio; CI, confidence interval; ref, referent.

Table 5. Primary causes of death before treatment completion in *Mycobacterium bovis* and *M. tuberculosis* TB case-patients (n = 110), San Diego County, California, 2001–2005*

Cause of death	No. (%) *Mycobacterium bovis* case-patients	No. (%) *M. tuberculosis* case-patients	p value†
Pulmonary TB	2 (11)	20 (22)	0.42
Extrapulmonary TB	3 (16)	8 (9)	0.58
Pulmonary NOS	0	8 (9)	0.41
HIV related	6 (32)	12 (13)	0.11
Cardiovascular	7(37)	18 (20)	0.19
Infectious (not TB)	1(5)	2 (2)	0.84
Other noninfectious disease	0	23 (25)	0.01
Total	19	91	

*TB, tuberculosis; NOS, not otherwise specified.
†Fisher exact test.

and the fluid population dynamics of the San Diego region, however, the true population at risk and what might be behind the trends observed are difficult to describe. The relative contributions of reactivated latent *M. bovis* infection compared with recent infection could not be discerned in our study, but prudent prevention strategies would include a focus on eliminating consumption of unpasteurized dairy products in both adults and children in the United States and Mexico.

Our study confirms earlier preliminary findings (*14*) that *M. bovis* case-patients appear to be more than twice as likely to die before TB treatment completion compared with *M. tuberculosis* patients, despite being treated for the same mean number of days. The association of higher mortality rates during *M. bovis* treatment persisted after HIV, multisite disease, age, and ethnicity were accounted for. Causes of death related to noninfectious disease, such as malignancy and noninfectious gastrointestinal pathologies, were underrepresented in the *M. bovis* cases (0 vs. 25%), but, overall, the *M. bovis* and *M. tuberculosis* cases were not significantly different with regard to all causes of death.

In mouse models, evidence indicates that certain strains of *M. bovis* are more virulent than *M. tuberculosis* strains (*24*), but those findings are not generally supported in the literature on human *M. bovis* TB (*7*). Although our mortality analysis partially controlled for extent of disease, it did not include information on coexisting conditions, stage of HIV disease, diagnostic delays, and prior access to medical care. Therefore, *M. bovis* deaths might be accounted for by other factors, such as health disparities or treatment differences, which warrant further investigation.

Public health measures to control TB are currently focused on interrupting person-to-person transmission by promptly identifying and treating infectious patients and ensuring that they do not expose new contacts until treatment has rendered them noninfectious. Based on our data, these strategies, which have proven to be effective at reducing *M. tuberculosis* cases in San Diego and most regions of the United States, appear to be less effective in controlling *M. bovis*, suggesting that human-to-human transmission of *M. bovis* is less likely an important mode of transmission in this community. The consumption of contaminated dairy products has been proposed to be the primary source of human TB from *M. bovis* (*25*). This hypothesis is supported by the findings of an investigation of *M. bovis* cases in New York that indicated the likely source of infection was unpasteurized cheese from Mexico (*10*). Additionally, San Diego pediatric *M. bovis* cases occur only after the age of weaning, when children are typically first exposed to dairy products (*6*), and *M. bovis* was also recently cultured from unpasteurized cheese seized at the San Diego–Mexico border (*26*,*27*).

Because of the widespread adoption of pasteurization of all commercially available dairy products in the United States, as well as the aggressive US state agricultural health programs designed to keep dairy cattle free from *M. bovis* disease, the threat of *M. bovis* in US dairy products was largely eliminated in the mid-20th century (*8*). The San Diego–Tijuana binational region, however, shares one of the busiest border crossings in the United States with Baja, Mexico (*28*), where *M. bovis* is prevalent in cattle and consumption of unpasteurized dairy products is a common cultural practice (*29*–*32*). Mexican dairy products, including the popular *queso fresco* (soft, unpasteurized cheese), may be brought into the United States for personal use and are sometimes distributed illegally (*27*). Given our finding that >90% of *M. bovis* cases in San Diego occurred in Hispanics, most of whom were born in Mexico, consumption of unpasteurized dairy products from Mexico is likely a major risk factor for *M. bovis* TB in San Diego. Collaboration with Mexico on prevention strategies, from education to regulation of the production of unpasteurized dairy products, and elimination of *M. bovis* from dairy cattle will be required in the long term to ensure that this mode of transmission is eliminated.

Limitations

A growing awareness of *M. bovis* as a cause of TB in San Diego since 1980 could have introduced a sampling bias into our trend estimates, but this possible bias is unlikely to have had a considerable effect in the years 1994

through 2005 as reported here. All suspected TB cases in San Diego County are reportable to the health department, and the county laboratory has consistently conducted testing to distinguish all *M. tuberculosis* complex isolates as either *M. bovis* or *M. tuberculosis* since 1994. The proportion of TB cases based only on clinical diagnosis and not species level culture has remained relatively level, at ≈20% of all reported TB cases. Increased efforts to obtain specimens for culture in pediatric TB cases in the years under study did not appear to change the proportion of culture-positive cases during the study period.

The cohort of TB case-patients who were not culture-positive and thus excluded from this analysis was significantly different from the study group. The <15-year age group (36% vs. 4%, respectively) and Hispanic ethnicity (57% vs. 45%) were both overrepresented in the excluded cases. Given that these are the groups most likely to have *M. bovis* TB, the total incidence of *M. bovis*, particularly in children, may be underestimated in our study.

Conclusions

San Diego, California, while unique in many respects because of its close proximity to Mexico, is possibly representative of other communities in the United States with large and growing Hispanic populations with ties to Mexico. The considerable and growing incidence of TB from *M. bovis*, especially in children, and the observed number of deaths during treatment in these cases is of serious concern. It raises the question of the importance of incorporating routine species-level identification into US TB surveillance as the national TB incidence shifts to persons born outside the United States. This surveillance will be greatly facilitated by the national genotyping project implemented by the US Centers for Disease Control and Prevention in 2004 (*33*), and its use will be particularly important for communities with strong ties to Mexico.

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Validation of Syndromic Surveillance for Respiratory Pathogen Activity

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Syndromic surveillance is increasingly used to signal unusual illness events. To validate data-source selection, we retrospectively investigated the extent to which 6 respiratory syndromes (based on different medical registries) reflected respiratory pathogen activity. These syndromes showed higher levels in winter, which corresponded with higher laboratory counts of *Streptococcus pneumoniae*, respiratory syncytial virus, and influenza virus. Multiple linear regression models indicated that most syndrome variations (up to 86%) can be explained by counts of respiratory pathogens. Absenteeism and pharmacy syndromes might reflect nonrespiratory conditions as well. We also observed systematic syndrome elevations in the fall, which were unexplained by pathogen counts but likely reflected rhinovirus activity. Earliest syndrome elevations were observed in absenteeism data, followed by hospital data (+1 week), pharmacy/general practitioner consultations (+2 weeks), and deaths/laboratory submissions (test requests) (+3 weeks). We conclude that these syndromes can be used for respiratory syndromic surveillance, since they reflect patterns in respiratory pathogen activity.

Early warning surveillance for emerging infectious disease has become a priority in public health policy since the anthrax attacks in 2001, the epidemic of severe acute respiratory syndrome in 2003, and the renewed attention on possible influenza pandemics. As a result, new surveillance systems for earlier detection of emerging infectious diseases have been implemented. These systems, often labeled "syndromic surveillance," benefit from the increasing timeliness, scope, and diversity of health-related registries (*1–6*). Such alternative surveillance uses symptoms or clinical diagnoses such as "shortness of breath" or "pneumonia" as early indicators for infectious disease. This approach not only allows clinical syndromes to be monitored before laboratory diagnoses, but also allows disease to be detected for which no additional diagnostics were requested or available (including activity of emerging pathogens). Our study assessed the suitability of different types of healthcare data for syndromic surveillance of respiratory disease.

We assumed that syndrome data—to be suitable for early detection of an emerging respiratory disease—should reflect patterns in common respiratory infectious diseases (*7–10*). Therefore, we investigated the extent to which time-series of respiratory pathogens (counts per week in existing laboratory registries) were reflected in respiratory syndrome time-series as recorded in 6 medical registries in the Netherlands. We also investigated syndrome variations that could not be explained by pathogen counts. As an indication for syndrome timeliness, we investigated the delays between the syndrome and pathogen time-series.

Methods

Syndrome Data Collection and Case Definitions

We defined syndrome data as data in health-related registries that reflect infectious disease activity without identifying causative pathogen(s) or focusing on pathogen-specific symptoms (such as routine surveillance data for influenza-like illness [*11*] or surveillance of acute flaccid paralysis for polio [*12*]).

Registries for syndrome data were included if they met the following criteria: 1) registration on a daily basis; 2) availability of postal code, age, and sex; 3) availability

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of retrospective data (≥2 years); and 4) (potential) real-time data availability.

Six registries were selected (Table 1) that collected data on work absenteeism, general practice (GP) consultations, prescription medications dispensed by pharmacies, diagnostic test requests (laboratory submissions) (*13*), hospital diagnoses, and deaths. In all registries, data were available for all or a substantial part of 1999–2004. For the GP, hospital, and mortality registry, definition of a general respiratory syndrome was guided by the case definitions and codes found in the International Classification of Diseases, 9th revision, Clinical Modification (ICD-9-CM), as selected by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (www.bt.cdc.gov/surveillance/syndromedef). For the laboratory submissions and the pharmacy syndrome, we selected all data that experts considered indicative of respiratory infectious disease (for detailed syndrome definitions, see online Technical Appendix, available from www.cdc.gov/EID/content/14/6/917-Techapp.pdf).

Respiratory Pathogen Counts

As a reference for the syndrome data, we included specific pathogen counts for 1999–2004 from the following sources: 1) Weekly Sentinel Surveillance System of the Dutch Working Group on Clinical Virology (which covers 38%–73% of the population of the Netherlands [*14*] for routine laboratory surveillance of respiratory syncytial virus [RSV], influenza A virus, influenza B virus, rhinovirus, *Mycoplasma pneumoniae*, parainfluenza virus, enterovirus, and adenovirus); 2) 6 regional public health laboratories for

Table 1. Registries from which syndrome data were obtained, the Netherlands, 1999–2004*

Data type	Period	% Coverage†	Respiratory syndrome definitions‡	Analyzed data	International code system	Registry
Absenteeism	2002–2003	80§	Reported sick employees; no further medical information	Sick leave reports of employees	–	Statistics Netherlands (CBS), www.cbs.nl
General practice consultations	2001–2004	1–2	Symptoms and diagnoses indicating respiratory infectious disease	Symptoms and diagnoses recorded in practice or telephone consultations and in home visits	ICPC	Netherlands Information Network of General Practice (LINH), www.nivel.nl/linh
Pharmacy dispensations	2001–2003	85	Prescribed medications indicative for respiratory infectious disease	Prescription medications dispensed in Dutch pharmacies, coded according to the WHO ATC classification	ATC	Foundation for Pharmaceutical Statistics, http://www.sfk.nl
Hospitalization	1999–2004	99	General respiratory symptoms/diagnoses; specific respiratory biologic agent diagnoses	Discharge and secondary diagnoses, date of hospitalization	ICD-9-CM	Dutch National Medical Register (LMR)
Laboratory submissions¶	2001–2004 (1999–2000 excluded due to unstable coverage)	16	All submissions for microbiologic diagnostic tests on respiratory materials; all submissions for serologic testing on known specific respiratory pathogens; all submissions for *Legionella* or *Streptococcus pneumoniae* antigen tests on urine	Laboratory submission requests for diagnostic testing	–	National Infectious Diseases Information System (ISIS) (*13*)
Mortality	1999–2004	100	General respiratory symptoms/diagnoses; specific respiratory biologic agent diagnoses	Date of death, primary cause of death, complicating factors, other additional causes of death	ICD-10	CBS

*ICPC, International Classification of Primary Care; WHO, World Health Organization; ATC, Anatomic Therapeutic Chemical Classification System; ICD-9-CM, International Classification of Diseases, 9th revision, Clinical Modification; ICD-10, International Classification of Diseases, 10th revision.
†Percentage of total population, 16.3 million.
‡For detailed syndrome definitions and codes, see online Technical Appendix, available from www.cdc.gov/EID/content/14/6/917-Techapp.pdf.
§Percentage of working population, 8 million.
¶Diagnostic test requests with both negative and positive results.

respiratory disease–related counts of *Streptococcus pneumoniae* (data in 2003–2004 were interpolated for 2 laboratories during short periods of missing data; total coverage 24%); and 3) national mandatory notifications of pertussis. The networks for respiratory pathogen counts are other networks than the earlier described laboratory submissions network for syndrome data.

Data Analysis and Descriptive Statistics

Data were aggregated by week and analyzed by using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). For the GP, pharmacy, and laboratory submissions registries, we expressed the respiratory counts as a percentage of total weekly counts to adjust for the influence of holidays and, for laboratory submissions, changes in the number of included laboratories over time. By looking at the graphs, we explored the relationship between the time-series of respiratory pathogens and syndromes and calculated Pearson correlation coefficients.

Linear Regression Models

To investigate whether the respiratory syndromes reflect patterns in respiratory pathogen counts, we constructed multiple linear regression models. These models estimated respiratory syndrome levels at a certain time with, as explanatory variables, the lagged (range of –5 to +5 weeks) pathogen counts as explanatory variables. We used linear regression of the untransformed syndrome to estimate the additive contributions of individual pathogens to the total estimated syndrome. We assumed a constant syndrome level attributable to factors other than the respiratory pathogens and constant scaling factors for each of the lagged pathogens. A forward stepwise regression approach was used, each step selecting the lagged pathogen that contributed most to Akaike's information criterion of model fit (*15*). Each pathogen entered the model only once and only if it contributed significantly ($p<0.05$). Negative associations (e.g., between enteroviruses, which peak in summer, vs. respiratory syndromes, which peak in winter) were excluded to avoid noncausal effects.

To discriminate between primary and secondary infections by *S. pneumoniae* (as a complication of respiratory virus infection) (*16–19*), we used the residuals from regressing *S. pneumoniae* counts on other pathogens as the variable for *S. pneumoniae* (instead of its counts) for all the earlier described models for respiratory syndromes.

We checked for autocorrelation in the residuals of the models with hierarchical time-series models (using SPLUS 6.2) (*20*,*21*). We calculated R^2 values to estimate to what extent respiratory pathogen counts explain variations in syndromes. To explore to what extent seasonal variation could be a confounder, we also calculated R^2 values of the models after adding seasonal variables (sine and cosine terms) and R^2 values for seasonal terms alone. We also investigated the pathogen-specific effects in the models, by calculating the standardized parameter estimates before and after adding seasonal terms.

The models were used to estimate the expected syndrome level with 95% upper confidence limits (UCLs). We considered distinct syndrome elevations that exceeded the UCLs, as unexplained by the models (for model details, see online Technical Appendix).

Timeliness

We investigated the timeliness of the registry syndromes in 2 ways: 1) as a measure of differences in timeliness between registries, we evaluated the time delays of the syndromes relative to each other by calculating for each of the syndromes the time lag that maximized Pearson correlation coefficient with the hospital registry (as a reference); 2) by estimating the time delays between each of the syndromes and the lagged pathogens included in its regression model.

Results

Data Exploration and Descriptive Statistics

Respiratory syndrome time series were plotted for all registries (Figure 1). The Christmas and New Year holidays coincided with peaks and dips in the pharmacy and absenteeism syndromes (not shown). Because these results were probably artifacts, we smoothed these yearly peaks and dips and censored them in the analyses performed on the absenteeism registry, in which they had a strong influence on outcomes. For all registries, the respiratory syndromes demonstrated higher levels of activity in winter, which overlapped or coincided roughly with the seasonal peaks of influenza A, influenza B, RSV, and (albeit less pronounced) *S. pneumoniae* laboratory counts (Figure 1). Infections with parainfluenza virus, *M. pneumoniae*, adenovirus, and rhinovirus were detected slightly more frequently during winter (data not shown). *Bordetella pertussis* and enterovirus showed seasonal peaks only in summer (data not shown).

The seasonal peaks in laboratory counts of influenza A, influenza B, and RSV corresponded with peaks in the GP, pharmacy, and hospital syndromes. Other syndromes did have less obvious correspondence. Each year, around October, the respiratory syndrome showed a peak in the GP (2001–2004), pharmacy (2001–2003), and absenteeism (2002–2003) registries (Figure 1, panels A–C) that was observed neither for the other registries nor in any of the laboratory pathogens.

We calculated Pearson correlation coefficients between the different unlagged time series of respiratory pathogens and syndromes (Table 2). Syndrome time series in all reg-

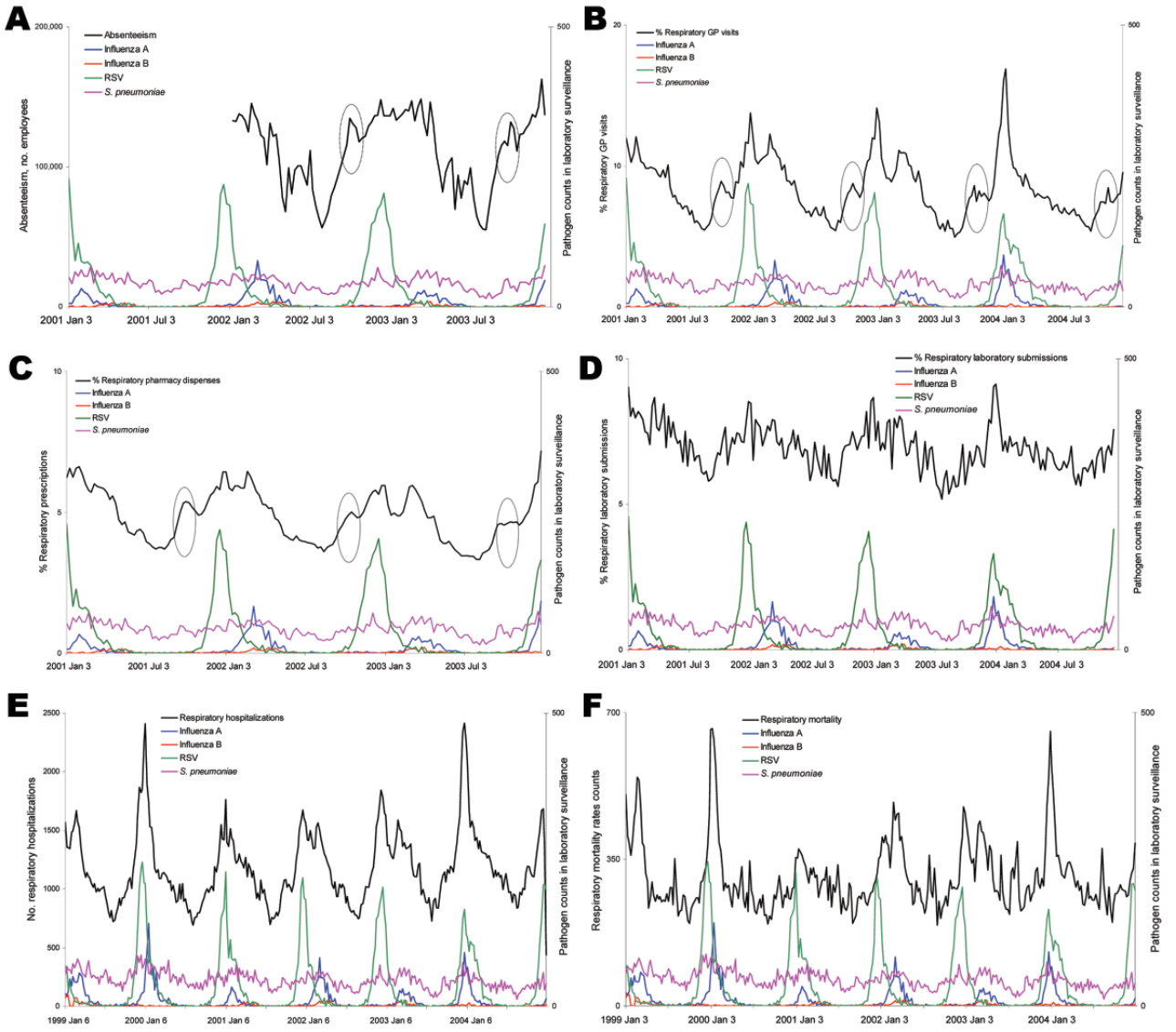

Figure 1. Respiratory syndrome time series and laboratory pathogen counts in the Netherlands. Respiratory syndromes were defined for the 6 registries defined in Table 1: A) absenteeism, B) general practice (GP) consultations, C) pharmacy, D) laboratory submissions, E) hospitalizations, and F) mortality counts. Pathogens plotted were respiratory syncytial virus (RSV), influenza A, influenza B, and *Streptococcus pneumoniae* [1999–2004 or part of this period, panels A–C]. Recurrent unexplained syndrome elevations in October are circled. Pathogen counts are daily counts of pathogens found in laboratory surveillence.

istries correlated strongly with *S. pneumoniae* (unadjusted total counts). The hospital, GP, pharmacy, and laboratory submissions data strongly correlated with RSV and influenza A counts (Table 2). Mortality data correlated strongly with influenza A (r = 0.65) and influenza B (r = 0.50) infections. The highest correlations between pathogen time series were between *S. pneumoniae* and the other pathogens (up to 0.51 with influenza A, Table 3).

Linear Regression Models

Table 4 presents, for each registry, the time lag (in weeks) that maximized the model fit of regressing syndrome on pathogens. For the GP, hospital, mortality, and pharmacy data, the respiratory pathogens explained the syndrome variation very well (78%–86%). Variations in the absenteeism syndrome could be explained for 68% by variations in the pathogen counts. Although the laboratory submissions syndrome had the lowest explained variance, still 61% of the variations in this syndrome were explained by variations in pathogen counts. Hierarchical time-series models did not show significant autocorrelation in the residuals of the models with pathogen counts as explanatory variables (*20*,*21*).

When seasonal terms were added to the model, the variations in the mortality syndrome were just as well ex-

Table 2. Pearson correlation coefficients between time series of syndromes and laboratory pathogen counts, the Netherlands, 1999–2004*†

Pathogen	Hospital	GP	Mortality	Pharmacy	Laboratory submissions	Absenteeism
RSV	**0.74**	**0.67**	0.41	**0.58**	**0.53**	0.47
Influenza A	**0.57**	**0.61**	**0.65**	**0.60**	0.47	0.35
Influenza B	0.31	0.39	**0.50**	0.42	0.34	0.33
Streptococcus pneumoniae	**0.73**	**0.71**	**0.56**	**0.75**	**0.58**	**0.69**
Rhinovirus	0.33	0.34	0.33	0.33	NS	0.35
Parainfluenza	0.20	NS	NS	NS	0.25	NS
Adenovirus	0.37	0.35	0.33	0.36	NS	0.34
Enterovirus	–0.65	–0.66	–0.59	–0.61	–0.57	–0.51
Mycoplasma pneumoniae	0.13	0.27	0.25	0.39	0.32	0.26
Bordetella pertussis	NS	NS	NS	NS	NS	NS

*GP, general practice; RSV, respiratory syncytial virus; NS, nonsignificant. Correlations ≥0.50 in **boldface**; p≥0.05.
†Unlagged.

plained as by the model with only pathogen counts (Table 5; R^2 remains 78%), while by the model with only seasonal terms, the explained variance was much lower (only 52%, Table 5). For the hospitalizations, laboratory submissions, and GP data, only slightly more syndrome variation was explained by adding seasonal terms. With only seasonal terms, the explained variance for these syndromes was clearly lower than with only pathogens in the models (8%–11% lower, Table 5). However, for the absenteeism and, to a lesser extent, the pharmacy data, the model with both pathogen and seasonal terms clearly explained more syndrome variations (Table 5, absenteeism 68% vs. 80%; pharmacy 80% vs. 87%). Furthermore, for the absenteeism data, the model with only seasonal terms had an even higher R^2 than the model with only pathogens, whereas for the pharmacy data, the R^2 with only seasonal terms was only slightly lower (3%, Table 5).

Table 6 shows that for mortality, hospitalizations, laboratory submissions, and GP data, the pathogens with the highest effect clearly were RSV, influenza A, and influenza B, with no or only modest decline in standardized parameter estimates after adding seasonal terms. For the GP and hospital data, some pathogens became insignificant after seasonal terms were added (GP: rhinovirus and adenovirus; hospital: parainfluenza virus). For the pharmacy data, half of all pathogen variables became insignificant after seasonal terms were added, whereas for the absenteeism data, almost all pathogens became insignificant (Table 6).

Several syndrome observations exceeded the 95% UCLs of the models (0–10/registry/year), which indicates that those syndrome observations deviated strongly from model predictions. The recurrent elevation in October of the absenteeism, GP, and pharmacy syndrome several times exceeded the UCLs (October 2001: pharmacy and GP; 2002: absenteeism; 2003: GP, absenteeism; not shown), which indicated that the model could not explain these elevations.

Timeliness

In Figure 2, for each registry, the difference in timeliness with the hospital registry is indicated by the lag that maximizes R^2. The absenteeism syndrome (green line) preceded the hospital syndrome by 1 week, followed by the GP-based and prescription-based syndromes at +1 week and the syndrome based on mortality and laboratory sub-

Table 3. Pearson correlation coefficients between time series in respiratory pathogen counts, the Netherlands, 1999–2004*†

Pathogen	*S. pneumoniae*	RSV	Influenza A	Influenza B	RV	PIV	Adenovirus	Enterovirus	*Mycoplasma pneumoniae*	*Bordetella pertussis*
S. pneumoniae	1.00	0.35	**0.51**	0.36	NS	0.32	0.32	–0.44	0.21	–0.31
RSV		1.00	0.23	NS	0.30	0.13	0.21	–0.30	0.19	NS
Influenza A			1.00	0.36	NS	0.12	0.24	–0.39	0.16	–0.25
Influenza B				1.00	NS	NS	NS	–0.30	0.25	–0.21
RV					1.00	NS	0.21	NS	NS	NS
PIV						1.00	NS	–0.19	NS	NS
Adenovirus							1.00	–0.21	NS	–0.14
Enterovirus								1.00	–0.15	0.21
M. pneumoniae									1.00	NS
B. pertussis										1.00

S. pneumoniae, Streptococcus pneumoniae*; RSV, respiratory syncytial virus; RV, rhinovirus; PIV, parainfluenza virus; NS, nonsignificant. Correlations ≥0.50 in **boldface; p value ≥0.05.
†Unlagged.

Table 4. All respiratory pathogen counts included as explanatory variables in the regression models, the Netherlands, 1999–2004*†

Syndrome data	RSV	Influenza A	Influenza B	*S. pneumoniae* (residual)	RV	PIV	Adenovirus	Enterovirus	*Mycoplasma pneumoniae*	*Bordetella pertussis*
Absenteeism	2	5	4	2	4	5	–	–	–	
GP	–1	1	2	–1	1	2	–2	–	–3	
Pharmacy	–1	0	2	0	2	5	–2	–	5	–3
Hospitalization	0	2	1	–	–2	3	–	–	–	
Laboratory submissions	–2	0	1	–3	–	2		–	5	
Mortality	–3	1	0	–	–	–	–	–	–	

**S. pneumoniae, Streptococcus pneumoniae*; RSV, respiratory syncytial virus; RV, rhinovirus; PIV, parainfluenza virus; GP, general practice; –, pathogen not included in model.
†The lag time (in weeks) is indicated, that showed optimal fit between syndrome time-series and lagged pathogen counts included in the linear regression model; e.g., according to the model, the trend in hospitalizations precedes the influenza A laboratory counts by 2 weeks.

mission data at +2 weeks after the hospital syndrome (projected on x-axis, Figure 2).

The differences in timeliness between the syndromes and the pathogen surveillance data were reflected by the regression models relating the syndromes to the (positive or negative) lagged pathogens (Table 4). Influenza A and influenza B had lags of 0–5 weeks, which suggests that the registry-syndromes were 0–5 weeks ahead of laboratory counts for these infections. Fluctuations in the time series of respiratory hospitalizations and the laboratory RSV counts seemed to appear in the same week (lag = 0). All other syndromes appeared to be 1–3 weeks later than the RSV counts, except absenteeism, which is 2 weeks earlier. Again, absenteeism seemed to be the earliest syndrome (2–5 weeks earlier than RSV, influenza A, and influenza B), followed by the hospital syndrome (0–2 weeks earlier), the GP-based and prescription-based syndromes (2 weeks earlier until 1 week later), the laboratory submission syndrome (1 week earlier until 2 weeks later), and the mortality syndrome (0–3 weeks later than RSV, influenza A, and influenza B).

Discussion

We explored the potential of 6 Dutch medical registries for respiratory syndromic surveillance. Although several other studies also evaluated routine (medical) data for syndromic surveillance purposes (*22*–*27*), most evaluated only 1 syndrome and correlated this only to influenza data. An exception is Bourgeois et al. (*24*), who validated a respiratory syndrome in relation to diagnoses of several respiratory pathogens in a pediatric population, and Cooper et al. (*27*), who estimated the contribution of specific respiratory pathogens to variations in respiratory syndromes. Both studies concluded that RSV and influenza explain most of the variations in these syndromes, consistent with our findings.

Our study shows that all syndrome data described in this study showed higher levels in winter, which corresponded to the seasonal patterns of RSV, *S. pneumoniae*, and influenza A and B viruses. Linear regression showed that the syndromes can be explained by lagged laboratory counts for respiratory pathogens (up to 86%, highest effect of influenza A, influenza B, and RSV), which indicates their potential usefulness for syndromic surveillance. Timeliness differed, with up to 5 weeks potential gain in early warning by syndromic data, compared with routine laboratory surveillance data.

A limitation of our study is the short duration of our time series, especially for absenteeism and pharmacy data. Therefore, whether our observed associations between syndromes and pathogen counts can be generalized remains unclear.

We relied on laboratory pathogen counts as a proxy for their prevalence and the illness they cause. Changes in test volume over time would result in misclassification bias (as noncausative pathogens will be detected as well). However, such changes are presumably dwarfed by changes during "truly" epidemic elevations of common respiratory pathogens. Additionally, laboratory diagnostics are mostly performed on hospitalized patients, and thus results inadequately reflect activity of pathogens that predominantly cause mild illness.

By adding seasonal terms, we observed that for the absenteeism and, to a lesser extent, the pharmacy registry, the associations between the respiratory syndromes and the pathogen counts might be biased to some extent. For the GP, hospital, laboratory submission, and mortality data,

Table 5. Syndrome variation that can be explained by either the pathogen counts, seasonal terms, or pathogen counts and seasonal terms together*

Syndrome data	Pathogens, %	Pathogens and seasonal terms, %	Seasonal terms, %
Absenteeism	68	80	79
GP	86	89	75
Pharmacy	80	87	77
Hospitalization	84	88	75
Laboratory submissions	61	63	53
Mortality	78	78	52

*Estimated by 3 different R^2 values for each registry: 1) for the syndromes explained by pathogen counts alone; 2) after adding seasonal terms to the pathogen model; and 3) for the syndromes explained by seasonal terms alone (sine and cosine parameters). GP, general practice.

Table 6. Standardized parameter estimates (βs) for all respiratory pathogen counts included as explanatory variables in the regression models, before and after adding seasonal terms to the models*†

Syndrome data	RSV	Influenza A	Influenza B	*S. pneumoniae* (residual)	RV	PIV	Adenovirus	Enterovirus	*Mycoplasma pneumoniae*	*Bordetella pertussis*
Absenteeism	0.31/ (NS)	0.27/ (NS)	0.33/ (NS)	0.28/ 0.12	0.19/ (NS)	0.20/ (NS)	–	–	–	–
GP	0.60/ 0.51	0.32/ 0.32	0.20/ 0.16	0.13/ 0.10	0.07/ (NS)	0.14/ 0.08	0.07/ (NS)	–	0.06/ 0.05	–
Pharmacy	0.51/ 0.54	0.27/ 0.22	0.24/ (NS)	0.25/ 0.11	0.16/ 0.08	0.16/ (NS)	0.08/ (NS)	–	0.12/ (NS)	0.11/ 0.11
Hospitalization	0.60/ 0.44	0.36/ 0.34	0.21/ 0.12	–	0.13/ 0.05	0.09/ (NS)	–	–	–	–
Laboratory submissions	0.49/ 0.47	0.19/ 0.20	0.22/ 0.18	0.28/ 0.22	–	0.17/ 0.08	–	–	0.10/ 0.10	–
Mortality	0.40/ 0.36	0.52/ 0.51	0.24/ 0.24	–	–	–	–	–	–	–

**S. pneumoniae, Streptococcus pneumoniae*; RSV, respiratory syncytial virus; RV, rhinovirus; PIV, parainfluenza virus; GP, general practice; –, pathogen not included in model; NS, the pathogen variable is no longer significant after seasonal terms are added.
†For example, 0.60/0.40 for RSV indicates a standardized β of 0.60 for RSV in the model with only pathogen variables and a β of 0.40 in the same model after adding seasonal terms.

season is probably not an important confounder for the association between the syndromes and pathogens, because including seasonal terms in the models resulted in the same or only slightly higher explained syndrome variance (measured by R^2). Models with seasonal terms alone mostly had lower explained variance than the pathogen models. For the GP and hospital data, some pathogens became insignificant after seasonal terms were added (Table 6) but not those pathogens with the largest effect estimates (RSV, influenza A and B). Therefore, we are confident in concluding that the GP, hospital, laboratory submission, and mortality syndromes do reflect pathogen activity sufficiently for use in syndromic surveillance.

The higher R^2 value of the absenteeism model with seasonal terms alone suggests seasonality of absenteeism caused by several nonrespiratory conditions (*28*,*29*). To some extent, this also applies to the pharmacy syndrome, which includes medications that are not specific for respiratory infections (e.g., antimicrobial drugs). This could be validated in future studies by linking medications to illness. However, for both the absenteeism and pharmacy syndromes, the variation explained by seasonal terms is probably overestimated to some extent because data for only 2 and 3 years were used. Consequently, these time series contained less information on variation between different years than for the other registries, which benefits fitting of a model with several sine and cosine terms.

To our knowledge, laboratory submission data (test requests) have not been evaluated before as a data source for syndromic surveillance. The modest explained variance for the laboratory submissions syndrome could possibly reflect the limited use in our country of laboratory testing algorithms, which leads to substantial differences in diagnostic regimes for patients with similar clinical symptoms. In addition, occasional extra alertness by clinicians can make these data unreliable for surveillance. For instance, an unusual peak was observed in the laboratory submissions syndrome in 1999, after the official announcement of an outbreak of Legionnaires' disease (*30*).

An unexpected increase was also observed in the absenteeism, GP, and pharmacy syndromes, which occurred consistently each year around October (2001–2004). These peaks preceded the syndrome peaks concurring with peaks in influenza A, influenza B, and RSV counts and may be caused by rhinovirus activity—and asthma exacerbations caused by rhinovirus—which usually rises in the fall (*31*–*33*). Rhinovirus might go undetected because GP physicians rarely ask for diagnostics if they suspect a nonbacterial cause for relatively mild respiratory disease. Although

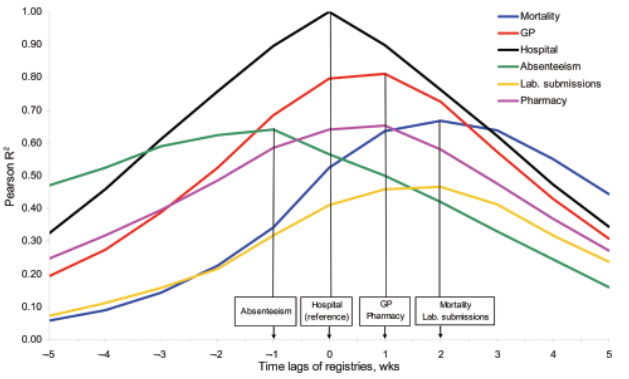

Figure 2. The (maximum) R^2 by the lagged syndromes with the hospital syndrome as a reference. Aggregated by week, univariate Pearson correlation coefficients were calculated of the hospital syndrome and each of the other syndromes. Note that the Pearson correlation coefficients are calculated over different periods for the different registries because not all registries cover the same period (Table 1). Measured by the syndrome lag with the maximized R^2, the timeliness differed between the registries in the following order: absenteeism, hospital, pharmacy/general practice (GP), mortality/laboratory submissions (as projected on the x-axis).

specific asthma diagnoses were excluded from the respiratory syndrome definitions, exacerbations of asthma might affect other respiratory categories in the GP or pharmacy syndrome. This observation illustrates that additional diagnostics are needed for identifying the causes of unexplained respiratory disease elevations. Several novel respiratory pathogens for which diagnostics are not yet widely available have been discovered in recent years, underlining that it is quite possible that "hidden" epidemics occur (*34*–*36*). The extra October peak and several other syndrome elevations above the 95% UCLs in our study may well reflect such hidden epidemics. The fact that these occur is supported by studies showing that many individual syndrome cases cannot be linked to known pathogens. For example, Cooper et al. (*37*), who investigated syndromic signals by using patient self-sampling (at home), could only obtain diagnostic results for 22% of these cases.

For early warning surveillance, timeliness is crucial. Absenteeism data seem to have the best timeliness, but their lack of medical detail complicates interpretation. Unexpectedly, the hospital data reflect respiratory pathogen activity earlier than the GP data. Although in the Netherlands patients are encouraged to consult their GP before going to the hospital, elderly persons, for whom respiratory infections are more likely to cause severe illness, may often go to a hospital directly. Therefore, hospital data may prove to be an earlier marker for respiratory disease than GP data, but this possibility needs further exploration.

An important concern when using syndromic surveillance is that it may generate nonspecific alerts, which, if they happen regularly, would lead to lack of confidence in a syndrome-based surveillance system. Here, we see a clear advantage of using data from multiple registries in parallel so that signal detection can be made more specific by focusing on signals that occur concurrently in >1 data source. To illustrate this we defined every exceeding of the UCLs of the regression models as a "signal," i.e., a syndrome elevation unexplained by known pathogen activity and therefore possibly reflecting activity of underdiagnosed or emerging infectious disease. Over 2002–2003 (the period that all 6 registries were in the study), only 5 "concurrent" signals occurred versus 34 "single" signals over all registries. We did not evaluate whether the syndromes indeed detect outbreaks of infectious diseases earlier than clinical or laboratory pathogen surveillance. Such an evaluation is often performed by testing the ability to detect historical natural outbreaks or simulated outbreaks (*10*,*38*). However, historical natural outbreaks are rare and simulated outbreaks may be unrealistic. Nevertheless, further research into the outbreak detection performance of these syndromes would be worthwhile.

The results of this study suggest that it might be best to combine syndromic data and pathogen counts in a prospective surveillance system. Such surveillance can identify distinct syndrome elevations that cannot be explained by respiratory pathogen activity as indicated by routine laboratory pathogen surveillance.

Conclusion

Overall, the GP, hospital, mortality and, to a lesser extent, laboratory submission syndromes reflect week-to-week fluctuations in the time-series of respiratory pathogens as detected in the laboratory. Registries monitoring trends of these syndromes will therefore most likely reflect illness caused by emerging or underdiagnosed respiratory pathogens as well and therefore are suited for syndromic surveillance. Further research would be required to assess to what extent absenteeism and pharmacy data reflect respiratory illness. Investigating the actual outbreak detection performance of the syndromes in this study would also be worthwhile.

Data from the registries in this study are not yet real-time available, although given modern information technology, this availability is clearly feasible. Our study can help prioritize which type of healthcare data to include in future syndromic real-time surveillance systems.

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Antibodies against Lagos Bat Virus in Megachiroptera from West Africa

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To investigate the presence of Lagos bat virus (LBV)–specific antibodies in megachiroptera from West Africa, we conducted fluorescent antibody virus neutralization tests. Neutralizing antibodies were detected in *Eidolon helvum* (37%), *Epomophorus gambianus* (3%), and *Epomops buettikoferi* (33%, 2/6) from Ghana. These findings confirm the presence of LBV in West Africa.

Bats host a range of lyssaviruses, depending on their species and locality. The genus *Lyssavirus* is differentiated into 7 genetically divergent genotypes: classical *rabies virus* (genotype 1), *Lagos bat virus* (LBV; genotype 2), *Mokola virus* (MOKV; genotype 3), *Duvenhage virus* (genotype 4), *European bat lyssavirus* (genotypes 5 and 6), and *Australian bat lyssavirus* (genotype 7) (*1*). All but MOKV have been isolated from bats.

LBV and MOKV are each distributed in Africa and are members of phylogroup 2 within the genus *Lyssavirus* (*1*). Because LBV isolates (*2*) from African bats are increasing, our goal was to determine the prevalence of virus neutralizing antibodies against LBV in bat populations in West Africa.

The Study

Bats were sampled in January and May 2007 at 6 sites in Ghana: the center of Accra (urban habitat); the wooded outskirts of Accra (savannah habitat); and forested habitats at Pra, Kibi, Adoagyiri, and Oyibi (a plantation with woodland/forest border). Bats were captured by using 6–18-m mist nets; roosting *Eidolon helvum* were captured by using nets on poles. A sample size of 59 would provide 95% confidence of finding at least 1 LBV-seropositive bat in a large population (>5,000), given a seroprevalence of 5% and assuming random sampling (*3*). Species were identified by using a dichotomous key (*4*). Captured bats were manually restrained and anesthetized by intravenous injection; ≈0.2–1.0 mL of blood was collected from the propatagial vein before the bat was released. Blood was centrifuged in the field at ambient temperature at 3,000 rpm for 15 min. Serum was heat treated at 56°C for 30 min and frozen at –70°C.

Two species, *Epomophorus gambianus* and *E. helvum,* were caught in sufficient numbers (117 and 66, respectively) for reasonable inferences to be made about LBV seroprevalence rates (Table). A standard approach was used to calculate 95% confidence intervals (CIs) for seroprevalence (*3*). Because of the relatively short distances between study sites and the likelihood of bats mixing between these sites, bats of each species were considered to belong to single populations. All but 3 *E. helvum* were derived from a colony in Accra, whereas *E. gambianus* were derived from all habitat types.

Bat serum samples were tested for virus neutralizing antibody against classical rabies virus (challenge virus standard) by using a standard fluorescent antibody virus neutralization (FAVN) test (*5*). Antibodies to LBV were measured by using a modified FAVN test (*6*). Because positive bat antiserum from naturally infected bats was not available, for positive controls we used human rabies immunoglobulin, LBV-positive rabbit serum, and serum from mice vaccinated with human diploid cell vaccine. Negative controls were negative rabbit and mouse serum. All samples were analyzed in duplicate and serially diluted by using a 3-fold series (representing reciprocal titers of 9, 27, 81, and 243–19,683) (*6*).

The modified FAVN test requires a cut-off threshold, which in prior bat lyssavirus studies has been a titer of 27, to avoid false-positive results (*6,7*). The first 121 samples collected were tested against the challenge virus standard; no tested bat was seropositive at 1:3 dilutions. A mean titer >9 was considered positive for LBV (Figure 1; [*8*]).

Levels of specific virus neutralizing antibodies against LBV were higher in *E. helvum* (seroprevalence 37%, 95% CI 24%–49%) than in *E. gambianus* (3%, 95% CI 0%–7%). Of 6 *Epomops buettikoferi*, 2 were seropositive (30%, 95% CI 0%–70%). No sex differences in *E. helvum* seroprevalence were evident (χ^2 1.0, p>0.9).

Because of the high level of seropositivity in *E. helvum*, we attempted to determine a possible case reproduction rate (R_0) for LBV infection in this species by using the equation $R_0 = 1/x^*$, where x^* = proportion of susceptible hosts in a population (*9*). We assumed that infection with each virus within the bat populations is endemic, stable, and randomly dispersed; that all seropositive animals have lifelong immunity that is detectable serologically; and that seropositivity is to 1 virus. On the basis of these assumptions, $R_0 = 1.6$ (95% CI 1.3–2.0).

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Table. Bat species and their respective seroprevalence rates against phylogroups 1and 2 lyssaviruses, Ghana, 2007*

Species	Habitat	No. caught	% Adults tested for LBV antibodies	Seroprevalence, % (95% CI, no. tested)	
				CVS rabies virus	LBV
Epomophorus gambianus	Savannah†	117	61	0 (49)	3 (0–7, 91)
Eidolon helvum	Urban‡	66	95	0 (57)	37 (24–49, 57)
Epomops franqueti	Forest‡	30	77	0 (3)	0 (31)
Epomops buettikoferi	Forest‡	9	83	0 (5)	30 (0–70, 6)
Hypsignathus monstrosus	Forest‡	18	56	0 (1)	0 (5)
Nanonycteris veldkampii	Forest‡	5	100	NT	0 (4)

*Phylogroup 1 contains challenge virus standard (CVS), genotype 1; phylogroup 2 contains Lagos bat virus (LBV), genotype 2; CI, confidence interval; NT, not tested.
†*E. gambianus* was caught in all habitats, including at the city colony and in plantations.
‡A small number of these species were caught in plantations.

Conclusions

We found antibodies against LBV in healthy *E. helvum* bats in Ghana. Previous studies have suggested that healthy bats develop antibodies to other lyssavirus infections (*7*,*10*,*11*), which may reflect past exposure, rather than survival from clinical disease. LBV likely co-evolved with its natural megachiropteran host until a genetic stasis had been reached in which the virus–host relationship was in equilibrium. This situation would result in high seroprevalence rates after a wave of virus circulation in a colony. Nine seropositive bats (8 *E. helvum*, 1 *E. buettikoferi*) were apparently healthy pregnant females. These results support theories that lyssaviruses are endemic within specific bat populations, that they may not cause high mortality rates, that exposure rates of LBV between megachiroptera in Old World African bats are high, and that bats may breed successfully after LBV exposure (*7*,*8*). The number of high reciprocal titers against LBV (Figure 1) and the history of LBV isolation in *E. helvum* suggest that LBV circulates in megachiroptera in Ghana. However, further work is needed to determine the specific phylogroup 2 virus and its prevalence within specific bat populations.

No previous estimate of R_0 for genotype 2 *Lyssavirus* has been calculated, and although anamnesis may lead to no detectable antibodies in bats with immunity and a consequent underestimate of R_0, this value indicates the potential R_0 and is comparable to values previously estimated for lyssavirus infections in bats (*7*,*11*). More detailed analysis relating to age-specific infection and survival rates or mode of transmission was precluded by the difficulty in determining the age of adult bats, the lack of juveniles in the sample, and the cross-sectional sample used.

The underlying cause of the difference in seroprevalence between *E. gambianus* and *E. helvum* with respect to LBV infection is unclear. Possible explanations include differential susceptibilities to infection; virus–host adaptation; different contact with the virus, including a recent epidemic in the *E. helvum* colony; or different population ecology. *E. helvum* resides in high-density populations (hundreds of thousands) (Figure 2, panel A) and migrates annually, compared with *E. gambianus*, which resides in less dense colonies of tens or hundreds (*4*). *E. helvum* commonly forms large colonies in African cities in close proximity to humans and domestic animals and is a food source in West Africa (Figure 2, panel B).

No investigations into infections of humans were made during these investigations, but lyssavirus infections in humans in Africa are underdiagnosed (*12*). Despite reduced pathogenicity of LBV in the laboratory, it has been isolated from dogs, cats, and a mongoose (*2*). Conversely, MOKV has caused a fatal case of encephalitis in a human (*1*). LBV and MOKV each have a substitution in the R333 glycoprotein residue (*1*). Although it is not the only protein to determine the pathogenicity of LBV, the R333 substitution still remains an important marker of rabies pathogenicity. In conclusion, the high seroprevalence to LBV in this population may pose a substantial public health risk because *E. helvum* is widely distributed in Africa and a food source in West Africa.

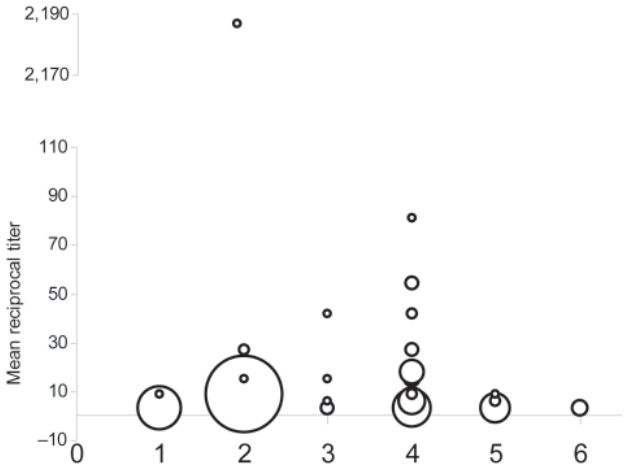

Figure 1. Antibody titers to Lagos bat virus (LBV) in 6 species of fruit bat in Ghana. An LBV-specific modified fluorescent antibody neutralization test was used to determine the level of antibody in bats; it used two 3-fold serial dilutions and derived a mean dilution at which the bats neutralized LBV. Bats with mean titers >9 were considered positive. The circle size represents the number of bats tested. 1, *Epomops franqueti;* 2, *Epomophorus gambianus*; 3, *Epomops buettikoferi*; 4, *Eidolon helvum*; 5, *Hypsignathus monstrosus*; 6, *Nanonycteris veldkampii.*

Figure 2. A) Density of a typical *Eidolon helvum* roost in the Accra colony. B) *E. helvum* as bushmeat in an Accra market.

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Increase in Adult *Clostridium difficile*–related Hospitalizations and Case-Fatality Rate, United States, 2000–2005

Marya D. Zilberberg,*† Andrew F. Shorr,‡ and Marin H. Kollef§

Virulence of and deaths from *Clostridium difficile*–associated disease (CDAD) are on the rise in the United States. The incidence of adult CDAD hospitalizations doubled from 5.5 cases per 10,000 population in 2000 to 11.2 in 2005, and the CDAD-related age-adjusted case-fatality rate rose from 1.2% in 2000 to 2.2% in 2004.

Clostridium difficile–associated disease (CDAD) represents a considerable public health hazard. In the United States, it is responsible for more deaths than all other intestinal infections combined (*1*). Incidence, hospitalizations, and deaths related to CDAD have been on the rise (*1*–*3*). Emergence of hypervirulent strains and in vitro resistance to third-generation cephalosporins and fluoroquinolones have also been reported (*4*). In view of these phenomena, it is unclear whether the recently reported 35%-per-year increase in CDAD-related deaths represents a rise in case-fatality rate or reflects increasing incidence of hospitalizations with this disease (*1*). We hypothesized the latter to be at least partially the cause. Given that 80% of all CDAD-related deaths occur in acute-care hospitals (*1*), we conducted a population-based analysis of CDAD-related adult hospitalizations in the period 2000–2005.

The Study

We identified CDAD-related hospitalizations for 2000–2005 from the National Inpatient Sample data (*5*), available on the Healthcare Costs and Utilization Project Net website, administered by the Agency for Healthcare Research and Quality (*6*). The National Inpatient Sample is a stratified 20% sample of US community hospitals, and the data are weighted to provide national estimates (*5*). CDAD was identified by the presence of the International Classification of Diseases, 9th revision, Clinical Modification (ICD-9-CM), diagnosis code 8.45 (intestinal infection with *Clostridium difficile*), and the numbers of discharges per year were age stratified. We obtained censal and intercensal data (numerical and demographic characteristics of the US population from 2000 through 2005) from the US Census Bureau (*7*). On the basis of these data, we calculated age-specific hospitalization incidence rates and fitted linear models, using the least-squares method, to describe this age-specific growth. Finally, using the population-based CDAD mortality numbers in the report by Redelings et al. (*1*), we computed case-fatality rates for hospitalized CDAD patients for the 5-year period from 2000 through 2004.

The number of adults discharged from US hospitals with a CDAD diagnosis rose by nearly 160,000, from 134,361 in 2000 to 291,303 in 2005 (Table) (*6*). This 117% rise in CDAD discharges over a 5-year period equates to an ≈23% average crude growth annually. As a benchmark, we examined the changes in overall hospital discharges and discharges with CDAD as the principal diagnosis over the same period. We found the overall hospitalizations rose ≈1.3% annually (from 36,417,565 in 2000 to 39,163,834 in 2005). Although the absolute change in volume of cases for which CDAD was the principal diagnosis mirrored those in all CDAD admissions, the relative contribution of CDAD primary diagnosis to all CDAD cases remained relatively stable over time at ≈25%.

The numbers of adult hospital patients discharged with a CDAD diagnosis from 2000 through 2005 by age group are presented in the Table (*6*). The Figure illustrates the age-specific growth in CDAD incidence for the same period. The rate of increase in the incidence of CDAD was steepest in the ≥85 age group, with the slope for the linear trend 11.3 (95% confidence interval [CI] 7.6–14.9, $p = 0.001$), and ranged from 0.2 (95% CI 0.1–0.3, $p<0.001$) among the 18–44 age group to 4.8 (95% CI 3.2–6.0, $p<0.001$) among the 65–84 age group; the overall CDAD hospitalization incidence rose from 6.4 cases per 10,000 in 2000 to 13.1 cases per 10,000 in 2005. When published population-based CDAD mortality estimates were applied to the annual CDAD hospitalization volumes, the crude case-fatality rate rose from 1.2% in 2000 to 2.3% in 2004; age-adjusting the 2004 estimate resulted in a similar case-fatality rate of 2.2% (*1*).

Conclusions

In our analysis we detected a 23% annual increase in CDAD hospitalizations in the 6-year period from 2000 through 2005. Moreover, the absolute number of CDAD hospitalizations more than doubled in all age groups except the youngest, for whom they increased by 74.1% over the study period. Additionally, we estimated that the age-ad-

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Table. Absolute numbers of adult hospitalizations with *Clostridium difficile*, by age group, United States, 2000–2005

Hospitalizations	2000	2001	2002	2003	2004	2005
18–44 y	14,738	15,001	18,747	19,393	22,168	25,662
45–64 y	28,280	29,527	39,421	43,290	50,898	61,757
65–84 y	69,018	74,010	98,148	105,404	122,875	147,675
≥85 y	22,325	25,194	31,899	35,363	43,341	56,209
All adult	134,361	143,732	188,215	203,450	239,282	291,303

justed case-fatality rate for CDAD hospitalizations nearly doubled from 1.2% in 2000 to 2.2% in 2004.

Our numbers help put in perspective the observed increasing mortality rates related to CDAD in the United States. The recent report by Redelings et al. noted an increase from 5.7 to 23.7 deaths with CDAD per million population from 1999 through 2004 in the United States, representing a 35% adjusted per annum increase (*1*). By observing a 23% per annum increase in the volume of hospitalizations with CDAD in the period 2000–2005, we demonstrate that at least half of the reported mortality increase with CDAD is due to an increase in the incidence of hospitalizations with this severe infection. Increased hospitalization may in turn be related to a simple increase in the overall volume of CDAD or reflect the increased virulence of the organism, leading to more cases of severe disease requiring hospitalization. We have also estimated that the unadjusted case-fatality rate did indeed increase from 1.2% in 2000 to 2.3% in 2004. While this doubling of deaths with CDAD is mirrored almost perfectly by the more-than doubling of CDAD admissions among all but the youngest age groups, who cumulatively represent 90% of all CDAD hospitalizations, age-adjusting the 2004 case-fatality estimate did not change it substantially. This finding indirectly confirms that the reported increase in CDAD deaths likely represents the effects of increased virulence of the organism (*1*,*4*).

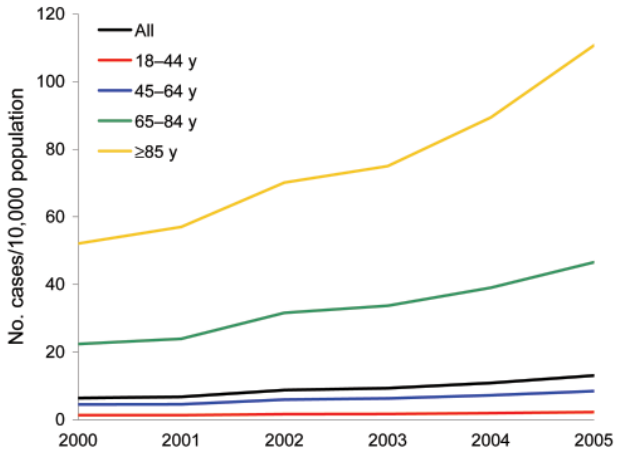

Figure. Changes in the age-specific *Clostridium difficile*–associated disease incidence rate per 10,000 population in the United States, 2000–2005.

Our analysis relied on ICD-9-CM coding to identify CDAD-related hospitalizations. Studies correlating the presence of the diagnostic code for CDAD to the presence of a laboratory confirmation of the disease have not suggested a clear over- or underdiagnosis trend in the administrative coding (*2*). However, the administrative nature of the data may have predisposed our case ascertainment to misclassification. Giving credence to our numbers, however, is the report by McDonald et al., who noted near-doubling of CDAD US hospital discharges, from 98,000 in 1996 to 178,000 in 2003 (*2*). Additionally, while exhibiting a similar absolute rise, CDAD primary diagnosis admissions as a fraction of all CDAD hospitalizations remained constant. Although it is possible that the observed rise in CDAD hospitalizations is due to changes in coding practices, evidence of an increase in microbiologic detection of this pathogen argues against this explanation for our observations (*8*).

The incidence in adult CDAD-related hospitalizations increased substantially in the period 2000–2005. In view of the aging US population, this rapid pace of growth is alarming. If this rate of rise, along with the increase in virulence and diminished susceptibility to antimicrobial drug treatments, persists, CDAD will result not only in a considerable strain on the US healthcare system (*9*,*10*) but also in rising numbers of deaths related to this disease (*1*). Allocation of public health resources aimed at prevention of CDAD is necessary to mitigate this growing epidemic. Research into the best preventive strategies, such as limiting the use of antimicrobial agents in both human disease and the food supply (*11*), is a public health imperative.

Dr Zilberberg is a health services researcher at the University of Massachusetts, Amherst, and president of Evi*Med* Research Group, LLC. Her interests include reducing complications and optimizing quality and efficiency of healthcare delivery in the hospital setting.

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Syphilis and Hepatitis B Co-infection among HIV-Infected, Sex-Trafficked Women and Girls, Nepal

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Sex trafficking may play a major role in spread of HIV across South Asia. We investigated co-infection with HIV and other sexually transmitted diseases among 246 sex-trafficked women and girls from Nepal. Those who were HIV positive were more likely than those who were HIV negative to be infected with syphilis and/or hepatitis B.

South Asia is currently home to >2.5 million HIV-infected persons, 95% of whom are from India (*1*). However, HIV seroprevalence in a subset of neighboring South Asian countries has rapidly increased in recent years, due in part to migration and human trafficking from these countries into India (*1*–*3*). Female sex workers, especially those who are victims of sex trafficking to India, are increasingly recognized as a major factor in Nepal's growing HIV epidemic (*2*,*4*,*5*). HIV seroprevalence among female sex workers in Nepal rose 24-fold (from <1% to 17%) from 1992 through 2002 (*6*). Women and girls trafficked for sexual exploitation from Nepal to India are considered particularly vulnerable to HIV infection because of their typically young age at trafficking, limited ability to negotiate condom-protected sex, and experiences of forced sex (*4*,*7*). Recent evidence documents high (38%) HIV seroprevalence among sex-trafficked women and girls returning from India to Nepal (*5*).

Despite high rates of HIV infection among sex-trafficked victims (*5*,*8*) and substantial prevalence of sexually transmitted infections (STIs) among female sex workers in South Asia and elsewhere (*9*,*10*), little is known about STI prevalence and co-infection with HIV among sex-trafficked women and girls. We therefore explored prevalence of syphilis and hepatitis B and co-infection with HIV among a sample of female sex-trafficking victims in Nepal.

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The Study

Data for this study were extracted from medical records of 395 women and girls examined at Maiti Nepal, a large nongovernment organization in Kathmandu, Nepal, which provides shelter and healthcare for sex-trafficking victims repatriated to Nepal. Upon intake and pending verbal consent, all sex-trafficking victims at Maiti Nepal are routinely tested for HIV and STIs. We excluded records of 149 because they lacked HIV test or accompanying syphilis or hepatitis B test documentation. Study protocols were approved by the Harvard School of Public Health Human Subjects Committee.

Standard HIV antibody testing was performed by using HIV ELISA, rapid testing, or Western blot. Syphilis testing was performed by using a nontreponemal serologic test, the Venereal Disease Research Laboratory test; all samples tested had titers ≥1:8 dilution, which strongly suggests true syphilis infection. (*11*) Serologic detection of the hepatitis B virus surface antigen was indicative of hepatitis B infection.

Age at time of HIV testing ranged from 13 to 40 years (median age 20 years), median age at the time of trafficking was 17 years (range 7–32 years), and median duration of brothel servitude was 12 months (range <1 month–13 years). A series of Fisher exact tests conducted to assess for potential biases in selection for diagnostic testing showed no differences in demographic or experiential variables (i.e., current age, age at trafficking, duration in brothel), and McNemar tests showed no relationship between likelihood of testing for syphilis or hepatitis B based on a positive HIV test result (all $p>0.05$). Because of the paired nature of the data, the McNemar test involving a continuity correction was used to assess associations between 1) HIV status and co-infection with syphilis, 2) HIV status and co-infection with hepatitis B, and 3) HIV status and co-infection with hepatitis B or syphilis.

Of the 246 women and girls in the study, 74 (30.1%, ≈1 in 3) had positive HIV test results. Syphilis infection was documented for 48 of 235 (20.4%, 1 in 5). Hepatitis B infection was documented for 8 of 210 (3.8%, 1 in 25). Those who were HIV positive were more likely than those who were HIV negative to be infected with syphilis (31.0% vs. 15.9%, respectively; odds ratio [OR] 1.88; 95% confidence interval [CI] 1.17–3.03) (Table 1). Similarly, those who were HIV positive were more likely than those who were HIV negative to be infected with hepatitis B (9.1% vs. 1.4%, respectively; OR 30.0; 95% CI 7.32–122.7) (Table 1). Finally, those who were HIV positive were more likely than those who were HIV negative to be infected with either syphilis or hepatitis B (35.1% vs. 15.1%, respectively; OR 1.78; 95% CI 1.11–2.85) (Table 2).

Table 1. Prevalence and likelihood of infection with syphilis or hepatitis B, by HIV serostatus, among sex-trafficked women and girls, Nepal

Syphilis (n = 235)	HIV status, no. (%)*	Syphilis positive, no. (%)†	Syphilis negative, no. (%)†	McNemar test statistic	OR (95% CI)†
HIV positive	71 (30.2)	22 (31.0)	49 (69.0)	6.45, p<0.05	1.88 (1.17–3.03)
HIV negative	164 (69.8)	26 (15.9)	138 (84.1)		Referent
Total	235	48 (20.4)§	187(79.6)§		
Hepatitis B (n = 210)		Hepatitis B positive, no. (%)†	Hepatitis B negative, no. (%)†		
HIV positive	66 (31.4)	6 (9.1)	60 (90.9)	52.4, p<0.0001	30.0 (7.32–122.73)
HIV negative	144 (68.6)	2 (1.4)	142 (98.6)		Referent
Total	210	8 (3.8)‡	202 (96.1)‡		

*Percentages based on HIV serostatus.
†OR, odds ratio, CI, confidence interval.
‡Percentages based on total sample tested for HIV and syphilis or hepatitis B.

Conclusions

Our findings demonstrate that HIV-infected sex-trafficking victims are more likely to be infected with other STIs, specifically syphilis and hepatitis B, than those not infected with HIV. Current evidence of HIV and STI co-infection implies a need to strengthen clinical practice among providers caring for persons at risk for HIV or other STIs, particularly high-risk populations such as those trafficked for sexual exploitation or otherwise exposed to commercial sex work. Our findings strongly indicate the need for syphilis and hepatitis B screening for HIV-infected persons and HIV screening for syphilis- and hepatitis B-infected persons. Clinical expertise alone may be insufficient to guide treatment decisions in the presence of undetected co-infection (*12*,*13*), resulting in missed case detection, incomplete or partial treatment, and suboptimal clinical follow-up.

Appropriate diagnosis of co-infection by comprehensive STI and HIV screening is also important for averting potential development of pathogen drug resistance, a disastrous scenario in a region that is already coping with high rates of syphilis, hepatitis B, and HIV infection. From a clinical perspective, accurate diagnosis of syphilis, hepatitis B, and HIV, alone or in combination, is critical for informed selection of medications to be used in combinations or regimens that reduce the likelihood of inciting drug resistance for the other pathogens. Furthermore, success of STI treatment depends not only on the potency of the antiviral medication but also on the patient's immunocompetence (*14*). A decision about when to modify a potentially failing or failed STI treatment regimen may thus be better informed by knowledge of HIV status.

Current data highlight prior calls for secondary prevention efforts (i.e., prevention of subsequent transmission) for this population because migration and repatriation of such women and girls has been described as a major factor in the spread of HIV and STIs across South Asia (*4*). Evidence from the World Health Organization shows that effective treatment of a variety of STIs can reduce HIV transmission rates because many STIs are increasing the risk for HIV acquisition (*15*). Therefore, treatment of prevailing STIs at the time of repatriation could potentially lessen risk for future HIV acquisition and reduce subsequent transmission to sex partners. The ability to properly treat and reduce the propagation of STIs represents an avenue by which to improve the health of the individual patients as well as potentially reduce rates of HIV in the region.

Finally, these data underscore the need for efforts by both government and nongovernment organizations to expand support for appropriate healthcare services to sex-trafficked women and girls and to develop comprehensive screening guidelines and treatment programs. Currently, most of the few nongovernment organizations serving this vulnerable population are unable to provide the quality of care indicated by our findings. Such improvements are urgently needed to help reduce the alarmingly high rates of HIV and co-occurring STIs among victims of sex trafficking and to curb the spread of these co-occurring epidemics throughout the region.

Table 2. Prevalence and likelihood of co-infection with syphilis or hepatitis B based on HIV serostatus (n = 246), among sex-trafficked women and girls, Nepal

HIV status	HIV status, no. (%)	Syphilis and/or hepatitis B positive, no. (%)*	Syphilis and hepatitis B negative, no. (%)*	McNemar test statistic	OR, 95% CI†
Positive	74 (30.1)	26 (35.1)	48 (64.9)	5.33, p<0.05	1.78 (1.11–2.85)
Negative	172 (69.9)	27 (15.7)	145 (84.3)		Referent
Total	246	53 (21.5)‡	193 (78.5)‡		

*Percentages based on HIV serostatus.
†OR, odds ratio, CI, confidence interval.
‡Percentages based on total sample tested for HIV, syphilis, or hepatitis B.

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Underreporting of Human Alveolar Echinococcosis, Germany

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We estimated the total number of human alveolar echinococcosis cases in Germany from 2003 through 2005 using the multiple source capture-recapture method. We found a 3-fold higher incidence of the disease than that shown by national surveillance data. We propose a revision of the reporting system to increase case ascertainment.

Human alveolar echinococcosis (AE), caused by the metacestode stage of the fox tapeworm *Echinococcus multilocularis,* is a rare zoonosis in Germany, mainly occurring in the south (*1*). The parasite predominantly develops in the liver of the human host, where the infiltrating growth can cause serious damage (*2*). Untreated AE has a very high fatality rate (*3*); when the patient survives, the cost of life-long treatment is substantial, projected at US $300,000 per patient (*4*). *E. multilocularis* infection is highly endemic among foxes in Germany, and studies indicate that the parasite's geographic range has widened in recent years (*5*,*6*). Growing fox populations in Europe, especially in urban zones, have drawn attention to a potential increased risk for humans (*7*–*9*).

In 2001, AE reporting became mandatory in Germany (*10*). Diagnosis of echinococcosis by serologic testing and histopathologic examination is reportable to the Robert Koch Institute (RKI) by microbiologic laboratories and pathologists, respectively. However, reports rarely come from pathologists. Referring physicians must provide additional diagnostic data (e.g., imaging findings and clinical information to confirm serologically diagnosed AE) but are not required to report cases independent of the laboratory diagnosis. Additionally, clinicians voluntarily report AE case-patients with active lesions to the European Echinococcosis Registry (EER) associated with the clinical referral center for AE in Ulm, Germany (*11*).

We conducted a 3-source capture–recapture analysis to generate an estimate of the true number of AE cases in Germany from 2003 through 2005. On the basis of this estimate, we assessed the sensitivity of the national surveillance system.

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The Study

The capture–recapture method estimates unascertained cases by comparing data from ≥2 different sources. It requires that persons have a correct diagnosis and equal probability of inclusion (catchability) and that the study population be closed. If only 2 sources are used, these should be independent (*12*).

We used 3 data sources: RKI, EER, and a pathologists' survey (PAS) conducted in June 2006 among all registered pathology laboratories in Germany (≈525). Pathologists were requested to complete a questionnaire reporting all echinococcosis cases diagnosed from 2003 through 2005 to RKI.

We defined confirmed AE case-patients as persons with positive results of histopathologic examination or with liver lesion showing typical morphologic features, identified by imaging techniques. Only case-patients with a first diagnosis from 2003 through 2005 were included. Because reporting of AE is anonymous, we used 3 proxy matching identifiers. Matching criteria were identical: 1) year and month of birth, 2) sex, and 3) year and month of diagnosis (±6 months to allow for time variability of different diagnostic methods). For case-patients for whom month of birth or month of diagnosis was missing in ≥1 source, the first 3 digits of the case-patient's postal code or the referring physician's postal code had to be identical in addition to the above criteria.

The distribution of matched and unmatched observed cases by source is displayed in a Venn diagram (Figure). To predict the frequency of unascertained cases, we constructed log-linear models. Each model included a variable for each source and up to 3 possible interaction variables between sources. The saturated model included all 3 interactions, whereas the independent model assumed no interactions (*13*).

We selected the final model using Akaike's Information Criterion (AIC), which indicates how well a model fits the data, considering the number of variables included. Small values of AIC correspond to a better adapted model (*12*). Ninety-five percent goodness-of-fit confidence intervals (95% CI) were calculated based on the likelihood ratio, to allow asymmetric intervals and avoid underestimation of the upper and lower limits (*14*).

The sensitivity of RKI data was estimated by dividing the number of cases reported to RKI by the total number of cases (*N*) from the selected model. Analysis was performed with Stata 9.0 (StataCorp, College Station, TX, USA) (*15*).

A total of 60 confirmed cases were reported to RKI; EER registered 59. The response rate for PAS was 64% (335 of 525 surveyed). Pathologists reported 49 AE cases in the survey, of which 25 were the first diagnosis, 5 were previously diagnosed, and 19 had no date of first diagnosis. Table 1 summarizes case-patient characteristics by source.

Table 1. Main characteristics of human alveolar echinococcosis case-patients by source, Germany, 2003–2005*

Characteristic	RKI (n = 60)	EER (n = 59)	PAS (n = 25)
Median age, y (range)	52 (15–92)	53 (17–81)	52 (18–81)
Female sex, no. (%)	32 (53)	29 (49)	14 (56)
Residence south Germany, no. (%)†	35 (76)‡	46 (77)	10 (53)§

*RKI, Robert Koch Institute; EER, European Echinococcosis Registry; PAS, pathologists' survey. Data as of March 2007.
†Case-patients for whom the 3 first digits of the residential postal code was ≥600.
‡Data available for 46 case-patients.
§Data available for 19 case-patients.

From 2003 through 2005, 114 confirmed cases were recorded by the combined sources, of which 28 could be matched (Figure).

Log-linear estimates for *N* ranged from 184 to 399 cases (Table 2). Model 5, with the single interaction term between RKI and PAS, was selected as the best fitting (AIC = –3.33) model. According to this model, 70 cases were missed, yielding 184 cases (95% CI 150–242) over 3 years. This corresponds to 61 cases (95% CI 50–81) annually, with an incidence rate of 0.07/100,000 persons. The lower estimate in model 5, compared with that of the independent model, suggested a negative dependence between RKI and PAS reports. Sensitivity of RKI was 33% (95% CI 25%–40%).

Conclusions

We estimated that the national surveillance system failed to detect 67% of AE cases in Germany over 3 years. Underreporting may occur for several reasons. Pathologists might be unaware of their obligation to report. Furthermore, reports almost exclusively come from microbiologic laboratories, and, consequently, case-patients who do not undergo serologic testing, or who have seronegative results, are likely to be missed. Finally, the reporting procedure is arduous because forms are detailed and must be first ordered from RKI.

Capture–recapture estimates can be biased if the underlying assumptions are violated. Because case identification was based on several variables, the potential for mismatching was considered small. However, the lenient criteria may have led to overmatching. Including more or fewer matching criteria had only a small effect on the estimate.

In the final models, we excluded cases reported through PAS when first-diagnosis status was unknown. Log-linear analysis that included these cases resulted in a higher estimate; therefore, we are confident that the exclusion did not overestimate the number of cases. Varying catchability can be addressed by stratification. Although the sources differed with regard to geographic distribution, we considered stratified analysis inappropriate due to missing postal codes for several case-patients, zero values in 1 stratum, and small numbers in general, which would increase the uncertainty around our estimate.

AE is not equally distributed in Germany, and the different geographic distribution of cases reported by PAS compared with RKI and EER indicated that PAS had missed case-patients mainly from the south. The number of histopathologically diagnosed cases was therefore likely underestimated. The importance of this for the estimated true number of AE case-patients presented here cannot be ascertained.

The negative dependence between RKI and PAS can be explained by diagnostic practices. Unpublished data from EER suggest that histopathologically diagnosed cases are less likely to have serologic test results than those without histopathologic examination. If a case-patient has had a histopathologic examination with positive results early in the diagnostic decision-making process, additional serologic testing is unnecessary, which reduces the chance of these case-patients being reported to RKI. Reporting to EER is independent of serologic testing, which could explain the greater overlap between EER and PAS than between RKI and PAS.

Despite the limitations, the study did demonstrate poor reporting of AE. To improve the national surveillance sys-

Table 2. Log linear estimates of the total number of alveolar echinococcosis cases, Germany, 2003–2005*

Models†	df	AIC	*x*	*N*	95% CI for *N*
Saturated: Interaction (RKI, EER) and (RKI, PAS) and (EER, PAS)	0	0.00	174	288	129–2020
Interaction (RKI, EER) and (RKI, PAS)	1	–1.49	83	197	143–358
Interaction (RKI, PAS) and (EER, PAS)	1	–1.33	70	184	148–253
Interaction (RKI, EER) and (EER, PAS)	1	–1.66	285	399	189–1961
Interaction (RKI, PAS)	2	–3.33	70	184	150–242
Interaction (RKI, EER)	2	–1.57	134	248	171–430
Interaction (EER, PAS)	2	–0.08	93	207	163–287
Independent (no interactions)	3	–1.94	89	203	163–268

*RKI, Robert Koch Institute; EER, European Echinococcosis Registry; PAS, pathologists' survey; df, degrees of freedom; AIC, Akaike Information Criterion (measures how well the model fits the data [small values indicate a better fit]); *x*, estimate of unascertained cases; *N*, estimate of total number of cases (total number of observed cases + *x*); CI, goodness-of-fit–based confidence interval.
†Each model includes all first-order terms. The first model (saturated) adjusts for dependencies between all 3 source pairs; the second model adjusts for possible dependencies between RKI and EER, and between RKI and PAS, etc.

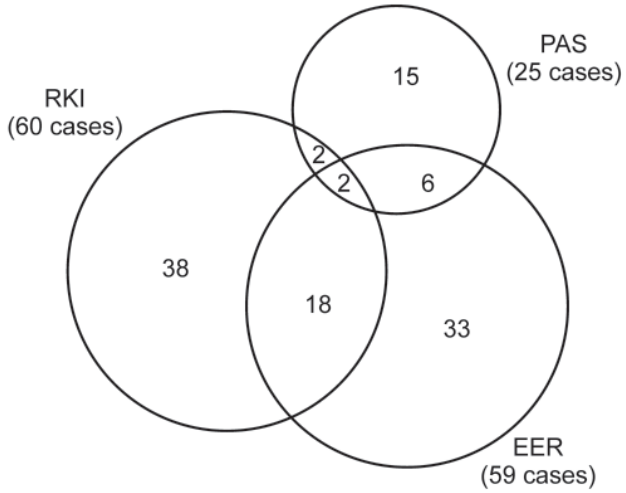

Figure. Venn diagram illustrating the distribution of confirmed first-diagnosis human alveolar echinococcosis cases from 2003 through 2005 in Germany by source and number of matches between sources. Data as of March 2007. RKI, Robert Koch Institute; EER, European Echinococcosis Registry; PAS, pathologists' survey.

tem, the focus of reporting should be shifted from microbiologic laboratories and pathologists to referring physicians, who usually collate the various diagnostic results.

Sustaining a surveillance system for AE in Germany is a major challenge because the disease is rare. However, a recent report on increasing human AE in neighboring Switzerland (*8*) underlines the importance of an effective surveillance system with adequate sensitivity to detect changes in disease incidence in order to guide strategies for prevention and control.

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Rickettsial Seroepidemiology among Farm Workers, Tianjin, People's Republic of China

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High seroprevalence rates for *Anaplasma phagocytophilum* (8.8%), *Coxiella burnetii* (6.4%), *Bartonella henselae* (9.6%), and *Rickettsia typhi* (4.1%) in 365 farm workers near Tianjin, People's Republic of China, suggest that human infections with these zoonotic bacteria are frequent and largely unrecognized. Demographic features of seropositive persons suggest distinct epidemiology, ecology, and risks.

Human granulocytic anaplasmosis and monocytic ehrlichiosis are emerging tick-borne rickettsial diseases (*1*,*2*). Like other rickettsial infections, these diseases are distributed worldwide but are predominantly reported in the United States or Europe (*2*). Despite evidence of *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* in ticks and rodents in the People's Republic of China (*3*–*7*), few investigations have been conducted. A pilot survey in Jiansu, Zhejiang, Shandong, and Hubei Provinces during 2004–2005 and an unusual cluster of cases in Anhui Province in 2006 identified human granulocytic anaplasmosis. As a result, a seroepidemiologic investigation was undertaken to assess exposure to *A. phagocytophilum*, *E. chaffeensis*, *Bartonella henselae*, *Coxiella burnetii*, and *Rickettsia typhi* among persons on 8 farms in 7 districts and rural counties near Tianjin, China.

The Study

Tianjin is located in the northeastern part of the Huabei plains in China. It has a temperate continental climate. The surrounding area comprises 13 districts and 5 rural counties. The study was conducted on 8 farms in 7 districts (Beichen, Dongli, Dagang, Xiqing, Jinnan, Tanggu, and Hangu) and Ninghe County.

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A. phagocytophilum (Webster strain) and *E. chaffeensis* (Arkansas strain) antigens were prepared from infected HL-60 and DH82 cells, respectively. The *E. chaffeensis* strain used was provided by the US Centers for Disease Control and Prevention (Atlanta, GA, USA), and the *R. typhi*, *C. burnetii*, and *B. henselae* strains and antigens used were provided by the World Health Organization Collaborating Center for Rickettsial Diseases (Marseille, France).

From May through July 2006, 365 healthy farm workers in close contact with domestic animals, vectors, or rodents were included in the analysis. A questionnaire was used to record demographic data, sex, age, occupation, length of service, and farm animal contact. The study was reviewed and approved by the Tianjin Institutional Review Board.

Serum samples were obtained from the 365 participants. Tests for antibodies to *A. phagocytophilum*, *E. chaffeensis*, *B. henselae*, *C. burnetii*, and *R. typhi* were performed on 220 samples at the National Institute of Communicable Disease Control and Prevention Laboratory. Serum samples from all 365 workers were separately tested for antibodies to *A. phagocytophilum* at Johns Hopkins University School of Medicine.

Serum samples were diluted 1:80 in phosphate-buffered saline, and 25 μL was placed on antigen slides and incubated for 60 min. Slides were washed, incubated with fluorescein isothiocyanate–conjugated goat antihuman immunoglobulin (Ig) (IgM plus IgG; Sigma, St. Louis, MO, USA) for 60 min at ambient temperature, washed again, and examined by fluorescent microscopy. Samples were considered reactive when fluorescent bacterial morphology was evident. Samples reactive at the 1:80 screening dilution were considered positive and not titrated further, except for samples reactive with *A. phagocytophilum* and *E. chaffeensis* antigens that were serially titrated to an endpoint titer to exclude cross-reactivity between these species. Statistical analysis was performed by using the χ^2 test to determine significant differences between groups. p values <0.05 were considered significant.

The median age of the 365 persons tested was 39 years (range 7–72 years) (online Appendix Table 1, available from www.cdc.gov/EID/content/14/6/938-appT1.htm) and the male:female sex ratio was 1.23 (205:160). All persons were engaged in livestock-rearing activities and spent substantial time outdoors. All 8 farms had pigs, sheep, horses, and cattle grazing on pastures. Among participants, 79.8% handled animals in pastures (graziers), 14.5% milked the animals (milkers), 2.9% worked in packing houses, 1.7% were veterinarians, and 1.2% assisted with animal birthing. The average length of service was 6.6 years (range 20 days to 45 years).

Online Appendix Table 2 (available from www.cdc.goc/EID/content/14/6/938-appT2.htm) shows the seroprevalence of infections by region and sex. Of 365 samples,

3 had insufficient volume for *A. phagocytophilum* testing. The highest *A. phagocytophilum* titer was 640; 32 (8.8%) had titers ≥80, 19 (5.3%) had titers ≥160, and 5 (1.4%) had titers ≥320. No significant differences between seroprevalence rates were found among the 8 communities surveyed (p>0.05). Tanggu (6/49, 12.2%) and Xiping (5/50, 10.0%) had the highest seroprevalence rates at a cutoff titer of 80; Xiping (5/50, 10.0%) and Hangu (4/45, 8.9%) had the highest seroprevalence rates at a cutoff titer of 160; and Xiping (2/50, 4.0%) had the highest seroprevalence rate at a cutoff titer of 320. Among 23 *A. phagocytophilum*–reactive sera tested for cross-reactivity, only 1 sample from Tanggu contained antibodies to *E. chaffeensis* at a titer of 160; this sample had an *A. phagocytophilum* titer of 80. Serologic analysis of 228 serum samples from all regions for *E. chaffeensis* showed no additional reactivity, with an overall seropositive rate of 0.4%.

A total of 220 serum samples showed seroprevalences of 9.5% (21/220) for *B. henselae*, 6.4% (14/220) for *C. burnetii*, and 4.1% (9/220) for *R. typhi*. The highest rates for *B. henselae* (22.9%) and *R. typhi* (18.8%) were found in Tanggu (both p<0.001); antibodies to *R. typhi* were not found in other locations. High seroprevalence rates for *B. henselae* were also identified in Xiqing (16.7%) and Jinnan (12.5%). The higher seroprevalence of *B. henselae* and *R. typhi* in Tanggu may be related to its low altitude, proximity to the Bo Sea (Bohai), or its port industry, which are environments conducive for fleas and their hosts (*8*). Antibodies to *C. burnetii* were found most often in Beichen (17.9%) and Xiqing (12.5%) (p<0.003), and seroprevalence was higher than that reported for the same area (*9*). Whether *C. burnetii* is an important pathogen in China needs further investigation.

There were no differences in seropositivity for antibodies to *A. phagocytophilum*, *B. henselae*, *R. typhi*, and *C. burnetii* by sex of the person tested. Seroprevalence of *A. phagocytophilum* was also similar across age groups, although the youngest group included all children ≤15 years of age, a potential bias given frequent exposure earlier in childhood (*10*). Antibodies to *B. henselae* and *R. typhi* were detected mainly in persons 20–50 years of age. Seroprevalence of *C. burnetii* was highest in persons 30–50 years of age. No differences in seroprevalence for any infections were found among graziers, milkers, packing house workers, veterinarians, or animal-birthing attendants, or among those with different lengths of farming service.

Conclusions

Rickettsioses are zoonoses for which risk factors include exposure to vectors carrying the pathogens (*11*); human infections occur often where such exposures are frequent. The emerging pathogens *A. phagocytophilum* and *E. chaffeensis* are transmitted by tick vectors (*1*,*2*), and *R. typhi* is transmitted by fleas of rats or other reservoirs (*12*). Because of similar risk factors and ecologic conditions, infections with *Bartonella* spp. and *Coxiella* spp. were historically considered rickettsioses and are often examined together. However, *C. burnetii* is generally acquired by aerosols from parturient farm animals or wildlife or by ingestion of contaminated foods (*13*), and transmission of *Bartonella* spp. pathogenic for humans occurs through body lice for *B. quintana* and between pets by fleas and possibly ticks for *B. henselae* (*14*). Such ecologic and epidemiologic conditions are common in Tianjin (*9*).

Our results show that *A. phagocytophilum* and *B. henselae* are emerging and may already be established in Tianjin, with seroprevalences similar to those in North America and Europe (*15*). In contrast, there is little evidence to identify human *E. chaffeensis* infections. These findings support those of a study that showed that arthropod-transmitted rickettsiae, such as *R. typhi,* are prevalent in Tianjin and surrounding areas (*9*). Studies are needed to investigate these pathogens, their local vectors and reservoirs, and their role in the transmission of these agents. Such information would better define human infection risk and establish evidence for an etiologic differential diagnosis of febrile illnesses among people in these areas.

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Persistence of *Yersinia pestis* in Soil Under Natural Conditions

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As part of a fatal human plague case investigation, we showed that the plague bacterium, *Yersinia pestis,* can survive for at least 24 days in contaminated soil under natural conditions. These results have implications for defining plague foci, persistence, transmission, and bioremediation after a natural or intentional exposure to *Y. pestis*.

Plague is a rare, but highly virulent, zoonotic disease characterized by quiescent and epizootic periods (*1*). Although the etiologic agent, *Yersinia pestis*, can be transmitted through direct contact with an infectious source or inhalation of infectious respiratory droplets, flea-borne transmission is the most common mechanism of exposure (*1*). Most human cases are believed to occur during epizootic periods when highly susceptible hosts die in large numbers and their fleas are forced to parasitize hosts upon which they would not ordinarily feed, including humans (*2*). Despite over a century of research, we lack a clear understanding of how *Y. pestis* is able to rapidly disseminate in host populations during epizootics or how it persists during interepizootic periods (*2–6*). What limits the geographic distribution of the organism is also unclear. For example, why is the plague bacterium endemic west of the 100th meridian in the United States, but not in eastern states despite several known introductions (*7*)?

Persistence of *Y. pestis* in soil has been suggested as a possible mechanism of interepizootic persistence, epizootic spread, and as a factor defining plague foci (*2*,*3*,*5*,*7*,*8*). Although *Y. pestis* recently evolved from an enteric bacterium, *Y. pseudotuberuclosis*, that can survive for long periods in soil and water, studies have shown that selection for vector-borne transmission has resulted in the loss of many of these survival mechanisms. This suggests that long-term persistence outside of the host or vector is unlikely (*9–11*). Previous studies have demonstrated survival of *Y. pestis* in soil under artificial conditions (*2*,*3*,*12–14*). However, survival of *Y. pestis* in soil under natural exposure conditions has not been examined in North America.

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The Study

As part of an environmental investigation of a fatal human plague case in Grand Canyon National Park, Arizona, in 2007, we tested the viability of *Y. pestis* in naturally contaminated soil. The case-patient, a wildlife biologist, was infected through direct contact with a mountain lion carcass, which was subsequently confirmed to be positive for *Y. pestis* based on direct fluorescent antibody (DFA) testing (which targets the *Y. pestis*–specific F1 antigen), culture isolation, and lysis with a *Y. pestis* temperature-specific bacteriophage (*15*). The animal was wearing a radio collar, and we determined the date of its death (October 26, 2007) on the basis of its lack of movement. The case-patient had recorded the location at which he encountered the carcass and had taken photographs of the remains, which showed a large pool of blood in the soil under the animal's mouth and nose. During our field investigation, ≈3 weeks after the mountain lion's death, we used global positioning satellite coordinates and photographs to identify the exact location of the blood-contaminated soil. We collected ≈200 mL of soil from this location at depths of up to ≈15 cm from the surface.

After collection, the soil was shipped for analysis to the Bacterial Diseases Branch of the Centers for Disease Control and Prevention in Fort Collins, Colorado. Four soil samples of ≈5 mL each were suspended in a total volume of 20 mL of sterile physiologic saline (0.85% NaCl). Samples were vortexed briefly and allowed to settle for ≈2 min before aliquots of 0.5 mL were drawn into individual syringes and injected subcutaneously into 4 Swiss-Webster strain mice (ACUC Protocol 00–06–018-MUS). Within 12 hours of inoculation, 1 mouse became moribund, and liver and spleen samples were cultured on cefsulodin-Irgasan-novobiocin agar. Colonies consistent with *Y. pestis* morphology were subcultured on sheep blood agar. A DFA test of this isolate was positive, demonstrating the presence of F1 antigen, which is unique to *Y. pestis.* The isolate was confirmed as *Y. pestis* by lysis with a *Y. pestis* temperature–specific bacteriophage (*15*). Additionally, the isolate was urease negative. Biotyping (glycerol fermentation and nitrate reduction) of the soil and mountain lion isolates indicated biovar *orientalis*.

Of the 3 remaining mice, 1 became moribund after 7 days and was euthanized; 2 did not become moribund and were euthanized 21 days postexposure. Culture of the necropsied tissues yielded no additional isolates of *Y. pestis*. Pulsed-field gel electrophoresis (PFGE) typing with *Asc*I was performed with the soil isolate, the isolate recovered from the mountain lion, and the isolate obtained from the case-patient (*16*). The PFGE patterns were indistin-

guishable, showing that the *Y. pestis* in the soil originated through contamination by this animal (Figure). Although direct plating of the soil followed by quantification of CFU would have been useful for assessing the abundance of *Y. pestis* in the soil, this was not possible because numerous contaminants were present in the soil.

Conclusions

It is unclear by what mechanism *Y. pestis* was able to persist in the soil. Perhaps the infected animal's blood created a nutrient-enriched environment in which the bacteria could survive. Alternatively, adherence to soil invertebrates may have prolonged bacterial viability (*17*). The contamination occurred within a protected rock outcrop that had limited exposure to UV light and during late October, when ambient temperatures were low. These microclimatic conditions, which are similar to those of burrows used by epizootic hosts such as prairie dogs, could have contributed to survival of the bacteria.

These results are preliminary and do not address 1) the maximum time that plague bacteria can persist in soil under natural conditions, 2) possible mechanisms by which the bacteria are able to persist in the soil, or 3) whether the contaminated soil is infectious to susceptible hosts that might come into contact with the soil. Answers to these questions might shed light on the intriguing, long-standing mysteries of how *Y. pestis* persists during interepizootic periods and whether soil type could limit its geographic distribution. From a public health or bioterrorism preparedness perspective, answers to these questions are necessary for evidence-based recommendations on bioremediation after natural or intentional contamination of soil by *Y. pestis*. Previous studies evaluating viability of *Y. pestis* on manufactured surfaces (e.g., steel, glass) have shown that survival is typically <72 hours (*18*). Our data emphasize the need to reevaluate the duration of persistence in soil and other natural media.

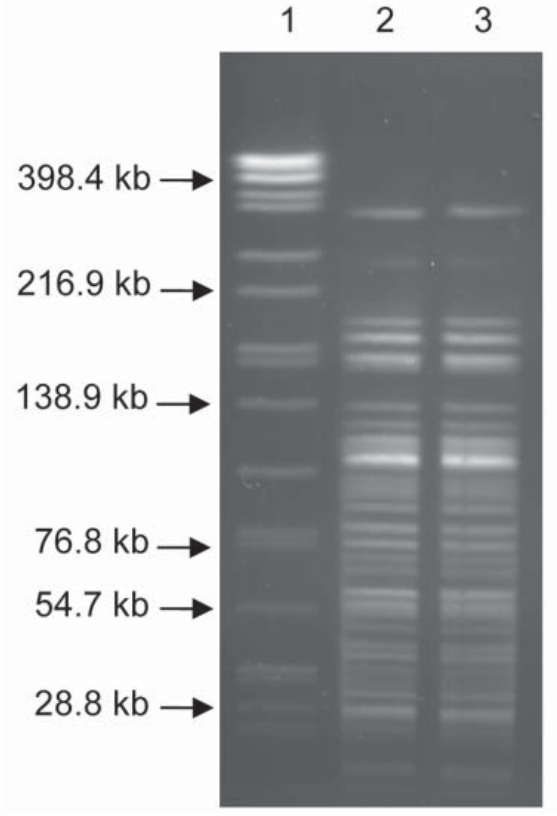

Figure. *Asc*I pulsed-field gel electrophoresis patterns for the *Yersinia pestis* isolates recovered from soil (lane 3) and the mountain lion (lane 2). Lane 1, *Salmonella enterica* serotype Braenderup standard.

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Plague Victims Catapulted Over Walls Into Besieged City

Thomas Lux

Early germ

warfare. The dead

hurled this way turn like wheels

in the sky. Look: there goes

Larry the Shoemaker, barefoot, over the wall, and Mary Sausage Stuffer, see how she flies, and the Hatter twins, both at once, soar over the parapet, little Tommy's elbow bent as if in a salute, and his sister, Mathilde, she follows him, arms outstretched, through the air, just as she did on earth.

From The Street of Clocks. New Yori: Houghton Mifflin Co.; 2001. Published with permission of the author.

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Global Distribution of Novel Rhinovirus Genotype

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Global surveillance for a novel rhinovirus genotype indicated its association with community outbreaks and pediatric respiratory disease in Africa, Asia, Australia, Europe, and North America. Molecular dating indicates that these viruses have been circulating for at least 250 years.

Acute respiratory illness (ARI) is the most frequent infectious disease of humans. Ordinary upper respiratory tract infections are usually self-limited; nevertheless, they result in major economic impact through loss of productivity and strain on healthcare systems. Lower respiratory tract infections (LRTIs) are among the leading causes of death in children <5 years of age worldwide, particularly in resource-poor regions (*1*). *Streptococcus pneumoniae* and *Haemophilus influenzae* are important bacterial causes of ARI, although their impact is expected to decline with increasing vaccine coverage. Collectively, however, viruses dominate as causative agents in ARI. Viruses frequently implicated in ARI include influenza virus, respiratory syncytial virus, metapneumovirus, parainfluenza virus, human enterovirus (HEV), and human rhinovirus (HRV).

HRVs are grouped taxonomically into *Human rhinovirus A* (HRV-A) and *Human rhinovirus B* (HRV-B), 2 species within the family *Picornaviridae* (International Committee on Taxonomy of Viruses database [ICTVdb]; http://phene.cpmc.columbia.edu). These nonenveloped, positive-sense, single-stranded RNA viruses have been classified serologically and on the basis of antiviral susceptibility profile, nucleotide sequence relatedness, and receptor usage (*2*). Phylogenetic analyses of viral protein VP4/VP2 and VP1 coding regions indicate the presence of 74 serotypes in genetic group A and 25 serotypes in genetic group B (*2*).

Isolated in the 1950s from persons with upper respiratory tract symptoms (*2*,*3*), HRVs have become known as the common cold virus because they are implicated in ≈50% of upper respiratory tract infections (*4*). Large community surveys, including the Virus Watch studies of the 1960–1970s (*5*), have shed light on some aspects of HRV biology and epidemiology. HRVs were also observed in LRTIs soon after their recognition (3), and data supporting a causative association have accumulated over the past decade (*6*,*7*). HRVs have also been implicated in exacerbations of asthma and chronic bronchitis and are increasingly reported in LRTIs of infants, elderly persons, and immunocompromised patients (*4*).

The Study

The advent of broad-range molecular assays, including multiplex PCR and microarray systems, promises new insights into the epidemiology and pathogenesis of respiratory disease (*8*,*9*), given that a laboratory diagnosis is not routinely achieved for a substantial portion of respiratory specimens from symptomatic patients. We recently described the application of a multiplex PCR method for microbial surveillance wherein primers are attached to tags of varying mass that serve as digital signatures for their genetic targets. Tags are cleaved from primers and recorded by mass spectroscopy, enabling a sensitive, inexpensive, and highly multiplexed microbial detection. We used the multiplex MassTag PCR system (*10*) to investigate respiratory samples that had tested negative during routine diagnostic assessment. This previous study yielded pathogen candidates in approximately one third of cases, and in 8 cases identified a novel genetic clade of picornaviruses divergent from the previously characterized clades, including HRV-A and-B (*8*). To assess whether this novel clade cir-

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culates outside New York state, where it was discovered in cases of influenzalike illness (ILI), we investigated respiratory specimens from Africa, Asia, Australia, and Europe. In most studies (Africa, Asia), the collecting laboratories performed MassTag PCR, and inert mass-tagged amplification products were sent for analysis by mass spectrometry (MS); in other instances (Europe, Australia), inactivated nasopharyngeal swabs or aspirates were sent to New York for MassTag PCR and MS analysis.

Samples in South Africa were collected through a program for comprehensive surveillance of causes of respiratory illness in hospitalized children in the Pretoria area. MassTag PCR was applied to 58 specimens collected during the 2006 season from symptomatic children in their first year of life with no diagnosis available from previous clinical laboratory evaluation. Analysis of amplification products by MS yielded positive signal for HEV/HRV in 14 (24%) samples. Independent amplification and sequence analysis of VP4/2 coding sequence (*8*) in both laboratories showed sequences that matched the novel genotype in 4 (29%) samples obtained from patients with LRTIs and respiratory distress (Table 1, Figure). Samples collected in Côte d'Ivoire, West Africa, were from symptomatic persons living in the vicinity of Taï National Park. This location was the most remote of our study; residents have limited contact with other human populations. In this location, 2 (10%) HRV-A were identified in the 52 samples available for analysis (Table 1, Figure).

In Nepal, viruses of the novel genotype were identified in specimens collected during ILI surveillance or outbreaks of respiratory disease. Samples from ILI surveillance activities were collected in Kathmandu and Bharatpur. Outbreak samples were collected in the summer months from camps of >100,000 refugees from Bhutan located in Jhapa, southeast Nepal. Samples represented all age groups and were collected from December 2005 through July 2006. The novel genotype was identified by independent molecular typing in both laboratories in 4 (5%) samples (Table 1, Figure). In India, samples from 50 children with ARI, submitted for routine laboratory analysis during the 2007 season, were evaluated by MassTag PCR. Independent molecular typing in both laboratories indicated the novel genotype in 3 (6%) samples (Table 1, Figure).

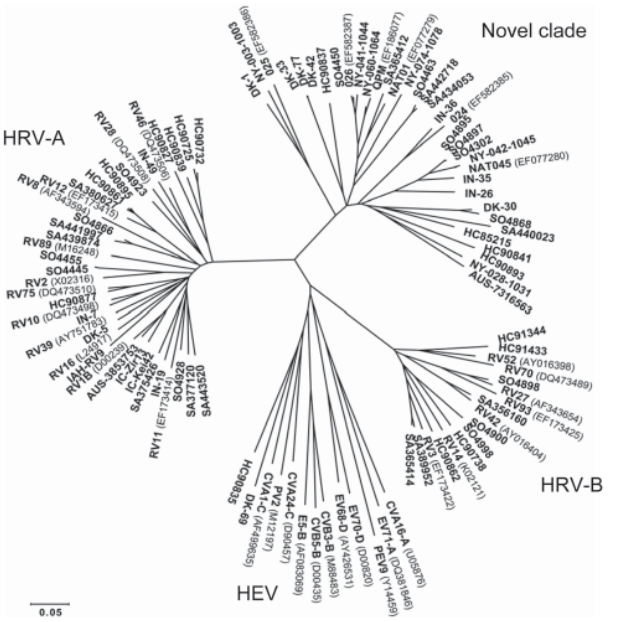

Figure. Phylogenetic analysis of VP4/2 coding region of viruses identified in association with acute respiratory illness (ARI) in South Africa, Côte d'Ivoire, Nepal, India, Western Australia, Denmark, and Spain (sequences deposited in GenBank under accession nos. EU697825–83). Phylogeny of VP4/2 nucleotide sequence (401 nt) was reconstructed by neighbor-joining analysis applying a Jukes-Cantor model; the scale bar indicates nucleotide substitutions per site. Included for reference are sequences belonging to the novel genotype identified in New York State (NY-003, –028, –041, –042, –060, and –074 [*8*]), similar viruses reported recently (QPM [*11*]; NAT01 and NAT045 [*12*]; and 024, 025, 026 [*13*]), and selected human rhinovirus A (HRV-A) serotypes (GenBank accession numbers for reference sequences are indicated in parentheses); HRV-B serotypes; human enterovirus C (HEV-C) viruses human coxsackievirus A1 and A24 (CV-A1, and CV-A24, respectively); human poliovirus 2 (PV-2); HEV-B viruses human echovirus 5 (E-5), human coxsackievirus B3 (CV-B3), and swine vesicular disease virus (CV-B5); HEV-D viruses human enterovirus 68 and 70 (EV-68, EV-70); porcine enterovirus B virus porcine enterovirus 9 (PEV-9); and HEV-A viruses human coxsackievirus A16 (CV-A16) and human enterovirus 71 (EV-71). SA, South Africa; IC, Côte d'Ivoire; HC, Nepal; IN, India; AUS, Australia; DK, Denmark; SO, Spain.

Table 1. Molecular diagnosis of ARI from 7 countries by using MassTag PCR* and VP4/2 sequencing†

Country	Season(s)	Samples	Picornavirus positive						
			Total	Novel clade	HRV-A	HRV-B	HEV	% Male	Age range (mean/median)
South Africa	2006	58	14	4	6	3	1	71	0.4–30 mo (5.6/3)
Côte d'Ivoire	2006	52	2	0	2	0	0	100	22–28 y (25/25)
Nepal	2005–06	80	17	4	7	5	1	56	0.25–56 y (8.5/3)
India	2007	50	6	3	3	0	0	83	4–36 mo (17.8/18)
Australia	2006	2	2	1	1	0	0	100	4–6 mo (5/5)
Denmark	2007	70	7	5	1	0	1	57	1–8 mo (2.9/2)
Spain	2003–2006	14‡	14	6	5	3	0	86	1–96 mo (23.2/15.5)

*See (*10*).
†ARI, acute respiratory illness; HRV, human rhinovirus; HEV, human enterovirus.
‡With previous HRV diagnosis.

Additional sample sets were obtained through main diagnostic laboratories in Western Australia, Denmark, and Spain, representing random respiratory specimens submitted for laboratory analysis. In 1 sample available from Western Australia, the novel genotype was identified in a preterm infant with undiagnosed, wheezy LRTI. The novel genotype was also found in 5 (7%) of 70 samples from Denmark and in 6 (43%) of 14 samples with previously diagnosed HRV infection from Spain (Table 1, Figure).

The 5% overall frequency of the novel genotype across our study samples, representing 34% of all detected picornavirus infections, and its observed global distribution, led us to analyze the accumulating sequence data for insights into their history. Rates of evolutionary change and the Time to the Most Recent Common Ancestor (TMRCA) of the novel clade were estimated by using the Bayesian Markov Chain Monte Carlo approach (BEAST package [*14*];), applying a relaxed molecular clock with an uncorrelated lognormal distribution of rates, a GTR + I + Γ_4 model of nucleotide substitution (determined by MODELTEST [*15*]), and exponential population growth. Statistical uncertainty in each parameter estimate is expressed as 95% highest probability density (HPD) values. The estimated mean rate of evolutionary change was 6.6×10^{-4} substitutions/site/y (95% HPD = $0.3–14.6 \times 10^{-4}$ substitutions/site/y; 38 dated samples collected over 32 mo (*8,16*) (S.R. Dominguez et al., unpub. data). Under this rate the mean TMRCA was estimated at 1,800 y, although with wide variance caused by the short sequence available (95% HPD = 279–5,201 y). Despite the inherent sampling error, this analysis suggests that this third clade of rhinovirus has been circulating for >250 years. The diversity observed within the novel clade and its genetic distance from other HRV/HEV were comparable to those seen for HRV-A, -B, or the HEV species (Table 2).

Conclusions

A clade of picornaviruses recently discovered in New York State is globally distributed and is found in association with community outbreaks of ARI and severe LRTIs of infants. These viruses contribute both to a substantial proportion of previously undiagnosed respiratory illness and to diagnosed, but nontyped cases of HRV infection. Similar viruses were recently characterized also in Queensland, Australia (*11*); California, USA (*12*); Hong Kong Special Administrative Region, People's Republic of China (*13*); and Germany (*16*). Our findings indicate the need for further investigation into this third (HRV-C) group of rhinoviruses with emphasis on epidemiology, pathogenesis, and strategies to prevent and ameliorate disease caused by HRV infection.

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Table 2. Percentage of intraspecies and interspecies conservation of VP4/2 nucleotide sequence*

Viruses	HEV-A	HEV-B	HEV-C	PV†	HEV-D	HRV-A	HRV-B	New clade
HEV-A	72	61	63	63	63	59	61	60
HEV-B		75	64	64	59	59	61	59
HEV-C			75	71	62	61	65	61
PV				81	60	60	62	61
HEV-D					83	59	61	61
HRV-A						80	61	63
HRV-B							80	60
New clade								75

*HEV, human enterovirus; PV, poliovirus; HRV, human rhinovirus.
†PV may be moved by the International Committee on Taxonomy of Viruses into HEV-C.

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Prevalence of *Cryptosporidium* spp. and *Giardia intestinalis* in Swimming Pools, Atlanta, Georgia

Joan M. Shields,*† Elizabeth R. Gleim,* and Michael J. Beach*

Cryptosporidium spp. and *Giardia intestinalis* have been found in swimming pool filter backwash during outbreaks. To determine baseline prevalence, we sampled pools not associated with outbreaks and found that of 160 sampled pools, 13 (8.1%) were positive for 1 or both parasites; 10 (6.2%) for *Giardia* sp., 2 (1.2%) for *Cryptosporidium* spp., and 1 (0.6%) for both.

Giardia sp. and *Cryptosporidium* spp. are gastrointestinal parasites spread through the fecal-oral route. In 2003–2004, these parasites were responsible for 61.2% (*Cryptosporidium* spp. 55.6%; *Giardia* sp. 5.6%) of gastroenteritis outbreaks associated with treated swimming venues (e.g., swimming pools, water parks) in the United States (*1*). *Cryptosporidium*'s key role in these outbreaks is likely because of its small size, low infectious dose (*2*), and high tolerance to chlorine (*3*), which is the major disinfectant used in swimming pools. Despite frequent outbreaks, little is known about these parasites' occurrence in swimming pools in the absence of outbreaks. Although the frequency of contamination is unknown, 4.4% of formed feces recovered from non–outbreak-related pools were positive for *Giardia* sp. and 0 were positive for *Cryptosporidium* spp. (*4*). In the Netherlands, 7 pools sampled for >1 year had a prevalence of 5.9% for *Giardia* sp., 4.6% for *Cryptosporidium* spp., and 1.3% for both pathogens (*5*). In Italy, 1 study found 28.6% (2/7) of tested pools were positive for both *Giardia* sp. and *Cryptosporidium* spp. (*6*) and another study found 40% (4/10) of tested pools positive for either parasite (*7*). No data exist on the occurrence of these parasites in US pools. Further data on pool contamination would reinforce existing US pool codes and support code changes designed to reduce the level of parasite contamination, particularly chlorine-resistant *Cryptosporidium* spp.

During the past 2 decades, *Cryptosporidium* spp. and *Giardia* sp. have been associated with increasing outbreaks of swimming-associated gastrointestinal illness in the United States; *Cryptosporidium* spp. is emerging as the leading cause of swimming pool-associated outbreaks of gastrointestinal illness (*1*). However, the baseline prevalence of contamination in non–outbreak-associated swimming pools is incomplete.

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The Study

A convenience sample of 160 public swimming pools from 2 metropolitan Atlanta, Georgia, counties was used to collect filter backwash samples for parasite examination during a 7-week period (late August–October 2006). Information on age of swimmers, pool type, pool size, and number of swimmers was gathered. No facility identifiers were assigned.

Filter backwashing is a cleaning process by which the water flow through the filter is reversed so that accumulated debris trapped in the filter is dislodged and directed to waste. Filter backwash therefore tends to contain more concentrated pathogens than does pool water. All selected pools had a sand filter (most public pools in the metropolitan Atlanta area use sand filters) and had been used by swimmers before the backwash cycle and sample collection began.

One-liter samples of filter backwash were collected in wide-mouthed plastic bottles shortly after the filter flow grew turbid and were transported and stored at 4°C before flocculation. The samples were calcium carbonate flocculated within 2 weeks, typically within a few days, after collection (*8*). Pellets were stored in DNase, RNase-free, sterile microcentrifuge tubes. DNA was extracted from 250–350 mg of each pellet by using a FastPrep DNA kit (MP Biomedical, Solon, OH, USA); 20 μL of polyvinyl pyrolidone (FW 40,000; Fisher Scientific, Pittsburgh, PA, USA) was added to the CLS-VF buffer provided in the kit. Final purification used a QIAquick spin column kit (QIAGEN, Valencia, CA, USA).

Real-time qPCR used the Stratagene Mx3000P thermocycler (Stratagene, La Jolla, CA, USA) and the triplex PCR reaction and amplification protocol described for *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. (primers and probe amplify both *C. parvum* and *C. hominis*) with a reported sensitivity and specificity of 100% (*9*). DNA from *E. histolytica* was added to each sample as a positive internal control. Sample inhibition was alleviated by repeating the qPCR with 4.7 μg/μL of bovine serum albumin (Sigma, St. Louis, MO, USA).

Of the 160 filter backwash samples collected, 13 (8.1%) were positive for 1 or both parasites; 10 (6.2%) were positive for *G. intestinalis*; 2 (1.2%) were positive for *Cryptosporidium* spp.; and 1 (0.6%) was positive for both pathogen genera. Because of the small amount of target DNA, speciation was not possible with most samples. However, 1 *C. hominis* positive sample was identified.

The Table summarizes parasite prevalence by age of swimmers, pool type, pool size, and number of swimmers. Although 117 (73.1%) of all pools tested were commonly used by children (28 were designated for children only, 89 for children and adults), these pools accounted for 12 (92.3%) of 13 positive pools sampled. In comparison, 43 (26.9%) of 160 pools designated for adult use were associated with 1 (7.7%) of 13 positive pools. Of the positive samples, 10 (76.9%) of 13 were found in community pools, although community pools accounted for 40% (64/160) of pools assayed. Small-volume pools (≤50,000 gallons) comprised 72 (45%) of 160 sampled pools but accounted for 9 (69.2%) of 13 positive samples. Similarly, pools with ≤75 swimmers per week comprised 42.5% of pools but accounted for 10 (76.9%) of 13 positive samples.

Although this study was small and the power low, estimates of the prevalence odds ratio (POR) were calculated. Those associations that were statistically significant for finding protozoa-positive samples were as follows: community pools (POR 5.7, 95% confidence interval [CI] 1.5–21.8) and weekly number of swimmers of <75 (POR 5.1, 95% CI 1.4–19.4). Although the positive parasite sample prevalence was higher in pools frequented by children and adults (10.1%) than in pools designated for adults only (2.3%), the POR was not significant (POR 4.8, 95% CI 0.6–38.1), likely because of the small sample size.

Prior analysis of 22,131 US pool inspections demonstrated that children's pools had an increased incidence of critical pool code violations perhaps because their smaller volumes and depths make maintaining appropriate levels of disinfectant more difficult (*4*,*10*). In addition, younger children may be more likely to contaminate recreational water as a result of being incontinent or having higher levels of perianal fecal contamination (*11*). This finding necessitates greater vigilance in maintaining water quality for this population because they are more likely to contaminate the water and are more vulnerable to the severe effects of diarrheal illnesses.

Table. Pathogen distribution in 13 *Cryptosporidium*- and *Giardia*-positive swimming pools (n = 13)

Characteristic	% (n/N)	Type of parasite*
Age of swimmers†		
Children	10.7 (3/28)	2 G, CG
Adults	2.3 (1/43)	G
Mixed	10.1 (9/89)	7 G, C, Ch
Total	8.1 (13/160)	
Pool type‡		
Community	15.6 (10/64)	7 G, CG, C, Ch
School	4.8 (1/20)	G
Health club	0 (0/25)	
Apartment	3.9 (2/51)	2 G
Total	8.1 (13/160)	
Pool size (x1,000 gallons)		
≤5	22.2 (4/18)	3 G, CG
6–50	9.3 (5/54)	4 G, Ch
51–100	0 (0/40)	
101–200	11.4 (4/35)	3 G, C
>200	0 (0/13)	
Total	8.1 (13/160)	
No. swimmers/bathers per week		
1–75	14.7% (10/68)	7 G, CG, C, Ch
76–200	7.4% (2/27)	2 G
201–500	2.9% (1/35)	G
>500	0% (0/30)	
Total	8.1% (13/160)	

*G, *Giardia intestinalis;* C, *Cryptosporidium* spp.; Ch, *C. hominis;* CG, both *G. intestinalis* and *Cryptosporidium* spp.
†Children defined as persons <16 y of age; adults defined as persons ≥16 y of age.
‡Residential and hotel/motel swimming pools were excluded from the study.

Conclusions

This study is a snapshot of contamination at the end of the swim season. Although an earlier sampling schedule may have detected more contamination, these findings suggest that contamination events in some pool types or with some swimmer compositions may be relatively common during the swim season. The prevalence of contamination found by this study is difficult to compare with that found by other studies that focus on serial samples from a small number of pools. However, the key finding, parasite detection, is repeated in all the studies cited (*5*–*7*). The risk for disease transmission is difficult to ascertain because most studies, including this one, have not measured viability of the parasites recovered from water or filter backwash. However, intact *Cryptosporidium* oocysts observed following hyperchlorination to inactivate the parasite are commonly noninfectious (M.J. Arrowood, pers. comm.).

This study is limited by having a small sample size, by being a convenience sample, and by using backwash collected from pools with a single filter medium (i.e., sand) exclusively. In addition, the sensitivity and specificity of PCR detection in pool-associated backwash samples is unknown, although positive and negative controls reacted appropriately. Although these deficiencies would likely lead to underestimates of the prevalence of parasites in this sample, clearly such study results are neither generalizable to all types of pools nor an accurate measure of national contamination levels. However, despite these deficiencies, the finding of swimming pool filter contamination by *Giardia* sp. and *Cryptosporidium* spp. is key and reinforces the need for continued emphasis on improving pool operation and maintenance (e.g., preventive hyperchlorination or flocculation on a routine basis). These improvements should also include consideration of supplementary inline disinfection systems known to inactivate *Cryptosporidium* spp. (e.g., ultraviolet light, ozone) and other pathogens (*3*,*12*–*14*). These data also underscore the need for the general public, particularly immunocompromised persons, to

understand recreational water–associated illness transmission and adopt healthy swimming habits (e.g., no swimming when ill with diarrhea, no swallowing of pool water, improved hygiene [*15*]) that are needed to reduce the risk for pathogen transmission.

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Spatial and Temporal Evolution of Bluetongue Virus in Wild Ruminants, Spain

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We confirmed the emergence of bluetongue virus (BTV) in 5 wild ruminant species in Spain. BTV seroprevalence was high and dispersed with time, with a south-to-north gradient. Our results suggest a complex epidemiology of BTV and underline the need for additional research on wildlife in Europe.

In October 2004, an outbreak of bluetongue caused by bluetongue virus serotype 4 (BTV-4) occurred in southern Spain (www.oie.int/hs2/zi_pays_mald.asp?c_pays=58&c_mald=10&annee=2004). Since then, several BTV-4 outbreaks have occurred in livestock in Spain. Recently, BTV-1 emerged in Spain and caused several outbreaks in livestock (www.oie.int/wahid-rod/public.php?page=single_report&pop=1&reportid=5799). In addition, BTV-8 emerged in central Europe in 2006 (*1*) (www.oie.int/esp/press/es_061023.htm). New outbreaks of BTV-8 occurred in 2007, and this virus serotype was recently detected in the United Kingdom (www.oie.int/wahid-prod/public.php?page=disease_immediate_summary).

BTV is a vector-borne pathogen; *Culicoides* species biting midges (Diptera: Ceratopogonidae) are its biologic vectors (*2*). The main BTV vector in Europe is *C. imicola* (*2*), a seasonal species that appears from late May to November (*3*). Other *Culicoides* species may also be vectors of BTV in Europe (*4*,*5*; www.oie.int/esp/press/es_061023.htm). BTV is distributed worldwide between the latitudes 42°30′N and 35°S (*6*), but it has recently spread northward (*1*,*7*). The potential for economic losses make BT a disease reportable to the World Organisation for Animal Health.

Several wild ruminant species are susceptible to BTV infection (*8*–*10*). Red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) are the most common wild ruminants in Spain; mouflon (*Ovis aries*), fallow deer (*Dama dama*), and aoudad (*Ammotragus lervia*) are less common (*11*). The distribution and density of wild ungulates have increased in recent decades in Spain (*12*), but the role of European wild ruminants in the epidemiology of BTV is unknown.

When diseases are transmitted between wildlife and livestock, wildlife disease research is an important tool in establishing effective disease control programs. The risk of wildlife acting as BTV reservoirs and relevant epidemiologic factors need to be explored (*13*). The situation in Spain, with well-distributed wild ruminant species and the presence of BTV vectors and BTV-4, led us to study the status of these ruminants in the epidemiology of BTV.

The Study

The study area included the southern half of peninsular Spain. This area was selected on the basis of the distribution of *C. imicola* and the geographic distribution of BTV-4 outbreaks in livestock and the associated animal movement restriction area (www.mapa.es).

Blood samples were obtained from 2,233 red deer, 106 fallow deer, 44 roe deer, 72 mouflon, and 10 aoudad during 2003–2007 in 62 locations (Figure 1). Most (n = 1,575) of the red deer samples and all samples from other ruminant species were collected from hunted animals; some samples (n = 658) from red deer were collected on 5 farms. These farms were located in the Alcornocales (ALC), Sierra Morena (SM), Guadiana Valley (GU), Montes de Toledo (MT), and Sistema Central (SC) areas. Most (69%) samples were collected during the hunting season (October–February). Samples were not obtained during certain periods because of logistic surveillance constraints (online Appendix Table,

Figure 1. Map of Spain showing wild ruminant sampling sites (black dots) and areas (gray) sampled along a south-to-north gradient during 2003–2007. SC, Sistema Central; TO, Toledo Province; MT, Montes de Toledo; GU, Guadiana Valley; SM, Sierra Morena; ALC, Alcornocales.

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available from www.cdc.gov/EID/content/14/6/951-appT.htm). Sex and age of red deer were determined, the latter according to tooth eruption patterns (*14*). Animals <1 year of age were classified as juveniles, those 1–2 years of age as subadults, and those >2 years of age as adults.

Blood was collected into sterile tubes from the heart or thoracic cavity from hunted animals and by cervical puncture from farm animals. Samples were centrifuged, and serum was stored at –20°C. Antibodies to BTV in sera were detected by using a competitive ELISA (Pourquier ELISA Bluetongue Serum; Institut Pourquier, Montpellier, France) according to manufacturer's instructions. The inhibition value of the ELISA for domestic ruminants has been found to be applicable for testing serum from wild ruminants (*15*).

We expected a south-to-north gradient in the expansion of BTV across Spain as observed in livestock. Thus, sampling sites were grouped into 6 areas according to a south-to-north gradient (Figure 1). These areas included southern (ALC), south-central (SM, GU, and MT), and central (Toledo Province and SC) Spain. On the basis of annual distribution of BT outbreaks in Mediterranean countries (*2*), a year was defined as the period July–June.

None of the samples collected in 2003–2004 and 2004–2005 had antibodies to BTV; antibody-positive samples were detected in 2005–2006 and 2006–2007. Mean seroprevalence values for ruminant species analyzed are shown in the Table. A total of 17% ±2% of red deer sampled in 2005–2006 and 29% ±4% of those sampled in 2006–2007 had antibodies to BTV. Northernmost red deer (SC area) were antibody positive only in 2006–2007. However, the number of samples tested in this area the previous year was low (n = 5).

BTV seroprevalence in red deer decreased along a south-to-north gradient and increased throughout the years sampled (online Appendix Table and Figure 2). Mean seroprevalence was slightly higher in males and adults but dependent on the sampling area (online Appendix Table). Because the first evidence of contact with BTV occurred in 2005, observed seroprevalences can be considered as incidence rates. Thus, in 2005–2006, the incidence rate was higher in subadults than in adults and juveniles.

Figure 2. Study areas in Spain showing wild ruminant sampling sites by study year. A) 2003–2004; B) 2004–2005; C) 2005–2006; D) 2006–2007. A year is defined as the period July–June. Animal movement restriction areas for each year (www.mapa.es) are shown (gray areas). White dots show wild ruminant seronegative sampling sites, and black dots show level of bluetongue virus seroprevalence in wild ruminants. Numbers of sampled wild animals per sampling site are shown in parentheses.

Conclusions

The presence of antibodies to BTV in wild ruminants has recently been studied in Belgium (A. Linden, pers. comm.) and Germany (www.efsa.europa.eu/EFSA/DocumentSet/appx6_bluetongue_S8_en.pdf). In 2006, BTV seroprevalence was 0.58% in Belgian cervids. The survey in Germany showed BTV seroprevalence rates of 0.09% in red deer, 5.7% in roe deer, and 4.9% in mouflon. Our study provides evidence that BTV is present in wild ruminants in Europe over a large area.

All ruminant species studied were positive for BTV. This result is not surprising because other wild ruminant species have been reported to be susceptible to BTV infec-

Table. Seroprevalence of bluetongue virus in wild ruminant species, Spain, 2005–2007*

	Red deer		Fallow deer		Roe deer		Mouflon		Aoudad	
Area	n/N	% (SE)	n/N	% (SE)	n/N	% (SE)	n/N	% (SE)	n/N	% (SE)
ALC	116/187	62 (0.04)	25/64	39.1 (0.06)	1/35	2.9 (0.03)	ND	ND	ND	ND
SM	111/580	19.1 (0.02)	9/17	52 (0.12)	ND	ND	0/1	0	ND	ND
GU	36/186	19.4 (0.03)	0/1	0	1/4	25 (0.22)	1/22	4.5 (0.04)	1/4	25 (0.22)
MT	34/435	7.8 (0.01)	0/14	0	ND	ND	8/42	19.1 (0.06)	ND	ND
TO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SC	12/21	57.1 (0.11)	ND	ND	ND	ND	0/3	0	ND	ND
Total	309/1,409	21.9 (0.01)	34/96	35.4 (0.05)	2/39	5.1 (0.04)	9/68	13.2 (0.04)	1/4	25 (0.22)

*n/N, no. positive/no. tested; SE, standard error; ALC, Alcornocales; ND, not determined; SM, Sierra Morena; GU, Guadiana Valley; MT, Montes de Toledo; TO, Toledo Province; SC, Sistema Central.

tion (*8–10*). We observed some differences in BTV seroprevalence between ruminant species (Table). However, if one considers the epidemic nature of BTV, differences in the number of seropositive ruminant species, and the unequal distribution of *C. imicola* in the study area (*2*; www.mapa.es), the observed results may be caused by differences in susceptibility to the vector/pathogen or differences in vector/pathogen distribution across the study area.

We observed similar spatial and temporal BTV patterns in red deer (Figure 2) and livestock. Nevertheless, we found the first evidence of contact with BTV 1 year later in red deer than in livestock. This delay may have been caused by larger numbers of samples from livestock than from wild ruminants. However, our findings suggest that wild ruminants, particularly cervids because of their wider distribution in Europe, could be used as sentinels for surveillance of BTV. Moreover, the high BTV seroprevalence in cervids from the southernmost sampling area suggests that cervids may not interfere with vaccinations given in this region.

This study shows an increased distribution of BTV across Spain and that wild ruminants in Europe can be infected with BTV. Our findings, combined with those of earlier studies, suggest a complex epidemiologic scenario of BTV in Europe with many susceptible hosts, an increase in its main vector because of climate changes, and the appearance of new competent vectors. Nevertheless, more information on the role of susceptible wild ruminant species is needed to clarify the complexity of BTV epidemiology in Europe.

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Ceftriaxone-Resistant *Salmonella enterica* Serotype Newport, France

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The multidrug-resistant (MDR) *Salmonella enterica* serotype Newport strain that produces CMY-2 β-lactamase (Newport MDR-AmpC) was the source of sporadic cases and outbreaks in humans in France during 2000–2005. Because this strain was not detected in food animals, it was most likely introduced into France through imported food products.

Third-generation cephalosporins are drugs of choice for treatment of persons with nontyphoidal *Salmonella* infections that require chemotherapy or when fluoroquinolones are contraindicated. A new public health concern is the emergence of third-generation cephalosporin–resistant *Salmonella* isolates (*1*). Multidrug-resistant (MDR) *Salmonella enterica* serotype Newport isolates that produce CMY-2, a β-lactamase that inactivates third-generation cephalosporins, were first reported in the United States in 1998 (*2*). These isolates, known as Newport MDR-AmpC, have quickly spread through the United States in cattle and humans (*3–5*). It has been hypothesized that use of ceftiofur, a third-generation cephalosporin licensed in the United States for use in cattle, could have selected for Newport MDR-AmpC (*2–4,7*). Several observations and case-control studies suggested beef and milk from dairy cattle were substantial sources of Newport MDR-AmpC infection in humans (*6–8*).

These isolates seem to be extremely rare in Europe. Two surveys performed in England and Wales (278,308 human *Salmonella* isolates tested, 1992–2003) and Spain (959 human *Salmonella* isolates, 1999–2000) did not detect Newport MDR-AmpC (*9*,*10*). In St. Petersburg, Russia, only 1 Newport MDR-AmpC isolate was reported among 1,078 *Salmonella* isolates during 2002–2005 (*11*). In France, a small outbreak (14 cases) of Newport MDR-AmpC was detected in 2003 and linked to consumption of imported horse meat (*12*). We undertook the present study to acquire more knowledge on circulation of Newport MDR-AmpC in humans, animals, and animal-derived food in France.

The Study

From 2000 through 2005, the French National Reference Centre for *Salmonella* at the Institut Pasteur in Paris reported 829 Newport isolates among 69,759 *Salmonella* clinical isolates. During this period and depending on the year, serotype Newport ranked between 6th and 10th in prevalence among human serotyped isolates. From 2000 through 2005, the Agence Française de Sécurité Sanitaire des Aliments reported 2,160 Newport isolates among 101,791 *Salmonella* isolates collected from animals and food products.

Antimicrobial drug susceptibility testing was performed on 585 human Newport isolates and 342 nonhuman Newport isolates by disk diffusion with 32 antimicrobial drugs (additional information available from fxweill@pasteur.fr). Data for Newport human isolates are shown in the Table. Of 585 isolates tested, 46 (7.9%) were resistant to third-generation cepalosporins. The geographic origin of the isolates was mainly the Paris metropolitan area and northern France (online Appendix Table, available from www.cdc.gov/EID/content/14/6/954-appT.htm). There was a high prevalence of third-generation cephalosporin–resistant isolates during 2000 (15%) and 2003 (17.5% caused by a small outbreak). No third-generation cephalosporin resistance was detected in any of the nonhuman Newport isolates tested.

Experiments were performed on the 46 third-generation cephalosporin–resistant Newport isolates (additional information available from fxweill@pasteur.fr). All but 1 of the Newport isolates were resistant to cefoxitin (online Appendix Table). These isolates showed 4 resistance phenotypes; most (41, 89.1%) were resistant to streptomycin, sulfonamides, chloramphenicol, and tetracycline. PCR and sequencing showed that the 45 isolates resistant to cefoxitin were positive for the $bla_{\text{CMY-2}}$ gene, and cefoxitin-susceptible isolates contained the extended-spectrum β-lactamase gene $bla_{\text{CTX-M-1}}$. Ceftriaxone MICs of Newport MDR-AmpC isolates ranged from 32 mg/L to >256 mg/L, and ceftazidime MICs ranged from 64 mg/L to >256 mg/L. No bla_{TEM} genes were detected. Three isolates with additional resistance to aminoglycosides contained a class 1 integron with the 1-kb gene cassette *aadA24* (known to encode resistance to strep-

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Table. Resistance to specific antimicrobial drugs in *Salmonella enterica* serotype Newport from humans in France, 2000–2005*

Drug	% Resistant isolates					
	2000 (n = 100) (N = 109)	2001 (n = 124) (N = 134)	2002 (n = 66) (N = 71)	2003 (n = 126) (N = 138)	2004 (n = 91) (N = 94)	2005 (n = 78) (N = 80)
Amoxicillin	27	9.7	1.5	19.8	8.8	3.8
Ceftriaxone/ceftazidime	15	4	1.5	17.5	2.2	0
Gentamicin	4	1.6	0	1.6	2.2	0
Nalidixic acid	23	7.3	4.5	1.6	4.4	2.6
Ciprofloxacin	0	0	0	0	0	0
Sulfonamides	29	10.5	4.5	19.8	8.8	0
Trimethoprim	10	4	3	1.6	4.4	0
Chloramphenicol	25	9.7	1.5	15.9	8.8	0
Tetracycline	27	11.3	3	19	9.9	3.8

*n, no. of isolates studied; N, no. of isolates received at the French National Reference Centre for *Salmonella* (1 per patient).

tomycin and spectinomycin) (*11*). The chloramphenicol/florfenicol resistance gene *floR* was detected in all but 1 CMY-2–producing Newport isolate.

Clonal relatedness of Newport isolates was assessed by multilocus sequence typing (MLST) and PulseNet standard method pulsed-field gel electrophoresis (PFGE) (Figure 1). All 16 Newport MDR-AmpC isolates tested had a common sequence type (ST), ST45. *Xba*I-PFGE identified 10 distinct profiles (similarity 76.7%) among all 45 Newport MDR-AmpC isolates. Single enzyme matches were found for 3 of the profiles (15 isolates) in the US PulseNet national database (www.cdc.gov/pulsenet; online Appendix Table; Figure 2). Two PFGE types (New6 and New8) were divided into 2–4 subtypes because of additional band(s) <100 kb. Isolates from the 2003 outbreak showed 4 similar but distinct PFGE profiles that differed by 1–2 bands, migrated between 60 and 100 kb, and were attributed to plasmid(s) (additional information available from fxweill@pasteur.fr). If only cases with indistinguishable PFGE profiles had been tested, potentially related cases would not have been linked to this outbreak. Therefore, during an outbreak investigation of Newport MDR-AmpC, analysis of plasmid content (either by alkaline lysis or S1 nuclease, depending on size of additional bands) might complete *Xba*I-PFGE profiles for isolates whose profiles differ by 1 or 2 additional bands of low molecular mass.

Alkaline lysis extraction showed that all but 1 of the Newport MDR-AmpC isolates harbored a plasmid >125 kb that hybridized with a bla_{CMY-2} probe; the remaining isolate harbored a plasmid of 100 kb (online Appendix Table). Analysis with S1 nuclease showed that these plasmids were 100 kb–370 kb. Up to 3 additional plasmids (3.5 kb–100 kb) that did not have bla_{CMY-2} were detected in most isolates (online Appendix Table). Cephalosporin resistance was transferred by electroporation of plasmid DNA to *Eschericha coli* DH10B for all 38 CMY-2–positive isolates tested. When present in the donor strain, resistance to sulfonamides, chloramphenicol, and tetracycline was also transferred. Restriction analysis of plasmids isolated from transformants showed 6 similar restriction profiles for Newport isolates (R1–R6) (Figure 2, online Appendix Table). R1 was predominant (found in 26 isolates among 35 tested, 74.3%). Newport plasmids R1–R6 and Agona plasmid R8 were shown by PCR to contain variant A/C_2 replicons (*13*), whereas Typhimurium plasmid R7 contained the I1 replicon.

*Pst*I-digested plasmids analyzed by Southern hybridization with a bla_{CMY-2} probe (Figure 2) showed 4 hybridization profiles among Newport isolates. Profile H1 corresponded to plasmid type C described by Carattoli et al. (*14*). Profiles H2, H3, and H4 differed from H1 by 1 additional band (>10 kb for H2, 3.2 kb for H3, and >18 kb for H4), which indicated that the bla_{CMY-2} gene was partially or totally duplicated.

Conclusions

Newport MDR-AmpC isolates have been the source of sporadic cases and small outbreaks in humans in France during 2000–2005. All isolates had the same MLST type, ST45, and highly similar *Xba*I-PFGE profiles. Their plas-

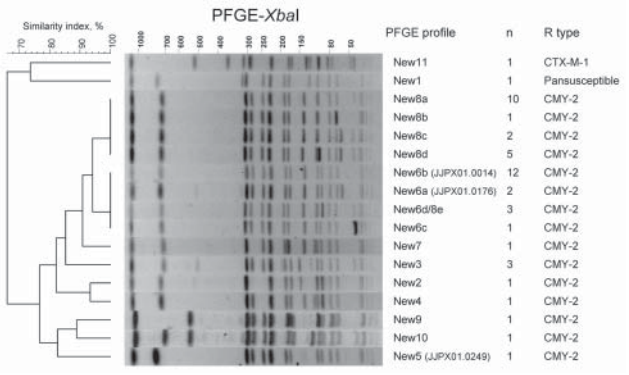

Figure 1. Representative *Xba*I pulsed-field gel electrophoresis (PFGE) profiles of third-generation cephalosporin–resistant *Salmonella* Newport isolates studied. A dendrogram was generated with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The PFGE profile (and if there were indistinguishable isolates in the PulseNet USA database [www.cdc.gov/pulsenet], the corresponding Centers for Disease Control and Prevention PulseNet profile), the number of isolates, and the β-lactamase genes are indicated.

mids carrying bla_{CMY-2} were homogeneous (same incompatibility group A/C_2, a main restriction type R1, and a main hybridization type H1). These results support clonal expansion of 1 Newport strain (or a limited number of genetically related Newport strains) able to acquire and maintain a large $incA/C_2$ MDR plasmid.

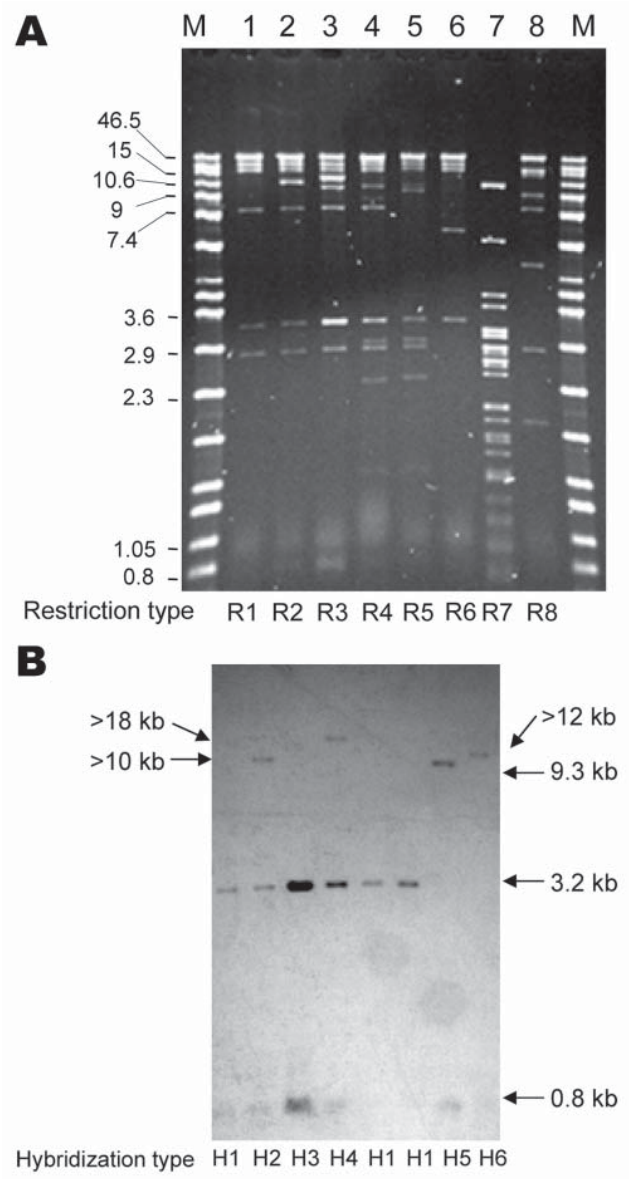

Figure 2. Representative *Pst*I restriction profiles (A) and bla_{CMY-2} Southern hybridization (B) of plasmids from *Escherichia coli* DH10B transformants of CMY-2–producing *Salmonella* spp. clinical isolates. Lane M, Raoul molecular mass marker (Qbiogene, Illkirch, France). Lane 1, DH10B/00-7490; lane 2, DH10B/03-3349; lane 3, DH10B/03-3367; lane 4, DH10B/00-3525; lane 5, DH10B/00-4165; lane 6, DH10B/03-9969; lane 7, DH10B/03-9243; lane 8, DH10B/02-2049. Values on the left of panel A are in kb. Restriction and hybridization profiles are indicated. The gel is focused on the resolution of high molecular mass bands; smaller bands (in particular, the 0.8-kb band) are not well visualized.

The source of the French isolates remains unknown. However, this strain was not found in French food animals or domestically produced food products (additional information available from fxweill@pasteur.fr). One outbreak during the study period was linked to imported horse meat. Further investigation identified the source as a wholesaler who imported meat from Belgium, the United Kingdom, Hungary, Canada, Brazil, Argentina, Uruguay, and Australia (*12*). In contrast to Europe, Newport MDR-AmpC has been frequently seen in the United States during the past decade. Furthermore, several characteristics were shared between US and French Newport MDR-AmpC isolates: ST45 (*15*), PFGE profiles New5, New6a, and New6b (displayed by 15 isolates among the 45 studied), and bla_{CMY-2} plasmid hybridization type H1 (*14*). We can reasonably hypothesize that during 2000–2005 some isolates likely entered France from North America through imported food. Alternatively, they could have come to France and North America from some other country.

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Avian Influenza A Virus (H5N1) Outbreaks, Kuwait, 2007

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Phylogenetic analysis of influenza A viruses (H5N1) isolated from Kuwait in 2007 show that (H5N1) sublineage clade 2.2 viruses continue to spread across Europe, Africa, and the Middle East. Virus isolates were most closely related to isolates from central Asia and were likely vectored by migratory birds.

Highly pathogenic avian influenza (HPAI) virus (H5N1) has been endemic in poultry in Asia since 2003 (*1*,*2*). From 2002 through 2005, influenza virus (H5N1) has also been sporadically isolated from dead wild birds in Hong Kong Special Administrative Region, People's Republic of China; however, these birds were considered dead-end hosts of viruses acquired from poultry (*3*,*4*). In April 2005, an influenza (H5N1) outbreak was detected in bar-headed geese (*Anser indicus*) at Qinghai Lake in western China (*5*). Following this outbreak, the Qinghai-like (clade 2.2) influenza virus (H5N1) lineage was detected in wild birds and domestic poultry in countries in central Asia, the Middle East, Europe, and Africa (*6*–*10*). The source of these introductions, while still debated, is likely through bird migration, although in some instances, the role of the poultry trade has not been ruled out (*6*–*12*).

The clade 2.2 influenza (H5N1) viruses continue to be detected throughout these regions; 69 human cases with 31 deaths were reported from Azerbaijan, Djibouti, Egypt, Iraq, Nigeria, Pakistan, and Turkey from January 2006 through December 2007 (*13*). Since early 2007, the Qinghai-like influenza (H5N1) lineage has continued its geographic spread and has been reported from more than 40 countries in Eurasia and Africa (*6*). The continued detection of these viruses in Africa, Europe, and the Middle East from mid-2006 onward suggests that the virus may now be endemic in these regions.

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The Study

On February 13, 2007, the Public Authority for Agriculture and Fisheries of Kuwait reported the initial outbreak of influenza (H5N1) in poultry in the Al Wafrah farm area in southern Kuwait. Subsequently, 131 influenza virus (H5N1)–infected poultry were confirmed from 20 farms throughout the country (Figure 1, panel A). The disease resulted in high mortality rates among infected flocks, especially in the commercial broiler farms in Al-Wafrah and among poultry raised in privately owned residential homes and backyard farms. Disease control measures were implemented beginning February 18, 2007, including control of poultry movement, vaccination, disinfection of infected premises, and culling of ≈500,000 birds. The final case of subtype H5N1 was detected on April 20, 2007, and

Figure 1. A) Kuwait, with location of subtype H5N1 virus outbreaks in 2007. Circles indicate location of farms with confirmed influenza (H5N1) infections in poultry; square indicates the Al Sulaibiya area where virus isolation was conducted. B) Eurasia, with location of subtype H5N1 isolates phylogenetically related to Kuwait isolates.

all restrictions were lifted on May 12, 2007. Kuwait was declared free of highly pathogenic avian influenza (HPAI) (H5N1) on July 21, 2007.

During these outbreaks, 20 samples were collected from small backyard farms in the Al Sulaibiya area (Figure 1, panel A). Among those samples, 10 throat and cloacal swabs were collected from chickens that showed signs of disease; 10 more samples were collected from internal organs (liver and spleen) of dead chickens. Seven of the 10 organ samples tested positive for subtype H5N1 by using the TaqMan Influenza A/H5 Detection Kit v1.0 on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

We sequenced the complete genome of these 7 subtype H5N1 strains isolated from poultry outbreaks in Kuwait during 2007. All sequences that were generated in this study have been deposited in GenBank (accession nos. CY029945–CY030000). To understand the developments of influenza A virus (H5N1) in Kuwait, we characterized and phylogenetically analyzed all 8 gene segments of these 7 viruses with all available influenza (H5N1) viruses previously isolated from Africa, Eurasia, Southeast Asia, and southern China, and with reference viruses belonging to each subtype H5N1 clade. Sequence assembly, editing, multiple sequence alignment, neighbor-joining, and Bayesian phylogenetic analyses were conducted as previously described (*11*).

Phylogenetic analysis of the hemagglutinin (HA) genes showed that all 7 subtype H5N1 isolates were derived from the Goose/Guangdong-like lineage and clustered together with other Qinghai-like (clade 2.2) viruses (Figure 2). The Kuwait isolates were most closely related to viruses from Germany and Krasnodar, in southwest Russia, which were also isolated in 2007 (Figure 1, panel B). Those viruses were mostly isolated from wild bird species (swan and grebe), although a single isolate was from chicken in Krasnodar. This group of viruses was in turn related to 2006 isolates from diverse geographic areas such as Afghanistan, Mongolia, and Siberian Russia (Figure 1, panel B). Phylogenetic analyses of the neuraminidase gene and all internal gene segments (data not shown) show that all of the viruses belong to subtype H5N1, genotype Z, and maintain phylogenetic relationships similar to the HA tree.

The HA protein of all 7 isolates maintained the motif of multiple basic amino acids (QGERRRKKR/G) at the HA-connecting peptide, a feature that is characteristic of HPAI virus. The receptor-binding pocket of HA1 retains Gln 222 and Gly 224 (H5 numbering) that preferentially binds avian-like α2,3-NeuAcGal linkages. However, a single Glu212Lys substitution occurred in the HA receptor binding site in all 7 Kuwait isolates, which has also been observed in all clade 2.2 influenza (H5N1) viruses characterized to date. The biological implications of this mutation

Figure 2. Phylogenetic relationships of the hemagglutinin (HA) gene of influenza virus (H5N1) isolates from Kuwait in 2007. Numbers at nodes indicate neighbor-joining bootstraps ≥60, and Bayesian posterior probabilities ≥95% are indicated by thickened branches. Analyses were conducted with nucleotide positions 1–963 of the HA gene. The HA tree was rooted to Gs/Guangdong/1/1996. Labels to the right of the tree refer to World Health Organization (H5N1) clade designations (*14*). Ck, chicken; Dk, duck; Gs, goose, MDk, migratory duck; Qa, quail; Ty, turkey.

remain to be investigated. None of the isolates had mutations in the M2 ion channel or the neuraminidase, conferring resistance to amantadine and oseltamivir, respectively. All isolates possessed Lys at position 627 of the PB2 gene, which is associated with increased virulence in mammals and is present in all known clade 2.2 viruses. Other virulence mutations were not recognized in any of the viruses characterized in this study.

Antigenic characterization of a representative virus from Kuwait (Ck/Kuwait/KISR2/2007) was conducted as previously described (*11*). These results demonstrate close antigenic relationship of Ck/Kuwait/KISR2/2007 to BHG/Qinghai/1A/2005 (Table), the prototype clade 2.2 virus, and a vaccine candidate virus that was isolated during the

Table. Antigenic analysis of influenza viruses (H5N1) by hemagglutinin inhibition test, 2007*

Virus	Clade†	Anti-VNM/1203‡	Anti-IDN/5	Anti-CDC357	Anti-QH/1A	Anti-Anhui/1
Vietnam/1203/2004	1	**160**	160	40	40	40
Indonesia/5/2005	2.1	<40	**640**	640	40	80
Indonesia/CDC357/2006	2.1	40	1,280	**1,280**	80	160
BHG/Qinghai/1A/2005	2.2	80	640	320	**160**	640
Dk/Laos/3295/2006	2.3.4	40	320	80	40	320
Ck/Kuwait/KISR2/2007	2.2	<40	640	160	320	<40

*BHG, bar-headed goose; Dk, duck; Ck, chicken. **Boldface** numbers indicate titers to prototype viruses.
†Based on the World Health Organization (H5N1) nomenclature system (*14*).
‡Ferret antisera dilution started at 1:40.

HPAI (H5N1) outbreak in wild birds in Qinghai Lake, China, in 2005 (*5*).

Conclusions

The results of this study confirm that clade 2.2 HPAI (H5N1) viruses were responsible for the poultry outbreaks recorded in Kuwait in early 2007. Notably, the viruses from Kuwait are most closely related to other 2007 subtype H5N1 isolates from Germany and Russia, but not to other 2007 isolates from Egypt, England, Ghana, and Hungary for which data are available (Figure 2). Furthermore, none of the current isolates from Europe or the Middle East has a close phylogenetic relationship with clade 2.2 isolates from China in 2005, although data on recent subtype H5N1 isolates from northern China are lacking (*5*,*12*). These relationships, along with reemergence of genetically similar viruses in widely distant geographic locations such as Germany, Krasnodar, and Kuwait (Figure 1, panel B), indicate that clade 2.2 influenza (H5N1) viruses may have become endemic in wild birds in central or eastern Asia (including Siberian Russia), from where they have been repeatedly introduced to Europe and the Middle East. Although it remains unclear in which hosts these viruses are maintained, the geographic distribution of closely related viruses suggests that migratory bird species are likely acting as vectors. Also, continued endemicity of clade 2.2 viruses in parts of Eurasia may result in the diversification of the virus in different geographic areas, as has been seen for subtype H5N1 lineages in eastern and Southeast Asia (*11*). Therefore, systematic surveillance in poultry and wild bird populations will be an important tool for tracking the evolution of clade 2.2 influenza (H5N1) viruses in this region.

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Land Use and West Nile Virus Seroprevalence in Wild Mammals

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We examined West Nile virus (WNV) seroprevalence in wild mammals along a forest-to-urban gradient in the US mid-Atlantic region. WNV antibody prevalence increased with age, urbanization, and date of capture for juveniles and varied significantly between species. These findings suggest several requirements for using mammals as indicators of transmission.

West Nile virus (WNV) is maintained in an enzootic bird-mosquito-bird cycle and is transmitted by numerous mosquito species, including many that feed on mammals (*1*). Several mammal species have been found to be naturally exposed to WNV, and it has been suggested that wild mammals could be used as indicators of transmission (*2*–*4*). WNV seroprevalence in wild mammals will be a useful indicator of WNV activity only if it differs between sites, if it reflects within-season transmission, and if other key confounding factors are accounted for.

To test 4 hypotheses about the exposure of mammals to WNV, we examined WNV seroprevalence in wild mammals in the eastern United States. First, we predicted that WNV seroprevalence would differ significantly among species because of differences in mosquito preferences, mammal behavior and survival, and other factors (*2*,*3*). Second, we predicted that seroprevalence would be higher for adults than for juveniles because adults have been exposed to WNV for at least 1 additional year. Third, we predicted that WNV exposure would increase with the date of capture over the transmission season because peak transmission occurs during late summer. Finally, we predicted that WNV seroprevalence would vary among sites and increase with urbanization because the abundance of *Culex pipiens*, the dominant enzootic vector in this region (*1*), increases with human population density (*4*).

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The Study

We trapped mammals at 7 sites along a forest-to-urban gradient in Maryland and Washington, DC, USA, from early June to late September 2005 and in April 2006. The sites included 1 forested area (Smithsonian Environmental Research Center, Edgewater, MD), 2 large wooded parks (Rock Creek Park, Rockville, MD; Fort Dupont Park, Washington, DC), 2 residential neighborhoods (Takoma Park, MD; Bethesda, MD), and 2 urban areas (Baltimore, MD; Washington, DC).

We quantified the land use around each site by calculating an urbanization index (UI) within a 1,000-m radius as follows:

$$UI = (100\% - \%\ \text{tree cover} + \%\ \text{impervious surface})/2$$

Impervious land and forest cover were estimated by using multitemporal (leaf-on and leaf-off) compilations of Landsat satellite images at 30-m spatial resolution, higher resolution satellite imagery, and digital orthophotography (*5*).

We ran trap lines of Tomahawk (models 201, 203, 204, 207; Tomahawk Live Trap Company, Tomahawk, WI, USA) and Sherman (model LFAHD; H.B. Sherman Traps, Inc., Tallahassee, FL, USA) traps for 2–5 days and nights at each site. Captured animals were chemically restrained and tagged, and age was determined by using body mass and/or reproductive characters (*6*). Blood samples (0.1 mL) were obtained, dispensed into tubes containing 0.9 mL BA-1 medium, and placed on ice packs until storage at –80°C. Blood samples were allowed to clot before antibody assays were run. We assayed the blood samples for neutralizing antibodies to WNV and Powassan virus (but not St. Louis encephalitis virus, which was absent in the local bird community at these sites [*7*]) by using the plaque-reduction neutralization test (*8*) at a 1:10 dilution, with 80% and 90% neutralization of plaques as cutoffs. We examined variation in WNV antibody prevalence by using binary logistic regression with species and age as categorical factors and capture date and urbanization index as covariates. We used October 15, 2005, as the capture date for the April 2006 samples because the abundance of WNV-infected mosquitoes falls precipitously after this date (*9*).

We obtained 244 samples from 11 mammal species (Table 1). The probability of being WNV antibody–positive varied significantly among species, was significantly higher for adults, increased with capture date for juveniles, and increased with the urbanization index (Table 2). The higher seroprevalence in samples collected in April 2006 showed that WNV exposure of juvenile eastern gray squirrels (*Sciurus carolinensis*) continued after the last trapping periods in September 2005 (Table 2).

Table 1. West Nile virus in wild mammals at 7 sites in Washington, DC, and Maryland, United States*

Capture site	UI	Age	% WNV seroprevalence (no. samples)					
			Tamias striatus	*Sciurus carolinensis*	*Didelphis virginiana*	*Peromyscus leucopus*	*Procyon lotor*	*Rattus norvegicus*
Baltimore, MD	91.2	J		0 (3)				
		A		64 (10)				50 (2)
Foggy Bottom, Washington, DC†	75.5	J		20 (11)		50 (2)		
		J‡		43 (7)				
		A		52 (23)		50 (2)		50 (6)
		A‡		100 (6)				
Fort Dupont Park, Washington, DC	38.8	J		100 (2)	20 (5)			
		A		75 (8)	60 (5)		50 (2)	
Takoma Park, MD§	50.4	J		0 (2)	71 (7)			
		J‡		50 (6)				
		A		65 (20)	50 (6)		100 (2)	
		A‡		100 (5)				
Bethesda, MD¶	41.5	J	0 (4)			100 (1)		
		A	22 (12)	67 (13)				
Rock Creek Park, Rockville, MD#	27.8	J		0 (5)		0 (1)		
		A	16 (6)	30 (20)		0 (3)	100 (3)	
SERC**	16.2	J			50 (4)	0 (11)	0 (1)	
		A		100 (1)	25 (4)	0 (6)	0 (1)	

*Mammals caught from June 14, 2005, through September 17, 2005, except where noted. WNV, West Nile virus; UI, urbanization index; A, adult; J, juvenile.
†Also sampled house mouse, *Mus musculus* (1 WNV-positive adult, 1 WNV -negative juvenile).
‡Samples from April 2006.
§Also sampled big brown bat, *Eptesicus fuscus* (1 WNV-negative adult), little brown bat, *Myotis lucifugus* (1 WNV-positive adult).
¶Also sampled little brown bat, *Myotis lucifugus* (1 WNV-positive adult).
#Also sampled groundhog, *Marmota monax* (1 WNV-negative adult).
**SERC, Smithsonian Environmental Research Center, Edgewater, MD; also sampled domestic cat (1 WNV-negative juvenile), groundhog, *Marmota monax* (1 WNV-negative adult, 1 WNV-positive adult), eastern cottontail rabbit, *Sylvilagus floridanus* (1 WNV-negative adult).

Seroprevalence rates were highest (and not significantly different) in 4 peridomestic species: eastern gray squirrels, Virginia opossums (*Didelphis virginiana*), raccoons (*Procyon lotor*), and Norway rats (*Rattus norvegicus*) (Tables 1, 2). Eastern gray squirrels were 5.5× more likely than eastern chipmunks (*Tamias striatus*) to have WNV antibodies and 4.5× more likely than *Peromyscus leucopus;* both differences were significant (Table 2).

Conclusions

Previous research on the exposure of mammals to WNV has shown patterns of antibody prevalence across several states and species (*2*,*3*,*10*–*12*). However, few studies have tested for statistical differences in the factors that influence the exposure of mammals to WNV, which thus would establish their usefulness as indicators of variation in WNV transmission. We found significant effects of age, species, site, and date of capture on WNV seroprevalence.

Table 2. Logistic regression analysis of West Nile virus seroprevalence in wild mammals*

Predictor	Coefficient	Odds ratio (95% CI)	p value
Constant	–7.52 ± 2.75		0.006
Age (adult)	7.83 ± 2.97	2,508.54 (7.48–8.4 x 10^5)	0.008
Juvenile date of capture†	0.024 ± 0.01	1.02 (1–1.05)	0.025
Adult date of capture	–0.004 ± 0.005	1 (0.99–1.01)	0.503
UI	0.015 ± 0.008	1.02 (1.0–1.03)	0.045
Species‡			0.007
Tamias striatus	–1.7 ± 0.68	0.18 (0.05–0.69)	0.012
Didelphis virginiana	0.46 ± 0.46	1.59 (0.64–3.94)	0.32
Peromyscus leucopus	–1.52 ± 0.68	0.22 (0.06–0.84)	0.026
Procyon lotor	0.88 ± 0.77	2.41 (0.53–11)	0.26
Rattus norvegicus	–0.7 ± 0.75	0.5 (0.11–2.19)	0.36

*Analysis used an 80% neutralization cutoff in plaque-reduction neutralization tests (PRNTs). Date refers to Julian date (January 1 = 1) and ranged from 165 (June 14) to 285 (October 15). All effects were significant when using a 90% cutoff in PRNTs at $p<0.05$ except urbanization index (UI) ($p = 0.10$). CI, confidence interval.
†Squirrel samples collected in September and April at Takoma Park, MD, and Foggy Bottom, Washington DC, were significantly different (logistic regression with age, site, and month as categorical factors; September coefficient –2.22 ± 0.85; $p = 0.009$).
‡Species effect χ^2 16.3; df 5; $p = 0.007$. Coefficients and odds ratios used eastern gray squirrels (*Sciurus carolinensis*) for the reference level.

The increase in seroprevalence with urbanization suggests that factors that increase WNV transmission, including mosquito abundance, WNV prevalence, or feeding on mammals, are higher in more urban areas. However, increased survival of mammals in urban areas could also result in increased seroprevalence associated with urbanization.

As in other studies, we found high WNV seroprevalence in 3 common peridomestic wild mammal species (*D. virginiana*, *P. lotor*, and *S. carolinensis*) and lower seroprevalence in *T. striatus* (Table 1) (*3*,*11*,*12*). Fatal WNV infection or low mosquito exposure for *T. striatus* could account for low seroprevalence in this species in areas where other species were often exposed (Table 1) (*7*,*9*,*13*). Additionally, we found that prevalence was significantly lower in juvenile *P. leucopus* in forested areas than in urbanized areas (Table 1; 0/11 vs. 2/3; Fisher exact test p = 0.032), which might explain some of the site variability found in previous studies (*3*). Finally, we found higher seroprevalence in rats than did previous studies (*3*) (4/7 vs. 2/36 [*1*]; Fisher exact test p = 0.004), which may have been a result of sampling rats from highly urban areas.

In addition to host death and spatial variation in prevalence and vector abundance, vector feeding preferences may also contribute to the observed variability in WNV seroprevalence. Previous studies have shown that WNV vectors do feed on *S. carolinensis*, *P. lotor,* and *D. virginiana* (*14*,*15*), but these studies do not show data on host abundance, so feeding preferences cannot be determined. Similarly, at our sites several mammal species, including *S. carolinensis* and *D. virginiana*, were sources of *Cx. pipiens* blood meals (*7*). Unfortunately, sample sizes of blood meals that came from mammalian hosts were too small for determining relative preferences for different mammals, and most species (except *S. carolinensis*) are at low enough abundances that substantial numbers of blood meals would be required to estimate feeding preferences. Mosquito preferences for different mammal species is an area for future research.

In our study, the probability of having WNV antibodies increased with capture date for juveniles but not for adults (Table 2), which suggests that juveniles experience higher exposure and would be more useful for WNV monitoring. Higher exposure of juveniles may result from increased attractiveness to mosquitoes or weaker defensive behavior.

Mammals have been proposed as sentinels for human WNV risk because infection would indicate transmission outside the enzootic bird cycle (*3*). Our study demonstrates that wild mammals satisfy 2 critical requirements: spatial and temporal variability in exposure. Our results also show that to estimate current year transmission at the site of capture, using wild mammals as sentinels will require adequate samples of young animals that year or a longitudinal approach (*10*). Our finding that mammalian WNV seroprevalence appears to be more intense in urban areas suggests that per capita risk for exposure is higher in these areas.

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High Failure Rates of Melarsoprol for Sleeping Sickness, Democratic Republic of Congo

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A retrospective chart review of 4,925 human African trypanosomiasis patients treated with melarsoprol in 2001–2003 in Equateur Nord Province of the Democratic Republic of Congo showed a treatment failure rate of 19.5%. This rate increased over the 3 years. Relapse rates were highest in the central part of the province.

Human African trypanosomiasis (HAT or sleeping sickness), caused by *Trypanosoma brucei gambiense,* is a slowly progressing fatal infectious disease that affects an estimated 100,000 persons in Central Africa each year. In the meningoencephalitic or second stage, melarsoprol, an arsenic derivative, and eflornithine are the only effective drug treatments available (*1*). High relapse rates for patients treated with melarsoprol were documented in Uganda (*2*,*3*) and in M'banza Congo in Angola (*4*). We investigated recent reports about very high relapse rates from the province of North Equator, in the Democratic Republic of Congo (DRC), where the largest HAT epidemic in recent history occurred (*5*).

The Study

We reviewed the records of all patients who received HAT treatment in the period January 1, 2001–December 31, 2003, in the 23 treatment centers operating in North Equator Province. We included only those case-patients who had received complete treatment with melarsoprol, obtained from Sanofi Aventis (Paris, France) under the World Health Organization donation program with 1 of the following regimens: 3 series of 3 injections (3.4 mg/kg) at 7-day intervals for patients with a cerebrospinal fluid (CSF) leukocyte count >20 leukocytes/mm^3, or 2 series of 3 injections (3.4 mg/kg) at 7-day intervals for patients with CSF leukocyte counts of 5–20 leukocytes/mm^3. Patients were asked to return for a routine follow-up visit to the HAT treatment center at 6, 12, 18, and 24 months after treatment or any time they felt unwell between visits.

Age, sex, disease stage, and results of parasitologic tests of the patients who experienced a relapse were recorded during the chart review. HAT relapse was defined as follows: trypanosomes found in body fluids at any follow-up assessment or a CSF leukocyte count >20/mm^3 and twice as high as the count at the previous follow-up visit. This case definition for relapse is used by the national program and in clinical trials (*6*). Patients who did not show up for suggested follow-up visits were not visited at home.

The relapse rate was calculated as the number of patients with HAT who experienced a relapse, divided by all patients who received full melarsoprol treatment during the study period. The study included 4,925 patients with second-stage parasitologically confirmed HAT; all were treatment-naïve for HAT, and none had been referred by another center. Relapse after melarsoprol treatment was noted for 959 (19.5%) patients. Table 1 shows the relapse rate by geographic area for the years 2001, 2002, and 2003. The patients from the central part of the province showed the highest relapse rates, and the trend increased over the 3 years (χ^2 for trend 22.3, $p<0.001$).

Table 2 shows characteristics of the patients with a relapse of HAT after melarsoprol treatment. Lumbar puncture was performed for 92% of patients at 6 months. This proportion dropped to 73% after 2 years, according to the annual reports for 2001–2005. Only 4.8% of patients with first-stage illness experienced a relapse; direct evidence of the parasite was found in 27% of these case-patients.

Conclusions

These data show that high failure rates with melarsoprol are no longer limited to Kasai Province in DRC. The HAT focus of the Equateur Nord Province does not border that in the Kasai Province, and given the limited contact between both provinces, resistant strains likely did not spread from Kasai to Equateur Nord Province.

The reported failure rate is certainly underestimated because some of the patients who did not return for follow-up visits probably died at home or sought treatment elsewhere. We cannot exclude the possibility that some retreated patients had been reinfected. However, the trend in observed incidence rates in Equateur Nord Province had been declining since 2004, and, regardless, the incidence was too low to contribute substantially to the observed 19.5% failure rate. The program used only basic parasitologic confirmation tests and none of the more sensitive concentration techniques, such as capillary tube centrifugation, quantitative buffy coat test, or the miniature anion-exchange column for trypanosomiasis during the study

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Table 1. Melarsoprol relapse rates in second-stage human African trypanosomiasis patients, Equateur Nord Province, 2001–2003

	Part of province							
	Northern		Central		Southern		Total	
Year	No. treated	No. (%) relapsed	No. treated	No. (%) relapsed	No. treated	No. (%) relapsed	No. treated	No. (%) relapsed
2001	708	90 (12.7)	1,154	284 (24.6)	506	46 (9.1)	2,368	420 (17.7)
2002	572	56 (9.8)	799	180 (22.5)	135	36 (26.7)	1,506	272 (18.1)
2003	362	57 (15.7)	570	171 (30.0)	119	39 (32.8)	1,051	267 (25.4)
Total	1,642	203 (12.4)	2,523	635 (25.2)	760	121 (15.9)	4,925	959 (19.5)

period, which explains the low proportion of relapses that were parasitologically confirmed.

Melarsoprol was used on a massive and unprecedented scale in Equateur Nord Province from 1996 through 2005, when 38,945 new patients received treatment with melarsoprol (annual reports of national program 2000 and 2005), so drug pressure (i.e., the use of a certain antimicrobioal agent potentially selecting out resistant strains) was certainly present in the region. Similarly high failure rates with melarsoprol in the Kasai Province led the national control program to introduce eflornithine as a first-line treatment there in 2006. The cause of these high relapse rates remains unclear because until now melarsoprol resistance could not be demonstrated in parasites in vitro or in animal models (*5*). The program used a shorter melarsoprol treatment regimen than that used by other countries, where 3 series of 4 injections are used. This difference is unlikely to be an explanation for the high relapse rates found, however, as in other provinces of DRC, relapse rates with the same regimen remained considerably lower (e.g., a 1.4% reported relapse rate for Bandundu Province, according to the 2006 Annual Report of the national control program).

Given this high failure rate, a switch to the safer eflornithine regimen is the most obvious solution. However, this drug is more complex to administer: patients receiving it require intravenous fluids and a high standard of nursing care. Eflornithine offers the additional advantage of lesser toxicity, which might enhance the acceptability of HAT treatment. A cost-effectiveness analysis showed that eflornithine is the more cost-effective option whenever relapse rates with melarsoprol treatment exceed 15% (*7*). Legitimate concerns have been raised regarding the use of this drug in monotherapy as first-line treatment because there are no alternatives if resistance to it emerges. A clinical trial on the use of the combination DFMO (alpha-difluoromethylornithine)–nifurtimox is in progress (*8*). Our data show the urgent need for novel drugs for stage-2 HAT. Public-private partnerships such as the Drugs for Neglected Diseases Initiative and others are currently investing in development of such drugs, but a new drug will not likely be available within the next decade. Therefore, rapidly controlling new outbreaks is essential to prevent large epidemics. These outbreaks may be more difficult to control if eflornithine resistance emerges.

Table 2. Characteristics of 959 patients who experienced relapse after treatment with melarsoprol, Equateur Nord Province, 2001–2003

Characteristic	No. (%)
Sex	
M	433 (45.2)
F	526 (54.8)
Age	
>1–11 mo	1 (0.1)
12–59 mo	13 (1.4)
5–59 y	936 (97.6)
≥60 y	9 (0.9)
Stage of disease	
First	46 (4.8)
Second	782 (81.5)
Unknown	131 (13.7)
Parasites found	259 (27.0)

Dr Robays is currently working for the Epidemiology and Disease Control Unit of the Institute of Tropical Medicine in Antwerp. His research focus is effective control and surveillance of HAT.

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Nosocomial Outbreaks Caused by *Leuconostoc mesenteroides* subsp. *mesenteroides*

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From July 2003 through October 2004, 42 patients became infected by strains of *Leuconostoc mesenteroides* subsp. *mesenteroides* (genotype 1) in different departments of Juan Canalejo Hospital in northwest Spain. During 2006, 6 inpatients, also in different departments of the hospital, became infected (genotypes 2–4). Parenteral nutrition was the likely source.

Leuconostoc species are catalase-negative, gram-positive microorganisms with coccoid morphology (*1*). In 1985, Buu-Hoi et al. (*2*) reported the first cases of *Leuconostoc* infection in humans. Since then, *Leuconostoc* spp. have been implicated in a variety of infections (*3*–*8*), particularly in patients being treated with vancomycin and in immunocompromised patients. However, these species have never previously been considered as agents that cause severe hospital outbreaks that threaten the lives of large numbers of persons.

Between July 2003 and October 2004, and between August and November 2006, 42 and 6 patients, respectively (Figure 1), in the Juan Canalejo Hospital (a tertiary-level, 1,400-bed hospital serving a population of 516,000 in La Coruña, northwest Spain) became infected by a strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* (LM). The patients had been admitted to 13 different, physically separated departments in the hospital (3 different hospital buildings), and 11 of the 48 were newborns. The aims of the present study were to characterize the epidemiologic features of the outbreak and to determine the risk factors associated with the infection.

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The Study

All bacterial isolates related to the outbreaks (1 per patient) were obtained from clinical samples. The strains were identified phenotypically by rapid ID 32 STREP (bioMérieux, Marcy l'Etoile, France), which yielded profile 22025001100 (*Leuconostoc* spp. 99.9%) and BIOLOG GP2 panels (Biolog, Hayward, CA, USA) (98%, T = 0.708). The results were confirmed by 16S rDNA sequence analysis, by a previously reported method (*9*), and the analysis of 1,420–1,500 bp showed 99% probability that the species were LM, when compared with GenBank database sequences.

Antimicrobial drug susceptibility was determined by microdilution, with DadeMicroscan system (Baxter Health Care, West Sacramento, CA, USA), and MICs were confirmed by E-test (AB Biodisk, Solna, Sweden). For interpretation of antimicrobial drug susceptibility, Clinical and Laboratory Standards Insitute criteria (*10*) for *Leuconostoc* spp. or when appropriate *Streptococcus* spp. other than *S. pneumoniae*, were used. The antimicrobial drug susceptibility profiles were almost identical for all genotypes and showed susceptibility to penicillin and gentamicin (MICs of 0.25 mg/L and <2 mg/L, respectively) and to levofloxacin, tetracycline, quinupristin-dalfopristin, linezolid, daptomycin, erythromycin, clindamycin, and chloramphenicol.

A pulsed-field gel electrophoresis (PFGE) technique was used to assess the possibility of a clonal relationship among the 48 LM strains. Genomic DNA was extracted, restricted with ApaI, and electrophoresed with CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The isolates were classified epidemiologically, according to published criteria (*11*). No differences in the band profile were observed among the 42 strains of the first outbreak (genotype 1). Analysis of the 6 strains isolated in the 2006 outbreak showed different DNA band patterns from those corresponding to genotype 1 (Figure 2). Of the 6 isolates, 4 shared the same genotype, designated genotype 2, whereas the remaining 2 isolates showed 2 new genotypes (genotypes 3 and 4). One LM strain, isolated from the parenteral nutrition catheter of a patient involved in the 2006 outbreak (genotype 2), was identical to those isolated from blood of the same patient (Figure 2) and from 3 other patients involved in the 2006 outbreak (data not shown).

Most of the 42 patients infected with LM genotype 1 in the first outbreak displayed severe underlying diseases (Table 1); 9 of the patients died, and 3 of the deaths (7.1%) were directly related to the *Leuconostoc* infection. The bacterial isolates were isolated from blood (52.1%), catheter (21.8%), or both (26.1%).

To assess risk factors related to acquisition of LM strains, we performed a case–control study. The first 42 patients (2003–2004) were designated as case-patients. Control-patients (n = 61) were randomly selected among

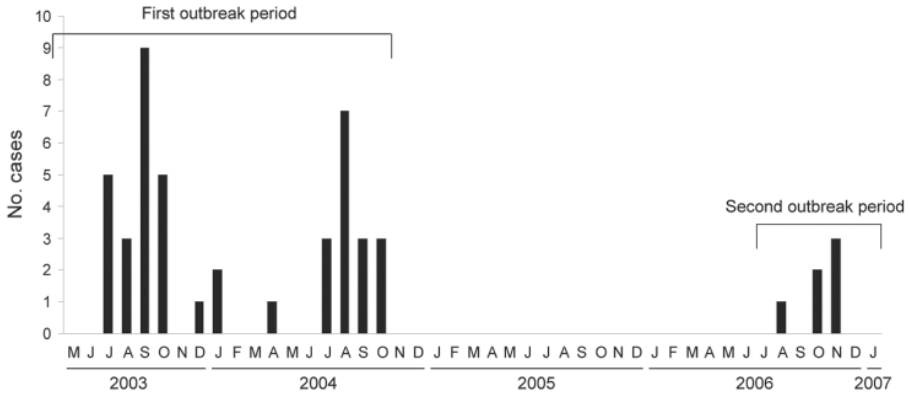

Figure 1. Epidemic curve of distribution of *Leuconostoc*-infected patients throughout the period of study. Two different outbreak periods were detected, July 2003 through October 2004 (42 patients) and August through November 2006 (6 patients). The first outbreak period was caused by a single epidemic strain and the second one was caused by 3 different strains.

remaining patients with another nosocomial infection caused by a non–*Leuconostoc* spp. microorganism isolated from a catheter, blood, or both, who were admitted to the same department and at the same time as the patients defined as case-patients. The variables analyzed are shown in Table 2.

Nosocomial infection criteria were those previously established by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*12*). A multiple logistic regression model was developed to identify potential independent factors associated with acquisition of LM strains. Predictor variables with $p<0.10$ in univariate analysis were included in the multivariate model to enable adjustment. Statistical analyses were conducted with SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

According to the multivariate analysis, previous infections (38.2% were bacteremias) (odds ratio [OR] = 4.2) and parenteral nutrition (OR = 27.8) were associated with *Leuconostoc* spp. infection (Table 2). After the case–control study, parenteral nutrition was suspected to be the source of the outbreak.

All case-patients received parenteral nutrition, with the exception of 2, although they received enteral nutrition. Parenteral nutrition is a putative source of the infection because all parenteral and enteral nutrition bags are prepared in the central hospital pharmacy and then distributed to the different medical units in the hospital. This possibility was further supported by 1 finding: PFGE analysis of isolates obtained from a parenteral nutrition catheter connected to a patient during the second outbreak yielded the same genotype as the isolates obtained from blood from the same patient (Figure 2) and from another 3 physically separated, infected patients. The physical distance between these patients as well as the impossibility of retrograde displacement of the bacterial isolate from patient's blood makes it unlikely that the LM strain was acquired by contamination from the blood and indicates parenteral nutrition as the main source of LM transmission in the hospital outbreak. Microbiologic controls of parenteral nutrition were reinforced during the second outbreak, and as stated, only 6 cases were detected. Moreover, during the second outbreak, microbiologic analysis

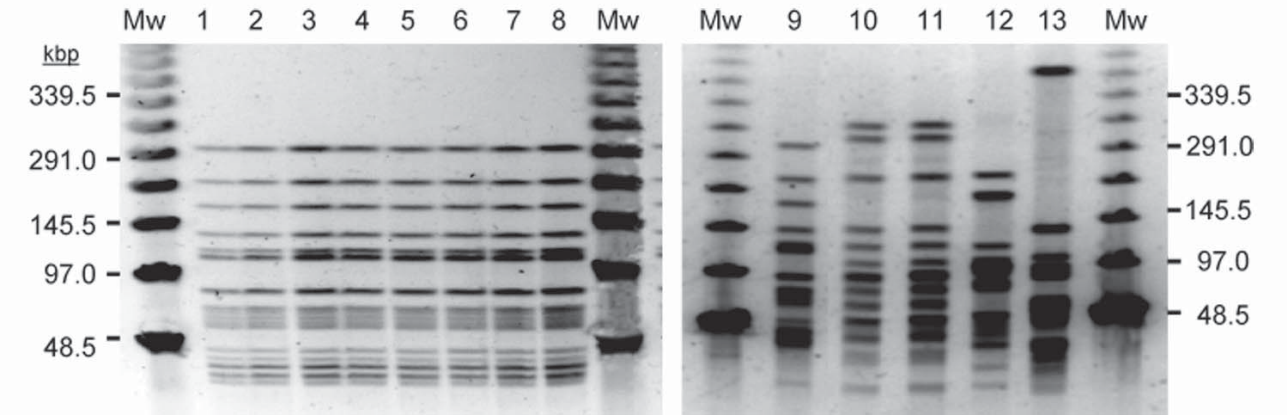

Figure 2. Band pattern obtained by pulsed-field gel electrophoresis of selected *Leuconostoc mesenteroides* subsp. *mesenteroides* (LM) isolates. Mw, molecular weight marker at indicated sizes; lines 1 to 9, representative LM isolates from the first outbreak (genotype 1); lines 10, 11, LM isolates obtained from parenteral nutrition catheter and blood from the same patient (genotype 2) and identical to those from 3 different patients infected in the second outbreak (data not shown); lines 12, 13, LM isolates from 2 different patients involved in the second outbreak (genotypes 3 and 4)

Table 1. Clinical features of the case-control patients in the first outbreak of *Leuconostoc mesenteroides* subsp. *mesenteroides* infection, La Coruña, Spain, 2003–2004

Diagnosis*	No. cases (%), n = 42	No. controls (%), n = 61
Newborns	11 (26.2)	17 (27.9)
Adults (>1 y)	31 (73.8)	44 (72.1)
Tumors		
Solid	9 (21.4)	13 (21.31)
Leukemia/lymphoma/myeloma	1/5/0 (2.38/11.9/0)	3/4/3 (4.92/6.56/4.92)
Digestive tract disease		
Pancreatitis	3 (7.14)	0
Necrotizing enterocolitis	2 (4.76)	1 (1.64)
Ulcerous colitis/Crohn disease	1/1 (2.38/2.38)	0/1 (0/1.64)
Cholecystitis	2 (4.76)	1 (1.64)
Bowel perforation	3 (7.14)	0
Bowel atresia	2 (4.76)	1 (1.64)
Bowel fistula	2 (4.76)	1 (1.64)
Prematurity	3 (7.14)	11 (18.03)
Infections†	3 (7.14)	2 (3.28)
Cardiopathy	2 (4.76)	3 (4.92)
Chylothorax	4 (9.52)	0
Brain vascular disease	3 (7.14)	8 (13.11)
Immunosupression	18 (48.9)	36 (59.0)
Others	3 (7.14)	9 (14.75)

*Each patient may have >1 diagnosis.
†Concomitant with *Leuconostoc* infection.

of environmental samples as well as samples from the digestive tract, skin, and throat of all patients involved did not yield any *Leuconostoc* strains.

Parenteral nutrition controls performed in the hospital pharmacy department are now routinely assayed for LM isolation. Since the last LM outbreak in November 2006,

Table 2. Model for predicting infection by *L. mesenteroides* subsp. *mesenteroides* (LM), La Coruña, Spain, 2003–2004*

	Cases, n = 42		Controls, n = 61				
Variable	No. (%)	Mean (SD)	No. (%)	Mean (SD)	Crude OR† (95% CI)	Adjusted OR (95% CI)	p value
Age, y		34.3 (28.2)		44.4 (31.7)	0.99 (0.98–1.0)		
Time between admission and infection		33.5 (38.4)		37.5 (100.9)	0.999 (0.994–1.0)	NS	
Charlson score		2.94 (2.13)		4.28 (2.38)	0.76 (0.61–0.96)	NS	
Previous surgery	29 (69)		23 (37.7)		3.7 (1.6–8.5)	NS	
Previous infections	31 (73.8)		15 (24.6)		8.6 (3.5–21.3)	4.2 (1.2–14.7)	0.023
Previous antimicrobial drug therapy	37 (88.1)		42 (68.9)		3.3 (1.1–9.9)	NS	
Teicoplanin	12 (28.6)		4 (6.6)		5.7 (1.7–19.2)	NS	
Vancomycin	5 (11.9)		3 (4.9)		2.6 (0.6–11.6)		
Central venous lines	39 (92.9)		41 (67.2)		6.3 (1.7–23.0)	NS	
Sex							
Male	24 (57.1)		41 (67.2)		0.7 (0.3–15)		
Urinary catheter	28 (66.7)		21 (34.4)		3.7 (1.6–8.6)	NS	
Enteral nutrition	18 (42.9)		23 (37.7)		1.2 (0.6–2.8)		
Parenteral nutrition	40 (95.2)		26 (42.6)		26.9 (6–121.6)	27.8 (5.5–141.1)	<0.000
Blood transfusion	24 (57.1)		31 (50.8)		1.3 (0.6–2.8)		
Intubation	18 (42.9)		17 (27.9)		1.9 (0.8–4.4)		
Tracheostomy	4 (9.5)		3 (4.9)		2.0 (0.4–9.6)		
Treatment with steroids	8 (19)		12 (19.7)		1 (0.4–2.6)		
Alteration of gastrointestinal barrier‡	29 (69)		30 (49.2)		2.3 (1.0–5.3)	NS	

*Values for 42 case patients and 61 control patients. SD, standard deviation; OR, odds ratio; CI, confidence interval; NS, variable did not meet criterion for remaining in the multivariate model. All these variables were considered as potential risk factors for LM infection. The LM infection was considered as an outcome variable.
†Predictor variables with p<0.10 in univariate analysis were included in the multivariate model to enable simultaneous adjustment.
‡Any process that modifies the gastrointestinal barrier (inflammation, atresia, resection, obstruction).

no cases of *Leuconostoc*-associated bacteremia have been reported in the hospital.

Conclusions

That 42 LM isolates from the first outbreak shared the same genotype and 4 of 6 isolates in the second outbreak also shared the same (another) genotype rules out the possibility of endogenous infections among patients and suggests a common source for each outbreak. The occurrence of cases in patients in areas that were physically separated rules out the possibility of indirect patient-to-patient spread through the hands of healthcare workers or contaminated hospital equipment (different departments do not share healthcare workers and equipment).

Enteral and parenteral nutrition has previously been described (*13*,*14*) as a risk factor associated with *Leuconostoc*-infections, although no microbiologic evidence was provided in any of the studies. With regard to previous infections in the multiple logistic regression model, this may be related to the alteration of the immune system caused by the microorganism that caused the previous infections. This alteration may play a role facilitating the subsequent *Leuconostoc* spp. infection.

Two previous reports have described hospital transmission of *Leuconostoc* spp (*7*,*15*); both outbreaks affected a small number of patients, and no epidemiologic studies were conducted to clarify the genetic relationship among the bacterial strains involved or the source of the nosocomial infection. Although up to 88 cases of *Leuconostoc* infection have been reported in the scientific literature in the past 25 years, these cases may not be comparable to those reported here, the largest nosocomial outbreak caused by *Leuconostoc* spp. worldwide.

This outbreak highlights the importance of LM as an emerging hospital pathogen in patients with underlying diseases and in whom parenteral nutrition may be the source of the initial infection and its spread. Every infection with LM could be a yet undetected outbreak and should result in an investigation that focuses on parenteral nutrition or products manufactured in a centralized hospital pharmacy.

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Anaplasma phagocytophilum Infection in *Ixodes ricinus*, Bavaria, Germany

Cornelia Silaghi,* Jérémie Gilles,*[1] Michael Höhle,* Volker Fingerle,* Frank Thomas Just,* and Kurt Pfister*

Anaplasma phagocytophilum DNA was detected by real-time PCR, which targeted the *msp2* gene, in 2.9% of questing *Ixodes ricinus* ticks (adults and nymphs; n = 2,862), collected systematically from selected locations in Bavaria, Germany, in 2006. Prevalence was significantly higher in urban public parks in Munich than in natural forests.

Anaplasma phagocytophilum, an obligate intracellular bacterium, causes a febrile disease in ruminants and granulocytic anaplasmosis in dogs, horses, and humans (*1*). A reorganization of the order Anaplasmataceae reclassified *Ehrlichia equi*, *E. phagocytophila,* and the human granulocytic ehrlichiosis (HGE) agent to the single species *A. phagocytophilum* (*2*), which in Europe is transmitted by the sheep tick, *Ixodes ricinus* (*3*). The agent is found among the *I. ricinus* population in Germany; average prevalence rates are 1% to 4.5% (*4*,*5*). The English Garden, a large (3.7-km^2) public park in Munich (state of Bavaria, Germany), has been suggested in 2 previous studies as a focal point for *A. phagocytophilum* (*5*,*6*). We investigated *A. phagocytophilum* in questing ticks in urban areas of Munich and focused on seasonal and geographic effects on the prevalence.

The Study

The sampling consisted of 2 phases. First, to gain an overview on the occurrence of *I. ricinus,* we collected questing ticks by the flagging method at 8 locations (labeled A1, A2, A3, B, C, D, E1, E2) close to the Isar River in the Munich area from May through September 2006 (Figure 1). Sites A1 and A2 were located in the city center part of the English Garden, which is enclosed by roads and houses. The vegetation of this heavily frequented area consists of groomed lawns, bushes, and deciduous trees. Site A3 was located in the northern part of the Garden, where vegetation was maintained by gardening, but bushes and trees were denser and grassland less frequently cut. The site was also used for horseback riding. Site B was a landscaped public green in the southern part of the city with groomed lawns and deciduous trees. Sites C, D, E1, and E2 were periurban riparian and deciduous forests. Three natural mixed forest sites (K, L, W) outside of Munich were sampled once (Figure 1). Ticks were registered and frozen individually at –26°C; adults were identified to species level by standard taxonomic keys (*7*). In the second phase, DNA was extracted from randomly chosen ticks (as available, 30 females, males, and nymphs, respectively, per month per site) with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to manufacturer's instructions with modifications. In individual 1.5-mL tubes, each tick was crushed mechanically with a metal spatula; sterile water (200 μL) was added, and the sample was kept overnight in a 55°C water bath for complete tissue lysis. At the beginning and end of each extraction line, a negative control was added. Quality and quantity of extracted DNA were tested with a spectrophotometer (NanoDrop ND-1000, PeqLab, Erlangen, Germany). A real-time PCR targeting the *msp2* gene of *A. phagocytophilum* (*8*) was performed with modifications in a Bio-Rad iCycler iQ (Bio-Rad, Munich, Germany). In a reaction volume of 25 μL, the HotStarTaq Buffer Set was used with 1.25 U HotStarTaq Polymerase (both QIAGEN, Hilden, Germany), 6 mmol/L $MgCl_2$, 200

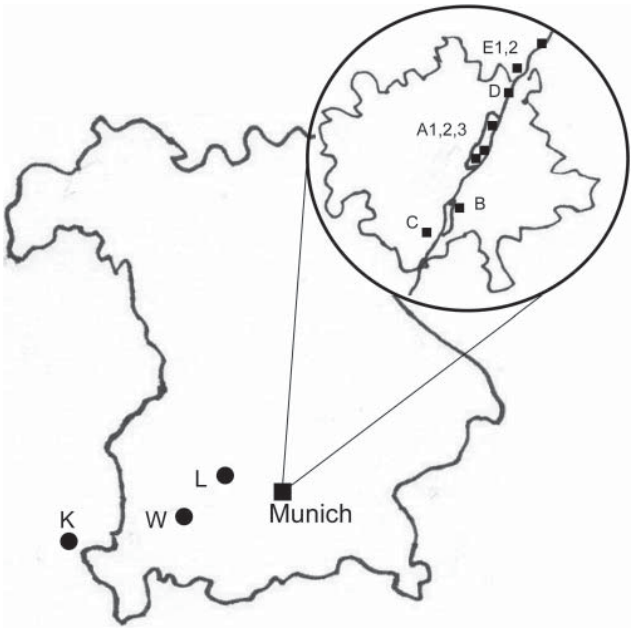

Figure 1. Location of collection sites. Large map, Bavaria, Germany; circled inset, city of Munich (with the Isar River). Sites in Munich area: A1, 2, 3, English Garden park; B, city park; C, D, E1, 2, riparian and deciduous forest; K, L, W, mixed forest areas outside of Munich.

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μmol/L each dNTP, 900 nmol/L each primer (ApMSP2f / ApMSP2r [*8*]), 125 nmol/L probe ApMSP2p-HEX (*8*), and 5.0 μL template DNA. Cycling conditions were as follows: initial activation (95°C, 15 min), 50 cycles denaturation (94°C, 15 s), and annealing–extension (60°C, 60 s). The original protocol was also used for part of the samples (*8*). Thirty-one DNA extracts, positive in real-time PCR, were amplified in a Thermocycler GeneAmp PCR System 2700 (Applied Biosystems, Weiterstadt, Germany) with a nested PCR (*9*) targeting the 16S rRNA gene, amplification of which is necessary to differentiate closely related strains (*8*). Negative and known positive controls were always included. After the final products were analyzed by 1.5% agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's instruction, the 497-bp fragments, without flanking primers, were sent for sequencing to MWG, Martinsried, Germany. The results were evaluated with ChromasLite (www.technelysium.com.au/chromas_lite.html), sequence homology searches made by BLASTn analysis of GenBank sequences (www.ncbi.nlm.nih.gov/BLAST), and multiple alignments (www.ebi.ac.uk/clustalw/index.html). The effects of month, location, stage, and sex of ticks on probability of infection were investigated with logistic regressions by using R version 2.5.0 (*10*); $p<0.05$ was regarded as significant. Due to low prevalence of *A. phagocytophilum*, odds ratios were interpretable as relative risks (RR). We calculated monthly prevalence with a weighted analysis, taking into account the sampling design: phase 1, a random sample, is stratified by sex, and in phase 2, a fixed number was drawn monthly at random within each sex stratum. Estimates were based on the Horvitz-Thompson estimator and corresponding 95% confidence intervals (CIs) computed by parametric bootstrap conditioning on phase 1 sample sizes (*11*).

A total of 9,507 ticks (4,932 adults, 3,573 nymphs, and 1,001 larvae) were collected, and adults were identified as *I. ricinus*. Real-time PCR was performed for 2,862 ticks (Table; online Appendix Table, available from www.cdc.gov/EID/content/14/6/972-appT.htm). With the modified protocol, atypical amplification occurred in ≈10% of samples, whereas with the original protocol, which had been tested on *I. scapularis* ticks, no amplification occurred. This difference suggests unspecific reactions in the modified protocol. *A. phagocytophilum* was detected in 5.67% of females, in 4.00% of males, and in 1.14% of nymphs (Table). The overall prevalence was 2.9% (95% CI 2.3%–3.5%). Significantly more females and males were infected than nymphs (RR = 4.906 for females, RR = 3.439 for males; $p<0.001$). Prevalence was significantly higher in the city parks (A1, A2, A3, B) than in natural forest areas (C, D, E1, E2, K, L, W; RR = 0.368, $p<0.001$). Prevalence was significantly lower in the riparian forest, Isarauen (E1, E2) in the north of Munich, than in the English Garden (A1, A2, A3) (RR = 0.314, $p<0.001$). Variations among the collection months, ranging from 0% to 20% for females and males and from 0 to 9.1% for nymphs (online Appendix Table), were not significant ($p = 0.40$).

Alignment of the partial 16S rRNA gene sequences showed that 30 sequences were 100% identical (GenBank accession no. EU490522); 1 sequence differed in 2 nucleotide positions (accession no. EU490523). The 30 homologous sequences were 100% identical to *Ehrlichia* sp. Frankonia 2 when compared with GenBank sequences (Figure 2) of *Ehrlichia* sp. Frankonia 2, *A. phagocytophilum* isolate X7, *A. phagocytophilum* isolate P80, and the prototype sequence of the HGE agent (GenBank accession nos. AF136712, AY281805, AY281794, and U02521, respectively). For Frankonia 2 and *A. phagocytophilum* isolate X7, the remaining sequence differed in 1 nt position. All differed in 1 nt position from the prototype HGE agent and *A. phagocytophilum* isolate P80 and in 2 more nt positions from P80.

Table. Total *Anaplasma phagocytophilum*–infected *Ixodes ricinus* ticks per site, southern Germany, 2006*

Study site	No. infected ticks/no. total ticks (%)		
	Females	Males	Nymphs
A1	11/87 (12.64)	5/88 (5.68)	3/104 (2.88)
A2	10/149 (6.71)	12/153 (7.84)	3/150 (2.00)
A3	7/114 (6.14)	4/105 (3.81)	1/83 (1.20)
B	8/80 (10.00)	5/65 (7.69)	0/42
C	1/68 (1.47)	1/60 (1.67)	0/96
D	5/150 (3.33)	5/152 (3.29)	2/142 (1.41)
E1	3/92 (3.26)	1/101 (0.99)	2/114 (1.75)
E2	5/122 (4.10)	1/134 (0.75)	0/140
K	1/30 (3.33)	1/31 (3.23)	0/30
L	1/30 (3.33)	2/30 (6.67)	0/30
W	2/30 (6.67)	1/30 (3.33)	0/30
Total	54/952 (5.67)	38/949 (4.00)	11/961 (1.14)

*A1, A2, A3, English Garden in Munich; B, other park in Munich; C, D, E1, E2, periurban forest areas of Munich; W, K, L, forests outside of Munich (compare Figure 1).

GenBank accession no.	Nucleotide at position				
	7	11	16	97	303
EU490522	A	G	T	C	A
EU490523	–	–	A	T	A
AF136712	A	G	T	C	A
AY281805	A	G	T	C	A
AY281794	G	A	T	C	G
U02521	A	G	T	C	G

Figure 2. Comparison of the 497-bp sequences of *Anaplasma phagocytophilum* obtained from *Ixodes ricinus* ticks, Bavaria, Germany, 2006, in relation to selected GenBank sequences.

Conclusions

Our results indicate that city parks of Munich may be focal points for *A. phagocytophilum*. Focal distribution depends mainly on mammalian reservoir hosts because of lack of transovarial transmission in ticks (*12*). Wood mice, yellow-necked mice, voles, roe, and red deer have been suggested as reservoirs in Europe (*13*,*14*). In the parks, a different reservoir host might be present. Large numbers of people and their domestic dogs pass through the parks, and the possibility of dogs acting as reservoirs for *A. phagocytophilum* should be investigated in further studies.

Ehrlichia sp. Frankonia 2 was first detected in adult ticks collected from domestic dogs in central Germany (*15*) and was later found in questing adults in Munich (*5*). However, neither *Ehrlichia* sp. Frankonia 2 nor the closely related *A. phagocytophilum* isolate X7 has been detected in humans or animals; thus, they can be regarded as strains of unknown pathogenicity. Future studies should aim at characterization of this strain and its possible role as a human or veterinary pathogen, as well as the identification of potential reservoir hosts in the city parks.

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Internet- versus Telephone-based Local Outbreak Investigations

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We compared 5 locally conducted, Internet-based outbreak investigations with 5 telephone-based investigations. Internet-based surveys required less completion time, and response rates were similar for both investigation methods. Participant satisfaction with Internet-based surveys was high.

Although the Internet has been increasingly used in epidemiologic research, its use for investigation of infectious disease outbreaks has been less frequently described. Most reports of Internet-based outbreak investigations have described large, single outbreaks conducted by national or state public health agencies. Examples of reported Internet-based outbreak investigations include a communitywide norovirus outbreak in Finland, a communitywide *Cryptosporidium* outbreak in Kansas, a multistate *Salmonella* outbreak, and a conjunctivitis outbreak at a university (*1–4*). These reports noted several advantages of Internet use, including reductions in resource use, workload, and time required for survey completion and data entry (*1–4*). However, these advantages are not generally quantified in outbreak reports. Moreover, Internet-based outbreak investigations are seldom reported from the local health department level, where resources are often constrained compared with those of state and national agencies. We offer an analysis of several small Internet-based outbreak investigations conducted at the local level. We describe response rates to Internet-based surveys with and without telephone follow-up, the time needed to complete Internet-based outbreak surveys in comparison with traditional telephone surveys, participant satisfaction with Internet-based surveys, and differences in Internet-based outbreak investigations based on the respondents' setting: professional versus household.

The Study

From April through September of 2006, the Tri-County Health Department (TCHD), a local health department in metropolitan Denver, Colorado, used Internet-based surveys to investigate 5 outbreaks. Three outbreaks involved respondents in professional settings: a norovirus outbreak at a teacher appreciation luncheon, a norovirus outbreak at a catered professional meeting, and a norovirus outbreak at an office staff luncheon. The other 2 outbreaks involved respondents in household settings: a norovirus outbreak at a Father's Day barbecue and a *Cryptosporidium* outbreak at a birthday pool party.

For all 5 outbreaks, a cohort study was conducted to ascertain illness and exposure information. Internet access among cohort members was assessed before Internet-based questionnaires were used. For 2 of the professional-setting outbreaks, all cohort members were sent by email a link to an Internet-based survey with directions on how to complete it and were asked to complete it by a certain deadline. In the third professional-setting outbreak, the office staff luncheon, the office requested that a link be sent to 1 office computer. Employees were individually given private access to that computer. In the 2 household-setting outbreaks, an email with a link to the Internet-based survey was sent to 1 household member, and household members took turns completing the survey. Cohort members of each outbreak were given the email address and telephone number of a TCHD contact, who was available to clarify survey-related questions. For all 5 outbreaks, an email reminder was sent out 1 day before the deadline. After the deadline had passed, follow-up telephone calls were made to nonresponders to improve response rates. Respondents were asked about their satisfaction with the Internet-based survey, as well as ease of use. Responses were entered directly into the Internet by the survey respondent, then downloaded by TCHD staff into Excel (Microsoft, Redmond, WA, USA) and analyzed with SAS (SAS Institute, Inc., Cary, NC, USA), thereby eliminating the need for data entry.

Response rates were then calculated, and the time required to complete each survey was tracked electronically. Response rates and survey completion time from the Internet-based investigations were then compared with those of 5 outbreaks in 2006 that were investigated by using traditional telephone surveys. All outbreaks in which TCHD conducted an epidemiologic investigation with a standardized questionnaire, from January through September 2006, were included in our study as either an Internet-based or telephone-based investigation. The questionnaires assessed illness-related symptoms, onset, and duration, as well as potential exposures. The number of questions ranged from 53 to 85 (median 67), depending on the number of possible exposures. The telephone-based investigations were similar to the Internet-based investigations in median sample size (Tables 1, 2), survey creation time, length, and content. Of

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Table 1. Response rates for telephone-based outbreak investigations, Denver, Colorado, 2006

Outbreak setting	N	Response rate, %
Elementary school	61	85
Italian restaurant	55	80
Childcare center	27	78
Japanese restaurant	14	86
Mother's Day brunch	7	100

TCHD employees who conducted all telephone interviews, 15 were surveyed to assess the time needed to complete the interviews. Statistical differences between the 2 investigation methods, for both median response rates and median survey completion time, were analyzed by using Wilcoxon rank-sum testing.

In this study, response rates to the Internet-based surveys were 60%–100%, before any telephone follow-up (Table 2). Response rates to the Internet-based surveys were lower in the professional-setting outbreaks but improved after follow-up telephone calls to nonresponders. In contrast, response rates in both household-setting outbreaks were 100% with the Internet survey alone; no follow-up telephone calls were necessary. No differences in completeness were found between those who took the survey online and those who completed the survey by telephone during a reminder follow-up call. In all 5 Internet-based investigations, most cohort members preferred the Internet-based survey to telephone or mailed surveys (online Appendix Table, available from www.cdc.gov/EID/content/14/6/975-appT.htm). Satisfaction with the Internet-based survey was lowest for those involved in the office staff luncheon outbreak (58%), for which employees took turns completing surveys on 1 computer. However, across outbreaks, most respondents (90%) found the Internet-based survey "very easy to use" (online Appendix Table).

Comparing the Internet-based investigations with the telephone-only investigations identified several differences. Telephone-based surveys took significantly more time to complete than Internet-based surveys (median of 30 min vs. 5 min, $p<0.01$). Median response rates to Internet-based surveys alone versus rates for telephone-based surveys alone were not significantly different (79% vs. 85%, $p = 0.69$). Median response rates improved when Internet-based surveys were combined with telephone follow-up; however, this improvement was not significantly different from telephone-only response rates (95% vs. 85%, $p = 0.28$).

Conclusions

Local health departments often deal with constrained resources when investigating outbreaks. In this article, we have shown the utility of conducting Internet-based investigations at the local level. Telephone interviews are often the most time-consuming aspect of an outbreak investigation. In this study, a median time of 30 min was needed for health department employees to complete a telephone interview, whereas the Internet-based survey took a median of 5 min of the respondent's time to complete and no health department employee time. Furthermore, fewer telephone calls were needed for the Internet-based investigations because follow-up telephone calls were made only to nonresponders, a small subset of cohort members. This was particularly true in the household-setting outbreaks, in which the response rate to the Internet-based survey was 100%, and no telephone calls were necessary. Moreover, because responses to the Internet-based survey were entered directly by the respondent, health department staff time for data entry was further reduced. Thus, Internet-based investigations can greatly reduce workload for local health departments. More studies are needed to further quantify the resource savings in Internet-based investigations and to analyze the effects of this data collection method on data quality.

We found that Internet-based surveys were associated with high levels of respondent satisfaction. Most respondents found the Internet-based survey preferable and easy to use. In professional-setting outbreaks, satisfaction may be higher when all participants have access to their own computers, rather than taking turns on a single computer. This finding contrasts with that of the household-setting outbreaks, in which, despite the sharing of a computer by household members, response rates and satisfaction rates were high. Thus, when Internet access is available, Internet-based surveys may be well received by respondents, particularly in household settings.

One limitation of this study is the small number of outbreaks analyzed and the small sample sizes. Another major limitation to any Internet-based outbreak investigation is participant access to the Internet. Internet-based investiga-

Table 2. Response rates for Internet-based outbreak investigations, by setting, Denver, Colorado, 2006

Outbreak setting	N	Response rate, %	
		Without follow-up telephone call	After follow-up telephone call
Professional			
Teacher appreciation luncheon	88	65	74
Office staff luncheon	20	60	95
Catered professional meeting	43	79	95
Household			
Father's Day barbeque	15	100	100
Child birthday pool party	21	100	100

tions can only be used when Internet access is widespread among the group being surveyed. Factors such as age, race, and income level may affect the familiarity with and availability of the Internet, as may geographic and language-related issues (*5*).

Public health agencies should assess these factors before initiating an Internet-based outbreak investigation. When access issues are not present, the Internet can be a useful and economical tool for investigating outbreaks.

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Lack of Serologic Evidence of *Neospora caninum* in Humans, England

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Retrospective testing of 3,232 serum samples from the general population and 518 serum samples from a high-risk group showed no evidence of human exposure to *Neospora caninum* in England. Results were obtained by using immunofluorescence antibody testing and ELISA to analyze frequency distribution.

The protozoan parasite *Neospora caninum* has recently emerged as a major cause of disease in cattle and dogs worldwide (*1*). An issue of concern is that *N. caninum* might be zoonotic because of its close biologic relationship to the common zoonotic parasite *Toxoplasma gondii* and because rhesus monkeys have been experimentally infected (*2*). Humans could become exposed to *N. caninum* by accidental ingestion of oocysts shed in the feces of canid definitive hosts or following the consumption of raw or inadequately cooked meat that contains tissue cysts. This retrospective study sought immunologic evidence of human exposure to *N. caninum* in England. Two cohorts of the population were examined: a convenience collection, which approximated the general population, and a putative high-risk group.

The Study

The first cohort comprised anonymized residues of serum samples submitted in 2000 for microbiologic or biochemical testing for diagnostic or screening purposes to 11 laboratories in England that were contributing to the Public Health Laboratories Service (PHLS) Serologic Surveillance Programme (now Health Protection Agency [HPA]) Seroepidemiology Programme (*3*). The second cohort comprised 518 samples collected in 1995 from the PHLS cohort of farm workers, which was recruited in 1991 to provide annual samples from a population at high risk for zoonotic infections from livestock (*4*,*5*). Ethical approval was granted by relevant ethics committees.

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Serum specimens were initially screened at a dilution of 1:10 in phosphate-buffered saline (PBS), pH 7.2, containing Tween 20 (PBS/Tween) with an inhibition ELISA developed in our laboratory and previously validated in cattle and dogs (*6*). Positive and negative bovine serum controls were used on each ELISA plate. In the absence of a human positive control, we used a primate serum sample as a further positive control. Optical density was read at 450 nm, and percentage inhibition (PI) values were calculated by using the formula 100 – [(test OD/negative control OD) ×100]. Without specific validation, a cut-off of 20% inhibition was chosen to indicate putative positives. This cut-off has been used for previous comparison of the inhibition ELISA with a conventional *N. caninum*–specific ELISA in bovine sera (*6*). All samples with inhibition ≥20% were subsequently tested in an immunofluorescence antibody test (IFAT) (*7*) with appropriate species-specific fluorescein isothiocynate conjugates. All samples were tested at a dilution of 1:50 with positive controls of bovine and primate *N. caninum* serum and bovine negative control serum as above. For the inhibition ELISA results, the distribution of the data was examined first by plotting percent inhibition, with the data aggregated into bands of 10%. The plots were repeated after logging the data and putting it into bins (equal width reactivity categories based on log 10% [$\log_{10}$] inhibition). The distribution of $\log_{10}$ inhibition was also examined according to person's sex and age and the submitting laboratory.

From the HPA collection, 3,232 samples from persons 20–70 years of age were tested; 1,889 (58.45%) were from women. The PHLS farm cohort, comprised 74% men and 26% women, with a median age range of 41–50 years (*5*). Six hundred and ninety-one (21.38%) of the HPA samples and 29 (5.56%) of the PHLS samples produced percentage inhibition of ≥20% in the inhibition ELISA test (Table). For the bovine-positive controls, mean percentage inhibition was 79.76% (5% confidence interval [CI] 78.94–80.58, n = 200). The primate-positive serum had a PI of 63.00% (average of 2 tests). When samples with a PI ≥20% in the inhibition ELISA were tested by IFAT, all failed to give positive fluorescence results, whereas the primate- and bovine-positive controls consistently gave positive fluorescence results.

The frequency distribution of actual percentage inhibitions for the HPA samples showed a single positively skewed distribution. After the data were logged, the plot showed a single log-normal distribution, with a mean close to 0% inhibition, i.e., similar to the negative control used. There was no evidence that the percentage inhibition differed by sex or region (results not shown) or by age (Figure). The log-transformed inhibition data for the PHLS cohort also produced a single normal frequency distribution about a mean close to 0% inhibition (Figure).

Table. Results of inhibition ELISA for *Neospora caninum*, England*

Population	% inhibition					No. samples tested
	<20	≥20–<30	≥30–<40	≥40–<50	≥50	
HPA seroepidemiology study	2,541	350	201	98	42	3,232
PHLS farm workers cohort	499	12	14	3	0	518

*HPA, Health Protection Agency; PHLS, Public Health Laboratory Service.

In this study, we sought evidence of human exposure to *N. caninum* infection by specific antibody detection in 2 populations. Since no serologic assay has been validated for *N. caninum* antibodies in humans, we used an inhibition ELISA and analyzed the frequency distribution of the percentage inhibitions. Although initial screening gave several putative positives, when the frequency distribution curves were plotted, no evidence was found showing that the samples were distributed discretely into those with antibody to *Neospora* spp. and those without. This provides strong evidence that no *Neospora*-specific immunoglobulin G was found in any of the samples and that the distribution observed describes a single population of nonexposed persons. Moreover, no change in distribution was observed with age, further evidence that the dataset is from a single large population for which results were negative. Finally, confirmatory testing with the IFAT of all samples with >20% inhibition in the inhibition ELISA, failed to detect any specific fluorescence indicative of true positives. The fact that the predictive value of a negative test is very high in low prevalence populations, even with tests of modest performance, further supports the conclusion that infection with *N. caninum* is unlikely. We therefore conclude that these sera show no evidence of exposure to *N. caninum*.

Selection bias in the HPA sera collection is unlikely because of the provision of free access to healthcare for all by the National Health Service. Moreover, a convenience collection has been shown to be comparable to a randomized sample in previous studies of population immunity (*8*). The collection may therefore be considered to approximate the general population of England in terms of its exposure to *N. caninum*. In contrast, the PHLS farm cohort may be regarded as a relatively high-risk group for *N. caninum* infection since those persons would be predicted to have greater than normal exposure to bovine placentas, fetal membranes, and fluids that are potentially infected, or to environments contaminated by feces of dogs, which have access to tissues from potentially infected cattle.

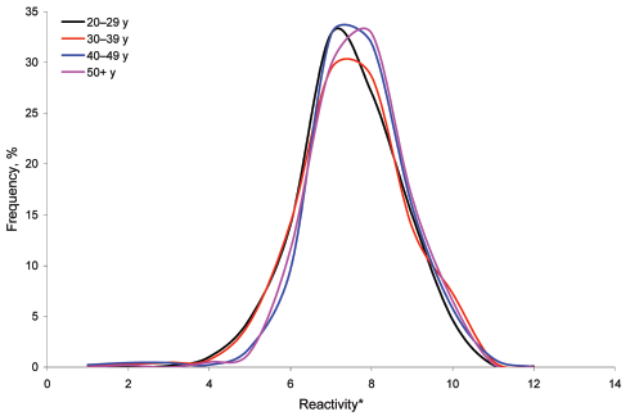

Figure. Frequency distribution of inhibition ELISA results for *Neospora caninum*, England (Health Protection Agency serum samples), stratified by age group. *Equal-width bands based on $\log_{10}$ percentage inhibition.

Analyses to detect parasites or parasite DNA have shown no definitive evidence of human infection with *N. caninum* (*1*), but seropositivity has been reported in 3 studies (*9–11*) although not in 2 other European surveys (*12*,*13*). Of the 3 reports describing seropositivity, 2 (*10*,*11*) noted a high correlation with seropositivity to *T. gondii*, and, in all, antibody titers versus *N. caninum* antigen were low (at ≤1:100 dilution). The interpretation of serologic results is difficult, and this study's approach, in which large numbers of samples were quantitatively assayed to give a frequency distribution, may provide a helpful means of addressing this uncertainty.

Conclusions

No evidence of human exposure to *N. caninum* was found in a high-risk population in England sampled in 1995 and in a sample of the general population of England collected in 2000. These results suggest that human infection is unlikely in England. However, given global variation in infection prevalence in cattle and possible regional differences in the incidence of oocyst shedding by dogs, there remains a need worldwide to remain vigilant to the possibility of human infection.

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etymologia

Bartonella henselae

[bär′′ tə-nel′ə henz′ ə-lā]

Bartonella is a genus of gram-negative bacteria named after Peruvian scientist Alberto Leonardo Barton. He identified a unique bacterium in 1905 during an outbreak among workers building a railway between Lima and La Oroya, a mining town in the Andes. The illness, usually fatal, was characterized by fever and severe anemia. Many of the sick were brought to Guadalupe Hospital in Lima, where Dr. Barton isolated the etiologic agent (which had been transmitted by sandflies) in patients' blood cells. It was later called *Bartonella bacilliformis*.

The species *B. henselae* was named after Diane Hensel, a technologist in the clinical microbiology laboratory, University Hospitals, Oklahoma City, who in 1985 observed a *Campylobacter*-like organism in blood cultures of HIV-infected patients. The organism was first named *Rochalimaea henselae* and then *B. henselae,* when sequencing showed identity with that genus.

Sources: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; http://www.whonamedit.com; Barton AL. Descripción de elementos endo-globulares hallados en las enfermos de fiebre verrucosa. La Crónica médica de Lima. 1909;26:7–10; http://sisbib.unmsm.edu.pe/BVrevistas/folia/Vol8_N4_dic97/bartonella.htm

In Memoriam: Joshua Lederberg (1925–2008)[1]

James M. Hughes* and D. Peter Drotman†

Dr Lederberg

The world of emerging infections lost a valuable friend and inspiring leader earlier this year with the death of Joshua Lederberg. The globally recognized scientist, educator, national and Presidential scientific advisor, and Nobel Laureate died of pneumonia on February 2, 2008, at the age of 82. Dr Lederberg's early work on bacterial genetics virtually established the discipline of molecular biology, earning him a Nobel Prize in Physiology or Medicine in 1958, when he was only 33 years of age. His contributions paved the way toward understanding microbial adaptation, including the development of antimicrobial drug resistance and the implications of these evolutionary changes for clinical medicine and public health. Equally impressive are his public service contributions; he served as a science advisor to 9 US presidents.

Dr Lederberg began his exploration of bacterial genetics as a doctoral student at Yale University, where he forged new paths and gained prominence in the developing field. Over the next 30 years, he held academic posts and chaired new genetics departments at the University of Wisconsin and Stanford University. In 1978, he joined Rockefeller University as its president. Over the next 12 years, his accomplishments and successes in this position were a testimony to his scholarly accomplishments and leadership skills; he remained affiliated with the university for the rest of his life. He offered science policy advice not only to 9 US administrations but also to the National Aeronautics and Space Administration and the Department of Defense. In addition to the Nobel Prize, his numerous prestigious awards included the National Medal of Science in 1989 and the Presidential Medal of Freedom in 2006.

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To those of us working in the field of emerging infectious diseases, Dr Lederberg will be most remembered for his critical role in recognizing the threats posed by emerging and reemerging infections and their implications for public health and national security. He is well known to readers of Emerging Infectious Diseases (EID) as a contributor to the journal (*1*,*2*) and for his leadership as co-chair with the late Robert Shope (*3*) of the Institute of Medicine (IOM) Committee on Emerging Microbial Threats to Health, which produced the 1992 report Emerging Infections: Microbial Threats to Health in the United States (*4*) (Figure 1, panel A). This landmark report defined the concept of emerging and reemerging infections, identified factors contributing to disease emergence and reemergence, and emphasized current and future challenges posed by infectious diseases. The report also highlighted deficiencies in our nation's public health infrastructure and made recommendations on the need to strengthen surveillance systems, address new

Figure 1. A) Emerging Infections: Microbial Threats to Health in the United States (*4*), a report of the Institute of Medicine (IOM) Committee on Emerging Microbial Threats to Health, published in 1992. B) Microbial Threats to Health: Emergence, Detection, and Response (*8*), a report of the IOM Committee on Emerging Microbial Threats to Health, published in 2003.

[1]All photographs used in this article were provided by the authors.

areas of research, provide multidisciplinary training for the next generation of scientists and public health workers, and establish new and enhance existing disease prevention and control programs. EID owes its genesis to this report.

Drs Lederberg and Shope were extremely effective in communicating the committee's observations and recommendations to the scientific, public health, and public policy communities, generating broad and renewed interest in infectious diseases. The report had a profound impact on the Centers for Disease Control and Prevention (CDC), the National Institute of Allergy and Infectious Diseases, the Department of Defense, and other federal agency programs involved in addressing emerging infectious disease threats. The report's emphasis on the need for interdisciplinary strategies and coordinated approaches led to the establishment of the Working Group on Emerging and Re-emerging Infectious Diseases under the auspices of the National Science and Technology Council Committee on International Science, Engineering and Technology. This Working Group was chaired by CDC Director David Satcher, and its deliberations led to vastly improved communication and collaboration among many federal agencies (*5*).

For CDC, the impact of the IOM report and Dr Lederberg's contributions were substantial. CDC worked with partners to develop new domestic and global strategies to address emerging infections, including specific efforts to respond to the IOM recommendations. Dr Lederberg served as an advisor on the development of these strategies (Figure 2), the first of which was published in 1994 (*6*), with an update 4 years later (*7*). From 1994 through 2004, CDC's funding for infectious diseases grew nearly 200-fold, from $1 million to >$190 million, enabling development and implementation of numerous programs to build epidemiology and laboratory capacity and improve preparedness and response capacity for infectious diseases and other health threats. Examples of these programs include the Emerging Infections Programs with activities such as FoodNet and Active Bacterial Core surveillance; the International Emerging Infections Programs; the Epidemiology and Laboratory Capacity for Infectious Diseases cooperative agreement; the Emerging Infectious Diseases Laboratory Fellowship Program; this journal; and the International Conference on Emerging Infectious Diseases, first held in 1998 with Dr Lederberg serving as the inaugural plenary speaker (*2*) and, most recently, in March 2008.

As an advocate for improvements in information systems and transparency, Dr Lederberg enthusiastically supported innovative approaches that included the establishment of ProMED and the Global Public Health Information Network. Internationally, he served as an advisor to the World Health Organization (WHO) on the creation of its Emerging Infections Program and personally advocated the need for establishing this program with WHO's then director general, Dr Hiroshi Nakajima.

Figure 2. Institute of Medicine co-chair Joshua Lederberg (left) in conversation with James M. Hughes, director, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), during a meeting in 1993 with expert consultants on development of the first CDC emerging infectious disease strategy.

Dr Lederberg also served as co-chair, with Dr Margaret Hamburg, of a second IOM committee that produced a follow-up to the 1992 report. Microbial Threats to Health: Emergence, Detection, and Response was published in March 2003 (*8*), less than 1 week after WHO issued its first global health alert in response to the outbreak of severe acute respiratory syndrome (SARS). This report had a broader global perspective than the 1992 report and identified additional factors contributing to disease emergence, reemergence, and global spread. The report also anticipated many issues that the 2003 SARS outbreak so starkly illustrated, including the need for increased collaboration among the human and animal health communities, a recommendation now supported by the One Health Initiative (*9*). The 2003 report's cover (Figure 1, panel B) depicts an artistic rendering of influenza virus, reflecting Dr Lederberg's longstanding interest in influenza research and his concern about national and global preparedness for the next pandemic.

As a charter member of the IOM, Dr Lederberg was the driving force behind the creation of its Forum on Emerging Infections (now the Forum on Microbial Threats), steering it effectively as chairperson through 2002. Consistent with his approach, the forum comprises representatives of the medical, academic, public health, veterinary, agricultural, environmental, national security, and pharmaceutical sectors. An important part of his legacy, the forum continues to address a broad range of issues in an interdisciplinary setting on a regular basis, recently focusing on vector-borne and zoonotic diseases and the potential impact of

climate change on infectious diseases. As Josh liked to say, there is an ongoing confrontation with the microbial world involving "our wits versus their genes." The forum held a workshop in May 2008 on microbial evolution and coadaptation in honor of Dr Lederberg (www.iom.edu/CMS/3783/3924/52347.aspx).

On a personal level, I (J.M.H.) first encountered Professor Lederberg in the late 1960s, when I was a medical student and he was the chairman of the Genetics Department at Stanford. His stature and accomplishments were legendary, and we were in awe of him. Over the course of my CDC career, I was fortunate to have many interactions with Josh and to greatly benefit from his insightful questions, keen observations, and constructive comments. He was very approachable and consistently available to discuss a broad range of issues, having an uncanny ability to readily span from basic science, to applied science, to public health, to policy, and to national security. He was a mentor, friend, and colleague to many.

For me (D.P.D.), Dr Lederberg was a great friend and early champion of EID, supporting the journal from its first issue in 1995. He helped us obtain credibility by recruiting editorial board members, supporting our application for early listing in national databases, and encouraging authors and reviewers of the nascent journal. We profiled him briefly when we published his plenary lecture at the inaugural International Conference on Emerging Infectious Diseases (Figure 3) (*2*) and compared his far-seeing work to that of Rudolf Virchow, one of the founders of modern medical science, because both wrote reports that called the attention of central governments to the manifold contributors to the web of causation of emerging diseases (*10*).

Dr Lederberg will be remembered for his scientific vision and contributions, his integrity and credibility, and his unwavering commitment to advocate for the highest quality science and evidence-based public policy. He was a firm believer in the need for scientists and public health officials to communicate clearly and concisely with policy makers and the public on scientific and public health issues. His influence and impact reached broadly, across areas of expertise and around the world. He will be greatly missed.

Figure 3. Keynote speakers at the inaugural International Conference on Emerging Infectious Diseases in Atlanta, March 8–11, 1998. Left to right: Anthony Fauci, David Heymann, Joshua Lederberg, Claire Broome, James Hughes, Guthrie Birkhead, D. Peter Drotman.

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Lethal *Mycobacterium massiliense* Sepsis, Italy

To the Editor: A strain of *Mycobacterium massiliense* was isolated from the blood of a kidney transplant patient in Italy at the same time she was diagnosed as having pulmonary tuberculosis. *M. massiliense* bacteremia appears to have played a role in her sudden death.

The patient, a 63-year-old woman who had had a kidney transplant 10 years earlier and was receiving immunosuppressive treatment with cyclosporine, azathioprine, and prednisolone, was hospitalized in an intensive care unit because of septic shock, a stuporous condition, hypotension, respiratory insufficiency, and acute renal failure. Results of initial microbiologic investigations (cultures of blood, urine, and bronchial aspirate, and nasal and pharyngeal swabs; tests for antigens of *Legionella pneumophila*, pneumococcus, and *Cryptococcus neoformans*; and serologic analysis for pneumotropic viruses and bacteria) were negative.

Hematologic analysis showed leukopenia with high neutrophil counts. Septic shock–specific therapy was given along with wide-spectrum antimicrobial drug therapy (levofloxacin, trimethoprim-sulfamethoxazole, piperacillin-tazobactam, and fluconazole). After moderate improvement, the patient's general condition worsened and prompted a new round of microbiologic tests, including those for mycobacteria. After acid-fast bacilli were detected in a bronchial aspirate and results of nucleic acid amplification (Amplicor; Roche, Basel, Switzerland) were positive for *M. tuberculosis* complex, she was given standard antituberculosis treatment. She died the next day.

Three blood cultures obtained 7 days before the death of the patient showed positive results in aerobic bottles but not in anaerobic bottles (BacT/ALERT SA; bioMérieux, Marcy l'Etoile, France). Subcultures spread onto blood agar yielded small white colonies of gram-positive bacilli, including branched forms, within 24 hours. One month earlier, the patient had been seen as an outpatient with intermittent fever, and unidentified gram-positive bacilli were observed in her blood culture.

Genetic sequencing of the first one third of the 16S rDNA gene (*1*) of the strain (GenBank accession no. EU370523) showed 99.8% identity with *M. abscessus*, *M. bolletii*, *M. chelonae*, and *M. massiliense*. To discriminate between these 4 species, a 723-bp fragment of the RNA polymerase β subunit (*rpoB*) gene (*2*) also was sequenced (GenBank accession no. EU370524). Sequencing showed 100% similarity with *M. massiliense*; the next most closely related species was *M. bolletii* (98.6% similarity).

An isolate of *M. tuberculosis* that was susceptible to all first-line antituberculosis drugs was recovered from this patient's bronchial aspirate in both solid and liquid media. Immunocompromised patients, including those with organ transplants, are known to be prone to mycobacterial infections (*3*). Mixed mycobacterial infections also have been reported (*4*). This newly described species has been isolated from pulmonary fluids (*5*), blood (*6*), intramuscular injection sites (*7*), and surgical wounds (*8*). Because sequencing 16S rDNA does not differentiate *M. abscessus*, *M. bolletii*, *M. chelonae*, and *M. massiliense* (*5*), use of the *rpoB* sequence is crucial.

As reported for isolates of *M. massiliense* (*5*–*8*), our isolate was characterized by high MICs (*9*) (broth microdilution by using the Sensititer RGMYCO; Trek Diagnostic Systems Inc., Cleveland, OH, USA) to most of the antimicrobial drugs tested. The isolate was sensitive only to clarithromycin and amikacin and showed borderline sensitivity to linezolid (Table).

Despite co-infection with *M. tuberculosis*, the patient's death was likely caused by *M. massiliense* bacteremia. The patient died before the isolate was identified as a mycobacterium, and unfortunately, none of the drugs used empirically was active against this organism.

Only speculations can be made about how this patient acquired the *M. massiliense* infection. However, 5 months before her hospital admission, the patient had received a coxofemoral arthroprosthesis as a result of a fall and had since complained of generalized bone pain and had remained bedridden. Although in this case no proof exists, infection caused by rapidly growing mycobacteria after surgical intervention (*10*) is well known and should be considered.

M. massiliense has been distinguished from *M. abscessus* by sequencing of the *rpoB* gene (*2*). Be-

Table. Antimicrobial drug resistance pattern of the strain of *Mycobacterium massiliense* isolated from the patient, Italy*

Drug	MIC (μg/mL)	Pattern
Amikacin	4	S
Amoxicillin/clavulanic acid	64/32	R
Cefoxitin	64	I
Ceftriaxone	>64	R
Ciprofloxacin	16	R
Clarithromycin	≤0.12	S
Gatifloxacin	>8	R
Imipenem	32	R
Linezolid	8	S
Tobramycin	16	R
Trimethoprim/sulfamethoxazole	8/152	R

*S, sensitive; R, resistant; I, intermediate resistance.

cause this technology is available in relatively few clinical laboratories, cases of infection with *M. massiliense* may be mistakenly attributed to *M. abscessus*. Although infections with *M. massiliense* may be underrecognized, reports of these infections are raising concern. The capacity of this bacteria to infect different body sites is further evidence for the pathogenic potential of a rapidly growing mycobacteria in human infections (*10*).

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Bovine Kobuviruses from Cattle with Diarrhea

To the Editor: A new species of kobuvirus, named U-1 strain, was first recognized in 2003 as a cytopathic contaminant in a culture medium of HeLa cells that had been used for >30 years in the laboratory (*1*). The RNA genome of the U-1 strain comprises 8,374 nucleotides; the genome organization is analogous to that of picornaviruses. Morphologically, the U-1 strain resembles the Aichi virus, but genetically it is distinct (*1*). Therefore, the U-1 strain is classified as a new species of genus *Kobuvirus* in the family *Picornaviridae*, and it is called *bovine kobuvirus* (*1*). To date, the genus *Kobuvirus* consists of 2 species, *Aichi virus* and *bovine kobuvirus* (*2*). The Aichi virus is associated with acute gastroenteritis in humans (*3–5*); bovine kobuvirus infection has been detected only in cattle (*1*).

Only 1 report has described the discovery and epidemiologic features of bovine kobuvirus (*1*). Of serum samples from 72 healthy cattle, 43 (59.7%) were positive for neutralizing antibody against bovine kobuvirus U-1 standard strain at a titer of ≥16. In addition, 12 (16.7%) of 72 stool samples collected from the cattle were positive for the bovine kobuvirus genome by reverse transcription–PCR (RT-PCR) (*1*). This finding suggested that bovine kobuvirus is common and that the virus particles could be detected in the stool samples of infected cattle. We therefore conducted an epidemiologic survey of bovine kobuvirus and report detection of this virus in stool samples from calves with diarrhea during 2001–2004 in Chiang Mai Province, Thailand.

From November 2001 to July 2004, a total of 72 fecal specimens were collected. The age of the calves ranged from 7 to 49 days. The presence of bovine kobuvirus in fecal specimens was detected by using RT-PCR with a protocol modified from the method described by Yamashita et al. (*1*). All the bovine kobuvirus strains detected in our study were analyzed further by direct sequencing of their PCR amplicons with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 3100, Applied Biosystems). The nucleotide sequences of these portions were compared with those of reference strains available in the GenBank database by using BLAST (*6*). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.1 (*7*). The nucleotide sequences of bovine kobuvirus strains described in this study were deposited in GenBank under accession nos. EF659450–EF659455.

The bovine kobuvirus was detected by the RT-PCR screening method in 6 (8.3%) of the 72 fecal specimens collected. The partial 3D regions of all 6 bovine kobuviruses exhibited highly conserved sequences of 99.3%–100% nucleotide and 100% amino acid

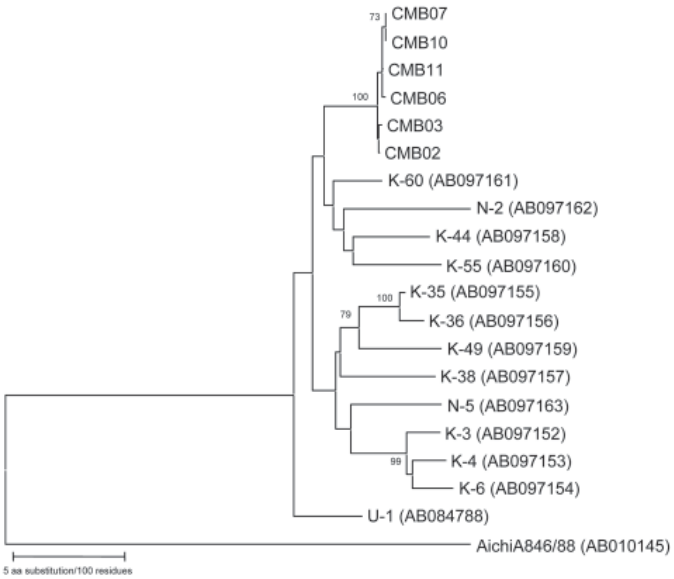

Figure. Phylogenetic analysis of the partial nucleotide sequence encoding the 3D region of bovine kobuviruses isolated in this study and other reference strains recognized to date. The tree was generated on the basis of the neighbor-joining method using the MEGA 3.1 program (*7*). Scale bar indicates branch length for a 5% nucleotide difference.

identities to each other. In addition, by searching for the closest sequences in the databank, we found that these sequences were closely related to all 13 bovine kobuvirus reference strains available in the GenBank database (U-1, K-35, K-36, K-49, K-38, N-5, K-3, K-4, K-6, K-60, N-2, K-44, and K-55); nucleotide and amino acid sequence identities ranged from 91.0% to 95.0% and 97.4% to 99.4%, respectively. Phylogenetic analysis of partial 3D nucleotide sequences of our bovine kobuvirus strains, together with those of all published bovine kobuvirus reference strains available in the GenBank database, is shown in the Figure. The Aichi virus standard strain was included in the tree as an outlier virus. The phylogenetic tree confirmed that all 6 virus strains belonged to bovine kobuvirus and formed a tight cluster in a monophyletic branch with other published bovine kobuvirus reference strains, but they were distantly related to the Aichi virus standard strain.

Detection and characterization of bovine kobuvirus strains from different geographic areas are important for understanding the worldwide distribution, heterogeneity, and association of bovine kobuvirus with enteric disease in cattle. Our findings indicate the role of kobuviruses in diarrheal disease in cattle and provide additional information on their relationship to bovine kobuviruses reported previously.

This research was supported by the Core University System Exchange Program under the Japan Society for the Promotion of Science, coordinated by the Graduate School of Medicine, the University of Tokyo, Japan, and Mahidol University, Thailand. The study was also supported in part by the Research Fund from the Faculty of Medicine, Chiang Mai University, Thailand.

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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.

Vibrio cholerae O1 Hybrid El Tor Strains, Asia and Africa

To the Editor: *Vibrio cholerae* is a water-borne pathogen that causes a severe watery diarrhea disease known as cholera. On the basis of variable somatic O antigen composition, >200 serogroups of *V. cholerae* have been recognized. Classical and El Tor are 2 well-established biotypes within the *V. cholerae* O1 serogroup, and they can be distinguished by differences in their biochemical reactions or phenotypic traits (*1*). In addition to phenotypic traits, genetic markers have recently been used in the identification of the biotypes of *V. cholerae*. For example, the major toxin-coregulated pilus (TCP) gene, *tcpA*, of the TCP cluster possesses classical- and El Tor–specific alleles that encode identical functions but differ in their DNA sequence composition; however, the *rtxC* gene of the repeat in toxin (RTX) cluster is present in El Tor strains only and absent in classical strains (*2*,*3*). The cholera toxin, encoded by the *ctxA* and *ctxB* genes, is the principal toxin produced by *V. cholerae* O1 and O139 and is responsible for the disease cholera. Heterogeneity within the *ctxB* gene and protein was first reported in the early 1990s, and, on this basis, 3 *ctxB* genotypes of the *V. cholerae* O1 strains have been identified. Based on amino acid residue substitutions at positions 39, 46, and 68, all classical and US Gulf Coast El Tor strains have been categorized as genotype 1, the Australian El Tor strains as genotype 2, and the El Tor strains of the seventh pandemic and the Latin American epidemic as genotype 3. Genotyping of *ctxB* has indicated that the classical strains harbor a unique cholera toxin gene that is not in the El Tor strains except for the US Gulf Coast El Tor clone (*4*). The US Gulf Coast hybrid El Tor strains that harbor the classical cholera toxin have been associated with sporadic outbreaks in the United States (*5*) and, until recently, had not been reported anywhere else in the world. Then in 2004, hybrid El Tor strains that encode the classical cholera toxin were isolated from cholera patients in Matlab, Bangladesh (*6*), and in Beira, Mozambique (*7*). In 2006, Nair et al. reported that the current seventh pandemic prototype El Tor strains had been replaced by hybrid El Tor strains in Bangladesh (*8*). We now report how far the hybrid El Tor strains have spread in Asia and Africa.

We examined 41 clinical *V. cholerae* strains from Asia and Africa that were isolated from 1991 through 2004 (Table) and confirmed as serogroup O1 by O-antigen biosynthesis gene (*rfbO1*)-specific PCR. Biotyping was performed by using standard procedures, and all strains were confirmed as El Tor (Table). All strains were PCR-positive for the El Tor–specific 451-bp *tcpA* and 263-bp *rtxC* amplicons but negative for the classical-specific 620-bp *tcpA* amplicon. All 41 strains were PCR-positive for *ctxAB* (1,037 bp) and produced cholera toxin, as demonstrated by the VET-RPLA Toxin Detection Kit (Oxoid, Basingstoke, UK). Sequence comparison of the PCR-amplified *ctxB* gene (460 bp) of each strain with the reference strains (569B and N16961) showed that 30 strains harbored classical cholera toxin (with histidine at position 39, phenylalanine at position 46, and threonine at position 68), whereas the remaining 11 strains carried the El Tor cholera toxin gene (with tyrosine at position 39, phenylalanine at position 46, and isoleucine at position 68) (Table). The overall analysis showed that all test strains are El Tor biotype but that most harbor the classical cholera toxin gene.

The major finding of this study is that El Tor strains that harbor the classical cholera toxin gene are not limited to the US Gulf Coast, Bangladesh, and Mozambique; they have spread to several other countries in Asia and Africa. Since 1817, 7 cholera pandemics have

Table. Phenotypic and genotypic traits of *Vibrio cholerae* O1 clinical strains isolated from Asia and Africa, 1991–2004*

Test strain origin (no. examined) or reference strain ID	Year(s) of isolation	*rfbO1*	Phenotypic tests			PCR amplicons			Cholera toxin type† (no. strains)
			CCA	Polymyxin B (50 U)	Voges-Proskauer	*tcpA* (El Tor)	*tcpA* (classical)	*rtxC*	
Japan (6)	1991–1997	+	+	Resistant	+	+	–	+	Classical (5), El Tor (1)
Hong Kong (18)	1998–2000	+	+	Resistant	+	+	–	+	Classical (11), El Tor (7)
Zambia (8)	1996–2004	+	+	Resistant	+	+	–	+	Classical (5), El Tor (3)
China (3)	1999	+	+	Resistant	+	+	–	+	Classical (3), El Tor (0)
Sri Lanka (1)	1998	+	+	Resistant	+	+	–	+	Classical (1), El Tor (0)
Vietnam (5)	1994–2002	+	+	Resistant	+	+	–	+	Classical (5), El Tor (0)
Classical 569B	1948	+	–	Sensitive	–	–	+	–	Classical
El Tor N16961	1971	+	+	Resistant	+	+	–	+	El Tor

**rfbO1*, O-antigen biosynthesis genes; CCA, chicken cell agglutination; tcp, toxin-coregulated pilus; rtx, repeat in toxin.
†Based on *ctxB* sequence. GenBank accession nos. for the *ctxB* sequences are EU156448–EU156488.

occurred around the world. Firm evidence indicates that the fifth and sixth cholera pandemics were caused by the classical biotype whereas the most extensive and ongoing seventh pandemic is caused by the El Tor biotype. Since the onset of El Tor dominance in 1961, the classical strains have been gradually replaced by the El Tor strains and are now believed to be extinct. However, reports from Bangladesh (*6*), Mozambique (*7*), and this study have provided sufficient evidence to indicate that the classical cholera toxin gene has reappeared but that for these cases its carrier has been El Tor. Although how the classical cholera toxin in El Tor strains would affect *V. cholerae* pathogenicity is unclear, cholera caused by the classical biotype is more severe, whereas the El Tor biotype is considered to be better able to survive in the environment (*1*,*9*). Given that cholera toxin is directly responsible for the major clinical sign of the disease, such a genetic change could result in substantial alteration in the clinical manifestation of cholera. Additionally, this subtle genetic change may also influence the effectiveness of current cholera vaccines, which could stimulate both antitoxic and antibacterial immunity.

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Mycobacterium avium subsp. *hominissuis* Infection in 2 Pet Dogs, Germany

To the Editor: The genus *Mycobacterium* contains various obligate and opportunistic pathogens of animals, which may also be transmitted to humans and cause disease in, thus exhibiting a considerable zoonotic potential (*1*,*2*). During the past few decades, members of the *Mycobacterium avium-intracellulare* complex (MAIC) emerged as pathogens of human diseases, including lymphadenitis in children, pulmonary tuberculosis-like disease, and disseminated infections (occurring predominantly in immunocompromised persons, particularly AIDS patients) (*1*,*2*). Similarly, important animal diseases are caused by members of this group, e.g., avian tuberculosis and paratuberculosis in ruminants (*1*). MAIC includes *M. intracellulare* and 4 subspecies of *M. avium*, namely, *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* (*3*,*4*). Whereas members of the *M. tuberculosis* complex are transmitted by direct host contact, MAIC species are acquired predominantly from environmental sources, including soil, water, dust, and feed. Subclinical infections are common among birds (*1*,*2*).

M. avium strains differ from *M. intracellulare* by containing the insertion sequence (IS) IS*1245* (*3*) and are further discriminated by terms of IS*901* (*4*). Avian isolates (*M. avium* subsp. *avium*) are usually positive for IS*901* and represent the main pathogen of avian tuberculosis (*5*). In contrast, mammalian isolates are IS*901*-negative and have been designated as *M. avium* subsp. *hominissuis* because of their predominant hosts. This subspecies is only weakly virulent for birds but causes disease in animals and humans (*5*).

Even though *M. tuberculosis* and *M. bovis* are the common etiologic agents of canine mycobacteriosis, dogs are reported to be relatively resistant to *M. avium* infection (*6*,*7*). Nonetheless, sporadic cases usually show nonspecific clinical signs, whereas necropsy consistently reveals granulomatous inflammation in numerous organs, including lymph nodes, intestine, spleen, liver, lung, bone marrow, and even spinal cord (*7*,*8*). The predominant involvement of the gastrointestinal tract indicates an oral route of infection (*7*,*8*), and simultaneously increases the risk for human infection by fecal spread of mycobacteria.

Our report concerns 2 young dogs, a 3-year-old miniature schnauzer and a 1-year-old Yorkshire terrier, that lived in different geographic regions in Germany. Both had had therapy-resistant fever, lethargy, progressive weight loss, and generalized lymphadenomegaly for several weeks and were euthanized after a final phase of diarrhea. Necropsy findings, similar in both dogs, included generalized enlargement of lymph nodes with a whitish, granular to greasy cut surface, leading to intraabdominal adhesions by extensive involvement of mesenteric lymph nodes. In the terrier, the greater omentum and a part of the right apical lung lobe showed changes similar to those in the lymph nodes. Furthermore, numerous white 1-mm nodules were found in the spleen (both dogs), liver (schnauzer) and costal pleura (terrier).

Histologic examination showed (pyo-)granulomatous inflammation of lymph nodes, tonsils, liver, spleen, and greater omentum. Additionally, pyogranulomatous pleuropneumonia was present in the terrier, and a granulomatous enteritis and pyelitis in the schnauzer. The granulomatous lesions frequently exhibited central necrosis surrounded by macrophages, epithelioid cells, and few neutrophils (Figure, panel A). However, multinucleated giant cells or mineralization was not observed. In both animals, Ziehl-Neelsen stain demonstrated large numbers of acid-fast bacilli within macrophages (Figure, panel B). Samples of lymph nodes and lung were processed for mycobacterial culture by using standard procedures (Löwenstein-Jensen, Stonebrink medium). Colonies emerging after 2-week incubation at 37°C were investigated by PCR targeting IS*1245* and IS*901* (*3*,*4*). In all samples, *M. avium* subsp. *hominissuis* was identified by growth characteristics as well as presence of an IS*1245*-specific and absence of an IS*901*-specific PCR product. Additionally, sequencing of *hsp65* was conducted (*9*), which indicated *M. avium* subsp. *hominissuis* in both dogs (GenBank accession nos. EU488724 and EU488725).

Despite improved therapeutic approaches, MAIC infection represents a frequent bacterial complication in persons with AIDS. However, several studies showed a very low incidence of *M. avium* subsp. *avium* infections in humans. Thus, most of these HIV-related infections are attributed to *M. avium* subsp. *hominissuis* (*2*,*5*). Unfortunately, the subspecies of *M. avium* was not identified in most canine cases reported in the literature (*7*,*8*). Nonetheless, different serotypes of *M. avium*, corresponding to either *M. avium* subsp. *avium* or *M. avium* subsp. *hominissuis*, have been identified sporadically (*6*,*10*). The source and route of infection were unclear in all reports including ours, albeit repeatedly observed enteritis strongly suggested an oral mode of infection. A common environmental or wildlife reservoir represents the most probable source of *M. avium* infection for both humans and animals. However, there is also evidence of direct transmission (*1*–*3*). Therefore, *M. avium* subsp. *hominissuis* infection in dogs may comprise a considerable zoonotic potential, particularly if pet dogs with close contact to the owner are affected and if prolonged nonspecific clinical signs and intestinal involvement occur, as demonstrated here.

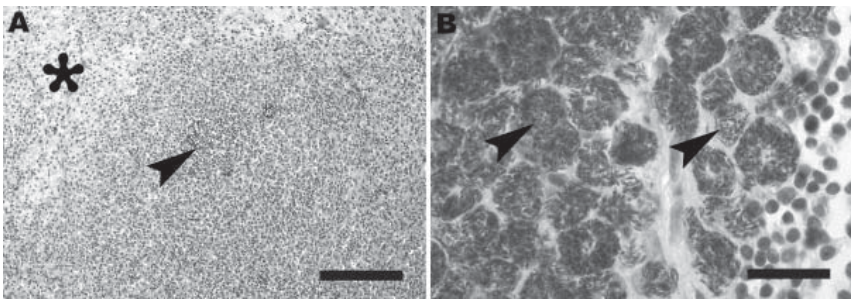

Figure. A) Mesenteric lymph node of Yorkshire Terrier shows diffuse granulomatous lymphadenitis with extensive infiltration of macrophages, foci of pyogranulomatous infl ammation (arrowhead), and focal necrosis (asterisk). Hematoxylin and eosin stain; scale bar represents 100 µm. B) Retropharyngeal lymph node of schnauzer shows innumerable acid-fast bacilli (arrows) within the cytoplasm of macrophages. Ziehl-Neelsen stain; scale bar represents 25 µm.

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Serogroup Y Meningococcal Disease, Colombia

To the Editor: *Neisseria meningitidis* is the etiologic agent of outbreaks, epidemics, and sporadic cases of meningitis or meningococcemia. Such infections have high illness and death rates, especially in children <5 years of age and adolescents. *N. meningitidis* serogroups A, B, C, Y, and W135 cause most meningococcal disease worldwide (*1*).

In Colombia, public health notification is required for all cases of invasive meningococcal disease. This reporting system is supported by a laboratory-based surveillance network for acute bacterial meningitis that has been coordinated by the Microbiology Group at the Instituto Nacional de Salud since 1994 (*2*,*3*). Clinical laboratories in Colombia submit isolates with associated information including geographic origin, specimen source, age, sex, and clinical diagnosis of the patient. Identification is confirmed by traditional phenotypic methods (*4*). Isolates are serogrouped by agglutination using commercial antisera (Difco, Detroit, MI, USA, and Becton Dickinson, Franklin Lakes, NJ, USA) and subtyped by dot blot with monoclonal antibodies (RIVM, Bilthoven, the Netherlands; and Institute Adolfo Lutz [IAL], São Paulo, Brazil) (*5*). Antimicrobial drug susceptibility testing for penicillin and rifampin is performed by the agar dilution, according to Clinical and Laboratory Standards Institute methods (*6*); for the breakpoints, we used those recommended by the Mesa Española de Normalización de la Sensibilidad y Resistencia a los Antimicrobianos (MENSURA) group (*7*). The reference laboratory participates in an external quality assurance program coordinated by the Pan American Health Organization (Sistema Regional de Vacunas [SIREVA] II, PAHO, Washington, DC, USA) with the Carlos III Institute, Madrid, Spain, and the IAL.

From 1994 through 2006, 434 *N. meningitidis* isolates were received by the Microbiology Group, from 22 of 35 departments (political divisions) and the Capital District: 119 (27.4%) from Antioquia, 117 (27.0%) from Bogotá, DC, 72 (16.6%) from Valle, 25 (5.8%) from Risaralda, 21 (4.8%) from Caldas, and 80 (18.4%) from 18 other departments. Distribution by department is published at the Institute's website (www.ins.gov.co) (*8*). According to public health reports, the reference laboratory is receiving ≈27% of the clinical case isolates. A slight majority (53.8%) were cultured from male patients. The age of patients was available for 396 isolates: 254 (64.1%) were <1–9 years of age, 71 (17.9%) 10–19 years, 41 (10.4%) 20–39 years, 21 (5.3%) 40–59 years, and 9 (2.3%) >59 years. Three hundred ninety-two isolates (90.3%) were recovered from cerebrospinal fluid and 42 (9.7%) from blood cultures. The diagnosis for 420 (96.8%) patients was meningitis; 11 (2.5%) patients had sepsis or bacteremia, and 3 (0.7%) had other invasive diseases (pneumonia, encephalopathy, or cellulitis).

Serogroup distribution was 338 (77.9%) group B, 42 (9.7%) group C, 40 (9.2%) group Y, and 2 (0.5%) group W135; 12 isolates were nongroupable. There was little annual variation for groups B and C, but there was an unexpected increase in serogroup Y (Figure), from 0% in 1994 to 50% in 2006. When the period 1994–2002 was compared with 2003–2006, this change was significant, increasing from 2.2% to 29.5% ($p<0.001$).

Antimicrobial drug–susceptibility testing showed that 17% of the isolates had intermediate resistance to penicillin (MIC 0.125–1.0 μg/mL) and 0.5% high resistance (≥2.0 μg/mL); only 1 isolate was resistant to rifampin (≥4.0 μg/mL). Penicillin resistance was not associated with any specific serogroup.

From the 40 serogroup Y isolates, 22 (55%) were from Bogotá, DC, 5 (12.5%) from Antioquia, 5 (12.5%) from Valle, and the remaining 8 (20%) from 5 other departments. Age distribution of patients who provided the isolates was as follows: 15 (37.5%) were <1–9 years of age, 7 (17.5%) 10–19 years, 11 (27.5%) 20–39 years, 4 (10%) 40–59 years, and 3 (7.5%) >59 years.

Subtyping of the 40 Y isolates showed that 30 (75.0%) were serotype 14 with 3 different subtypes: 23 were Y:14:NST; 6 were Y:14:P1.5,2; and 1 was, Y:14:P1.10. Four (9.8%) were nontypeable (NT) with 2 subtypes: 3 were NT:P1.5,2, and 1 was NT:NST; the remaining 6 (14.6%) belonged to 5 other serotypes. Intermediate resistance to penicillin was found in 5% of the serogroup Y isolates, and one was resistant to rifampin.

From 1994 through 2005, the laboratory-based surveillance program identified serogroup B as the most frequently isolated serogroup that caused acute bacterial meningitis in Colombia. In 2003, there was an unexpected increase in serogroup Y (Figure), and by 2006 it was the most common serogroup in Colombia. Seventy-five percent of the isolates collected during 2002–2006 were recovered from male patients younger than 14 years.

An increase in serogroup Y has also been reported in Chicago, Illinois, where one third of meningococcal disease cases are caused by this serogroup (*9*). A similar increase has been reported in Canada (*10*). Both the United States and Canada have investigated genetic similarity, and circulating clonal types have been determined (*9*,*10*). Similar molecular studies with Colombian isolates are under way in collaboration with the Carlos III Institute from Spain under PAHO coordination. Our data demonstrate the importance of laboratory-based surveillance programs supported by active participation of clinical and public health laboratories.

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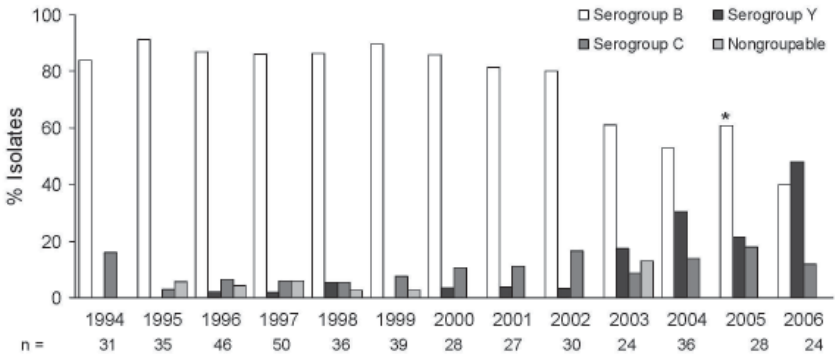

Figure. *Neisseria meningitidis,* serogroup distribution by surveillance year, N = 434. *p<0.001.

Extensively Drug-Resistant Tuberculosis, Lesotho

To the Editor: Reports of extensively drug-resistant tuberculosis (XDR TB) in the Republic of South Africa have generated concern in the medical and public health literature (*1*,*2*). Given that XDR TB is spread through the air, it is not surprising that cases have been reported in multiple countries throughout the world (*3*). We report a documented case of XDR TB in Lesotho. This case is closely tied to transnational work in South Africa, thus raising concern about the spread of this disease across highly porous borders and the need for international attention to migrant worker health.

Lesotho is a mountainous nation that is home to 2 million people and completely surrounded by South Africa. It has the third highest rate of HIV in the world; an estimated 24%–30% of the population is infected (*4*). Lesotho also has a high prevalence of TB with an estimated 695 cases per 100,000 population (*5*). Ten percent of patients with smear-positive TB are estimated to have multidrug-resistant TB (*6*). The US Centers for Disease Control and Prevention is undertaking a national reporting registration survey in Lesotho.

In 2007, the Ministry of Health and Social Welfare began working with Partners In Health and the Foundation for Innovative and New Diagnostics, Geneva, Switzerland, to document and treat drug-resistant TB in Lesotho. Hundreds of cases of drug-resistant TB have been reported in the country, and the patient we describe in this letter was reported to have XDR TB.

The patient was a 44-year-old man who had been working in the gold mines in South Africa. He had a history of receiving multiple treatments for TB while he was working in one of the mines. His condition was declared a treatment failure in July 2007. The patient was discharged from medical care in South Africa with no follow-up plan or medical records and was told, per his report, to "return home." He traveled by road and bus to Lesotho and easily crossed the border. He was originally seen at a public TB clinic in Lesotho but, given his reports of prior TB treatment, his sputum was sent for culture and drug susceptibility testing. He was followed up at his home with a daily visit from a village health worker trained in the management of drug-resistant TB. When XDR TB was confirmed (in vitro resistance to at least isoniazid, rifampin, a fluoroquinolone, and an injectable agent [*7*]), he was admitted to the hospital for drug-resistant TB patients in Lesotho and placed in a negative-pressure, single isolation room.

When the patient sought treatment from our program in October 2007, he exhibited severe wasting and dyspnea. An HIV test result was positive; his CD4 count was 36 cells/μL. First-line drug susceptibility testing (carried out by the Medical Research Council [MRC], Pretoria, South Africa) showed resistance to isoniazid, rifampin, and pyrazinamide. On the basis of these results, on October 26, 2007, he was empirically prescribed a regimen of second-line drugs: capreomycin, para-aminosalicylic acid, cycloserine, ethionamide, and ciprofloxacin. One month later, second-line drug susceptibility testing, sent by the medical team in Lesotho (none was ever sent during his treatment in South Africa) but carried out at MRC, showed additional resistance to amikacin (MIC 1.0 μg/mL), capreomycin (MIC 2.5 μg/mL), and ofloxacin (MIC 1.0 μg/mL) but susceptibility to ethionamide (5.0 μg/mL). The patient's regimen was changed to kanamycin, moxifloxacin, ethionamide, para-aminosalicylic acid, and cycloserine. Unfortunately, he died of his disease in December 2007. His known contacts are being monitored closely for signs and symptoms of TB. Reports have been made to the mine in which he was working, the Ministry of Health of South Africa, and the Ministry of Health of Lesotho. As of the writing of this letter, the South African Ministry of Health has made no formal response regarding this patient.

The report of this case of XDR TB in Lesotho raises many concerns. First, XDR TB was readily found (along with other forms of drug-resistant TB) in this small country that already has high prevalence of HIV. The potential for spread in the community as well as in hospital settings is substantial. The link to working in South Africa is also a major factor. Given the patient's prior treatment while employed by a mining company and the literature reporting XDR TB in South Africa (*8*), XDR TB likely developed while he was working in the mines, and he brought the disease back to his home in Lesotho. Because South African mines rely heavily on migrant labor from countries such as Lesotho, Swaziland, and Mozambique, transnational spread of drug-resistant TB, including XDR TB, is quite probable and calls for a concerted international approach and institutional collaboration for management and control of this epidemic. Infection control in the mines needs to be addressed, and international efforts to communicate that migratory populations are at risk for XDR TB need to be a priority in controlling the spread of this disease.

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Inquilinus limosus and Cystic Fibrosis

To the Editor: *Inquilinus limosus*, a new multidrug-resistant species, was reported in 1999 as an unidentified gram-negative bacterium in a lung transplant patient with cystic fibrosis (CF) (*1*). This species was later characterized by the description of 7 new isolates of *I. limosus* and 1 isolate of *Inquilinus* sp. (*2*). Infections and colonizations by *I. limosus* have been documented mainly in adolescent or adult patients with CF. To date, 8 clinical cases have been described in Germany (*3,4*), 1 case in the United States (*1*), 5 cases in France (*5*), and 1 case in the United Kingdom (*6*) (Table). Only 1 isolate of *Inquilinus* sp. has been recovered from blood samples of a patient without CF who had prosthetic valve endocarditis (*7*).

Because this bacterium is not recorded in all commercial identification system databases currently available, a longitudinal study for *I. limosus* detection with a new real-time PCR assay with a Taqman probe (Applied Biosystems, Foster City, CA, USA), that targets the 16S rRNA gene, has been developed and compared with the culture isolation. Primers il1d (5′-TAATACGAAGGGGGCAAGCGT-3′) and il1r (5′-CACCCTCTCTTGGATT CAAGC-3′) and probe ilProbe (6FAM-GGTTCGTTGCGTCAGATGTGAAAG-TAMRA), which were used in this study, were designed on the basis of multisequence alignment of all *I. limosus* 16S rDNA sequences available in the GenBank database.

To confirm specificity, the primers and probe were checked by using the BLAST program (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and also by using suspension of several bacteria recovered habitually in patients with CF. For sensitivity of the Taqman PCR assay (Applied Biosystems), the minimal CFU detectable was 2 CFU/PCR. From January 2006 through June 2007, 365 sputum samples recovered from 84 children and 61 adults with CF and 71 sputum samples recovered from 54 patients without CF were screened blindly for *I. limosus*. By using our real-time PCR, we detected 9 *I. limosus*-positive samples from 4 patients with CF (Table); 8 of these samples were also culture positive. However, all sputum samples from patients without CF were negative. In 1 patient (Table, case 17), *I. limosus* was detected by using real-time PCR 3 months before the culture was positive. Retrospectively, the patient's medical file was rechecked and his clinical respiratory condition worsened briefly at that stage, which indicates an infection by this bacterium. Thus, in our study, the incidence of *I. limosus* was 2.8% (4.9% for adults with CF and 1.2% for children with CF). The incidence of *Burkholderia cepacia* complex during the same period and in the same patients was 2.1% (3 adults with CF were positive, data not shown).

The genus *Inquilinus* belongs to the α-*Proteobacteria*; the genus *Azospirillum* is the most closely related bacteria (*2*). This cluster of bacteria contains several strains that are able to grow under saline conditions and in biofilms (*8,9*). The mucoid phenotype of *I. limosus* may contribute to its colonization and resistance to many antimicrobial drugs. Recently, the exopolysaccharides (EPS) produced by *I. limosus* were studied. The authors indicated that *I. limosus* produces mainly 2 EPSs that exhibit the same charge per sugar residue present in alginate, the EPS produced by *Pseudomonas aeruginosa* in patients with CF. This similarity may be related to common features of the EPS produced by these 2 opportunistic pathogens that are related to lung infections (*10*). Transmission of *I. limosus* between patients with CF is not known, but in the report from Chiron et al., 1 of the 5 patients with *I. limosus* had a brother who had never been colonized with this bacterium despite living in the same home (*5*). Schmoldt et al. reported that 3 patients were treated in the same outpatient CF clinic during overlapping time periods and each patient was infected/colonized by an individual *I. limosus* clone, which suggests that there was no transmission among these patients (*4*). This bacterium has been recovered mainly from sputum of adolescents (mean age 17 ± 6.47 years, range 8–35), except in our study with a 2-year-old boy, which suggests that this emerging bacterium may be hospital acquired, as recently suggested (*7*). Because this bacterium is multiresistant to several antimicrobial drugs, particularly colistin, which is widely

Table. Clinical and epidemiologic features of cystic fibrosis (CF) patients with *Inquilinus limosus**

Case no.	Age, y/sex	Lung transplant	Positive samples	Clinical manifestation, first isolation	Growth on MacConkey agar	Growth on selective agar (d)	Phenotypic identification†	Other associated pathogens	Reference
1	22/F	Yes	Lung explant, BAL, sputum	Pneumonia	Poor	ND	AR	PA, PM	(*1*)
2	17/M	No	Sputum	Stable	No	Yes (6 d)	SP	SA, PA, CA	(*3*)
3	14/F	No	Sputum	Stable	No	Yes (5 d)	SP	PA, AF, CA	(*3*)
4	12/M	No	Sputum	Stable	ND	Yes (ND)	SP	PA, SM, SA, AX	(*5*)
5	13/F	No	Sputum	Exacerbation	ND	Yes (ND)	SP	PA, SA, AF	(*5*)
6	8/M	No	Sputum	Stable	ND	Yes (ND)	SP	PA	(*5*)
7	10/M	No	Sputum	Stable	ND	Yes (ND)	SP	None	(*5*)
8	18/M	No	Sputum	Exacerbation	ND	Yes (ND)	AR	PA, SA, AF	(*5*)
9	16/F	No	Sputum	Severe exacerbation	ND	ND	PA	PA	(*4*)
10	19/M	No	Sputum	Stable	ND	ND	ND	PA	(*4*)
11	17/F	No	Sputum	Exacerbation	ND	ND	ND	PA, CA, AF	(*4*)
12	20/F	No	Sputum	Exacerbation	ND	ND	ND	PA, SA, CA, AF	(*4*)
13	17/F	No	Sputum	Stable	ND	ND	ND	PA, SA, SM, CA, AF	(*4*)
14	35/M	No	Sputum	Respiratory decline	ND	ND	PA	PA, SM, SMA	(*4*)
15	17/F	No	Sputum	Stable	No	Yes (4 d)	SP	CA	This study
16	2/M	No	Sputum	Productive cough	No	Yes (3 d)	SP	SA, HI	This study
17	21/M	No	Sputum	Exacerbation	No	Yes (3 d)	AR	PA, AF	This study
18	15/M	No	Sputum	Fever and thoracic pain	No	Yes (3 d)	AR	SA	This study

*BAL, bronchoalveolar lavage; ND, not determined; AR, *Agrobacterium radiobacter*; PA, *Pseudomonas aeruginosa*; PM, *Proteus mirabilis*; SP, *Sphingomonas paucimobilis*; SA, *Staphylococcus aureus*; CA, *Candida albicans*; AF, *Aspergillus fumigatus*; SM, *Stenotrophomonas maltophilia;* AX, *Achromobacter xylosoxidans*; SMA, *Serratia marcescens;* HI, *Haemophilus influenzae*.
†Phenotypic identification was obtained by using the BIOLOG GN MicroPlate assay (BIOLOG Inc., Hayward, CA, USA) for case 1 and the API 20NE kit system (bioMérieux, Marcy l'Etoile, France) for cases 2–8 and 15–18.

used for treatment for *P. aeruginosa* colonization (as was the case for our 4 patients), we hypothesize that this bacterium is selected during the evolution of the disease.

We have developed a real-time PCR molecular method that is faster and easier than amplification-sequencing for prompt detection and accurate identification of *I. limosus* with good specificity and sensitivity. By using this screening assay, we identified 4 additional cases of patients with CF who were also infected with this bacterium, including a 2-year-old child. In addition, by using this technique, we were able to detect *I. limosus* in a patient with deteriorated respiratory function 3 months before the culture-based isolation, indicating that a low bacterial load, insufficient for being isolated in culture, can be detected by PCR in the lower respiratory tract of patients with CF.

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Mr Bittar is a PhD student at URMITE UMR, Faculty of Medicine, Marseille. His research interest is detection and description of new or emerging pathogens in cystic fibrosis patients.

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Splenic Rupture and Malignant Mediterranean Spotted Fever

To the Editor: Mediterranean spotted fever (MSF) is a *Rickettsia conorii* infection endemic to the Mediterranean. In this case, a 55-year-old man was referred to the Necker-Enfants Malades Hospital, Paris, France, for fever, myalgia, and hypotensive shock. The patient had been in Southern France (Montpellier) 6 days before symptom onset and had been bitten by a tick on the left hand. Four days later, he reported fatigue, fever (39°C), and myalgia. His medical history showed polycystic kidney disease, which had necessitated hemodialysis and a kidney transplant. He was receiving ongoing treatment with an immunosuppressive regimen of cyclosporine, prednisolone, and tacrolimus; his baseline hemoglobin level was 15 g/dL, and creatinine level was 230 μmol/L.

At admission, the patient's temperature was 39.5°C, blood pressure 55/40 mm Hg, and heart rate 104 beats/min. Physical examination showed a diffusely tender abdomen with guarding, no hepatosplenomegaly, a nontender renal transplant, and no lymphadenopathy. Results of cardiovascular, respiratory, and neurologic examinations were unremarkable. A diffuse maculopapular cutaneous eruption was noted on the lower limbs; no eschar was detected.

Laboratory analyses showed the following values: hemoglobin 7.9 g/dL, platelet count 115 × 10^9/L, leukocyte count 6.7 × 10^9/L (neutrophils 5.2 × 10^9/L, lymphocytes 1.4 × 10^9/L); serum creatinine 466 μmol/L, and C-reactive protein 156 mg/L. Blood cultures were negative. Serologic study results were negative for HIV, hepatitis viruses, Epstein-Barr virus, cytomegalovirus, *Legionella*, *Mycoplasma*, *Coxiella*, *Bartonella*, *Leishmania*, and *Toxoplasma* spp. Serologic testing obtained at day 1 was negative for spotted fever group (SFG) rickettsiosis.

A computed tomographic scan showed hemoperitoneum secondary to a ruptured subcapsular splenic hematoma (online Appendix Figure, available from www.cdc.gov/EID/content/14/6/995-appG.htm), and an emergency splenectomy was performed. Histopathologic evaluation of the spleen showed white pulp atrophy; the red pulp indicated congestion and ill-defined nodules, varying in size and comprising macrophages, polymorphonuclear neutrophils, and necrotic cells (Figure, panels A, B). Skin biopsy of the macular eruption on day 2 demonstrated a leukocytoclastic vasculitis with nonocclusive luminal thrombi in the dermal capillaries (Figure, panel C).

Universal 16S rRNA gene PCR amplification on spleen and skin tissue samples and direct sequencing identified an *R. conorii*–specific 16S rRNA sequence match. We confirmed this by using primers for *gltA* and *ompA* specific for *R. conorii*. Immunohistochemical staining demonstrated *Rickettsia* in endothelial cells and macrophages in the spleen and skin (Figure, panels D–F). Blood culture, skin biopsy specimens, and splenic tissue cultures were subsequently *R. conorii* positive. Doxycycline therapy (100 mg intravenously twice a day) was instituted at day 2 because rickettsiosis was suspected. The patient dramatically improved within 72 hours and remained well 36 months after diagnosis.

MSF is a rickettsiosis belonging to the tick-borne SFG caused by *R. conorii*, an obligate intracellular bacteria transmitted by the dog tick *Rhipicephalus sanguineus*. Endemic to Mediterranean countries, MSF generally results in a benign febrile illness accompanied by a maculopapular rash, myalgia, and local black eschar at a tick bite inoculation site. A minority of persons seeking treatment display a malignant form, which results from disseminated vasculitis associated with increased vascular permeability, thrombus-mediated vascular occlusion, and visceral perivascular lymphohistiocytic infiltrates (*1*). Focal thrombi have been identified in almost all organs of patients with fatal cases. Manifestations of MSF include neurologic involvement, multi-organ failure, gastric hemorrhage, and acute respiratory distress syndrome; the case-fatality rate is 1.4%–5.6%.

Splenic rupture has been reported in the course of infection with several microbial agents, including Epstein-Barr virus (*2*), HIV, rubella virus, *Bartonella* spp. (*3*), *Salmonella* spp., mycobacteria (*4*), and *Plasmodium* spp. (*5*). Splenomegaly as a result of MSF has also been documented previously (*6*);

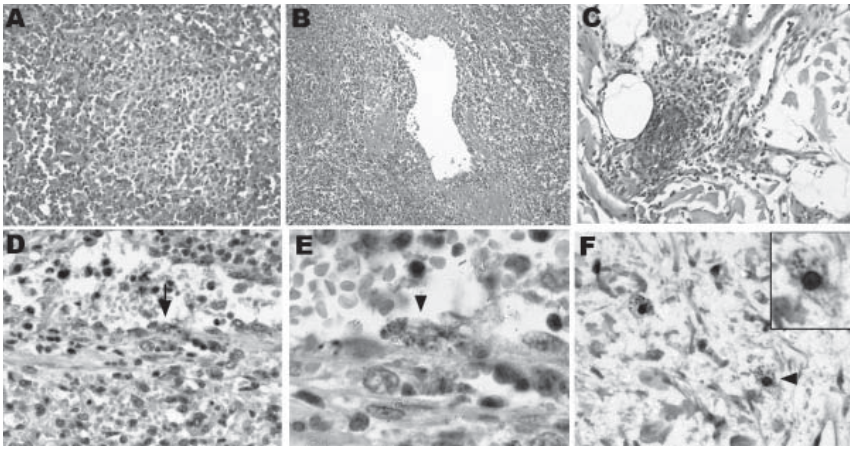

Figure. Histopathologic and immunohistochemical labelings of spleen and skin tissue samples. Tissue samples were fixed in 10% formalin, paraffin-embedded, and examined after hematoxylin-eosin staining, Gimenez staining, or immunostaining with the R47 anti-*Rickettsia conorii* polyclonal rabbit antibody. The spleen red pulp indicated congestion and ill-defined nodules varying in size and comprising macrophages, polymorphonuclear neutrophils, and necrotic cells (A, magnification ×100). A diffuse macrophage infiltration with abundant hemophagocytosis (not shown) and venulitis (B, magnification ×50) was also observed. In the skin, leukocytoclastic vasculitis with focal vascular necrosis and nonocclusive luminal thrombi were noted in dermal capillaries (C, magnification ×100). Intracellular images evocative of rickettsiae were observed in the splenic arteriolar endothelium upon immunohistochemical staining (D, arrow, magnification ×200; magnified view shown in E, arrowhead, magnification ×500). No infected cells were observed in nodular inflammatory splenic lesions. Immunohistochemical staining also disclosed intracellular immunolabeled dots in cells that could correspond to infected dermal macrophages (F, arrowhead, magnification ×300; magnified view shown in inset, magnification ×600), at a distance from the vascular alterations. Endothelial cells of dermal capillaries were also immunolabeled (not shown). A color version of this figure is available online (www.cdc.gov/EID/content/14/6/995-G.htm).

however, splenic rupture in the context of tick-borne illness has only previously been reported for *R. typhi* (*7*) and *Coxiella burnetii* infections (*8*).

SFG rickettsioses have rarely been described in transplant recipients. Barrio et al. reported a case of MSF in a liver transplant recipient with clinical resolution of infection (*9*), and a case of Rocky Mountain spotted fever after heart transplantation has been described (*10*).

Seroconversion remains the principal diagnostic tool for the rickettsioses, but often no detectable antibody is found in the early phase of the disease. Spleen and skin tissue samples allowed rapid 16S rRNA gene PCR and sequencing before the results of other diagnostic procedures were obtained. Immunostaining allowed detection of *R. conorii* in spleen and skin tissue samples and illustrated the cell tropism of this intracellular bacterium for cells morphologically similar to endothelial cells and possibly macrophages. Although *R. conorii* infection of postmortem human splenic samples from patients with fatal cases has been documented by immunohistochemical testing, *R. conorii* has not been described previously in spleen tissue of those who have survived malignant MSF.

This case expands the spectrum of infectious agents associated with spontaneous splenic rupture and solid organ transplantation. Rickettsioses are a significant risk both for those living in disease-endemic regions and for international travelers. To facilitate early detection and treatment, physicians must be vigilant for atypical symptoms, especially in immunocompromised persons.

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Acetobacter indonesiensis Pneumonia after Lung Transplant

To the Editor: Unusual and multiresistant bacterial infections are increasingly reported in cystic fibrosis (CF) patients (*1*). On January 25, 2007, a 31-year-old man with CF (mutation ΔF 508 and I 507) was admitted to our institution in Marseille, France, for lung transplantation. His immunosuppressive regimen included IV cyclosporin A (for the first 6 days with conversion to oral tacrolimus thereafter), azathioprine, and corticosteroids. Induction therapy that used antithymocyte globulin was administered for the first 3 days (Thymoglobuline, Genzyme Corporation, Naarden, the Netherlands). Since 2003, the patient was chronically colonized by methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (susceptible only to colistin sulfomethate), and *Candida albicans*. Preemptive treatment with antimicrobial agents including colistin sulfomethate, tobramycin sulfate, ceftazidime, and linezolid was administered, starting on posttransplant day 1; prophylactic caspofungin, followed by inhaled amphotericin B, was given for the first month. Six and 9 days, respectively, after surgery, sputa from the patient showed *P. aeruginosa* and MRSA.

On postoperative day 11, the patient's clinical condition worsened. Leukocytes increased to 13.84 × 10^9/L. In addition to *P. aeruginosa* (10^4 CFU/mL) and MRSA (10^3 CFU/mL), culture of later sputum samples yielded the growth of 10^4 CFU/mL of gram-negative, catalase-positive, and oxidase-negative bacillus (isolate 7120034) on CEPACIA agar (AES, Combourg, France) after 72 hours of incubation at 30°C. API 20NE, API 20E, and VITEK 2 Auto system (bioMérieux, Marcy l'Etoile, France) did not identify the bacillus. This bacterium was multiresistant to antimicrobial agents, including colistin, and was susceptible only to imipenem, rifampin, and aminoglycosides. The final identification of this isolate as *Acetobacter indonesiensis* was achieved after partial sequencing of 16S rRNA gene, as previously described (*2*) (GenBank accession no. AJ199841, 99% similarity). The sequence of our isolate has been deposited in GenBank under the accession no. EF681860. The phylogenetic position of isolate 7120034 among other gram-negative bacteria is shown in the Figure.

Tobramycin was stopped at day 11, colistin and ceftazidime were stopped at postoperative day 14, lin-

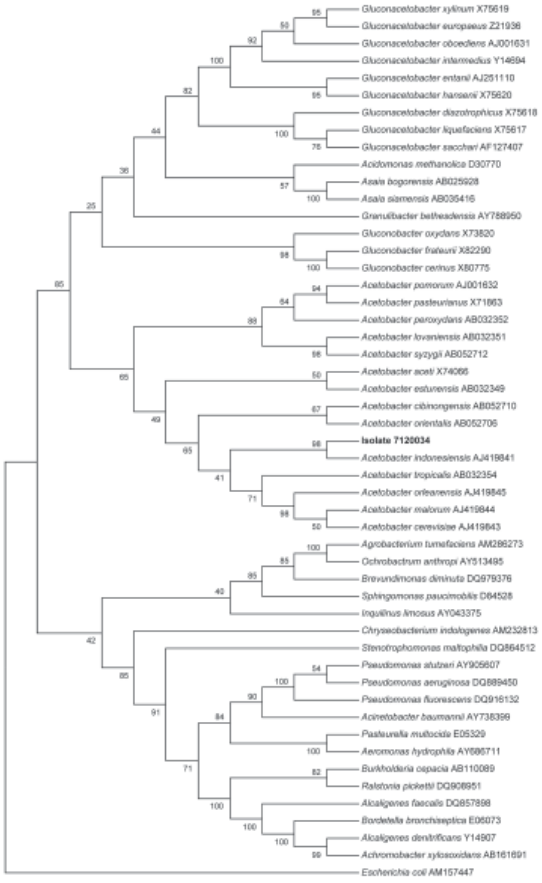

Figure. Phylogenetic tree showing the position of *Acetobacter indonesiensis* (isolate 7120034, GenBank accession no. EF681860), in **boldface**, within acetic acid bacteria and other gram-negative rods. The tree was based on 16S rDNA comparison by the neighbor-joining method. Numbers along the branches indicate bootstrap values.

ezolid was maintained for 10 additional days, and gentamicin (for 13 days) was added to the patient's drug therapy on February 10. Despite this treatment, *A. indonesiensis* was cultured from sputa obtained on February 16 (10^4 CFU/mL) and February 20 (10^2 CFU/mL), respectively. Six days later, leukocytes decreased to 8.62×10^9/L, and the patient's condition improved. He was discharged at the beginning of March. During 7 months of follow-up, the *A. indonesiensis* strain was not found again.

Acetic acid bacteria are gram-negative bacilli classified into the genera *Acetobacter, Gluconobacter, Gluconacetobacter, Acidomonas,* and the recently described genus *Asaia*; these bacteria belong to the α subgroup of *Proteobacteria* (*3*). Isolates of this family are recognized as food-associated bacteria and are able to grow at acidic pH (*4*). Three species have been reported as emerging pathogens in humans: *Asaia bogorensis* (in a case of peritonitis in a patient with a peritoneal dialysis catheter [*5*]); *Granulibacter bethesdensis* (in 3 cases of lymphadenitis associated with chronic granulomatous disease [*6*]); and *Acetobacter cibinongensis* (a recent case of bacteremia in a patient receiving chronic hemodialysis for end-stage renal failure [*7*]).

A. indonesiensis has been isolated from fruits and flowers in Indonesia (*8*); only 3 reports on it have been published (*3*,*8*,*9*). In our patient, we believe that this multidrug-resistant bacterium was the primary cause of the infection because the patient was eventually cured after an adapted antimicrobial drug therapy. Because this bacterium grows easily at acidic pH (*4*), a classic condition in the CF airway surface liquid, acidity might contribute to bacterial adhesion and colonization (*10*). Because acetic acid bacteria have never been isolated from human flora, the source of the contamination for our patient remains unknown.

We also report the antimicrobial drug susceptibility of this bacterium. It was multiresistant, especially to colistin. Antimicrobial drug susceptibility results were obtained by using Vitek 2 Auto system because of the absence of growth on Mueller-Hinton agar. This pattern was also the case for *G. bethesdensis* (*6*). The antimicrobial susceptibility of *A. cibinongensis* could not be validated because of the lack of interassay reproducibility (*7*). Initial antimicrobial drug therapy for this patient with amoxicillin, pristinamycin, and cefazolin did not cure the patient; he was eventually cured after the therapy was switched to tobramycin (*7*).

Our findings reemphasize the emergence of new colistin-resistant pathogens in CF patients, as recently reported for *Inquilinus limosus* (*1*). The increased clinical use of nebulized colistin for *P. aeruginosa* infection in CF patients may select specific colistin-resistant bacteria in such populations. In summary, this report of a respiratory tract infection caused by *A. indonesiensis* after lung transplantation in a French CF patient supports that this multidrug-resistant bacterium may be an emerging opportunistic pathogen in immunocompromized patients, including CF patients with a lung transplant.

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Coronavirus Antibodies in Bat Biologists

To the Editor: Severe acute respiratory syndrome–associated coronavirus (SARS-CoV) is a new coronavirus that caused an epidemic of 8,096 cases of SARS and 774 deaths during 2002–2003 (*1*). Attempts are ongoing to identify the natural reservoir of SARS-CoV. Several horseshoe bat species (*Rhinolopus* spp.) from Asia (*2*,*3*) and a sample of bats from Africa (*4*) have been found to be infected by and potential reservoirs for various SARS-like CoVs and various CoVs that are not SARS-like (*2*–*4*). However, transmission of bat SARS-CoV from bats to humans has not been reported.

During October 2005, we looked for serologic evidence of infection among bat biologists attending an international meeting in the United States. After giving informed consent, volunteer biologists completed an anonymous survey and provided 10 mL of blood. Serum samples were tested at the Centers for Disease Control and Prevention (CDC) for antibodies against inactivated human SARS-CoV and against recombinant, expressed SARS-CoV nucleocapsid protein (SARS-CoV N) by enzyme immunoassays (EIAs) as described (*5*,*6*). This study was approved by the CDC Institutional Review Board.

Of 350 registered biologists, 90 (26%) participated. Of participants, 89% had worked with or studied bats in North America, 21% in South America, 11% in Africa, 8% in Asia, 7% in Europe, and 6% in Australia. The primary genera studied by participants were *Myotis* (24%), *Tadarida* (13%), and *Eptesicus* (10%). A total of 20 (23%) participants had worked with or had contact with horseshoe bat species (*Rhinolopus* spp.). Because this genus has 69 species, distributed from Australia to Europe, some participants who indicated that they worked with the *Rhinolopus* spp. may likely have worked with species found outside of Asia. Involvement with bats most often consisted of capturing or handling them in the field (90%), followed by capturing or handling them in the laboratory (36%). Urine and feces were encountered most frequently ("always" or "most of the time" by 66%–68% of participants); contact with blood, saliva, or tissues and bites or scratches reportedly occurred less often ("always" or "most of the time" by 4%–28% of participants).

The serum samples from all 90 participants were negative for antibodies against inactivated SARS-CoV, and samples from all but 1 were negative for SARS-CoV N protein. The 1 positive sample gave a strong signal (optical density 1.08 at 405 nm at a 1:400 dilution) by SARS-CoV N protein EIA and against SARS-CoV N by Western blot but gave no reactivity against recombinant SARS-CoV spike protein or inactivated SARS-CoV by either EIA or Western blot. Because the N protein has a region that is relatively conserved among all known coronaviruses (*7*), the antibodies against SARS-CoV N protein could have been induced by other CoVs. Previous studies have demonstrated that SARS-CoV N protein can cross-react with polyclonal antiserum induced by group 1 animal CoVs (*8*).

To address the possibility that the antibodies from this serum sample were not specific to SARS-CoV, we tested it against recombinant N proteins of human CoVs, HCoV-229E, HCoV-OC43, NL63, and HKU-1. The serum reacted to all 4 N proteins, by EIA and Western blot, at titers of 400–1,600. We then tested the sample against 3 recombinant fragments of the N protein from each of 3 viruses: SARS-CoV, HCoV-229E, and HCoV-OC43. One of these fragments, N2, contains a highly conserved motif (FYYLGTGP) that should detect cross-reacting antibodies; the other 2 fragments should detect antibodies specific to the strain or group. The serum reacted to 2 of 3 fragments from HCoV-OC43 and -229E but to only the N2 fragment with the conserved motif from SARS-CoV (Figure), which suggests that the antibodies against SARS-CoV N were likely induced by a CoV that was not SARS-like.

If the antibodies were induced by a SARS-like CoV infection, we would expect to have also detected antibodies against recombinant S protein (*9*) or recombinant fragments representing antigenically distinct regions of the N protein of SARS-CoV. We did not detect either; instead, we detected antibodies against the antigenically distinct N fragments from group 1 and 2 human CoVs. Thus, this survey of a sample of bat biologists, who were exposed primarily to North American bats but also to bats from Asia and Africa, showed no evidence of SARS-like CoV infection.

Our survey found no evidence of SARS-CoV transmission from bats to humans. However, since the conclusion of this study, Dominguez et al. found coronavirus RNA in bats in North America, particularly *Eptesicus fuscus* and *Myotis occultus* (*10*), 2 species of the genera handled by 25% of the participants in our survey. Of interest is whether the bat biologists who worked with these bats might be at risk for infection with group 1 bat CoVs. Unfortunately, the high likelihood of infection with human group 1 CoVs will make it difficult to address this question. Additional studies of bat SARS-CoV infections in a larger number of persons who have been in contact with the species found to be positive for SARS-like CoV are needed before the risk for SARS-like CoV transmission from bats to humans can be clearly understood.

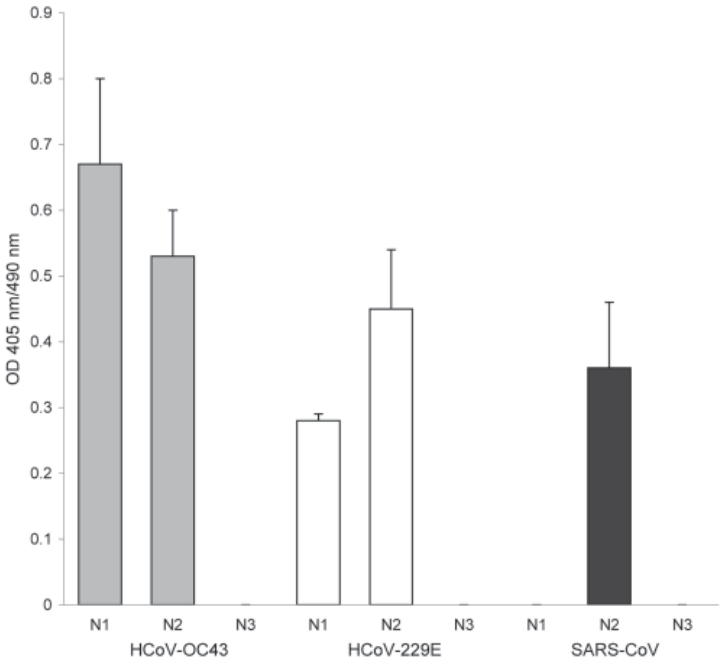

Figure. Antibody reactivity to coronavirus (CoV) nucleocapsid (N) protein fragments by ELISA. A set of recombinant protein fragments covering the N protein sequence of human CoV [HCoV]–OC43, HCoV-229E, and severe acute respiratory syndrome (SARS)–CoV were used as antigen; the serum (1:400 dilution) from the participant was tested by ELISA. The fragments include the following HCoVs: HCoV-OC43 N1 (aa 1–119), HCoV-OC43 N2 (aa 120–332), HCoV-OC43 N3 (aa 333–448), HCoV-229E N1 (aa 1–74), HCoV-229E N2 (aa 75–311), HCoV-229E N3 (aa 312–389), SARS-CoV N1 (aa 1–105), SARS-CoV N2 (aa 106–324), and SARS-CoV N3 (aa 325–422). The HCoV-OC43, HCoV-229E, and SARS-CoV fragments were coated at 4×10^{-7} M, 2.5×10^{-3} M, and 8×10^{-8} M, respectively. The N-terminal of the N protein contains a highly conserved motif (FYYLGTGP) found in all CoVs (*7*). This conserved motif is found in HCoV-OC43 N2, HCoV-229E N2, and SARS-CoV N2 recombinant protein fragments. The sizes of the expressed protein fragments used in this study were confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In addition, the reactivity of each protein fragment was confirmed by using Western blot with the anti-His antibody and the respective convalescent-phase serum. The mean optical density (OD) of absorbance at 405 nm (490-nm reference) of duplicate wells is shown. Error bars represent the standard deviation of duplicate wells.

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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.

Chagas Disease in Ancient Hunter-Gatherer Population, Brazil

To the Editor: Chagas disease, caused by the protozoan *Trypanosoma cruzi,* and first described by Carlos Chagas in 1909, is endemic to Latin America. As a results of multinational control initiatives launched in the 1990s, the disease prevalence has been reduced. This campaign was focused on the interruption of *T. cruzi* vectorial transmission by eliminating domiciled triatomines. In 2006, Brazil was declared to be free from *T. cruzi* transmission by *Triatoma infestans* (*1*). *T. cruzi* is a heterogeneous taxon with multiple mammal hosts and vectors, besides alternative routes of infection and infective forms. In the Brazilian Amazon region, where domiciled triatomines have not been reported, human cases of Chagas disease have been increasing (*2*). This increase has been attributed to uncontrolled migration and deforestation (*2*). Additionally, recent outbreaks of Chagas disease attributed to oral transmission in previously non–disease-endemic areas out of the Amazon region (*3*) indicate that a new epidemiologic profile is emerging in Brazil.

T. cruzi has 2 main genotypes, *T. cruzi* I and *T. cruzi* II, and these subpopulations display distinct biologic, biochemical, and genetic profiles. In Brazil, *T. cruzi* I is widespread among wild mammals and sylvatic vectors of all biomes. Moreover, this genotype is commonly isolated from humans and wild mammals in the Amazon Basin. In contrast, *T. cruzi* II, has a focal distribution in nature but is the main agent of human infection in other Brazilian regions (*4*).

In this report, we describe the finding of *T. cruzi* in human remains dating back 4,500–7,000 years that were obtained from a Brazilian archeological site and, the recovery of an ancient DNA (aDNA) sequence corresponding to the parasite lineage type I. The mummy, called AM1, was a woman ≈35 years of age from a hunter-gatherer population. She was found in Abrigo do Malhador archeological site, Peruaçu Valley, Minas Gerais State (*5*). This region, where the semiarid ecosystem is predominant, has a dry climate, karst relief (an area of limestone terrain characterized by sinks, ravines, and underground streams), and soil with a basic pH. These conditions have contributed to the preservation of specimens.

The remains were excavated in 1985 and maintained in an environment protected from light and humidity. In 2005, after taking precautions to avoid contamination with exogenous DNA or cross-contamination between samples, we collected ≈6 cm of a rib fragment from AM1. All experiments were conducted in a restricted area that was isolated from the major laboratory, where post-PCR experiments were performed. *T. cruzi* had never been used in either laboratory. Mitochondrial DNA haplotypes of laboratory staff were determined to control contamination. PCR-positive controls (*T. cruzi*) were not used. The rib surface was gently scraped to remove impurities and decontaminated with bleach (6% NaOCl) and UV light (15 min for each side). We processed 300 mg of bone powder according to the extraction protocol of dehybernation B solution of the Geneclean Kit for Ancient DNA (Bio101, La Jolla, CA, USA). By using specific set of primers (*6*), a 350-bp miniexon gene fragment was successfully recovered by PCR (Figure); the fragment corresponded to the *T. cruzi* I lineage, according to miniexon gene typing assay (*6*). Moreover, nucleotide sequence analysis (341 bp) (GenBank accession no. EF626693), showed 98% similarity with *T. cruzi* I sequences in GenBank. Additionally, total aDNA hybridization with *T. cruzi* probes (miniexon and total kinetoplast DNA) confirmed the infection. Sequence analysis of the

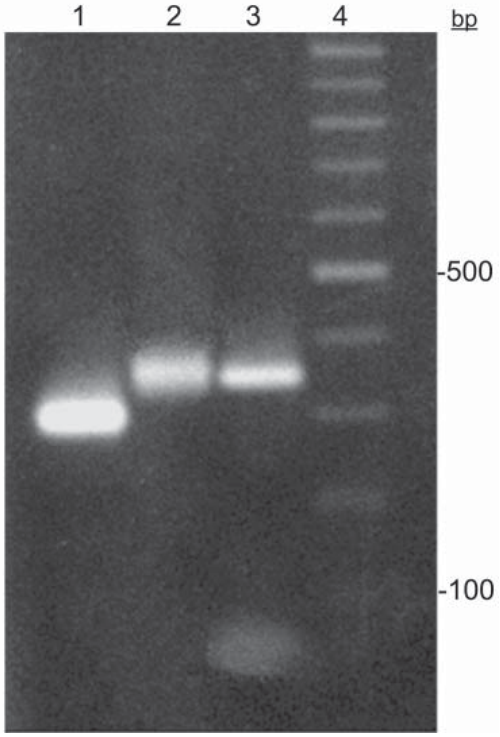

Figure. *Trypanosoma cruzi* miniexon gene typing assay. Lanes 1 and 2, *T. cruzi* II (300 bp) and *T. cruzi* I (350 bp) markers, respectively; lane 3, *T. cruzi* I cloned fragment recovered from Brazilian mummy; lane 4, 100-bp ladder.

human mitochondrial DNA HVS-I region characterized this person as belonging to haplogroup B (GenBank accession no. EU359272), one of the founder human haplogroups in the Americas.

The antiquity of human *T. cruzi* infection in South America has been demonstrated on the basis of paleonthologic studies. Clinical manifestations of Chagas disease were observed in Chilean mummies from pre-Columbian times (*7*). Moreover, a *T. cruzi* kinetoplast DNA region was recovered in Chilean and Peruvian mummies from up to 9,000 years ago (*8*,*9*).

In Brazil, the current epidemiologic scenario concerning Chagas disease in indigenous populations involves ecologic aspects of their settlements, along with nomad habits, which prevent triatomine nesting and, therefore, the infection. The beginning of *T. cruzi* transmission to humans is attributed to the domiciliation of *T. infestans* as a consequence of precarious mud dwellings, built after European colonization (*10*). In this report, we showed that *T. cruzi* human infection in Brazil is ancient, dating back at least 4,500 years, and therefore occurring in hunter-gatherer populations largely preceding *T. infestans* domiciliation. The presence of the *T. cruzi* I genotype infecting humans 4,500–7,000 years ago in Minas Gerais State, where this genotype is currently absent (*6*), suggests that the distribution pattern of *T. cruzi* genotypes in humans has changed in time and place. Moreover, the recovery of an aDNA sequence and the possibility of genotyping parasites from human remains make it possible to reconstruct the early dispersion patterns of *T. cruzi* subpopulations. On the basis of our results, one may speculate that the current outbreaks of human *T. cruzi* infection, independent of triatomine domiciliation, are the reemergence of the ancient epidemiologic scenario of Chagas disease in Brazil.

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Coxiella burnetii in Wild-caught Filth Flies

To the Editor: *Coxiella burnetii*, the agent of Q fever, is a bacterium and a potential agent of bioterrorism. The most frequent signs of infection in domestic animals are abortion and reduced fertility (*1*). Clinical signs of Q fever in humans vary from mild fevers to pneumonia, hepatitis, or death; atypical cases occur as other disorders, such as cholecystitis (*1*,*2*). Aerosols are the most common route of exposure, but oral transmission occurs (*1*).

Some flies feed on the feces, milk, carcasses, or blood of domestic animals that can be infected with *C. burnetii*. These flies regurgitate and defecate when feeding and are mechanical vectors of bacteria (*3*,*4*). Flies have been shown to harbor, mechanically transport, and even support the growth of *C. burnetii* (*4*–*6*). It is known that house flies (*Musca domestica*) are possible mechanical vectors of *C. burnetii* because this organism survived 32 days in house flies and viable bacteria were shed by flies for 15 days (*4*). There are no studies of *C. burnetii* in field-collected flies. To examine the prevalence of *C. burnetii* in field-collected flies, we tested flies from farms, forests, ranches, and zoos.

Flies that develop on animal dung, carcasses, feces, blood, or garbage are often called filth flies. Adult

Calliphoridae, Hippoboscidae, Muscidae, and Sarcophagidae were collected from forests, zoos, ranches, and farms (Table). Flies were killed in 95% ethanol or by freezing. DNA was extracted from individual flies as described (*7*,*8*). A distilled water negative control was used for each extraction.

Individual DNA samples were tested, in duplicate, with a previously described TaqMan assay with a lower limit of detection of 1 *C. burnetii* organism (*8*). Positive and negative controls were used for all assays. Positive flies were verified by PCR and sequencing of 16S rRNA gene as described (*9*). Vouchers for each insect species were deposited in the Clemson University Arthropod Collection (Clemson, SC, USA), the University of Georgia Museum of Natural History (Athens, GA, USA), or the University of Wyoming Insect Collection (Laramie, WY, USA).

Five of 363 flies were positive for *C. burnetii* DNA (Table). These flies included *Stomoxys calcitrans*, in which the adults feed on animal and human blood, and the blowflies *Lucilia coeruleiviridis* and *L. sericata*. *C. burnetii*–positive flies were obtained from carrion (1/12, 8.3%), a garbage bin of elephant feces (3/18, 16.7%), and a barn at a ranch (1/55, 1.8%). We sequenced 1,100 bp of the 16S rRNA gene from select DNA extracts, which were 99% identical with that of *C. burnetii* strain NC 002971.

We detected DNA from *C. burnetii* in flies from a zoo, a ranch, and carrion in a forest. Laboratory data on house flies, which shed live *C. burnetii* for 15 days after exposure, suggest that related flies (e.g., *S. calcitrans* and *Lucilia* spp.) might also harbor viable *C. burnetii*. On the basis of our field data, *S. calcitrans* and *Lucilia* spp. should be studied as mechanical vectors of *C. burnetii*. Unlike many enteric bacteria, which require large inocula to cause disease, *C. burnetii* can be infectious at the level of 1 bacterium (*10*). If flies transmit *C. burnetii*, they pose an additional threat to human and animal health.

The role of the sheep ked (*Melophagus ovinus*) in maintenance or transmission of *C. burnetii* is unknown. This fly is an obligate ectoparasite of sheep. It feeds on sheep blood, and feces from sheep keds can accumulate in the wool of sheep. Testing of sheep keds from infected sheep would help understand whether keds play a role in the epidemiology of *C. burnetii*.

Table. Flies from the United States and Dominica assayed for *Coxiella burnetii*, 2004–2007

Species	Collection site and collector	Date of collection	No. positive for *C. burnetii*/no. collected
Calliphora vicina (Robineau-Desvoidy)	Elephant dung, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2006 Apr 18	0/1
Lucilia coeruleiviridis (Marquart)	Trapped on carrion near Pickens, SC, USA, by K.D. Cobb and W.K. Reeves	2004 Jul 3	1/12
L. coeruleiviridis (Marquart)	Elephant dung, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2006 Jul 18	0/13
L. coeruleiviridis (Marquart)	Elephant dung, Greenville Zoo, Greenville County SC, USA, by M.P. Nelder	2005 Aug 17	0/3
L. sericata (Meigen)	Garbage bin, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2005 Aug 17	3/18
Melophagus ovinus (Linnaeus)	Sheep, Bozeman, Gallatin County, MT, USA, by J.E. Lloyd	2007 Jun 27	0/154
Musca domestica (Linnaeus)	Cow, Springfield Estate, St. Paul Parish, Dominica, by W.K. Reeves	2005 May 18	0/6
M. domestica	Goat pens, petting exibit, Greenville Zoo, Greenville County, SC, USA, by W.K. Reeves and M.P. Nelder	2005 Aug 1	0/5
M. domestica	*Colobus* spp. monkey dung; Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2005 Oct 19	0/6
Ravinia stimulans	Lion dung, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2005 Oct 16	0/10
Ravinia new sp.	Lion dung, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2005 Oct 17	0/11
Stomoxys calcitrans (Linnaeus)	Goat pens, petting exibit, Greenville Zoo, Greenville County, SC, USA, by W.K. Reeves and M.P. Nelder	2005 Aug 1	0/20
S. calcitrans	Fly trap, Greenville Zoo, Greenville County, SC, USA, by M.P. Nelder	2006 Apr 28	0/20
S. calcitrans	Cow, Riverbanks Zoo, Richland County, SC, USA, by M.P. Nelder	2006 Apr 5	0/17
S. calcitrans	Cow, goats, horses, and llama, Riverbanks Zoo, Richland County, SC, USA, by M.P. Nelder	2006 Apr 6	0/12
S. calcitrans	Cattle and elk hay barn, Sybille Canyon, Albany County, WY, USA, by W. Yarnell	2007 July 14–Aug 3	1/55

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Conflict and Emerging Infectious Diseases

To the Editor: In the November 2007 issue of Emerging Infectious Diseases, Gayer et al. (*1*) describe how conflict leaves populations in dire poverty, internally displaced or seeking asylum, having poor access to essential services, and consequentially vulnerable to infectious diseases.

Cholera, caused by the bacterium *Vibrio cholerae,* is a disease that seems particularly sensitive to conflict and deserves more consideration. Major risk factors for cholera—poverty, overcrowding, poor hygiene, contaminated food, and lack of safe drinking water (*2*,*3*)—largely resemble the consequences of war and civil fighting. Yet little is known about the relationship between cholera and conflict. This lack of information may be because cholera tends to be epidemic, affecting hundreds to thousands of people across vast, war-torn regions, making it impossible for local governments, nongovernment organizations, and aid workers to control, let alone collect and analyze data.

Examination of data sources listed by Gayer et al. (*1*) and recent reviews (*2*,*3*) indicate that cholera occurs 1) in countries during war and civil unrest, as exemplified by the latest outbreaks among displaced populations across northern Iraq; 2) in neighboring countries, where temporary camps accommodate masses of political refugees under poor conditions, such as those in eastern Chad near Darfur, Sudan; and 3) during the postwar period when large numbers of repatriated persons return home and consequently place undue pressure on an eroded and fragile national infrastructure, as evident in Angola in recent years.

Moreover, all the countries affected by conflict shown in the Figure by Gayer et al. (*1*) (available from www.cdc.gov/EID/content/13/11/1625-G.htm) have reported cholera outbreaks (*2*–*4*). They are also among the poorest countries in the world; the latest statistics on human development (*5*) indicate that compared with all developing countries, on average they have higher rates of undernourishment, refugees, child deaths, and less adequate water and sanitation facilities. Thus, more information is needed about conflict and cholera, especially in Africa.

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In Response: We agree with Kelly-Hope on the propensity for cholera outbreaks to occur in conflict-affected countries and the need to monitor and respond more effectively to such events. In 2006, cholera was reported from 33 countries in Africa, and 88% of all reported cases were from conflict-affected countries (*1*).

As highlighted in our November 2007 article on conflict and emerging infectious diseases (*2*), conflict situations present a multitude of risk factors that enhance disease emergence and transmission, over and above those in other resource-poor countries. Many such conflicts facilitate the occurrence of cholera outbreaks.

More precise research on cholera and conflict is indeed necessary. However, despite cholera being a disease that has been around for a long time and that causes frequent outbreaks to this day, much information about this disease, beyond its relationship with conflict, remains unknown. For example, although *Vibrio cholerae* persists in the environment, little is known about the exact conditions that trigger a cholera outbreak at a particular time. Further elucidation is needed about the factors that influence the duration of an outbreak, disease severity, and duration of individual protective immunity after an episode of cholera.

Cholera, which is closely linked to a country's social and economic development (*1*,*3*), ceased to be of concern in Europe, for example, when access to potable water and sanitation improved although its cause was still unknown and antimicrobial drugs were not yet available. Today, renewed interest from the international public health community is urgently warranted, and strong initiatives are needed to help developing countries (conflict-affected or not) fight against cholera and control this easily preventable disease on a global level.

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Legionella and the Prevention of Legionellosis

Jamie Bartram, Yves Chartier, John V. Lee, Kathy Bond, and Susanne Surman-Lee, editors

World Health Organization Press, Geneva, Switzerland, 2007

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Pages: 276; Price US $40

Legionella and the Prevention of Legionellosis is much less about the former and more about the latter. The book is essentially a risk-management manual for legionellosis, modeled on the World Health Organization's framework for providing safe drinking water. Introductory chapters on the disease and the ecology of *Legionella* spp. and a concluding chapter on laboratory aspects of *Legionella* spp. serve as bookends for 7 chapters on risk management of legionellosis. The intervening chapters discuss known sources of risk for the disease: potable water; cooling towers and evaporative condensers; healthcare facilities; hotels and ships; and natural spas, hot tubs, and swimming pools. A chapter on disease surveillance and outbreak management and another on regulatory aspects complete the core.

Although this book is more a manual than a text, it has much to recommend and little to criticize. An international group of 58 experts contributed to the book, assuring consensus, completeness, and accuracy. Also, unlike many multiauthored texts, which typically suffer from duplication, frequent omissions, and widely varying writing styles, the book's careful editing has averted these common pitfalls. However, an effort to ensure uniformity in some chapters has led to too much rigid conformity to style. An identical template is used for all risk-management chapters, and frequent use of bulleted lists is not particularly engaging and may prove insufficient for some readers.

The text is generously supplemented, perhaps overly so, with 33 tables, 14 figures, and 24 call-out boxes. However, the book's front matter gives a listing of these illustrations for handy reference. Three appendixes are included: a sample water system checklist, a form for compiling relevant epidemiologic information about patients with Legionnaires' disease, and an example of a national surveillance form. The list of references is impressive, and the glossary of terms will be valuable to many readers.

Notably, this disease-specific treatise arrives at a time when public health officials in some countries are moving toward an all-hazards approach to public health preparedness. Even within this context, this text will remain an authoritative reference for many years to come, and the generic algorithm for ensuring water safety has utility beyond the immediate scope of the book.

The brochure accompanying the book recommends it to environmental and public health officials, healthcare workers, workers in the travel industry, certain researchers, and perhaps some special interest groups. I concur with that general assessment, although the book will be used more frequently by some of those groups than by others.

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Global HIV/AIDS Medicine

Paul Volberding, Merle Sande, Joep Lange, Warner Greene, and Joel Gallant, editors

Elsevier, New York, New York, USA, 2007

ISBN-10: 1-4160-2882-X
ISBN-13: 978-1-4160-2822-6
Pages: 846; Price US $99.00

In 1988, Paul Volberding and Merle Sande published the first edition of The Medical Management of AIDS. The 6th and last edition of this authoritative reference was published in 1999, leaving a void on the reference shelves of HIV care providers. Twenty years after the original book, Volberding et al. now offer Global HIV/AIDS Medicine. Their aim is to make this "The first textbook aimed at a comprehensive approach to the management of what is truly a global problem." The first edition of Global HIV/AIDS Medicine has been extensively revamped from the previous textbook. Three new editors have been added, the text has been expanded from 38 to 71 chapters, and the 135 expert authors have been recruited from throughout the world.

The text is divided into 6 major sections: Epidemiology and Biology of HIV Infection; Prevention, Diagnosis, and Treatment of HIV Infection; Diseases Associated with HIV Infection; Prevention and Management in Resource-Rich Settings; Prevention and Management in Resource-Poor Settings; and Economic and Social Consequences of the HIV Epidemic. Advances in HIV medicine since the publication of the last edition are extensively reviewed in the first and second sections. The chapter on the molecular biology of HIV provides an excellent overview of how HIV and cellular proteins interact. Current practices in antiretroviral treatment are nicely summarized in chapters 15–18. In the last 2 sections of the book, the

authors address problems facing HIV care providers practicing in the developing world. Many new chapters have been written for these sections, including chapters on antiretroviral therapy in resource-poor settings, and malaria and HIV; also included is an updated section on parasitic infections among patients residing in the developing world.

Limitations of this book include the predictable minor duplications and contradictions between chapters written by different authors. Given the tome's comprehensive nature and global purpose, certain diseases are inevitably given short shrift to limit the likelihood of back strain among readers. Any person hoping to learn about progressive multifocal leukoencephalopathy, biliary tract diseases, outcomes of surgery, or manifestations of thrombotic thrombocytopenic purpura in HIV-infected patients will be disappointed. In addition, the well-written chapter on dermatologic diseases is distinguished by the baffling lack of any images of the manifestations it describes. These shortcomings are dwarfed by the positive aspects of this book.

In summary, this is a magnificent work by a group of expert editors and world-class authors. This volume should be a part of every reference collection and an essential tool for any serious provider of HIV care.

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Immigrant Medicine

Patricia F. Walker and Elizabeth D. Barnett, editors

Saunders Elsevier, Philadelphia, Pennsylvania, USA, 2007

ISBN-10: 0-323-03454-8
ISBN-13: 978-0-323-03454-8
Pages: 765 (plus CD-ROM);
Price: US $79.95

The United States is experiencing its largest wave of immigration since the beginning of the 20th century, and immigrants are making their way to every region, city, and town. As a result, medical professionals are increasingly called on to care for persons of diverse cultures and ethnicity. Providing care that is culturally sensitive and appropriate is a challenge. In addition to substantial language, cultural, legal, and financial barriers, physicians are faced with medical conditions that they may not have seen before, including unusual infectious diseases and complex, heartbreaking mental health and social issues.

Immigrant Medicine provides, in 1 handy, compact reference, a comprehensive discussion of the issues involved in the compassionate and competent care of refugees and immigrants. The 78 contributors to this reference represent a "Who's Who" of experts in their respective fields. The book is divided into 8 sections on immigrant medicine: 1) introduction; 2) medical screenings and immunizations; 3) epidemiology of diseases and disorders; 4) major diseases and disorders; 5) additional diseases and disorders; 6) chronic illness; 7) mental health and illness; and 8) special issues.

The editors begin with an excellent introduction on the magnitude and scope of immigrant health. The second chapter, "Compassion," is unique in that the author, David R. Shim, proposes that compassion can be learned. He challenges the reader to contemplate the suffering that so many in the world are forced to endure and the difference between acting more compassionately and genuinely being more compassionate. For the most part, subsequent chapters and content flow in a logical sequence to such key areas as cultural competence before moving on to the diagnosis, treatment, and prevention of specific diseases and disorders.

Topics are relevant to practitioners new to immigrant medicine as well as those who are more experienced. For the clinician outside of academic medical centers or special immigrant clinics, language services and payment often present substantial barriers to care. The chapters on language assistance and communicating with patients who have a limited understanding of English describe a more "nuts and bolts" approach to accessing and financing interpreter services, in addition to the legal requirements for language assistance.

Sections 3–6 are particularly helpful for physicians who treat immigrants. Provided in these sections are expanded differential diagnoses by organ system, country of origin, latency period, and race and ethnicity for syndromes such as fever, diarrhea, eosinophilia, and skin problems. Also included are diseases not normally considered in residents of North America. Specific infectious diseases are described in detail, although some diseases are intermingled with syndromes in one section, and additional diseases and disorders are listed alphabetically in another. Chapters on individual diseases are well written. Summaries of therapeutic options are provided, but understandably do not address all of the nuances of treatment. No one textbook can stay abreast of recent advances and changing recommendations. Physicians lacking experience in the diagnosis and treatment of these diseases may need to access additional therapeutic information in the literature or request the recommendations of infectious diseases experts.

A notable strength of this book is the inclusion of the full range of other conditions likely to be seen by those caring for immigrants and refugees. Included are discussions on women's health, dental diseases, and preventive healthcare, which are often overlooked. Because psychiatric and other mental health resources are limited in many locations, the chapters on mental illness and on survivors of torture and violence toward women are of considerable value. Finally, Section 8 provides information on topics frequently missing from other publications, such as school readiness, health literacy, and healthcare risks that immigrants face when they visit friends and relatives abroad. The last chapter includes an excellent table that lists pretravel health resources (websites). It would have been useful to have more of this type of web-based resource information highlighted in other chapters. Overall, we highly recommend Immigrant Medicine for clinicians, other healthcare providers, and public health departments who care for immigrants and refugees.

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Imported Skin Diseases

William R. Faber, Rod J. Hay, and Ben Naafs, editors

Elsevier Gezondheidszorg, Maarssen, the Netherlands, 2006

ISBN-13: 9789035228047
Pages: 304; Price: US $69.95

The foreword to Imported Skin Diseases states, "This book was written and illustrated for the health professional in order to help in the diagnosis and management of patients with diseases acquired in another, often tropical, environment." The book identifies an important clinical need for practitioners whose patients are traveling to an ever greater degree to more distant and exotic locales.

This book is neither an encyclopedic compendium of all tropical skin diseases nor a simple handbook for the house officer or medical student. The breadth of topics is broad, but some topics are discussed in great depth with good authority. The photographic images are clear and well chosen. Chapters that stand out include those on Pigmentary Disorders in Black Skin by J.P.W. van der Veen, M.F.E. Leenarts, and W.W. Westerhof; Coloured versus White Skin by B. Naafs; Buruli Ulcer by F. Portaels and W.M. Meyers; Tungiasis by H. Feldmeier and J. Heukelbach; Myiasis by D.A. Burns; and Beetle Dermatitis by P. Schmid-Grendelmeier and S. Haug.

The chapter Persistent Insect Bites by C.L.M. van Hees assumes that all mosquitoes are night biters. The author states that one can prevent mosquito bites by "avoiding being outside from dusk till dawn." But this message misses the point because anopheline and other species bite from dusk to dawn, and *Aedes* and other species are day biters. This advice is really intended to help avoid contracting malaria, not being bitten.

At times, the writing is puzzling. For example, the chapter, Fever and Rash, by H.G. Schipper and P.A. Kager discusses loiasis and indicates that "fever is absent." It is unclear why a chapter on fever would include a disease that has no fever. Loiasis is more fully covered in the later chapter, Onchocerciasis/Filariasis, by M. Murdoch, so its discussion in Fever and Rash is superfluous and out of place.

Many of the chapters were written by authorities whose first language was probably not English. These chapters could have been edited more carefully because grammatical errors interfere with comprehension. The same applies to the book's typographical errors, which are too frequent to count.

Imported Skin Diseases is organized primarily by diagnosis rather than by syndrome. The disease descriptions are generally complete, with adequate sections on diagnosis and treatment. I discovered at the back of the book, almost by accident, some rather skimpy tables organized by syndrome (fever and rash, ulceration, eschar). I would have preferred this approach to be covered in greater detail because it would be much more useful to the Western practitioner who is confronted with a patient returning from the tropics with an undiagnosed skin disorder. For example, I have a patient in my office with an eschar. The patient has returned from a trip to Africa. What are the diagnostic considerations? Or, a patient has a rash and fever and has traveled widely throughout Asia; how can I approach a proper diagnosis?

As the book is written it could be useful as preparation for the Diploma in Tropical Medicine and Hygiene, certification examination of the International Society of Travel Medicine or American Society of Tropical Medicine and Hygiene, or perhaps as a primer for a physician who will travel to the tropics to practice medicine.

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Festival Banner, Nepal, 17th century. Ground mineral pigment on cotton (60.325 cm × 44.45 cm). Rubin Museum of Art, New York. C2007.19.1 (HAR 69050)

"As the Dew Is Dried Up by the Morning Sun, So Are Mankind's Sins at the Sight of Himalaya"

—The *Puranas*, scriptures of ancient India

Polyxeni Potter*

The Himalaya, "abode of snow," dominates the landscape of Nepal. Home of the highest peaks in the world, including Mount Everest, the country is traversed and bound by the massive range. "In a hundred ages of the gods, I could not tell thee of the glories of the Himalaya," elegize Hindu texts. A barrier to the movement of people for thousands of years, the slopes, glaciers, rivers, and lakes associated with the range discouraged outside interference and military adventures. In the remote gorges, valleys, and high plateaus, flourished instead the individual and unique cultures of a diverse population.

Landlocked in the south by India, the north by Tibet, present-day Nepal historically comprised the kingdoms of the Kathmandu Valley. At the crossroads of trade and multiple religious traditions (Hinduism, Buddhism, Jainism, Sikhism), the valley became a shared sacred site, a focus of social and cultural exchange, and the creative locus of much Himalayan art. The symbiosis of Hinduism and Buddhism is reflected in the art of the indigenous Newar.

When famed Chinese envoy Wang Hsüan-tse visited Nepal in the 7th century, he wrote with admiration of the metalwork in King Narendradeva's palace in Kathmandu and mentioned the king's elaborate belt decorated with golden images of Buddha (*1*). He also noted many architectural marvels and houses embellished with sculptures and paintings.

Around the same time, with Mahayana Buddhism new to Tibet having been introduced through Nepal during the reign of Angshuvarma, demand was high for icons and sacred texts. Many, including scriptures referred to as the Prajñāpāramitā, were copied in the Kathmandu Valley. Surviving texts contain images of religious figures. Gifting manuscripts to monasteries or temples was valued as high virtue, so manuscript illumination persisted for centuries (*2*).

The same colorful, theatrical, and detailed style perfected in manuscripts is also found in larger paintings on

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cloth. These religious paintings, used both to enhance and to record the contemplative experience, were known as *paubha*, the Newari term for the kind of painting called *thangka* in Tibet (*3*). Paubha may have originated with Newar artists who worked in Tibet producing metalwork, murals, and illuminated manuscripts. To meet increased demand for religious scenes, they created a new type of painting on cloth that could be easily rolled up for transport. Paubha may have also originated when Songtsän Gampo, founder of the Tibetan empire, married Nepalese princess Bhrikuti Devi. As legend has it, she took paubha artists, renowned throughout Asia for the high quality of their craft, with her to Tibet.

Early paubhas were representations of religious traditions. To promote meditation, they were hung in private altars, temples, and monasteries or were carried by monks in ceremonial processions. They were rectangular, mostly uniform in size and, as they were intended for three-dimensional presentation, free of rigid frames or easels. Content, color, and proportion followed prescribed rules. Floral borders were common as was brilliant red. A main deity heavy with symbolic elements occupied the center of the composition and was surrounded by smaller less symbolic figures. The intent of the artist was iconography that would ease the path toward enlightenment. Painters for the most part remained anonymous.

Festival Banner on this month's cover of Emerging Infectious Diseases is a scroll painting in the paubha tradition. It was likely paraded in one of the many festivals popular in Nepal to celebrate Hindu or Buddhist gods or goddesses, honor relatives, or mark the beginning or end of the agricultural cycle. The banner was part of a set depicting the Seven Mothers, important *shaktis* (feminine energies) motivating and empowering male Hindu gods. This banner shows Vaishnavi, the shakti consort of Vishnu. Also known as Adh Kanwari (eternal virgin), she attained great powers through meditation and prayer. Shaded by a multiheaded cobra hood, she traveled through the universe on the back of a mythical water monster (*makara*) (*4*).

A striking feature of Hindu and Buddhist art is the portrayal of gods with several arms and heads, symbols of multiple powers and responsibilities. Vaishnavi lifts her primary right hand in a gesture of blessing. Her remaining hands hold various symbols, among them a wheel emblazoned with the *Shri Yantra* (configuration of triangles representing the male and female principles), a lotus (sign of spiritual perfection) (*5*).

In creating sublime images of the gods, artists drew from nature. Eyes were shaped like the curve of little fish, eyebrows like an archer's bow, lips like lotus blossoms, chin like an elephant's trunk, female arms tapered like a root. Intricate details of lavish jewelry, headdresses, imperial robes, and garlands created a pleasing contrast against the spare stylized figure.

"The observing of figures of objects and the making of likenesses of them, which are often looked upon as idle occupation, are for a well-regulated mind a source of wisdom and an antidote against the poison of ignorance," wrote historian and theologian Abu'Fazl Allami (1551–1602) (*6*). The unknown painters of the paubhas transformed observation of the world around them into bodies that were transcendent as well as human and created both a model and the vehicle for enlightenment.

"As the dew is dried up by the morning sun, / So are mankind's sins at the sight of Himalaya," read the *Puranas*. That was before global travel reached the remotest peaks and before climate change threatened the glaciers. And that was when artists painted Vaishnavi as an idealized form without evidence of muscle or bone.

Now, HIV-infected sex-trafficked women and girls from Nepal are more likely than those not infected to also have syphilis and hepatitis B (*7*). In one of her many manifestations, Vaishnavi protected her devotees from fear and gave them peace. The task now falls on global public health and its multiple hands.

Acknowledgment

Special thanks to Lloyd Nick, Director Oglethorpe University Museum of Art (http://museum.ogelthorpe.edu), who facilitated permissions from the Rubin Museum of Art, New York, for use of Festival Banner on the cover of Emerging Infectious Diseases.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Spread of Vector-Borne Diseases and Neglect of Leishmaniasis, Europe

Worldwide Distribution of *Rickettsia felis* and Potential Human Infection

Household Responses to School Closure Resulting from Influenza Outbreak

Coccidioidomycosis Testing among Patients with Community-Acquired Pneumonia

Cluster Size in Large, Population-Based Study of Tuberculosis, Malawi

Spotted Fever Group Rickettsiae in Ticks, Morocco

Distribution of Highly Virulent *Borrelia burgdorferi* Clone in Europe and North America

Toxinotype V *Clostridium difficile* in Humans and Food Animals

Alcaligenes xylosoxidans Bloodstream Infections in Outpatient Oncology Office

Transmission of *Bartonella henselae* by *Ixodes ricinus*

Attributable Outcomes of *Clostridium difficile*–associated Disease in Nonsurgical Patients

Etiology of Childhood Acute Bacterial Meningitis, Turkey

Optimizing Use of Multistream Influenza Sentinel Surveillance Data

AIDS Patient Death Caused by Novel *Cryptococcus neoformans* × *C. gattii* Hybrid

Pneumocystis jirovecii Transmission from Immunocompetent Carriers to Infant

Sudden Onset of Pseudotuberculosis in Humans, France, 2004–05

Molecular Typing of *Trypanosoma cruzi* Isolates, United States

Proficiency of Nucleic Acid Tests for Influenza Viruses, Australasia

Anaplasma phagocytophilum Infection in Hosts of *Ixodes* Ticks, United States,

Complete list of articles in the July issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

June 19–22, 2008
13th International Congress on Infectious Diseases
Kuala Lumpur, Malaysia
http://www.isid.org

June 24–27, 2008
ANAEROBE 2008
The 9th Biennial Congress of the Anaerobe Society of the Americas
Marriott Hotel
Long Beach, CA, USA
http://www.anaerobe.org

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

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Article Title

Transmission of Human Papillomavirus in Heterosexual Couples

CME Questions

1. Which of the following was the most common human papillomavirus (HPV) status at baseline in the current study cohort?

A. Both partners HPV-negative
B. One partner HPV-negative and one HPV-positive
C. Both partners HPV-positive with the same HPV type
D. Both partners HPV-positive with different HPV types

2. What was the most common means of HPV transmission in the current study?

A. Male-to-female transmission
B. Female-to-male transmission
C. Male auto-inoculation
D. Female auto-inoculation

3. Which of the following statements about anatomic sites of transmission of HPV in the current study is *most* accurate?

A. Most women obtained infection from the glans of the penis
B. The female anus was not a significant site of transmission to men
C. There were no cases of transmission from the women's hands to the men's genitals
D. Among men, the rate of auto-inoculation was comparable to the rate of transmission from women

4. Which of the following factors from the current study was *most* significant in the risk for HPV transmission?

A. Frequency of condom use
B. Length of relationship
C. Any history of anal intercourse
D. A history of genital herpes

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

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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.