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On the Cover

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From The Seasons (1565)
Return of the Herd
Oil on panel (160 cm × 120 cm)
Kunsthistorisches Museum
Vienna, Austria

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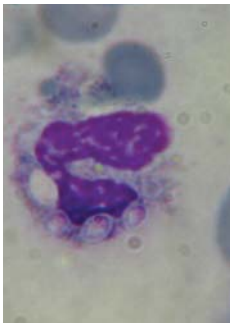

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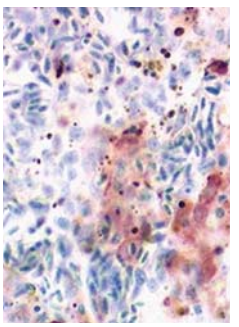

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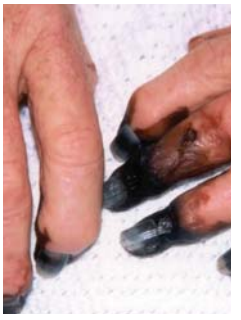

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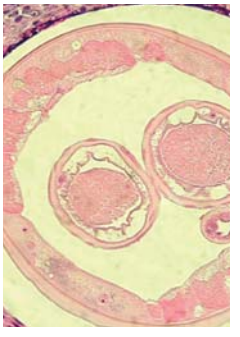

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Conflict and Emerging Infectious Diseases

Michelle Gayer,* Dominique Legros,* Pierre Formenty,* and Maire A. Connolly*

Detection and control of emerging infectious diseases in conflict situations are major challenges due to multiple risk factors known to enhance emergence and transmission of infectious diseases. These include inadequate surveillance and response systems, destroyed infrastructure, collapsed health systems and disruption of disease control programs, and infection control practices even more inadequate than those in resource-poor settings, as well as ongoing insecurity and poor coordination among humanitarian agencies. This article outlines factors that potentiate emergence and transmission of infectious diseases in conflict situations and highlights several priority actions for their containment and control.

An emerging infectious disease is one that is either newly recognized in a population or involves a recognized pathogen affecting new or larger populations or geographic areas (*1*,*2*). Disease emergence is influenced by ecologic and environmental changes (e.g., agriculture, deforestation, droughts, floods), human demographics and behavior (e.g., population migration, urbanization, international trade and travel), technology and industry, microbial adaptation, and breakdown in public health measures (*1*,*2*).

Conflict situations are characterized by war or civil strife in a country or area within a country. Affected populations may experience defined periods of violence (weeks to months), ongoing or recurrent insecurity in a protracted conflict (years to decades), or long-term consequences of a previous (usually prolonged) war.

Conflict may lead to the displacement of large populations into temporary settlements or camps with overcrowding and rudimentary shelters, inadequate safe water and sanitation, and increased exposure to disease vectors during the acute phase of the emergency. In protracted and postconflict situations, populations may have high rates of illness and mortality due to breakdown of health systems, flight of trained staff, failure of existing disease control programs, and destroyed infrastructure. These populations may be more vulnerable to infection and disease because of high levels of undernutrition or malnutrition, low vaccine coverage, or long-term stress. Long-term consequences of civil war can affect entire countries (such as Angola, the Democratic Republic of the Congo [DRC], or Afghanistan) because of chronic lack of investment in health, education, and public works. These conditions, which are encountered during or after war and conflict, favor emergence of infectious diseases. Examples of emerging infectious diseases in conflict situations, where several overlapping risk factors are often involved, are numerous (Figure).

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Risk Factors Enhancing Disease Emergence and Transmission in Conflict Situations

Population Displacement and Environmental Conditions

Malaria had been virtually eliminated in Tajikistan in the early 1960s, and before 1992 only 200–300 malaria cases were reported annually (*3*). Civil strife during 1992–1993 led to massive population displacement and deterioration in living conditions. More than 100,000 persons fled to Afghanistan, reintroducing malaria parasites when they returned in 1994. An outbreak ensued, which reestablished *Plasmodium falciparum* malaria in Tajikistan for first time in 35 years (*4*). By 1997, 29,794 annual cases were reported, although estimates were 200,000–500,000 for that year (*3*). During 1998–1999, a reemphasis on malaria control activities reduced the incidence of malaria by 50% within 2 years (29,794 registered cases in 1997, 19,351 in 1998, and 13,493 in 1999) (*5*).

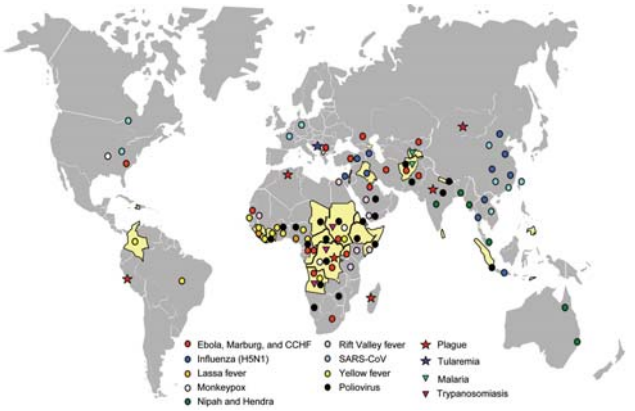

Figure. Geographic distribution of recent emerging or reemerging infectious disease outbreaks and countries affected by conflict, 1990–2006. Countries in yellow were affected by conflict during this period (source: Office for the Coordination of Humanitarian Affairs, World Health Organization, www.reliefweb.int/ocha_ol/onlinehp.html). Symbols indicate outbreaks of emerging or reemerging infectious diseases during this period (source: Epidemic and Pandemic Alert and Response, World Health Organization, www.who.int/csr/en). Circles indicate diseases of viral origin, stars indicate diseases of bacterial origin, and triangles indicate diseases of parasitic origin. CCHF, Crimean-Congo hemorrhagic fever; SARS-CoV, severe acute respiratory syndrome coronavirus.

Lassa fever containment requires control of the rodent vector, good surveillance, and infection control in healthcare facilities. In West Africa, surveillance has been poor and the extent of Lassa fever is unknown. However, in the 1980s an estimated >200,000 cases and 3,000–5,000 deaths occurred annually across this region (*6*). In disease-endemic areas of Sierra Leone and Liberia, Lassa fever causes an estimated 10%–16% of hospitalizations (*7*). Civil war in the Mano River Union countries (Guinea, Liberia, and Sierra Leone) in the 1990s led to >2 million displaced persons and is likely to have provided new opportunities for rodents to proliferate when persons were forced to abandon villages and relocate in overcrowded camps. However, numbers of new cases related to the conflict are unavailable. Emergence of Lassa fever in camps in non–disease-endemic areas has been documented (World Health Organization [WHO], unpub. data) and is probably related to the poor condition of dwellings and storage of grain rations in nonsecure canvas sacks, which attracts rodents.

Similarly, unsanitary environmental conditions led to the proliferation of rats in postwar Kosovo and resulted in a tularemia outbreak among the displaced population from August 1999 through April 2000, with 327 serologically confirmed cases in 21 of 29 municipalities (*8*). The population had fled their villages because of bombings, and on their return several weeks later, they found destroyed buildings, contaminated food stores and wells, and a greatly increased rodent population. Control measures included appropriate case management, improving water and waste management, health education on hygiene, and protection of food and water sources from rats.

Breakdown in Infection Control

Poor infection control practices in healthcare facilities have enabled amplification of outbreaks of viral hemorrhagic fevers (*9*). Medical settings have been the foci for several outbreaks of Ebola hemorrhagic fever (EHF) in Yambuku, DRC, in 1976, in Sudan in 1976 and 1979, in Kikwit, DRC, in 1995, and in Gulu, Uganda, in 2000 (*9*). Compared with other resource-poor settings, conflict situations, because of disrupted health services, may have even more substandard infection control, insufficient trained staff, and personal protective equipment (PPE), which make EHF containment difficult. The natural reservoir for this disease is present in countries affected by prolonged civil strife, and 11 of the 17 EHF outbreaks from 1976 through 2006 occurred in conflict-affected countries (*10*). Two of the largest outbreaks of EHF have been in conflict-affected countries, with nosocomial transmission playing a major role. The EHF outbreak in Kikwit, DRC, was the second largest to date with 315 cases and had a case-fatality rate (CFR) of 81% (*10*). Before infection control procedures were instituted in the hospital, 79 healthcare workers were infected compared with only 1 afterwards. These procedures included establishing an isolation facility; ensuring safe water, sanitation, and waste disposal; and providing PPE for staff (*11*). The Ebola outbreak in Gulu was the largest recorded to date (425 cases, CFR 53%), with nosocomial transmission being 1 of 3 mechanisms of spread (the others were attendance at burials and unsafe home care of EHF patients) (*12*).

The outbreak of Marburg hemorrhagic fever in Angola from October 2004 through July 2005 was the first outbreak in an African urban setting and the most lethal (374 cases, CFR 88%) (*9*,*13*). Thirty years of civil war had destroyed infrastructure, left roads mined, and left medical services with untrained staff and a persistent lack of supplies (*9*,*13*). Healthcare centers were primarily responsible for amplification of the outbreak through reuse of needles and syringes and use of multidose vials in healthcare centers due to poor training in safe injection practice (WHO, unpub. data).

Years of war in Sierra Leone during the 1990s weakened health systems and led to a long-term deterioration in infection control practices. As a result, a nosocomial outbreak of Lassa fever occurred in Kenema District Hospital from January through April 2004. A total of 410 cases occurred with a CFR of 30% (Ministry of Health Sierra Leone and WHO, unpub. data). The outbreak started in the pediatric ward, where nosocomial transmission likely resulted from use of contaminated multiuse vials and reuse

of contaminated needles and syringes. Children discharged into the community were readmitted with suspected Lassa fever into the Lassa ward and comprised most of the pediatric cases in this outbreak (*14*). A total of 50% of the case-patients were <15 years of age and several deaths occurred among healthcare workers (*14*). The CFR was particularly high in young children (50% in those <5 years of age [132 cases] and 71% in those <1 year of age [41 cases]). The average CFR for Lassa fever is 1% and can be as high as 15% in hospitalized patients (*15*). During outbreaks, the CFR can reach 50% among hospitalized patients (*7*).

Disruption of Disease Control Programs and Collapse of Health Systems

Malaria had virtually been eliminated in Afghanistan by the end of the 1970s after implementation of vector control programs in the 1960s and 1970s. However, with the onset of civil war in 1978, which continued almost without interruption until 1995, control programs collapsed and enabled malaria reemergence, including *P. falciparum* malaria; >50% of the population now live in malaria-endemic areas (*16*). The number of cases has been decreasing since the introduction of artemisinin-based combination therapy in the national malaria treatment protocol in 2003 (Table 1) (*17*).

There was a significant recrudescence of sleeping sickness (human African trypanosomiasis) in the 1990s, predominantly in conflict-affected Angola, DRC, and Southern Sudan. In particular, the DRC has had a dramatic resurgence of this disease as a direct consequence of conflict. In 1930, >33,000 new cases were detected; by 1958, after active case finding and treatment, this incidence decreased to ≈1,000 new cases. Control measures were interrupted in the 1990s because of conflict, which resulted in >150,000 new cases from 1989 through 1998, with 26,000 cases in 1998 (*18*). Since 1998, detection and treatment have been reinforced in Africa, and new cases have decreased substantially amid larger populations being screened in the DRC (Table 2) (*19*). However, despite intensification of control measures, all major outbreaks in 2005 occurred in conflict-affected countries (Angola, DRC, and Southern Sudan) (*20*).

Inadequate Surveillance and Early Warning and Response Systems

Surveillance systems are often weak in conflict situations, which results in delays in detection and reporting of epidemics. Limited laboratory facilities and lack of expertise in specimen collection may delay confirmation of the causative organism. Outbreak investigation and implementation of control measures may be hampered by fighting, impeded access to populations, destroyed infrastructure, limited coverage of healthcare services, poorly trained health staff, and difficult logistics that prevent delivery of drugs.

Table 1. Officially reported malaria cases in Afghanistan, 2002–2005

Year	No. cases	*Plasmodium falciparum* confirmed
2002	629,839	83,783
2003	586,602	44,243
2004	261,456	9,212
2005	281,888	5,017

An outbreak of Marburg hemorrhagic fever in Durba in northeastern DRC from October 1998 through September 2000 was the first large outbreak in rural areas under natural conditions (154 cases, CFR 83%). The area had been affected by civil war since 1997 and was controlled by Congolese rebels and Ugandan soldiers when the outbreak occurred. Although the outbreak was first reported to the national authorities in October 1998 by the chief medical officer for the health zone, an investigation was only launched after the medical officer died of this disease on April 23, 1999 (*21*). This Marburg fever outbreak was confirmed on May 6, and an international team arrived at the government's request on May 8. Given that the area was difficult to access because of security problems and poor communications and transport infrastructure, the outbreak was already decreasing by the time the international team arrived. Only 8 cases were laboratory confirmed, and 68 were identified retrospectively by the team, which left after 3 weeks (*21*). Sporadic cases continued to occur until September 2000, although data were collected retrospectively by a second international team.

Before the implementation of the Early Warning and Response Network in Southern Sudan in 1999 by WHO in collaboration with local authorities and nongovernmental organizations (NGOs), it took 6 months to respond to a relapsing fever outbreak in 1998, which resulted in >400,000 cases and >2,000 deaths. In 2000, alerts of a relapsing fever outbreak were received within 1 week and responded to by a local team; the outbreak was contained within 2 weeks, resulting in only 154 cases and 8 deaths (*22*).

Impeded Access to Populations

Ongoing conflict can hamper access to populations for timely delivery of supplies and implementation of control measures during an outbreak. Several outbreaks of pneu-

Table 2. New cases of trypanosomiasis per year, total population screened, and no. mobile teams for active case finding, Democratic Republic of the Congo

Year	New cases	Total screened	Mobile teams
1930	>33,000	3,000,000	Unknown
1958	1,218	6,000,000	250
1992	5,825	525,464	4
1998	26,318	1,472,674	33
2003	10,900	2,700,000	40

monic plague have been documented in Oriental Province in northeastern DRC, where war has hampered control efforts. Outbreaks occurred in a camp for mine workers in the Bas-Uele District (134 cases, CFR 43%) from December 2004 through March 2005 (*23*) and in the Ituri District (100 cases, CFR 19%) from May through June 2006 (*24*). In these outbreaks, achieving humanitarian access to relevant sites was difficult because of security problems, which delayed travel by response teams for investigation and implementation of control.

Access to populations to conduct vaccination campaigns may also be interrupted for months to years during protracted conflict due to long-term inadequacies in cold chain and logistics or ongoing insecurity. Low vaccine coverage has played the major role in reemergence of poliomyelitis in conflict-affected countries and has also pushed back global polio eradication targets. Conflict in Somalia since 1991 resulted in polio vaccination coverage for the required 3 polio doses being only 35% in 2005 (*25*). Somalia had been free of polio since 2002 when a large outbreak occurred in Mogadishu in 2005. By September 2006, 14 of the 19 regions in Somalia were affected with 215 cases (*26*). In May 2004, a patient infected with poliovirus was confirmed during the Darfur conflict, the first case in Sudan since 2001. By January 2005, a total of 105 cases had been confirmed in 17 of the 26 states in Sudan (*27*). Six rounds of national immunization campaigns vaccinated 8.1 million children <5 years of age in 2005, with the last case reported in June 2005. A total of 154 cases were reported in the 2004–2005 outbreak (*28*).

Interruption of routine immunization programs combined with forced migration of populations caused by conflict has also contributed to the resurgence of yellow fever in Africa (*29*). This resurgence began with the 1990 epidemic in Cameroon, then spread into conflict-affected West Africa, which since 1995 has been the most affected African region. Ten countries in Africa at risk from yellow fever have been affected by conflict, and multiple outbreaks have occurred in 6 of them: Angola (1988), Liberia (1995, 1996, 1997, 2000, 2001, and 2004), Sierra Leone (2003), Côte d'Ivoire (2000 and 2001), Guinea (2001 and 2005), and Sudan (2003 and 2005). The 2005 outbreak in Sudan resulted in a high CFR of 25% (*30*).

Development of Drug Resistance

Pathogen resistance to drugs can contribute to disease emergence. Resistance may develop more rapidly in conflict situations because of inappropriate diagnoses or inappropriate drug regimens and outdated drugs. Treatment compliance may be poor because of purchase of insufficient quantities of drugs, selling or saving of them by patients, or interrupted treatment with sudden displacement or irregular access to healthcare facilities. In addition, private pharmacies, which can flourish in conflict situations because of no regulation, can compound this problem with drugs of unknown quality and acceptance of prescriptions from unqualified prescribers.

In an outbreak of *Shigella dysenteriae* type 1 infection in a Rwandan camp for Burundian refugees fleeing civil war in 1993, <50% of patients complied with their 5-day antimicrobial drug treatment. A high attack rate of 32% was observed among 20,000 people in that camp, with a CFR of 4%. *S. dysenteriae* type 1 isolated from 3 of 7 stool samples was resistant to nalidixic acid (*31*). Refugee populations had higher anti-tuberculosis (TB) drug resistance rates than nonrefugee populations in northeastern Kenya. Drug resistance to ≥1 drug was observed in 18% of newly diagnosed sputum-positive TB patients (with multidrug resistance in 3%) in refugee populations compared with 5% (and no multidrug resistance) in nonrefugee populations (*32*). A study of patients receiving short-course therapy for TB in an active war zone in Somalia during 1994–1995 showed that although treatment completion or cure was achieved in 70% of pulmonary TB patients, 14.5% of patients defaulted treatment (*33*), which is almost double the acceptable default rate limit for TB control programs in such settings (*34*).

Movement of Refugees and Aid Workers

International spread of infectious diseases from conflict situations may occur through movement of refugees, relief workers, animals, goods, and private sector employees working in mining, oil, logging, or construction industries. A prolonged outbreak of hepatitis E virus in a camp in Darfur, Sudan, in May 2004 had >2,600 cases in 6 months, an attack rate of 3.3%, and a CFR of 1.7% (*35*). The outbreak occurred during an acute conflict in a setting with >1 million displaced persons crowded into camps with little access to safe water because of drought and inadequate sanitation. The outbreak subsequently spread into neighboring eastern Chad in June 2004 because of movement of Sudanese refugees fleeing Darfur.

Rebuilding and rehabilitation efforts in postconflict Sierra Leone have placed aid workers, United Nations peacekeeping forces, and businessmen at risk for contracting Lassa fever and enabled importation of cases to industrialized countries. Deaths from Lassa fever occurred in humanitarian workers in 2000, including United Nations peacekeepers (*36*,*37*). An imported case of Lassa fever was confirmed in Germany in July 2006, after the patient, a Sierra Leonean resident, flew from Freetown to Frankfurt through Abidjan and Brussels, 5 days after symptom onset (*36*). A businessman born in Liberia and residing in the United States died of Lassa fever in 2004 after traveling between Sierra Leone and Liberia before his illness (*38*). Aid workers and British soldiers have imported Lassa fever

into the Netherlands (2000) and the United Kingdom (2000 and 2003) after postings in Lassa-endemic areas of Sierra Leone (*36*).

There is also a hypothetical possibility that aid workers returning from a containment zone of an emerging infectious disease, such as novel pandemic influenza, may introduce the virus causing this pandemic into conflict settings. This introduction may reduce the time for preparedness, which can lead to increased illness, death, and social disruption in these already vulnerable populations.

Improving Detection and Control of Infectious Diseases in Conflict Situations

Detection and control of many emerging infectious diseases primarily require a functional healthcare system. This system involves investment in primary healthcare infrastructure, human resources, training, and provision of essential drugs, supplies, vaccines, and equipment. NGOs, United Nations agencies, and international organizations are providing crucial humanitarian assistance to many conflict-affected populations in coordination with relevant authorities.

In such settings, good hygiene and standard infection control precautions in health facilities are needed to reduce the potential for nosocomial transmission and amplification of disease. Correct guidance must be given on the rationale for infection control and use of PPE and isolation according to an assessment potential exposure and risk for infection. This guidance must be supported by ensuring a sustained supply of PPE, soap, disinfectants, sterilizing material, and single-use injection supplies so that shortages do not occur and force breaches in infection control.

It is imperative that the technical capacity of all humanitarian health partners and ministries of health regarding disease surveillance, prevention, and control in conflict-affected countries be enhanced to ensure effective implementation of infectious disease interventions. This implementation can be achieved through availability of internationally accepted standards, guidelines, and tools adapted to conflict situations, which can be supported by specific training of health planners and health facility staff, and rapid mobilization of international experts to provide technical field support as required. As in resource-poor settings, building the capacity of national staff must be an integral part of program implementation, especially in times of heightened insecurity, when staff often remain behind in areas and continue working.

Data on disease incidence and trends are essential for prioritizing risks and planning interventions and should be obtained through disease surveillance and early warning and response systems. Several of these systems have been implemented in conflict situations. These systems include those in Southern Sudan and for Kosovar refugees in Albania in 1999, in Darfur, Sudan, in 2004, and in Basrah Governorate, Iraq, in 2003, and resulted in early detection and response to outbreaks of EHF in Yambio in Southern Sudan in 2005, hepatitis E in Darfur in 2004, and cholera in Basrah in 2003.

Surveillance systems rely on close partnerships with NGOs, international organizations, and community groups and are built on resources and capacities of all organizations present. Effective surveillance systems in emergencies have involved selecting a small number of syndrome-based priority events, using standard surveillance forms, simplifying case definitions, health facilities weekly reporting of data, immediate reporting if set alert thresholds are passed, and establishing community mechanisms for identifying disease clusters.

Epidemic preparedness measures to be taken should involve training staff to use surveillance tools and manage cases of epidemic-prone diseases and equipping them with reliable means of communication. Isolation facilities and laboratories for pathogen confirmation must be identified in advance, and support must be provided to local institutions regarding training and supplying equipment and reagents. Mechanisms should be formulated for specimen transport and stockpiling of essential drugs, supplies, and outbreak investigation kits. Data should be analyzed locally and regular feedback provided (e.g., a weekly bulletin) to health partners. A rapid response mechanism for investigation alerts and implementation of control measures as outlined in outbreak preparedness plans (e.g., by an interagency outbreak control committee) are also crucial.

Revised International Health Regulations of 2005 provide a global legal framework to guide response to public health events of international concern. Conflict-affected countries represent one of the weakest links in global health security and should be prioritized by the international community in provision of technical and operational support to implement core capacities for detection and response to epidemics.

Military forces are increasingly implementing aid programs for conflict-affected populations. These programs have a crucial role and are a valuable resource. However, military aid can affect the neutrality of humanitarian aid. A consistent and transparent policy is needed for military humanitarian interventions, as well as extensive civil-military liaisons and close cooperation with other humanitarian agencies (*39*).

Given that healthcare in conflict situations is delivered by a wide range of national and international agencies, extensive collaboration between relevant health authorities and implementing partners should be encouraged. During an international response to an outbreak, coordination between partners and national authorities is usually ensured by WHO, which can also mobilize international experts

from various institutions belonging to its Global Outbreak Alert and Response Network.

Detection, containment, and control of emerging infectious diseases in conflict situations are major challenges because of multiple risk factors that promote disease transmission and hinder control even more than those in many resource-poor settings. Beyond the global public health imperative to prevent the emergence and international spread of infectious diseases, there is also a moral imperative to alleviate the effects of these diseases on already vulnerable conflict-affected populations.

Dr Gayer works for WHO in Geneva. Her research interests include infectious disease surveillance, monitoring, and control in conflict situations and after natural disasters.

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Danish Integrated Antimicrobial Resistance Monitoring and Research Program

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Resistance to antimicrobial agents is an emerging problem worldwide. Awareness of the undesirable consequences of its widespread occurrence has led to the initiation of antimicrobial agent resistance monitoring programs in several countries. In 1995, Denmark was the first country to establish a systematic and continuous monitoring program of antimicrobial drug consumption and antimicrobial agent resistance in animals, food, and humans, the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP). Monitoring of antimicrobial drug resistance and a range of research activities related to DANMAP have contributed to restrictions or bans of use of several antimicrobial agents in food animals in Denmark and other European Union countries.

In 1969, the Swann Committee recommended to the British government that antimicrobial agents used for human therapy, or antimicrobial substances that selected for resistance to these agents, should not be used for growth promotion in food animals (*1*). In 1993, the first report of nonhospital and nonhuman reservoirs of *vanA* vancomycin-resistant *Enterococcus faecium* (VREF) in the United Kingdom was published (*2*). This finding was surprising because no therapeutic glycopeptide (vancomycin or teicoplanin) had been used in food animals. However, another glycopeptide, avoparcin, had been used for decades as a feed additive for growth promotion; it was suggested that the occurrence of VREF might be related to this usage. The finding of VREF in the United Kingdom led to similar investigations and subsequent findings in Germany and Denmark in 1994 and 1995 (*3*,*4*). In 1995, one of the only antimicrobial agents available for treatment of multidrug-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) infections was vancomycin. On May 18, 1995, the Danish Minister of Agriculture and Fisheries banned the use of avoparcin nationally because new scientific evidence showed that avoparcin used as a growth promoter in food animals constituted a potential threat to human health (Article 11 of Council Directive 84/587/EEC). In July 1995, the ban became effective in all countries in the European Union (EU) after a decision by the EU Council of Ministers.

The avoparcin ban called attention to the wide array of antimicrobial substances being used in food animals for growth promotion or disease control and to the risk for transfer of other resistant bacteria or resistance genes from animals to humans through the food chain. A systematic approach was needed to generate the data necessary to determine the magnitude of current or potential future public health hazards from nonhuman use of antimicrobial agents. In September 1995, the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) was established at the initiative of the Danish Ministry of Health and the Danish Ministry of Food,

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Agriculture and Fisheries, as a coordinated national monitoring and research program. Participants in the program are Statens Serum Institut, the National Food Institute and National Veterinary Institute at the Technical University of Denmark, the Danish Veterinary and Food Administration, and the Danish Medicines Agency. DANMAP has 4 objectives: 1) monitor the consumption of antimicrobial agents for food animals and humans; 2) monitor the occurrence of antimicrobial agent resistance in bacteria isolated from food animals, food of animal origin, and humans; 3) study associations between antimicrobial agent consumption and antimicrobial agent resistance; and 4) identify routes of transmission and areas for further research.

Denmark was the first country to establish systematic and continuous monitoring of antimicrobial agent consumption and resistance in animals, food, and humans. Other antimicrobial agent resistance monitoring programs are now established in other countries: Norway (Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway [NORM/NORM-VET]) (*5*), Sweden (Swedish Veterinary Antimicrobial Resistance Monitoring [SVARM] and Report on Swedish Antibiotic Utilisation and Resistance in Human Medicine [SWEDRES])(*6*), the Netherlands (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands [MARAN] and Consumption of Antimicrobial Agents and Antimicrobial Resistance among Medically Important Bacteria in the Netherlands [NETHMAP]) (*7*,*8*), Canada (Canadian Integrated Program for Antimicrobial Resistance Surveillance [CIPARS]) (*9*), and the United States (National Antimicrobial Resistance Monitoring System. [NARMS]) (*10*).

The first results covering all 3 reservoirs from DANMAP were published in 1997 (*11*); annual reports have subsequently been published (www.danmap.org). We present selected results and experiences from 11 years of monitoring and reporting of antimicrobial agent consumption and antimicrobial agent resistance in bacteria isolated from animals, food, and humans in Denmark.

Description

DANMAP collects and presents data on consumption of antimicrobial agents and the occurrence of resistance in indicator bacteria, zoonotic bacteria, and pathogenic bacteria from animals, food, and humans. The setup for sampling of isolates and data for DANMAP are briefly described below. A schematic description of sampling of isolates and data flow is presented in Figure 1. A more detailed description can be found in the DANMAP reports (www.danmap.org).

Isolates from Animals

Bacterial isolates are collected from healthy animals at slaughter (*Escherichia coli*, enterococci, and *Campylobacter* spp.) as well as from diagnostic submissions (*Staphylococcus hyicus* from pigs and *E. coli* from cattle and pigs with diarrhea). *Salmonella* isolates from subclinical as well as clinical cases of salmonellosis are included. *Salmonella* isolates from subclinically infected pigs and poultry are collected on farms as part of a national *Salmonella* monitoring program.

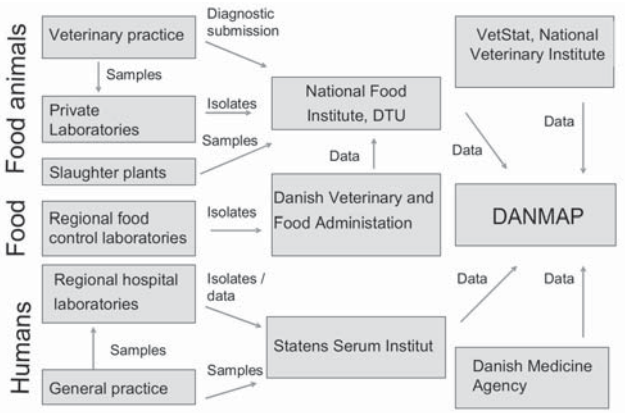

Figure 1. Data flow. DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Program; DTU, Technical University of Denmark.

Isolates from Food

All food samples are collected at wholesale and retail outlets during routine inspections by the Regional Veterinary and Food Control Authorities (*Salmonella* and *Campylobacter* spp.) or on request from DANMAP (enterococci and *E. coli*). Bacterial isolates included in the monitoring program originate from food from Denmark as well as imported food.

Isolates from Healthy Persons in the Community

To monitor the level of resistance among healthy persons, ongoing surveillance was initiated in 2002. Currently, 1 isolate each of *E. faecium*, *E. faecalis,* and *E. coli* is sampled from each fecal sample, if isolated. Furthermore, a selective method is used to detect vancomycin-resistant enterococci.

Isolates from Patients in the Community and in Hospitals

Isolates of *Salmonella* and *Campylobacter* included in the monitoring program originate from diagnostic submissions sent to Statens Serum Institut (SSI). For *S. aureus* testing, all blood isolates from 15 of 16 Danish counties and all MRSA isolates nationwide are sent to SSI. For *Streptococcus pneumoniae* testing, all isolates from blood and spinal fluid found nationwide by clinical microbiology laboratories are sent to SSI. For *E. coli,* coagulase-negative staphylococci, and *Streptococcus pyogenes*, data on

all isolates from blood samples (*E. coli*, coagulase-negative staphylococci), urine samples (*E. coli*), and clinical samples (*S. pyogenes*) submitted for susceptibility testing are provided by clinical microbiology laboratories participating in the Danish Study Group for Antimicrobial Resistance Surveillance.

Changes after 11 Years of DANMAP

Ban of Antimicrobial Growth Promoters

In 1994, the consumption of antimicrobial growth promoters constituted more than half of the total antimicrobial consumption by animals in Denmark (Figure 2). Antimicrobial growth promoters were used as an in-feed supplement for nearly all broiler chicken and pig farms in Denmark. At the time avoparcin was banned, a high level of resistance to several antimicrobial growth promoters was observed among bacteria from production animals (*11*). Although use of avoparcin was banned in 1995, the total use of growth promoters increased until 1998. In the spring of 1998, the Danish pig and poultry producers voluntarily discontinued use of all antimicrobial growth promoters in finisher pigs and broiler chickens. DANMAP measured and documented the effect of this marked change in antimicrobial consumption in animal production.

By 2006, 11 years of monitoring data were available (Table; Figure 3) (*12*). The 1995 ban on avoparcin had a substantial effect on lowering the occurrence of VREF isolated from fecal samples from broiler chickens (Figure 3). In 2005, <3% of the *E. faecium* isolates from broiler chickens were resistant to vancomycin (*12*). To avoid bias in the selection of the isolates, the VREF isolates included in DANMAP were detected by using a nonselective isolation method. Although studies that used selective enrichment for isolation of VREF have documented that VREF could still be isolated from a high percentage of poultry flocks several years after the ban of avoparcin (*17*,*18*), the quantity of VREF isolated from Danish poultry has been substantially reduced.

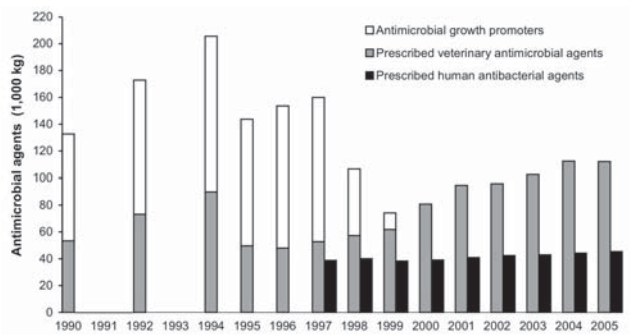

Figure 2. Consumption of prescribed antimicrobial agents and growth promoters in animal production and prescribed antibacterial agents in humans, Denmark, 1990–2005 (*12*).

In contrast, no significant change in the occurrence of VREF in pigs was observed in the first years after the ban of avoparcin. Nearly all VREF isolated from pigs in Denmark belonged to the same clone; whereas VREF isolates from broilers were polyclonal (*17*,*19*). In the pig VREF clone, genes encoding resistance to glycopeptides (*vanA*) and macrolides (*erm*[B]) were shown to be located on the same mobile DNA element (*19*). The consumption of macrolides (tylosin) for growth promotion decreased substantially in 1998, and a statistically significant decrease in the occurrence of VREF among *E. faecium* isolates from pigs was observed in 1999 and 2000, which suggests that persistence of VREF among the pig population was caused by the continued use of macrolides, mainly tylosin, for growth promotion and therapy.

Since 2002, fecal samples from 485 healthy human volunteers in Denmark have been screened for vancomycin-resistant enterococci. Only 3 VREF isolates (*12*,*20*) and 2 vancomycin-resistant *E. faecalis* isolates have been detected by using a selective isolation method (*21*).

Transfer of VREF from animals to humans can be difficult to demonstrate, especially if only the *vanA* gene encoding vancomycin resistance is transferred. Several animal studies and 1 human study have shown that gene transfer between enterococcal isolates is possible in mouse and human intestines, which indicates that gene transfer can take place in intestines of humans that have eaten meat containing enterococci (*22*,*23*). Transfer of the *vanA* gene is cause for concern if it is transferred to an *E. faecium* isolate belonging to an invasive clone, e.g., clonal complex 17, which causes *E. faecium* infections in humans (*24*).

In January 1998, another antimicrobial growth promoter, virginiamycin, was banned from use in Denmark. Resistance to virginiamycin confers cross-resistance to pristinamycin and quinupristin/dalfopristin, a new antimicrobial with a wide gram-positive spectrum including MRSA and VREF. The Danish ban on virginiamycin was based on the same concerns for human health as the ban on avoparcin. The ban on virginiamycin had an effect also on the occurrence of streptogramin-resistant *E. faecium* in broiler chickens and pigs (*12*).

In July 1999, virginiamycin, together with 3 other growth promoters—tylosin, spiramycin, and bacitracin—was banned in the entire EU. The final step in the termination of the use of antimicrobial agents for growth promotion was taken in December 2002 when the EU Council of Ministers, with a Danish president, decided that all use of antimicrobial growth promoters should be terminated within the EU starting January 1, 2006 (*25*).

VetStat Monitoring Program

In Denmark, all antimicrobial agents used in animals, except coccidiostats used in poultry, are available by pre-

Table. DANMAP's contributions to decreasing antimicrobial agent resistance in Denmark, 11 years*

Sector	Problem	Intervention (reference)	Type of intervention	Intervention had effect
Food animal	High occurrence of vancomycin-resistant *Enterococcus faecium* isolates in food and production animals	Banned avoparcin: Denmark May 1995 and EU Dec 1997. Provided data for national and EU ban. Monitored effect of ban in animals, food, and healthy humans.	Regulatory	Yes
	High occurrence of streptogramin-resistant *E. faecium* isolates in food and production animals	Banned virginiamycin: Denmark Jan 1998 and EU Jul 1999. Provided data for national and EU ban. Monitored effect of ban in animals, food, and healthy humans.	Regulatory	Yes
	High use of fluoroquinolones in animal production	Restricted use of fluoroquinolones in animal husbandry, by Danish law in 2002.	Regulatory	Yes
	High use of antimicrobial agents in swine production	Implemented new guidelines for veterinary practitioner prescription of antibacterial agents for swine production in 2005.	Guideline	Not yet known
Food	Higher levels of resistance in *Salmonella* and *Campylobacter* isolates from imported food than from Danish food	Implemented evaluation of safety of food products, by November 2006.	Regulatory	Not yet known
Human	Increasing macrolide resistance in *Streptococcus pneumoniae*	Published report to prescribers in EPI-NEWS (*13*).	Awareness campaign	Yes
	Increasing use of newer, broad-spectrum antibiotics, especially in hospitals	Published report to prescribers in EPI-NEWS (*14*).	Awareness campaign	No, use of newer, broad-spectrum antibiotics still increasing
	Higher levels of resistance in travel-associated *Salmonella* and *Campylobacter* infections	Published report to prescribers in EPI-NEWS (*14*).	Awareness campaign	Not yet known
	Increasing no. of methicillin-resistant *Staphylococcus aureus* (MRSA) cases	Published report to prescribers in EPI-NEWS (*15*). Informed national reference center, which reported to physicians and other prescribers (*15*). Made notification mandatory Nov 2006 (*16*).	Mandatory notification	Not yet known
	Increasing use of antimicrobial agents, outside and inside hospitals	Published report to prescribers in EPI-NEWS (*14*).	Awareness campaign	No, antibiotic use still increasing
	Increasing ciprofloxacin resistance related to increasing ciprofloxacin use	Published report to prescribers in EPI-NEWS (*14*).	Awareness campaign	No, ciprofloxacin use and resistance still increasing, but low compared with that of other countries

*DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Program; EU, European Union.

scription only. To collect information about veterinary use of antimicrobial agents, the Danish Medicines Agency obtained data from the pharmaceutical industry and importers during 1996–2001. These data were used in the first DANMAP reports (1996–2000). In addition, the consumption of antimicrobial agents from 1990 through 1994 was estimated by data collected from the pharmaceutical industry. However, these data did not contain information on antimicrobial agent use within the different animal species. On the basis of recommendations from the EU conference The Microbial Threat, held in Copenhagen in 1998, the Danish government decided that a monitoring system of all veterinary use of prescription medicine on a detailed level should be developed (*26*). The implementation of this monitoring program, VetStat, was initiated in 2000. VetStat data on prescription medicines used in animals are collected from pharmacies, feed mills, and veterinary practitioners. From these 3 sources, detailed data comprising farm identity, species, age group, disease group, identity of medicine, amount, date of purchase, and identity of the prescribing veterinarian are obtained for all antimicrobial agents used in production animals. Valid data by animal species and age group level were reported for the first time in the DANMAP 2001 report.

Use of Fluoroquinolones in Animal Production

In 2002, the Danish regulation restricted the use of fluoroquinolones, e.g., enrofloxacin, difloxacin, and marbofloxacin, in animal husbandry. Fluoroquinolone use is legal in food animals only when susceptibility tests performed in an approved diagnostic laboratory show that the infecting bacteria are resistant to all other antimicrobial agents registered for treatment in the animal species concerned (*27*). Subsequently, consumption of fluoroquinolones for production animals (pigs, poultry, and cattle) was reduced from 114 kg in 2001 to 18 kg in 2005 (*12*).

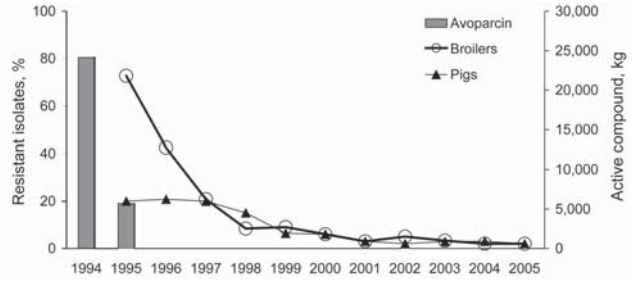

Figure 3. Trends in glycopeptide resistance among *Enterococcus faecium* from broiler chickens and pigs and the consumption of the growth promoter avoparcin in animals, Denmark, 1994–2005 (revised from *12*).

New Guidelines for Veterinary Prescription of Antimicrobial Agents for Pigs

Pigs constitute the largest volume of animals produced in Denmark. In 2005, a total of 25.7 millions pigs were produced, and pig production accounted for 82% of the total veterinary consumption of antimicrobial agents (*12*). From 2002 through 2004, antimicrobial use increased by 27%; large regional differences indicated that the increase was related to local factors (*27*). To reduce the use of antimicrobial agents and encourage specific substitutions of different agents in pig production, in 2005 the Danish Veterinary and Food Administration published a set of new guidelines for veterinary prescription of antimicrobial agents for pigs. The guidelines were developed in cooperation with relevant national institutions. They exclude quinolones and cephalosporins from the list of recommended agents. The effect of these guidelines on antimicrobial agent use in pig production cannot yet be determined.

Increased Resistance in Zoonotic Bacteria from Imported Meat

Since 1998, levels of resistance have been higher in *Salmonella* isolates from imported food than in isolates from Danish food (*28*). In 2005, the occurrence of resistance in *Salmonella* Typhimurium isolated from imported pork generally exceeded that of corresponding isolates from Denmark (*12*). A similar tendency was observed for *Campylobacter jejuni* isolated from poultry meat: imported poultry meat showed a higher resistance frequency than Danish poultry meat. The high frequency of antimicrobial agent resistance in *Salmonella* from imported pork and in *Campylobacter* spp. from imported poultry meat probably reflects differences in the use of veterinary antimicrobial agents in the countries of origin as compared with Denmark (*12*). The higher occurrence of antimicrobial agent resistance in zoonotic bacteria in imported meat products is important because a large part of meat consumed in Denmark is of foreign origin. In November 2006, case-by-case evaluation of the safety of imported meat was implemented.

In the DANMAP 2000 report, higher levels of resistance in travel-associated *Salmonella* and *Campylobacter* infections were reported for the first time (*29*). Increased ciprofloxacin resistance in isolates from infections acquired abroad was particularly significant. Similarly, *C. jejuni* isolates from human infections acquired abroad generally had a higher frequency of resistance to ciprofloxacin and tetracycline (*12*). From 2001 through 2005, the number of long holidays (>4 overnight stays) for Danish travelers increased by 14%. The most popular destinations were Spain, France, and Italy, which accounted for >30% of the long holidays abroad in 2005. Travel to the southern part of Europe might be associated with risk of acquiring infections due to resistant zoonotic pathogens because *Salmonella* and *Campylobacter* spp. isolated from meat produced in southern Europe are generally more resistant to antimicrobial agents than isolates obtained from meat produced in Denmark (*30*). Thereby, foodborne zoonotic infections acquired in southern Europe may offer limited therapeutic options. Another high-risk area for acquiring antimicrobial-resistant foodborne zoonotic infections is Asia (*31*).

Increased Human Consumption of Therapeutic Antimicrobial Agents

Since the early 1980s, data on use of antimicrobial agents for human therapy have been available from pharmaceutical industry sources or from the Danish Medicines Agency. DANMAP systematically reports these data in conjunction with resistance data, which has enabled identification of specific problems linked to antimicrobial consumption for human therapy (Table).

Increasing use of antimicrobial agents by humans, both outside and inside hospitals, was reported by DANMAP in 2000 (*29*). At the time, antimicrobial consumption by outpatients was among the lowest in Europe and was similar to that in Germany, Sweden, and Austria (*32*). Since then the increase has continued for outpatients (data not shown) and hospitalized patients. The mean antimicrobial consumption in hospitals has increased by 39%, from 421 defined daily doses (DDD)/1,000 bed-days in 1997 to 585 DDD/1,000 bed-days in 2004 (*14*). Much of this increase can be explained by increased hospital activity, i.e., an increased number of patients treated in hospitals concomitant with shorter lengths of hospital stay. Another reason for the increased consumption has been an increase in doses, based on better understanding of the pharmacokinetic and pharmacodynamic properties of antimicrobial agents. Still, parts of the increase are unaccounted for, and much remains to be understood to explain this increase in antimicrobial consumption in humans and how it may be

controlled. In addition to increased total use of antimicrobial agents, newer antimicrobial agents are being used, e.g., cephalosporins, fluoroquinolones, and carbapenems, at the expense of extended-spectrum penicillins (except pivmecillinam), aminoglycosides, and macrolides (*14*). Although use of quinolones remains lower in Denmark than in European countries (*33*), a small but statistically significant increase in the frequency of ciprofloxacin-resistant *E. coli* isolates from urine has been observed since 2003 in primary healthcare facilities and in hospitals. The increase in ciprofloxacin resistance has occurred concurrently with a recent increase in the consumption of fluoroquinolones, primarily ciprofloxacin (*14*,*34*).

Publication of MRSA Guidelines

An increase in MRSA cases was observed in 2000 (97 cases) and continued in 2001 (104 cases) (*29*,*35*). The number of MRSA cases, including infection and colonization, reached 856 in 2005 (*12*). As a response to this increase, new national guidelines for the control and prevention of MRSA were issued by the National Board of Health (*35*). The guidelines enforce use of the search-and-destroy policy in hospitals as well as in other healthcare institutions such as nursing homes. Additionally, to maintain a low colonization pressure in Denmark and thus reduce cross-transmission, all MRSA-positive persons are offered eradication treatment (*35*). The guidelines also recommend that MRSA-positive persons be given a personal "MRSA card," which must be shown at each contact with healthcare providers to ensure proper treatment and to prevent further transmission (*35*). To better monitor the new MRSA situation in Denmark and to facilitate implementation of control measures in connection with outbreaks, reporting MRSA cases has been mandatory since November 1, 2006 (*16*).

Increased Pneumococcal Resistance to Macrolides

In 2000, susceptibility testing performed on pneumococcal isolates from blood and cerebrospinal fluid sent to SSI showed that the frequency of erythromycin resistance in pneumococci slowly increased from ≈0% in 1990 to 3.4% in 1999 (*36*). This increase in macrolide resistance of pneumococci was probably related to a relative high consumption of macrolides combined with a change in the distribution of the macrolides used (*13*). Since 2000, macrolide resistance in pneumococci from blood and spinal fluid has been ≈5% (*12*).

The Danish experience shows that even if antimicrobial agent consumption is generally low and the frequency of resistance is correspondingly small, a temporary rise in consumption of even a single class of antimicrobial agent can shift this balance in an unfavorable direction. In Denmark, rational antimicrobial therapy is the tool to ensure optimum treatment of patients with bacterial infections and low levels of antimicrobial agent resistance. However, continuous training and efforts are essential to keep general practitioners as well as hospital specialists updated on the rational use of antimicrobial agents (*14*).

Possible New Areas for DANMAP Monitoring

Unlike the monitoring programs in Sweden and Norway, DANMAP never included bacteria obtained from companion animals (*5*,*6*), and rational therapy guidelines for companion animals have not been promoted or implemented in veterinary university clinics or private veterinary practices. Fluoroquinolone and cephalosporin consumption by companion animals in Denmark is substantial compared with that of food animals (*37*). Considering the shared environment of humans and companion animals, transfer of resistant bacteria or of mobile resistance determinants from companion animals to humans seems possible. Thus, emergence of resistance to fluoroquinolones and cephalosporins in companion animals should be a matter of concern and could be considered a new area for surveillance. Recently, MRSA has been detected in companion animals and in food-producing animals in other countries, the potential importance of which should also be monitored (*38*).

A substantial proportion of human *Salmonella* isolates belongs to serotypes other than *S.* Enteritidis and *S.* Typhimurium. The occurrence of more uncommon serotypes is increasing and in 2005 represented ≈30% of all human *Salmonella* isolates (*12*). Prevalence of antimicrobial agent resistance varies greatly among less frequent *Salmonella* serotypes isolated in Denmark, and specific serotypes showed a high level of resistance (*39*). These *Salmonella* serotypes, as well as other emerging or reemerging pathogens such as *Mycobacterium tuberculosis* (*40*), represent a potential focus area.

In future DANMAP reports, more attention should be given to the presence of extended-spectrum β-lactamase (ESBL)–producing *E. coli* and *Salmonella* from animals and humans. The emergence of ESBL resistance is a new threat for human therapy.

Conclusions

DANMAP has led to changes in the use of antimicrobial agents in Denmark and other countries. Until now, the effect in Denmark had been seen mostly in animals, but awareness has been raised for humans as well (Table). One of the strengths of DANMAP is cooperation between veterinary and human healthcare providers, thus offering a broad range of viewpoints and professionals. This integrated program was made possible because access to all relevant data and samples that were already systematically collected from animals, food, and humans has been shared. To complete these data, a random sampling of indicator bacteria "from farm to fork" was implemented, which has

made follow-up of antimicrobial agent resistance for zoonotic and indicator bacteria possible.

The relationship between antimicrobial agent resistance in the food supply and human foodborne infections is complex. It depends on the level of resistance of bacteria in domestic food, level of resistance in imported food, and influence of travel abroad. The need for surveillance of antimicrobial consumption and resistance in animals and humans is universal because food, humans, and even livestock travel. Solid scientific data are needed for an evidence-based debate and to facilitate further regulation regarding antimicrobial agent resistance and consumption.

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Growing Problem of Multidrug-Resistant Enteric Pathogens in Africa

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Control of fecal–orally transmitted pathogens is inadequate in many developing countries, in particular, in sub-Saharan Africa. Acquired resistance to antimicrobial drugs is becoming more prevalent among *Vibrio cholerae*, *Salmonella enteritidis*, diarrheagenic *Escherichia coli,* and other pathogens in this region. The poor, who experience most of the infections caused by these organisms, bear the brunt of extended illness and exacerbated proportion of deaths brought about by resistance. Improved antimicrobial drug stewardship is an often cited, but inadequately implemented, intervention for resistance control. Resistance containment also requires improvements in infectious disease control, access to and quality assurance of antimicrobial agents, as well as diagnostic facilities. Structural improvements along these lines will also enhance disease prevention and control as well as rational antimicrobial drug use. Additionally, more research is needed to identify low-cost, high-impact interventions for resistance control.

The adverse effects of infectious diseases in many developing countries is considerable and, within those countries, economically disadvantaged persons are most likely to contract communicable diseases and least likely to access appropriate treatment (*1*,*2*). Many bacterial and parasitic diseases could, until recently, be treated with inexpensive antimicrobial agents, but treatment has recently been made more expensive and less successful by the emergence and spread of resistant organisms. Drug resistance is a large and growing problem in infections that account for most of Africa's disease burden, including malaria, tuberculosis (TB), HIV infection, and respiratory and diarrheal diseases. The proportion of malaria infections resulting in death has increased in Africa, largely due to resistance, and the cost of effective antimalarial agents is higher than the health budgets of malaria-endemic countries can accommodate (*3*). Similarly, a recent outbreak of extensively drug-resistant TB in rural South Africa illustrated that resistant organisms pose an enormous and costly threat to HIV-infected persons and their HIV-negative contacts (*4*).

Much of the current discourse on infectious disease and drug resistance as it affects sub-Saharan Africa is limited to the pressing problems associated with HIV, TB, and malaria. Resistance, however, equally compromises the management of acute respiratory infections, sexually transmitted diseases, and diseases spread by the fecal–oral route, such as typhoid fever, cholera, dysentery, and other diarrheal diseases, which are the focus of this perspective. Moreover, young children are especially likely to acquire resistant enteric infections, from which they can experience less obvious, but long-term adverse effects.

Increased Antimicrobial Drug Resistance in Enteric Bacteria

Cholera toxin–producing *Vibrio cholerae* cause the characteristic life-threatening gastroenteritis, cholera. At least 7 pandemics of the disease, originally designated "Asiatic cholera," have occurred in recent history. The ongoing pandemic has seen the emergence of O139 *V. cholerae*, as a non-O1 pandemic strain and, importantly, the emergence and spread of drug-resistant O1 strains. The current focus of the cholera pandemic is Africa, which has

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seen two thirds of all cholera outbreaks in the last decade (*5*). The primary treatment for cholera is rehydration. Most patients will overcome the infection if they are rehydrated promptly and properly, even if they do not receive antimicrobial drugs. Antimicrobial drugs, however, shorten the course of infection and prevent person-to-person transmission, which may be crucial for slowing outbreaks because organisms from infected persons may be more virulent than those acquired in the wild (*6*). Antimicrobial agents may also be life-saving for malnourished and other immunocompromised patients who have cholera.

Tetracycline was the empiric drug of choice for cholera in Africa and elsewhere for many years. At the end of the 1970s, however, incompatibility group C tetracycline-resistant plasmids were isolated from *V. cholerae* isolates in Tanzania, Kenya, and other parts of Africa (*7*,*8*). In each case, resistance emerged during an ongoing epidemic where tetracycline was being used intensively for prophylaxis as well as treatment. Tetracycline has sequentially been replaced by trimethoprim-sulfamethoxazole and, more recently, quinolones, because of the emergence and spread of resistant strains. Molecular evaluation of more recent resistant *V. cholerae* isolates typically found regionally conserved plasmids, some of which carried class 1 integrons bearing multiple resistance cassettes (*9*,*10*). A chromosomally integrated transferable resistance element, SXT, has also spread worldwide and has been recently reported from Africa (*9*).

Emergence of resistance in *V. cholerae* has been linked to increased mortality rates in recent African outbreaks. Similar experiences have also been reported with *Shigella dysenteriae* type 1, another enteric pathogen that causes life-threatening disease and has epidemic potential. The impact of resistance in both pathogens is illustrated by an overwhelming outbreak in July 1994 at the Goma camp, which resulted in the deaths of ≈12,000 Rwandan refugees (*11*). More recently, Dalsgaard et al. (*12*) observed a marked increase in case-fatality rate during the 1997–1998 phase of a Guinea-Bissau cholera outbreak, compared to an overlapping 1996–1997 outbreak. A major feature of the latter wave of cholera was the presence of strains simultaneously resistant to ampicillin, erythromycin, tetracycline, furazolidone, aminoglycosides, trimethoprim, and sulfamethoxazole. These multidrug-resistant strains were not present during the first wave and probably arose following the acquisition of a 150-kb–pair resistance plasmid bearing a class 1 integron and genes encoding resistance to all antimicrobial agents commonly used in empiric management of cholera.

The emergence and spread of multidrug-resistant *Salmonella enterica* subsp. Typhi worldwide has had important consequences for mortality rates from typhoid fever (*13*). There are very few reports from Africa; nonetheless, available data suggest that although the problem may not be as intense as in other parts of the world, resistance has emerged, and alternatives to current treatment protocols are often not available or unaffordable. Multidrug-resistant nontyphoidal *Salmonella* spp. (NTS) have emerged as a global public health threat. In industrialized countries, they are most commonly associated with foodborne gastroenteritis. In parts of sub-Saharan Africa, however, NTS are important causes of life-threatening bacteremia. Studies from Kenya have found that community-acquired NTS are among the top 3 causes of death among children <5 years of age (*14*,*15*). Moreover, pulsed-field gel electrophoresis data suggest that most life-threatening disease is caused by isolates that are clonal in origin (*14*). In a recent study, children from poor slums of Kenya were significantly more likely to be infected with multidrug-resistant NTS than were children from middle-income families (*14*). The patterns of resistance among these strains suggest that third-generation cephalosporins should be the drug of choice for empiric management of these infections, but in most cases, these drugs are too expensive.

Antimicrobial drug resistance is a large and growing problem among organisms that cause diarrheal disease. Although most diarrheal diseases are self-resolving and should not be treated with antimicrobial agents, invasive or protracted infections require chemotherapy and are typically managed empirically. Recent data from Gabon, Nigeria, and Tanzania suggest that resistance among causative organisms of these infections, such as enterotoxigenic, enteropathogenic, and enteroaggregative *Escherichia coli,* is high and appears to be rising (*16*–*18*). Although oral rehydration therapy has drastically reduced deaths from the disease, prolonged infectious bouts of diarrhea have long-term consequences for physical and cognitive development. Very few reports have examined the epidemiology of diarrheal pathogens and even fewer have looked at drug resistance. Notable drug-resistant enteropathogenic *E. coli* outbreaks and sporadic cases have been reported from several African countries, including Kenya and Tanzania (*16*,*19*). The more recently defined enteroaggregative *E. coli* are typically multidrug-resistant and are one of the most common causes of childhood diarrhea, particularly persistent infections (*20*). Antimicrobial drug–resistant diarrheagenic *E. coli* pathotypes, including enteroaggregative *E. coli*, are also emerging as important diarrheal pathogens in AIDS patients (*21*).

Surveillance in healthy populations has demonstrated that commensals constitute a rich reservoir of genetic material from which pathogens can readily acquire resistance on mobile elements. A long-term study in Nigeria showed that resistance of commensal *E. coli* to almost all agents studied increased rapidly over time (*22*). Additionally, urban residents in Nigeria, Ghana, and Zimbabwe were more likely

to carry multidrug-resistant *E. coli* than were rural or provincial residents (*23*,*24*). This finding has important consequences in light of the rapid rate of urbanization in these countries and other parts of the continent. Travel networks have become more efficient and are more extensively used. Therefore, just as Africa has had to deal with imported resistant organisms, resistant strains that emerge or are amplified in Africa will be exported (*25*,*26*).

Two overlapping problems are worsening the situation regarding diarrheal disease within Africa: the failure to control the spread of diarrheal pathogens, due to unclean water, poor sanitation, and malnutrition; and the failure to contain resistant organisms and resistant genes so that, when infections occur, they produce more adverse consequences. It is perhaps obvious, if unaddressed, that poor and displaced persons in Africa are least likely to be able to access potable water, safe sanitation, and other factors to prevent fecal–oral infection and that public health facilities need to be strengthened to protect the poor (*27*,*28*). However, the poor also disproportionately bear consequences from drug resistance, and interventions to curb the current trend are sorely needed. Economists describe a situation in which the decision to use a commodity that produces a deleterious byproduct that imposes costs on other persons, who are not part of the decision, as a negative externality. Because resistance is an externality, the poor are victims of the resistance-predisposing activities of the affluent, including the presence of visitors from other countries. Furthermore, resistance-promoting activities and the consequences of resistance more often than not occur at a different time and place. It is therefore unrealistic to expect that the poor will mount resistance-curbing interventions as a priority without prompting and support.

Overcoming Roadblocks to Containing Drug Resistance in Africa

There are difficulties associated with monitoring resistance in many parts of Africa, but sufficient published data exist to suggest that resistance rates are high and rising (*22*,*29*). In 2001, the World Health Organization issued a strategy for resistance containment (*30*). Most developing countries, particularly those in sub-Saharan Africa, have yet to implement any of the recommended interventions in spite of a mounting resistance crisis. Special risk factors for resistance, and roadblocks for evaluating and implementing interventions, are linked to patient poverty and health system poverty. This is evident in sub-Saharan Africa, where we study resistance, but also in other developing countries.

Industrialized countries that are actively addressing community-acquired resistance have typically prioritized those interventions predicted to lower total antimicrobial agent consumption and therefore selective pressure (*31*,*32*). An important concern for poor countries is not the total amount of antimicrobial agents consumed, whose need exceeds the resources available, but the way these drugs are used. Diagnostic imprecision spurs overprescribing, particularly of broad-spectrum agents, and low antimicrobial diversity promotes emergence and spread of potentially epidemic resistant clones. In Africa, medicines are available from unorthodox sources and persons earning low daily wages often procure them 1 dose at a time. Sources include itinerant vendors, often encountered on buses in many African countries, who cannot always be located should a patient choose to purchase more doses later (Figure). Poor patients have little personal incentive to purchase more medicine than is needed to produce short-term relief. Additionally, regimen fragmentation comes without sanction and allows for large price mark-ups and consequent exploitation. Therefore, distributors have every incentive to encourage antimicrobial drug misuse.

Quality Assurance

The relative scarcity of antimicrobial drugs in poor countries with a high prevalence of infectious diseases means that the demand for antimicrobial agents exceeds their supply. This imbalance, coupled with poor purchasing power, makes sub-Saharan Africa and other developing regions a counterfeiters' paradise. Substandard products with lower-than-stated doses promote resistance, and those containing no antimicrobial drug at all promote microbial dissemination. At least 30% of medicines sold in Africa are estimated to be counterfeit, with antimicrobial agents the most popular target (*33*). However, fake drugs are not the only poor quality pharmaceuticals on the market (*34*). Substandard drugs also include medicines that were appropriately manufactured but improperly stored. Proper storage in the tropics requires expensive electrical equipment, a constant electricity supply, pharmaceutical handling expertise, and an efficient supply chain, which do not exist in many parts of Africa. It has long been known that antibiotics are unstable at ambient tropical conditions, but shelf lives and packaging are not adapted to preserve drug potency or mark their degradation in countries where these drugs are most needed.

Antimicrobial Drug Supply and Distribution

Prescribing health workers and their patients, particularly those who are poor, in sub-Saharan Africa continually battle a "drug is out of stock" syndrome. Rational antimicrobial drug policies, essential decision-support tools in the battle against resistance, are impossible to develop or to implement without an ensured supply of a reasonable range of drugs. Antimicrobial cycling has been piloted for antimalarial drugs in some parts of Africa, but more data are needed to gauge its effectiveness. Instituting and expanding

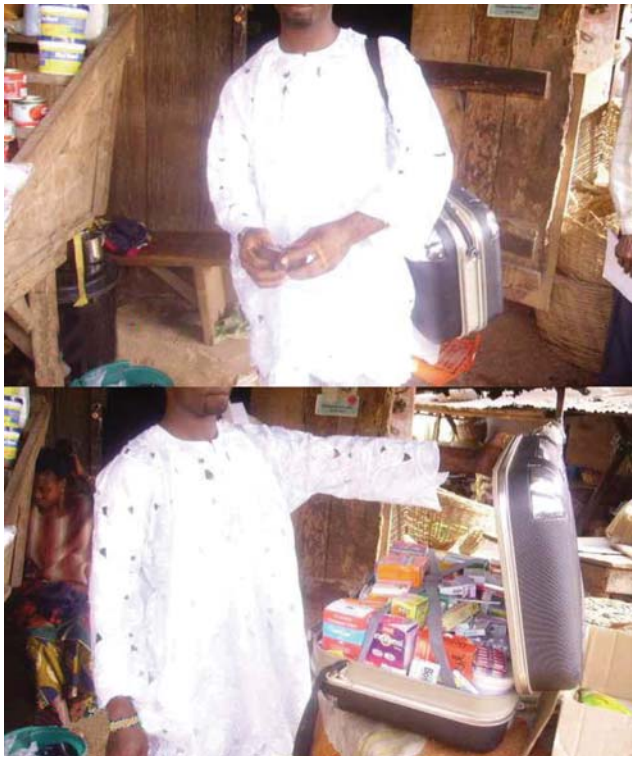

Figure. Itinerant medicine vendor in Oja-tuntun marketplace, Ile-Ife, Nigeria.

pilot programs have been hampered by drug supply issues, particularly the difficulty in removing cheaper, resistance-compromised drugs, which are all the poor can afford, from the market (*35*). As long as demand for antimicrobial drugs exceeds their supply, uncontrolled and inadequate regimens will be the norm. To combat resistance in poor countries, antimicrobial agents will have to be made more available. For best results, when improved supply increases selective pressure, drug misuse and resistant-strain dissemination must decline. Thus, antimicrobial drugs need to be made available along with the infrastructure to monitor their utility and improve selection. Special considerations are needed to encourage patients to procure and consume a complete regimen and to ensure antimicrobial drug quality as close to the point of care as possible. These are formidable challenges because antimicrobial agents are available from unsanctioned, as well as sanctioned, providers and the former may have little or no training and unorthodox means of drug distribution.

Unrestricted access to antimicrobial drugs is perhaps the most favorable acknowledged predisposing situation for development of resistance. Ideas for promoting supervised, or at least informed, access, however, need to advance beyond proposing improved legislation because most countries already have laws proscribing unsanctioned distribution. Illegal peddling is a function of inadequate law enforcement and the reality that drug selling provides a livelihood for otherwise underemployed persons. Notably, unofficial outlets are the only source of life-saving medications for many rural residents in locations where enforcing laws modeled on wealthy societies could do more harm than good. Until the infrastructure to effectively abolish unsanctioned drug distribution is available, incorporating informal distributors into a containment strategy may be the wisest option. Focusing interventions predominantly on formal health delivery systems enhances the quality of care available to the wealthy but neglects the supply chain on which the less privileged majority are largely dependent.

Enhanced Infectious Disease Control

The poverty–resistance cycle operates within a larger cycle of poverty and disease. Selective pressure for resistance is, in almost all cases, a response to actual or supposed infection, and resistant bacteria are largely spread through the same routes as pathogens. One of the most effective means of conserving antimicrobial drugs is therefore preventing infections. The effects of enteric infections in Africa are almost entirely driven by poor access to safe water and sanitation. Thus, once resistant pathogens emerge, they are easily spread. The commensal reservoir of resistance genes spreads through the same channels, undetected but providing a ready source of resistance genes and elements that can be transmitted to pathogens. Middle-class and affluent Africans typically reside in the few areas where piped water is available or have private water supplies. Providing safe water and sanitation to those who cannot afford these capital-intensive options and to public institutions such as schools, health centers, and markets is the single most important intervention for preventing outbreaks and sporadic cases of diarrheal disease, including those caused by resistant organisms.

Interventions that affect disease prevalence attack resistance at the root of the problem and therefore have the greatest chance of success. Effective implementations of vaccination and drug use policies, such as the World Health Organization's Integrated Management of Childhood Illnesses, represent examples that address community-acquired infections that disproportionately affect economically disadvantaged populations (*29*). Unfortunately, even though the effectiveness of some of these interventions has been demonstrated, their access by poor populations is often not assured (*36*).

Malnourished or otherwise immunocompromised patients are more likely to have inadequate economic resources, and they become the target of resistant pathogens when these organisms are prevalent (*37*). Patients infected with resistant strains pay more for cure, lose more from extended illness (in terms of time away from work and other

activities, i.e., productivity, costs of supportive therapy), and are more likely to be disabled. In wealthy countries, hospitals are often the sites where resistance emerges, and then it slowly, but eventually, seeps into the community. In poor countries, often no barrier exists between the hospital and the community. A patient's relatives, who must be on hand to assist overstretched health systems with care, sleep under beds and in hospital corridors. In hospitals, costly infection control measures are often compromised. The potential for organisms to be transmitted into, within, and beyond the hospital is very high.

Diagnostic Development

Diagnostic development represents a potentially powerful strategy to simultaneously improve healthcare delivery and contain resistance (*38*,*39*). The cause of bloodstream and enteric infections has diversified considerably in recent years, in part due to the definition of previously unrecognized etiologic agents but also due to the spread of HIV and the emergence of new pathogens. This increased diversity makes syndromic diagnosis of many conditions less accurate, particularly in areas where surveillance does not occur. Better systems are needed to provide laboratory support for serious cases, outbreaks, and routine surveillance. As disease control efforts begin to yield fruit, syndromic diagnosis will become increasingly inaccurate and laboratory diagnosis even more essential.

If diagnostic tests for poor patients are subsidized as well as, or better than, medicines, health professionals and patients would be more likely to use them. Additionally, sentinel laboratory facilities will also provide data on local causes of infections and prevailing susceptibility patterns to inform local prescribing and alleviate the prevailing poverty of information. Currently, studies that claim a global or worldwide coverage often exclude Africa so that as in the case of poor populations, the plight of poor countries, particularly as relevant to resistance, is underdocumented (*40*). Although programs outside Africa collate decades worth of susceptibility data, only 1 or 2 tertiary-level care centers in Nigeria and Ghana can produce complete records of susceptibility data from the past 5 years. Many African laboratories that perform susceptibility testing often cannot collate, store, or disseminate surveillance data, even though open source software is available for the purpose (*41*). Much of the data they do generate is produced with antibiotic disks donated by pharmaceutical companies with a repertoire that does not necessarily reflect the best choices for patients or even available stock. Diagnostic development could also help alleviate the pressing need for antimicrobial drug quality assurance. Precise assessment of drug content is beyond the capabilities of a basic microbiology laboratory, but rudimentary diagnostic laboratories can be equipped to identify outright counterfeits and severely degraded antimicrobial agents. Finally, the weak laboratory capacity makes it difficult to evaluate the success of interventions targeting resistance or other aspects of global disease control. Diagnostic microbiology laboratories should be considered an integral part of healthcare delivery in parts of the world where most patients visiting a health center have a microbial infection.

Conclusion

Resistance is encountered with virtually every infectious disease. Few proven mechanisms exist for resistance control, and almost none have been validated in the developing-country setting. The dearth of intervention study data is particularly acute in the context where infectious disease prevalence is high and access to antimicrobial agents is low, which best describes the situation faced by low-income persons in Africa. Most intervention studies in developing countries have focused on relatively inexpensive and easily piloted educational interventions. Educational interventions push against the strong influence of unregulated distribution, sometimes accompanied by unscrupulous counteradvertising, and their value has not been evaluated in the long run. Importantly, although educational interventions typically yield positive results, these results are modest (*31*). Other methods may in fact be more cost effective, or might boost the value of education. Strategies that have been evaluated and found to deal with the problem of resistance need to be further diversified in poor countries. Safe water and sanitation, addressing the imbalance between antimicrobial drug supply and demand, and building realistic infrastructure for rational antimicrobial use are priority areas for resistance control that could address the short- and long-term disease effects on the poor.

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Literature Review and Case Histories of *Histoplasma capsulatum* var. *duboisii* Infections in HIV-infected Patients

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African histoplasmosis caused by *Histoplasma capsulatum* var. *duboisii* is an invasive fungal infection endemic in central and west Africa. Most of its ecology and pathogenesis remain unknown. *H. capsulatum* var. *capsulatum* is an AIDS-defining opportunistic infection in HIV-infected patients who are living in or have traveled to histoplasmosis-endemic areas. In contrast, reports concerning African histoplasmosis during HIV infection are rare, although both pathogens coexist in those regions. We report 3 cases of imported African histoplasmosis diagnosed in France in HIV-infected patients and a literature review on similar cases.

Human histoplasmosis is caused by 2 varieties of *Histoplasma*. The most common variety worldwide is *H. capsulatum* var. *capsulatum*, which has been reported from many disease-endemic areas where HIV infection is prevalent. Histoplasmosis is more frequent in the United States (Ohio and Mississippi River valleys), but it is not unusual in other parts of the world, such as Africa (*1*,*2*). In the western and central regions of sub-Saharian Africa, *H. capsulatum* var. *capulatum* coexists with another variety, *H. capsulatum* var. *duboisii*, whose ecology and pathogenesis remain almost unknown. Cases due to *H. capsulatum* var. *duboisii* are scarce in Europe, and all are imported (*3*).

Before the era of highly-active antiretroviral therapy (HAART), the prevalence of *H. capsulatum* var. *capsulatum* infections reached up to 30% of HIV-infected patients in hyperendemic areas of the southeastern part of the United States (*4*). The infection occurs more often in patients with a CD4 count <50/mm^3 and is usually disseminated. For unknown reasons, although HIV infection and *H. capsulatum* var. *duboisii* coexist in Africa, this coinfection remains rare (*5*). We report 3 imported cases of the potentially emerging histoplasmosis due to *H. capsulatum* var. *duboisii* diagnosed in France during the course of HIV infection and a literature review on similar cases.

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Case 1

A 37-year-old man from the Democratic Republic of Congo, who had lived in France since 1980, was infected by HIV-1 since 1987. He was admitted to the hospital in 1992 because of a fever of unknown origin. His physical examination showed a left axillary tumefaction 2 inches in diameter. This mass had already been explored 5 months before. At that time, histopathologic examination disclosed a necrotizing lymphadenitis with epithelioid cells but without caseum. No microorganism was seen after Ziehl, periodic acid–Schiff, and Grocott stainings, but culture was not performed. When the patient was hospitalized in 1992, laboratory tests showed an erythrocyte sedimentation rate of 104 mm, fibrin 4.5 g/L, C-reactive protein 74 mg/L, and a CD4 count of 100/mm^3 (9%). The adenopathy was surgically removed. Histopathologic examination showed necrosis and large yeasts, and culture grew *Histoplasma* sp. on day 12. Anti-*Histoplasma* antibody detection was negative. No other lymph node, bone, skin, or bone marrow involvement was found.

Itraconazole treatment was started (400 mg/d), but it was switched to amphotericin B (1 mg/kg/d) after 3 weeks because local symptoms persisted. The total dose of amphotericin B was 1,200 mg. Itraconazole (400 mg/d) was then restarted for 1 year. The clinical course was satisfactory, so itraconazole was lowered to 200 mg/d for 3 years. The patient died in 1995 of HIV-related encephalitis despite antiretroviral therapy, including nucleoside reverse transcriptase inhibitors, without recurrence of histoplasmosis.

Case 2

A 41-year-old man from the Democratic Republic of Congo, who had lived in France since 1981, was infected by HIV-1 in 1994. He received AZT (3′-azido-3′-deoxythymidine) and diethyldithiocarbamate (ddC) when *Pneumocystis jiroveci* pneumonia was diagnosed in December 1995. Despite the introduction of HAART in 1996 (AZT, lamivudine [3TC], and ritonavir), his CD4 count remained <50/mm^3. In mid-1996, nodular cutaneous lesions, a right cervical adenopathy, and a right Bell's palsy developed. Direct examination of the lymph node showed numerous yeasts with a typical lemon shape and a narrow budding, suggestive of *H. capsulatum var. duboisii*. The culture grew *Histoplasma* sp.

The patient did not respond to itraconazole (400 mg/d). After 1 month, he was given conventional amphotericin B (1 mg/kg/d); severe renal insufficiency developed within 8 days. Treatment was switched to liposomal amphotericin B (3 mg/kg/d) with a dramatic improvement of the symptoms and partial regression of the renal insufficiency. Immune reconstitution inflammatory syndrome also developed; its characteristics were reported previously (*6*). After 1 month, treatment was switched to itraconazole, 400 mg/d, for long-term therapy. Because of a persistent low CD4 count despite undetectable viral load, the patient benefited from several courses of interleukin-2 (IL-2) therapy, which allowed a marked and sustained increase of the CD4 count. Itraconazole was stopped in 1996. His condition remains stable 11 years later, and no recurrence of histoplasmosis has been observed.

Case 3

A 2-year-old girl from the Democratic Republic of Congo was referred to the hospital in June 2001 for a fever of unknown origin. (She arrived in France in 2001 at the age of 18 months.) Investigations showed *Escherichia coli* pyelonephritis. HIV-1 serologic test results were positive, and her CD4 count was 45/mm^3. HAART was started quickly, combining AZT, 3TC, and nelfinavir. This treatment resulted in a decrease in, but not elimination of, the viral load and a CD4 count <200/mm^3 despite appropriate nelfinavir serum concentrations.

In August 2001, a frontal swelling appeared, associated with fever and generalized weakness. Direct examination of a skin biopsy specimen showed large, lemon-shaped yeasts suggestive of *H. capsulatum* var. *duboisii.* Culture of this specimen grew *Histoplasma* sp. Diffuse bone involvement (several lytic lesions of the right humerus, left ulna, both tibias, right fibula) was found on radiographs. Culture of the buffy coat was concomitantly positive for *Fusarium verticillioides.* No involvement of the lungs or lymph nodes was found.

Treatment with liposomal amphotericin B was started but switched to itraconazole after 1 month. Fever relapsed shortly thereafter, as well as the facial tumefaction. Radiographic examination showed several lesions of the skull, and a bone biopsy demonstrated large yeasts on direct examination. Amphotericin B was restarted for 4 months. The patient's status improved dramatically, and the treatment was switched to fluconazole until September 2003. She did not experience any relapse, and antifungal prophylaxis was discontinued because of the improvement of her immunologic status (CD4 count >200/mm^3 and undetectable viral load). In July 2007, she is doing well with a CD4 count of 700/mm^3 and a still-undetectable viral load.

Discussion

H. capsulatum var. *duboisii* is also known as African histoplasmosis because it has only been described on that continent, mostly in central and western Africa. The prevalence of histoplasmosis due to variety *duboisii* has not been established in countries in these regions in HIV-negative patients. Fewer than 300 cases are reported in the literature (*7*). The reason it remains rare, despite the major HIV pandemic in Africa, is unknown. Potential explanations are that patients die from other causes before histoplasmosis develops (*8*) or that variety *capsulatum* is more virulent than variety *duboisii*. This situation is reminiscent of *Cryptococcus gattii* and *C. neoformans*. *C. gattii* is rarely identified in HIV-infected patients, in contrast with *C. neoformans,* whereas both are present in the environment in countries where the prevalence of HIV infection is high (*9*). However, variety *capsulatum* is frequent in Africa. No data on the relative frequency of those 2 varieties has been published. Skin reaction to histoplasmin in histoplasmosis-endemic areas showed a 3% prevalence (*10*), but variety *capsulatum* and variety *duboisii* were not able to be differentiated. Higher prevalence (≈35%) was found in rural populations, especially among farmers, traders, and cave guides (*11*). Histoplasmosis due to variety *duboisii* may be misdiagnosed in those areas because of physicians' lack of awareness.

The pathogenesis of African histoplasmosis remains unclear. The main route of acquisition could be airborne contamination from the soil, rarely direct inoculation. Variety *duboisii* is classically associated with cutaneous lesions

(nodules, ulcers) and osteolytic bone lesions, especially affecting the skull, ribs, and vertebrae (Table 1) (*12*,*13*). Histopathologic examination shows granuloma with necrosis and suppuration. Disseminated disease is not uncommon and can involve every organ; however, the heart and central nervous system are unusual locations. A total of 17 cases have been reported thus far among HIV-infected patients, including the 3 cases described here (*14*–*19*). An additional case has been reported, but without detailed description, in a Ugandan patient diagnosed in Japan (*20*). Among the well-described cases (Table 1), most involved patients with poor immunologic status (mean CD4 count 55/mm^3), which also occurs with histoplasmosis due to variety *capsulatum* (*21*). Most patients had disseminated infections, and only 4 patients died. The prognosis of disseminated infection in this context is close to the 20% mortality rate reported for disseminated histoplasmosis due to variety *capsulatum* among AIDS patients (*21*), but the few number of cases does not allow us to extrapolate the mortality rate related to variety *duboisii.* Epidemiologic information, clinical manifestations, and outcomes of immunocompetent versus HIV-infected patients infected with variety *duboisii* are compared in Table 2 (*13*). These data confirm the tropism of variety *duboisii* for lymph nodes, skin, and bones. It is noteworthy that the disease is often located in the lungs in HIV-negative patients, whereas HIV-infected patients have

Table 1. Description of HIV-infected patients with histoplasmosis due to *Histoplasma capsulatum* var. *duboisii**

Case no.†	Age, y	Sex	Country	Clinical findings	CD4 count/mm^3	Pathology	Positive fungal culture	Treatment	Outcome
1	20	F	Congo	Skin lesions	NR	Skin	–	AmB 1 mg/kg/d, Itr 300 mg/d	Relapse
2	44	M	Congo	Skin lesions, weight loss, lymph nodes, peritonitis	NR	Skin, pus	–	Ketoconazole 600 mg/d, AmB, Itr 300 mg/d	Relapse
3	41	M	Congo	Skin lesions, weight loss, lymph nodes, hepatomegaly, splenomegaly	NR	Skin	–	AmB	Death
4	65	M	DRC	Fever, weight loss, anemia	NR	Bone marrow	Bone marrow, blood	AmB	Death
5	28	M	DRC	Fever, skin lesions, lymph nodes, weight loss, bone lesions	NR	Skin	Skin	Ketoconazole 600 mg/d	NR
6	31	F	Cameroon	Septic shock	2	Bone marrow	Bone marrow, blood	ABLC 5mg/kg/d, Itr 400 mg/d	No relapse
7	29	M	Liberia	Skin lesions	NR	Skin	Skin	Itr 200 mg/d	NR
8	43	F	Guinea-Bissau	Fever, weight loss, anemia, abdominal pain	68	Colon	–	Itr 400 mg/d	No relapse
9	30	M	Nigeria	Fever, skin lesions, lymph nodes, anemia	2	Skin	Skin	AmB 1 mg/kg/d, Itr 400 mg/d	Relapse
10	38	M	DRC	Fever, weight loss, lymph nodes	160	Lymph nodes	Bone marrow, lymph nodes	AmB	No relapse
11	26	M	Congo	Fever, skin lesions, lymph nodes	NR	Lymph nodes	–	AmB 1 mg/kg/ 48 h	No relapse
12	30	M	Côte d'Ivoire	Fever, weight loss, lymph nodes	6	Bone marrow	–	Itr 400 mg/d	No relapse
13	50	F	Nigeria	Skin lesions, bone lesions	NR	Skin, bone	–	Fluconazole 100 mg/d	No relapse
14	45	M	Ghana	Fever, weight loss, splenomegaly	24	Blood	–	AmB 0.7 mg/kg/d	Death
15	37	M	DRC	Fever, lymph nodes	100	Lymph nodes	–	Itr 400 mg/d	Death
16	41	M	DRC	Lymph nodes, skin lesions	50	Lymph nodes	Lymph nodes	Liposomal AmB, Itr 400 mg/d	No relapse
17	2	F	DRC	Fever, skin lesions, bone lesions	45	Skin, bone	Skin	Liposomal AmB, fluconazole	No relapse

*NR, not reported; AmB, amphotericin B deoxycholate; Itr, itraconazole; DRC, Democratic Republic of Congo; ABLC, amphotericin B lipid complex.
†Cases 1–14 are from the literature review; cases 15–17 are personal cases; see text.

Table 2. Comparison of clinical and microbiologic findings of HIV-infected and immunocompetent patients with histoplasmosis due to variety *duboisii**†

Characteristic	HIV positive (n = 17)	HIV negative (n = 20)
Age, y (range)	35 (2–65)	34 (8–62)
Sex (M:F)	12:5	19:1
Visceral localizations		
Lymph nodes	53	65
Skin	59	40
Bones	18	25
Lungs	0†	35†
Gastrointestinal	12	5
Disseminated	85†	55†
Clinical manifestations		
Fever	58†	15†
Weight loss, asthenia, anorexia	54	30
Respiratory symptoms	0	20
Hepatosplenomegaly	12	15
Diagnosis sites		
Lymph nodes	24	45
Skin	48	35
Bone marrow	18	0
Bone	12	5
Gastrointestinal	6	5
Pus	6	25
Lung	0†	25†
Mycologic diagnosis		
Direct examination	100	40
Culture	64	65
Blood culture	12	0
Treatment		
Amphotericin B	66	80
Ketoconazole	12	35
Itraconazole	64	20
Fluconazole	12	0
Outcome		
Relapse	12	40
Death	24	5

*Except where indicated, all values are percentages. HIV-negative patients are from Dupont et al. (*13*).
†p<0.05.

substantially more disseminated disease. The latter finding may be explained by immunodepression, poor access to the healthcare system for HIV-infected persons in Africa, and late diagnoses of histoplasmosis.

Despite its rarity, African histoplasmosis should be kept in mind as a diagnosis in Africa-born patients or travelers to sub-Saharan West and central Africa who have compatible signs or symptoms, even if they are HIV-infected, because the saprophytic phase of this dimorphic fungus should be manipulated in a Biosafety Level 3 cabinet. The laboratory diagnosis is performed by direct examination and culture. Cultures of tissue samples or body fluids are made onto Sabouraud dextrose agar, incubated at 25°C; incubation could be prolonged for up to 6 weeks. The success rate depends on the extent of infection, the source of the sample, and the prompt processing of the sample.

In addition to differences in clinical manifestations and epidemiology, the 2 varieties can be easily distinguished on observation of the yeast phases present in infected fresh or fixed tissues, whereas the saprophytic phase is identical. Variety *capsulatum* presents as small (3-μm) oval yeasts free or inside histocytes or macrophages (Figure 1), whereas yeasts of variety *duboisii* are large (7–15 μm), globose to ovoid, thick-walled, and typically lemon-shaped with a narrow budding (Figure 2). They are often seen in the cytoplasma of giant cells (*1*).

Diagnoses such as cryptococcosis and blastomycosis can be easily ruled out by direct examination or histopathology, but blastomycosis is unlikely in central and western African patients (*22*). The differential diagnosis is rarely difficult with cryptococcosis because of the shape and size of yeasts, presence of capsule, and lack of inflammation in the surrounding tissue. In any event, cryptococcal antigen testing and culture will easily ascertain the diagnosis.

Antigen detection in serum and urine is a sensitive test but has been developed for the variety *capsulatum*. It is validated in HIV-infected patients with disseminated diseases (*8*,*23*,*24*). *H. duboisii* is a cause of false-positive test results for antigen detection in urine. Antibody detection is useful for the retrospective diagnosis of histoplasmosis caused by variety *capsulatum*. Since variety *duboisii* antigens may cross-react with those of the variety *capsulatum*, serologic tests are potentially useful for diagnosis of African histoplamosis.

Although some PCR assays have been developed, they are not yet routinely used (*25*). Real-time and semi-nested PCR seem promising for the diagnosis of histoplasmosis due to variety *capsulatum* in blood and tissue samples (*26*–*28*). No PCR has yet been developed for variety *duboisii*, but a specific PCR assay could be helpful for this underdiagnosed disease.

Treatment of African histoplasmosis can be extrapolated from the guidelines of the Infectious Diseases Society of America established for histoplasmosis due to variety *caspulatum* (*29*). No clinical trial or efficacy studies have been performed for histoplasmosis due to variety *duboisii*, but as mortality rates are similar for the 2 species with the same management, the guidelines can be extrapolated to African histoplasmosis. In patients with AIDS, recommended therapy includes an intensive phase of 3 months with amphotericin B replaced by itraconazole (400 mg/d) for the severe forms, or itraconazole alone (600 mg/d for 3 days, then 400 mg/d) for mild forms. Fluconazole (800 mg/d) can be an alternative, but it has lower efficacy and a higher recurrence rate with isolates harboring higher MICs (*30*). Moreover, new azoles such as voriconazole require careful biologic and clinical monitoring when used for treating histoplasmosis in HIV-infected patients because of increased risk for in vitro resistance, especially in patients who had

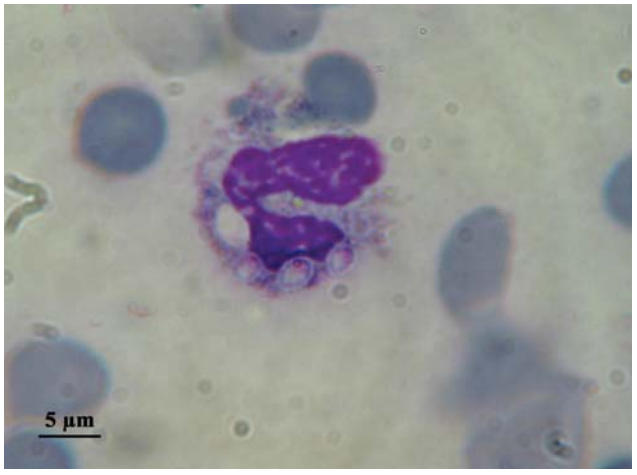

Figure 1. Direct examination of bone marrow smear. Intracytoplasmic *Histoplasma capsulatum* var. *capsulatum.*

fluconazole (*31*). Nothing is known, however, about development of resistance for variety *duboisii*. Managing AIDS by HAART is an essential part of the treatment. The availability of HAART in Africa is increasing, but it may be absent in areas where histoplasmosis is endemic. This is a real concern for optimal management of such patients.

Maintenance therapy with itraconazole (200 mg or 400 mg/d) is recommended. Fluconazole (400 mg/d) should be avoided because of its reduced capacity to prevent relapses. However, as for many other opportunistic infections, maintenance therapy can be discontinued if the immunologic status of the patient improves, as described for case-patient 3. This patient's prophylaxis was stopped 3 years ago, and she experienced no relapse and her CD4 count has always been >200/mm^3. The stability of immune improvement has to be confirmed for several months before prophylaxis is stopped (*32*). Recent data suggest that the risk for relapse is rare after 12 months of treatment with a sustained immunologic improvement (CD4 >150/mm^3) (*33*). However, in our experience based on the management of 20 cases of histoplasmosis due to variety *duboisii* in patients considered immunocompetent (*13*), relapses may be observed several years after the first episode. Thus, prolonged follow-up is mandatory for every patient with histoplasmosis due to variety *duboisii*.

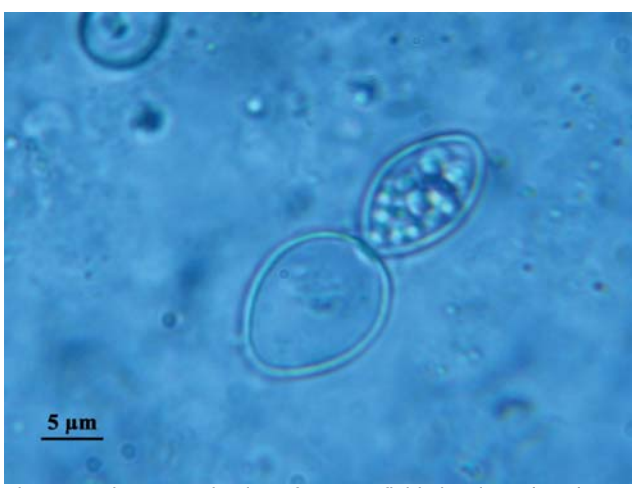

Figure 2. Direct examination of sputum fluid showing *Histoplasma capsulatum* var. *duboisii.*

Since HAART was introduced, the clinical and immunologic conditions of HIV-infected patients have dramatically improved, but physicians should now be aware of immune reconstitution inflammatory syndrome (IRIS) (*34*). As for many pathogens, both varieties of *H. capsulatum* can induce IRIS in HIV-infected patients, as recently reported by our group (*6*). The importance of the inflammatory reaction during IRIS contrasts with the mild one observed in the initial phase of the disease in severely immunocompromised patients and may require specific treatment.

Thus, histoplasmosis due to variety *duboisii* in HIV-infected patient remains a rare clinical entity but diagnosis should not be discounted because of the HIV status of the patient. Physicians working in Africa should be aware of *H. capsulatum* var. *duboisii* as a potentially emerging infection in HIV-infected patients.

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Mycobacterium ulcerans in Mosquitoes Captured during Outbreak of Buruli Ulcer, Southeastern Australia

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Buruli ulcer (BU) occurs in >30 countries. The causative organism, *Mycobacterium ulcerans*, is acquired from the environment, but the exact mode of transmission is unknown. We investigated an outbreak of BU in a small coastal town in southeastern Australia and screened by PCR mosquitoes caught there. All cases of BU were confirmed by culture or PCR. Mosquitoes were trapped in multiple locations during a 26-month period. BU developed in 48 residents of Point Lonsdale/Queenscliff and 31 visitors from January 2001 through April 2007. We tested 11,504 mosquitoes trapped at Point Lonsdale (predominantly *Aedes camptorhynchus*). Forty-eight pools (5 species) were positive for insertion sequence IS*2404* (maximum likelihood estimate 4.3/1,000), and we confirmed the presence of *M. ulcerans* in a subset of pools by detection of 3 additional PCR targets.

Buruli ulcer (BU), also known as Bairnsdale ulcer (*1*), Daintree ulcer (*2*), and Mossman ulcer in Australia, is an emerging disease of skin and soft tissue with potential to cause scarring and disability (*3*). It is caused by *Mycobacterium ulcerans* (*4*), an environmental pathogen that produces a destructive polyketide toxin, mycolactone (*5*); the genes for the production of this toxin are encoded on newly described plasmid *p*MUM001 (*6*). BU occurs in >30 countries worldwide, but it affects mainly children in sub-Saharan Africa, where it is now more common than tuberculosis and leprosy in some regions (*7*). This disease occurs in people of all ages and races who live in or visit BU-endemic areas, but the precise mode of transmission remains unknown.

Analysis of the recently sequenced *M. ulcerans* genome has shown that in addition to *p*MUM001, there are unusually high copy numbers of 2 independent insertion sequences (IS*2404* and IS*2606*) and a high incidence of pseudogene formation (*8*). These data suggest that *M. ulcerans* is unlikely to be free-living in the environment but is instead undergoing adaptation to a specific ecologic niche in which the products of some ancestral genes are no longer essential. One such niche may be in aquatic insects because *M. ulcerans* has recently been reported to colonize the salivary glands of carnivorous water bugs (Naucoridae) under laboratory conditions (*9*), and mycolactone production appears to be necessary for this colonization (*10*). Studies from disease-endemic areas in Africa have reported that farming activities near rivers (*11*) and swimming in rivers or marshes (*12*) may be risk factors for BU; bites from contaminated water bugs may transmit the infection.

In temperate southeastern Australia, outbreaks of *M. ulcerans* infection occur in localized areas, but few patients report direct contact with environmental water other than the ocean, which led to the proposal that aerosols from contaminated water may cause human infections (*13*). However, these low-lying disease-endemic areas also harbor large populations of mosquitoes, and some patients have reported that BU first appeared at the site of what may have been a mosquito bite (Figure 1). These observations, and

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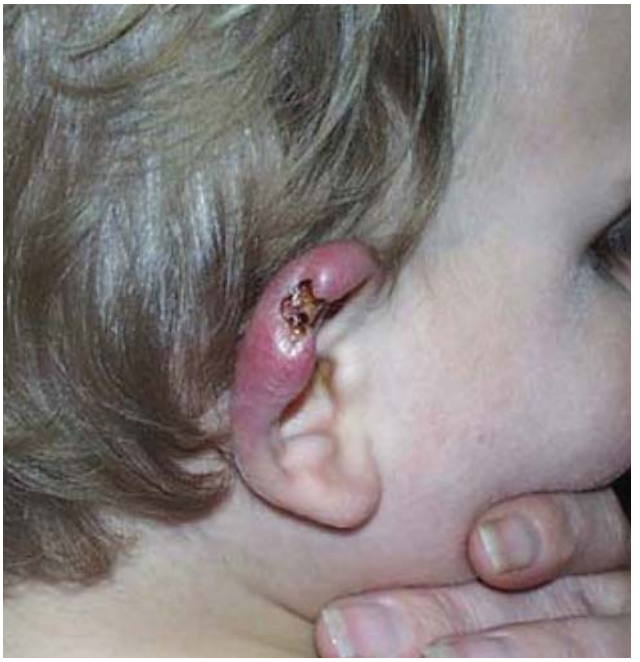

Figure 1. Ear of a 18-month-old child with culture- and PCR-confirmed Buruli ulcer who briefly visited St. Leonards, Australia, in 2001 (Figure 2). The initial lesion resembled a mosquito bite or that of another insect.

knowledge from field studies in Africa implicating insects as either a reservoir or mode of transmission, led us to capture and screen mosquitoes during our investigation of a large outbreak of BU in humans in a small coastal town in southeastern Australia (Point Lonsdale), ≈60 km south of Melbourne (Figure 2).

Methods

Outbreak Investigation

M. ulcerans infection has become increasingly common in the southern Australian state of Victoria since the early 1990s (*14*,*15*) and characteristically causes localized outbreaks (*16*). In 1995, a research group at the Royal Children's Hospital in Melbourne developed an IS*2404* PCR to improve speed and accuracy of diagnosis of BU (*17*). This method has now become the initial diagnostic method of choice in Australia and elsewhere (*18*). All PCR- and culture-positive cases of *M. ulcerans* infection in Victoria have been unofficially reported to the Victorian Department of Human Services (DHS) since the 1990s, and investigators from DHS began to routinely contact and interview all new reported case-patients in 2000. All new cases of *M. ulcerans* infection were made legally reportable in Victoria in January 2004 (*19*).

Case Definition

For this study, a case of BU was defined as a patient with a suggestive clinical lesion from which *M. ulcerans* was identified by PCR or culture from a swab or tissue biopsy specimen from January 2002 through April 2007; the patient must have been either a resident of, or a visitor to, Point Lonsdale or Queenscliff (adjacent coastal towns on the Bellarine Peninsula) who did not report a recent history of contact with another known BU-endemic area. Australian Bureau of Statistics data derived from the 2001 Australian Census for Point Lonsdale/Queenscliff (postcode 3225) were used to obtain the resident population numbers and age distribution in the outbreak area (*20*).

Mosquito Trapping

A total of 8–13 overnight mosquito traps were placed at Point Lonsdale on 22 occasions from December 2004 through January 2007. Adult mosquito sampling was conducted with CO_2-baited miniature light traps (*21*). Traps were 2-L, cylindrical, insulated containers designed to hold CO_2 pellets that continuously produce CO_2, which then diffuses through holes in the bottom of the container. A small electric light and fan at the base of the container deflected attracted mosquitoes into a holding container. The traps were set before dusk and then retrieved several hours after dawn the next morning. The catches were transported to Primary Industries Research in Attwood, Victoria, where they were counted, sorted, and pooled by sex and species. Mosquito species were identified by using the key of Russell (*22*). All captured mosquitoes were tested except in February–March 2005 and again in October 2005 when recent rains led to large spikes in mosquito numbers.

Screening of Mosquitoes by PCR

DNA was extracted from pools of ≤15 individual mosquitoes (occasional pools had ≤23 mosquitoes) of the same

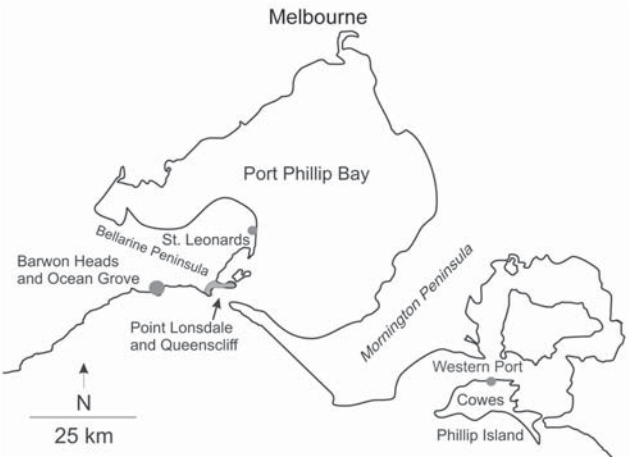

Figure 2. Map of central coastal Victoria, Australia, showing towns and places referred to in the text or in associated references.

sex and species by using the FastDNA Kit (revision no. 6540-999-1D04) and the FastPrep Instrument (Qbiogene Inc., Irvine, CA, USA) according to the manufacturer's instructions. We adapted fluorescence-based real-time PCR technology to screen mosquitoes for 3 *M. ulcerans*–specific DNA sequences as described (*23*). Briefly, oligonucleotide primers and TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA) labeled with fluorescent dyes 6FAM or VIC were designed that targeted 3 independent high-copy number repetitive sequences (IS*2404* and IS*2606* [*24*] and the ketoreductase B domain [KR] from *p*MUM001 [*6*]). The copy number of these targets per bacterial cell in the published sequenced of *M. ulcerans*, to which the outbreak strain is phylogenetically closely related, is 213 for IS*2404*, 91 for IS*2606*, and 30 for KR (*8*). Assays were conducted with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Each pool was first tested for IS*2404* with an internal positive control to test for PCR inhibition and separate negative and positive controls. Samples were considered positive for a given target when they had a result above a previously determined critical threshold (*23*). Pools that were positive for IS*2404* were then screened in duplicate with confirmatory assays to detect IS*2606* and KR. For pools with sufficiently high signal strength, amplification and sequencing of variable number tandem repeat (VNTR) locus 9 were conducted by using a nested PCR. The first round PCR used 2 primers, MUVNTR9NF (5′-ACTGCCCAGACATGGCGA-3′) and MUVNTR9NR (5′-ACGCGAGGTGGAACAAAGC-3′), designed to flank the published VNTR locus 9 primer. First-round PCR products were used as template for a second-round PCR performed as described by Ablordey et al. (*25*). PCR products of the expected size were sequenced by using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Precipitated reaction products were tested in a 3730S Genetic Analyzer (Applied Biosystems) (*23*). The maximum likelihood estimate (MLE) per 1,000 mosquitoes tested (bias corrected MLE) was calculated by using software recommended for this purpose by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*26*).

Results

Description of Outbreak

The climate in Point Lonsdale is temperate with a mean daily maximum temperature of 12.8°C in July (winter) and 22.4°C in January (summer). Average annual rainfall is 660 mm and is spread throughout the year (e.g., average 41.3 mm in January and 59.1 mm in July) (*27*). Most of the town is low-lying and close to sea level, and there are several natural and human-made swamps and water features in the vicinity (Figure 3). Natural vegetation includes dense clumps of coastal tea trees (*Leptospermum laevigatum*).

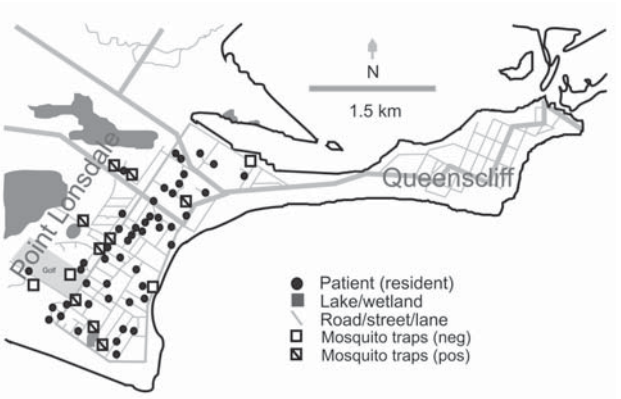

Figure 3. Map of Point Lonsdale/Queenscliff, Australia (postcode 3225), showing location of houses of affected permanent residents, mosquito traps, and other features mentioned in the text. Not all traps yielded PCR-positive mosquitoes during the trapping period. Neg, negative; pos, positive.

Point Lonsdale shares a beach with Queenscliff (Figure 3), a neighboring town 4 km to the east. Point Lonsdale and Queenscliff (postcode 3225) were included in the 2001 Australian Bureau of Statistics Census and had a resident population of 3,851 at that time, but there are also large numbers of visitors to this scenic area during summer holiday periods (e.g., 54,000 people visited the Queenscliff Visitor Information Centre in 2005; pers. comm.).

M. ulcerans infection was not found in the area before 2002. From January 2002 through April 2007, BU developed in 79 persons (48 residents and 31 visitors). Initially, all patients were local residents, but in 2004 the outbreak increased in intensity and began to include visitors as well as residents (Figure 4). All case-patients who could be accurately located either lived in or visited Point Lonsdale and the western edge of Queenscliff, and none were linked solely to the main township of Queenscliff.

Most case-patients were adults and many were elderly (Figure 5), although 14 of the 79 were children ≤18 years of age. Among visitors, there was a bimodal age distribution, with relatively low numbers of adults 20–50 years of age. An estimate of the age-specific attack rate for residents of Point Lonsdale/Queenscliff was obtained with reference to the 2001 Australian census. Because census data were not available for the 2 towns separately, the calculation assumes that the age distribution of Point Lonsdale and Queenscliff is similar. A similar analysis for visitors was not performed because appropriate denominators could not be determined. The risk appeared to increase strongly with age and was ≈7× higher for those ≥55 years of age than in those <55 years of age ($p<0.001$) (Figure 6).

The incubation period for residents and for most visitors could not be assessed because exposure to the BU-en-

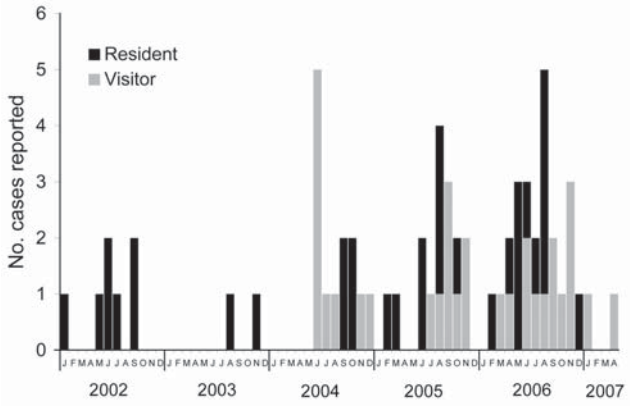

Figure 4. Epidemic curve of cases of Buruli ulcer linked to Point Lonsdale/Queenscliff, Australia, by resident/visitor status and month of reporting, 2002–2007.

demic area occurred repeatedly over a prolonged period. However, in 2006, one patient reported a 1-week contact history with Point Lonsdale (P.D.R. Johnson, unpub. data). Her case was diagnosed and reported 7 months after this exposure.

Mosquito Testing

A total of 23,692 mosquitoes were captured in Point Lonsdale during a 25-month period; 96% were *Aedes camptorhynchus* (Thomson). Twelve other species comprised the remaining 4% (Table 1). Of 11,504 mosquitoes tested, 48 pools were positive for IS*2404*; of these, 13 pools were also positive for KR and IS*2606*. Forty-one of 48 pools were female *Ae. camptorhynchus*, 4 were positive pools of *Coquillettidia linealis* (Skuse), and 1 each were *Anopheles annulipes* (Walker s.l.), *Culex australicus* (Dobrotworsky and Drummond), and *Ae. notoscriptus* (Skuse). For 2 positive pools with particularly high *M. ulcerans* DNA concentrations, VNTR locus 9 was amplified and the sequence was identical to that of the local outbreak strain cultured from case-patients (*23*).

Thirty-five IS*2404*-positive pools did not contain IS*2606* and KR. However, the cycle threshold (Ct) values for IS*2404* were lower for those pools that did have IS*2606* and KR, which suggested that failure to detect KR and IS*2606* in some pools was caused by low DNA concentration, rather than lack of specificity for *M. ulcerans*. This finding is consistent with known differences in copy number per cell of targets used for PCR screening and confirmation (*23*). A total of 124 pools of mosquitoes that were negative for IS*2404* by PCR were screened with probes for KR and IS*2606*. None were positive, which indicated that these 2 loci are consistently linked to IS*2404* and do not occur independently.

The MLE (bias corrected) for all mosquitoes over the entire testing period at Point Lonsdale was 4.3 *M. ulcerans* PCR-positive mosquitoes/1,000 tested (95% confidence interval [CI] 3.2–5.6). However, mosquito numbers varied widely between trappings, as did proportions of positive pools. On 1 occasion, only 269 mosquitoes were trapped, but 6 of the pools were positive (December 2005; MLE 22.4, 95% CI 10.3–50.3). Most PCR-positive pools had relatively high Ct values for IS*2404* PCR, which indicated low numbers of contaminating *M. ulcerans* cells. With reference to spiking experiments under laboratory conditions, ≈10–100 *M. ulcerans* were likely to have been present per contaminated mosquito (*23*).

Mosquito Numbers, Proportion PCR Positive, and Reporting of BU

Trapping was conducted at Point Lonsdale between December 2004 and January 2007. Mosquito numbers varied during the period, and traps were not set when local reports suggested low mosquito numbers (online Appendix Figure, available from www.cdc.gov/EID/content/13/11/1653-appG.htm). There appeared to be a qualitative relationship between PCR-positive mosquitoes in spring and summer (September–February) and reporting of new cases of human disease in autumn and winter (March–August). The exposure-to-reporting interval is typically longer than the actual incubation period because patients do not always seek medical assistance immediately and doctors do not always diagnose BU when a patient is first seen (*28*).

Mosquitoes Caught at Other Locations in Victoria

To test that the observed association between *M. ulcerans* and mosquitoes only occurs in outbreak areas, we tested 3,385 mosquitoes from several inhabited areas with lower BU endemicity than Point Lonsdale. From October 2005 through January 2007, a total of 2,119 mosquitoes (89% *Ae. camptorhynchus*) were trapped in townships on the Bellarine Peninsula where 30 cases of BU have been reported in the past 5 years; 3 pools of *Ae. camptorhyn-*

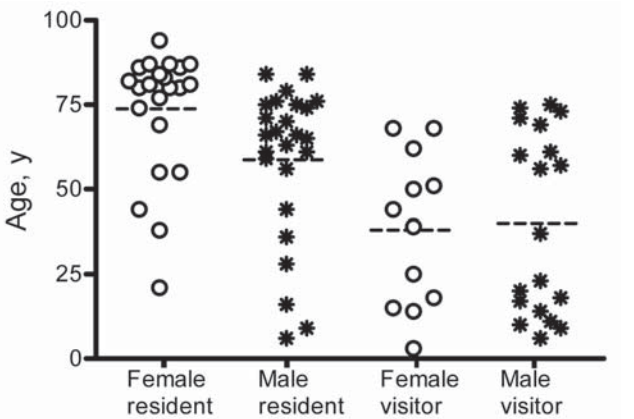

Figure 5. Cases of Buruli ulcer epidemiologically linked to Point Lonsdale, Australia, by resident/visitor status, age, and sex. Dashed lines are medians.

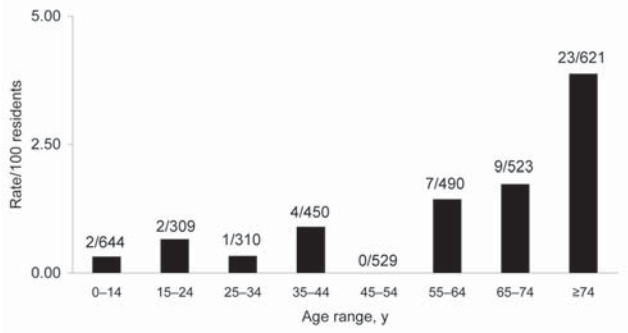

Figure 6. Estimated age-specific attack rates of Buruli ulcer for residents of Point Lonsdale/Queenscliff, Australia (postcode 3225). Values above the bars are cases per total no. residents in each age group.

chus were positive by IS*2404* PCR. In January and June 2006, a total of 795 mosquitoes (82% *Ae. camptorhynchus)* were trapped in the Bass Coast Shire, which includes Phillip Island, a region that has previously been endemic for *M. ulcerans* (*14*) but has only reported 2 cases in the past 5 years. One pool of *Ae. notoscriptus* was positive for IS*2404*. From February through April 2006, 471 mosquitoes were captured from inhabited areas in northern and central Victoria where no human cases of *M. ulcerans* have been reported. Ten different species were trapped, including 226 *Ae. camptorhynchus* (48%), but all pools were negative for IS*2404*. When analyzed together, an association was observed between degree of endemicity and probability of trapping mosquitoes that are positive by PCR for *M. ulcerans* (Table 2), but this association did not show statistical significance (p = 0.07).

Discussion

To our knowledge, the outbreak of BU in Point Lonsdale is the largest ever recorded in Australia and has now caused more than twice as many cases as the well-described outbreak at Phillip Island a decade earlier (*16*,*29*). A striking feature of both outbreaks is their intensely localized nature. We identified 79 cases that were epidemiologically linked to Point Lonsdale and the western edges of Queenscliff, but the town of Queenscliff, only 4 km to the east along the same beach, has so far remained disease free. The cumulative attack rate for both towns is estimated to be 1.2% of the resident population, but it could be up to twice as high if only the population of Point Lonsdale, where all transmission appears to have occurred, were considered. Although Queenscliff remains unaffected, the nearby towns of Barwon Heads and Ocean Grove, ≈12 km west of Point Lonsdale, began reporting their first cases in 2005.

The first case at Point Lonsdale was reported in January 2002. In 2004, the outbreak increased in intensity and began to involve visitors as well as residents, which suggested that environmental contamination with *M. ulcerans* has steadily increased over 5 years. Among local residents, we found a higher attack rate in the elderly, with 3.7% of residents of Point Lonsdale/Queenscliff >75 years of age with BU. The reasons for this age distribution are not known, but increasing risk with age could be caused by an age-related immune defect or an unrecognized behavioral factor. Among visitors, there was a pronounced bimodal age distribution, which probably represents a skewing of the exposed population (e.g., young children going to stay with their retired grandparents over the summer while their parents stayed at work) but may also reflect increased susceptibility in young persons. This bimodal pattern, which included increased incidence in young persons and the elderly, has also been reported in Africa (*30*).

During our investigations at Point Lonsdale, we focused initially on several marshy areas and obtained positive PCR results for plant material from 2 small ornamental lakes and soil from storm water drains (*23*). However, case-patients did not report direct contact with these lakes or drains (these sources of water are not used for swimming or wading). Thus, how people were exposed is not clear. In

Table 1. Maximum likelihood estimate (MLE) per 1,000 mosquitoes trapped in Point Lonsdale, Australia, and tested by PCR for insertion sequence IS*2404* of *Mycobacterium ulcerans*

Species	No. mosquitoes	No. positive pools/no. pools tested	MLE*	95% Confidence interval
Aedes camptorhynchus	10,558	41/757	3.98	2.90–5.35
Coquillettidia linealis	480	4/57	8.53	2.80–20.34
Ae. notoscriptus	221	1/49	4.47	0.26–21.37
Culex australicus	76	1/27	12.78	0.77–59.74
Ochlerotatus alboannulatus	52	0/16	0	0.00–58.19
Anopheles annulipes s. l.	49	1/18	22.44	1.24–115.30
Cx. globocoxitus	43	0/20	0	0–76.19
Cx. annulirostris	10	0/2	0	0–161.60
Cx. molestus	6	0/6	0	0–390.33
Cx. quinquefasciatus	5	0/2	0	0–319.26
Oc. australis	3	0/2	0	0–499.14
Cx. pipiens gp.	1	0/1	0	0–793.45
Total	11,504	48/957	4.28	3.20–5.62

*MLE bias was corrected when ≥1 pool was positive; otherwise uncorrected.

Table 2. Relationship between cases of Buruli ulcer, mosquitoes tested, and maximum likelihood estimate (MLE) per 1,000 mosquitoes trapped in Victoria, Australia, and tested by PCR for insertion sequence IS*2404* of *Mycobacterium ulcerans**

Region	No. cases past 5 y	No. mosquitoes tested (% *Aedes camptorhynchus*)†	No. pools positive	MLE (95% CI)
Point Lonsdale	79	11,504 (91.8)	48	4.2 (3.08–5.53)
Bellarine Peninsula (excluding Point Lonsdale)	30	2,119 (88.7)	3	1.42 (0.37–3.85)
Bass coast Shire including Phillip Island	2	795 (82.1)	1	1.25 (0.07–6.03)
Central and northern Victoria (Mildura, Swan Hill, Moira, Shepparton)	0	471 (48.0)	0	0 (0–7.34)
Total	111	14,889 (89.4)	52	3.57 (2.70–4.64)

*MLE bias was corrected when ≥1 pool was positive, otherwise uncorrected. CI, confidence interval
†p value = 0.07 (χ^2: 4 × 2 table; pools positive/no. tested).

an outbreak in Phillip Island, many cases were clustered around a newly formed wetland and a golf course irrigation system, and we proposed transmission from these sites by aerosol (*16*,*29*). However, this hypothesis may not be supported by our new evidence, which suggests that *M. ulcerans* may not be free-living in the environment but may have adapted to specific niches within aquatic environments, including salivary glands of some insects. Thus, we investigated whether *M. ulcerans* could be detected in mosquitoes, which had been reported in higher than usual numbers at Point Lonsdale. We also investigated behavior in a case-control study (the subject of a separate report), which found that being bitten by mosquitoes increased the odds of having BU (*31*).

A total of 14,889 mosquitoes obtained over a 25-month period (11,504 from Point Lonsdale) were tested for *M. ulcerans* by using a highly sensitive and specific real-time PCR (*23*). We used PCR because direct culture of *M. ulcerans* from the environment is extremely difficult and was only achieved when IS*2404* PCR screening of environmental samples accurately directed researchers to specific microenvironments that include water insects and aquatic plants (*32*). Although IS*2404*, IS*2606*, and the mycolactone-producing virulence plasmid have been detected in mycobacteria other than *M. ulcerans* (*33*–*35*), identification of these targets in expected relative proportions and the VNTR locus 9 sequence identical to that of the outbreak strain in a subset of mosquito pools with sufficiently high DNA concentrations confirms that we identified the outbreak strain (*23*).

We also demonstrated that over a 2-year cycle at Point Lonsdale absolute numbers of mosquitoes and PCR-positive mosquitoes increased in spring and summer followed by a cluster of new human cases in autumn and winter. This pattern is consistent with recent point estimates that suggest the incubation period for BU in Australia is 3–7 months (2 cases) (*36*) and 1–4 months (3 cases) (*28*), and that an additional 1–6 weeks may elapse before cases are diagnosed and reported (*28*).

The predominant species trapped was *Ae. camptorhynchus*; however, identification of *M. ulcerans* in 4 other species suggests that *M. ulcerans* contamination of mosquitoes is not species specific. *Ae. camptorhynchus* is a salt marsh species, an aggressive biter, and a major pest in coastal areas of southeastern Australia that has been linked to transmission of Ross River virus. The mosquito appears in large numbers after rain as minimum temperatures begin to increase, with a lag time of ≈1 month (*37*). Of the other species from which at least 1 PCR-positive pool was identified, *An. annulipes* and *Cq. linealis* are fresh water species (*38*). *Ae. notoscriptus* is a peridomestic species that breeds in containers (e.g., in roof gutters) (*39*), can transmit dog hookworm, and has a limited flight range (e.g., <200 m) (*40*). In contrast, *Cx. australicus* may have a flight range of many kilometers (*41*). A limited number of other biting or aquatic insects were also tested and none were positive for *M. ulcerans*. However, larger numbers must be screened before it can be concluded that they do not transmit *M. ulcerans*.

Our results do not demonstrate viability or transmissibility of *M. ulcerans* at the time mosquitoes were captured, and the method we used does not answer questions about location of *M. ulcerans* within the insect. Because *M. ulcerans* is an environmental pathogen, PCR-positive mosquitoes may only be indicators of its presence in the environment and not linked to transmission. The Ct values obtained for mosquito pools suggest that only 10–100 organisms were present per positive pool, which is more consistent with organisms being acquired on outer surfaces of mosquitoes when resting or feeding in storm water drains (*23*), rather than mosquitoes being a true productive reservoir and vector. However, if some bacterial cells were present on the proboscis, they could have been injected beneath the keratin layer during feeding. Although the inoculum size required to cause a human infection is unknown, the long incubation period suggests a low initial inoculum. Our findings do not demonstrate that mosquitoes are responsible for transmission, but this possibility should be investigated. Studies are underway to artificially infect mosquito larvae with *M. ulcerans* and initiate infection in a mouse model, as has been conducted with naucorids (*9*).

Although our findings may not apply to the situation in Africa, the close genetic relationship of Australian isolates

of *M. ulcerans* with strains from humans with BU in Africa (*35*) should encourage similar search on *M. ulcerans* in mosquitoes from the primary BU-endemic regions of West Africa. We have shown that a small proportion of mosquitoes of 5 species captured in a BU-endemic area during an intense human outbreak of BU can carry *M. ulcerans*; PCR-positive mosquitoes are likely present at times of peak transmission and mosquitoes captured in areas with few human cases appear less likely to be positive for *M. ulcerans*. We hypothesize that transmission by mosquitoes offers a partial explanation for the outbreak at Point Lonsdale and possibly at other sites in southeastern Australia.

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Dr Johnson is deputy director of the Infectious Diseases Department at Austin Health, a University of Melbourne teaching hospital. His research interests include epidemiology, transmission, and treatment of *M. ulcerans* infections, drug resistance in *Staphylococcus aureus*, and hospital infection control.

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Risk Factors for *Mycobacterium ulcerans* Infection, Southeastern Australia

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Buruli/Bairnsdale ulcer (BU) is a severe skin and soft tissue disease caused by *Mycobacterium ulcerans.* To better understand how BU is acquired, we conducted a case–control study during a sustained outbreak in temperate southeastern Australia. We recruited 49 adult patients with BU and 609 control participants from a newly recognized BU-endemic area in southeastern Australia. Participants were asked about their lifestyle and insect exposure. Odds ratios were calculated by using logistic regression and were adjusted for age and location of residence. Odds of having BU were at least halved for those who frequently used insect repellent, wore long trousers outdoors, and immediately washed minor skin wounds; odds were at least doubled for those who received mosquito bites on the lower legs or lower arms. This study provides new circumstantial evidence that implicates mosquitoes in the transmission of *M. ulcerans* in southeastern Australia.

Mycobacterium ulcerans is an environmental pathogen that is most commonly associated with water and soil (*1–4*). In humans, *M. ulcerans* causes an ulcerative skin disease known as Buruli ulcer in Africa and Bairnsdale ulcer in southeastern Australia (BU). Although BU is usually regarded as a disease of subtropical climates, a slowly increasing number of cases have been recorded in temperate southeastern Australia over the past 15 years. In sub-Saharan Africa countries such as Côte d'Ivoire (*3*), Uganda (*5*), Benin (*6*), and Ghana (*7*), BU has been responsible for considerable suffering and disability, and the disease has become more common than tuberculosis and leprosy in some highly disease-endemic regions (*8*).

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M. ulcerans infection was first definitively described in 1948 (*9*). In the state of Victoria, in southeastern Australia, BU cases appeared to be confined to the original BU-endemic region of Bairnsdale until 1982, when a new disease focus was noted 150–200 km west of Bairnsdale (Figure). Since 1991, incidence has progressively risen, and outbreaks of BU have occurred at Phillip Island and on the Mornington Peninsula (*10*). In 1998, BU was reported >50 km farther west on the Bellarine Peninsula, and since then, a sustained outbreak has involved at least 3 Bellarine Peninsula towns. The annual number of cases in Victoria has continued to increase; 41 cases were reported to the Victorian Department of Human Services in 2005, compared with 25 in 2004 and 12 in 2003. Although the incidence is still low overall, the disease is becoming a substantial public health concern in affected coastal communities. Treatment of BU is not straightforward and usually requires surgery combined with prolonged courses of antimicrobial drugs. The average cost of diagnosing and treating a case of BU in Australia in 2004 was estimated to be AUD $14,608 (*11*).

Although the mode of *M. ulcerans* transmission is unknown, epidemiologic evidence suggests that transmission occurs through being in close proximity to slow-moving waters contaminated with the bacteria (*1–3*,*12*,*13*). Previous case–control studies have reported an increased risk for BU for those who regularly swim or wade through rivers (*12*,*13*) and those who farm near a BU-endemic town's main rivers (*3*). More recently, results from laboratory experiments have suggested a new hypothesis that aquatic insects, fish, and plants may be reservoirs for *M. ulcerans* (*14–17*) and that aquatic insects may even be involved in transmission to humans (*14*,*16*,*18*).

Because the ecologic factors that are driving the emergence of *M. ulcerans* infection in newly disease-endemic

Figure. Bellarine Peninsula region, southeastern Australia.

countries are unknown, the most effective means of reducing the incidence of BU may be to identify modifiable behavior associated with the risk for infection. Therefore, we designed a case–control study to investigate risk factors that could be used to assist public health authorities in reducing the effects of BU.

Methods

Study Area

This study was conducted on the Bellarine Peninsula, in southeastern Australia, on which *M. ulcerans* infection is newly endemic (Figure). Most residents live in small towns along the coast of the Peninsula (e.g., Point Lonsdale, St Leonards, Queenscliff, Barwon Heads), while the more sparsely populated interior is used for primary agricultural production and industry.

Participants

BU has been a reportable disease in Victoria since 2004. Patients with BU were identified retrospectively from the records of the treating physicians at Barwon Health and Austin Health from 1998 through 2005 and from the Victorian Department of Human Services reportable diseases database. The preliminary search found 73 confirmed BU case-patients, defined as patients from whom a clinical specimen (swab or biopsy) was positive for *M. ulcerans* by culture, PCR, or both (*19*). All patients in this study were either permanent residents of the Bellarine Peninsula or persons who had visited the area before first noticing the lesion that was subsequently diagnosed as BU. Patients were excluded if they were <20 years of age (n = 9), had moved with no forwarding address (n = 2), died (n = 1), or had a diagnosis of dementia (n = 1). Of the remaining 60 case-patients who were contacted, 49 responded (82% participation rate) and median age was 70 years (interquartile range 58–81 years).

A total of 609 controls participated in the study (78% participation rate). Control participants were randomly selected from the Commonwealth electoral roll (Australian Electoral Commission, 2005) for Point Lonsdale and St Leonards (which have each experienced separate outbreaks of BU since 1998) and other towns on the Bellarine Peninsula (e.g., Clifton Springs, Curlewis, Whittington, Portarlington, Queenscliff, Ocean Grove, Indented Head, and Drysdale). The entire adult population of the Bellarine Peninsula is captured by the Australian electoral roll because it is compulsory for all Australian citizens to vote in federal elections. We were therefore able to randomly select controls from the entire adult population of 32,480 on the Bellarine Peninsula (*20*).

Data Collection

Case-patients and control participants were mailed a written questionnaire with questions about their medical history; outdoor lifestyle and behavior; and soil, animal, and insect exposure on the Bellarine Peninsula. Case-patients were asked to restrict their responses to the 12-month period before their diagnosis of BU. We investigated outdoor-related behavior and habits such as types of clothing worn, measures taken to protect against insect bites, types of treatment given to skin trauma, parts of their bodies most frequently bitten by insects, natural fauna and household pets with which they had regular contact, and types of soil products they were exposed to in the previous year while on the Peninsula. For activities associated with water or located near specified lakes or marshes on the Bellarine Peninsula, and for exposure to mosquitoes (Family Culicidae), March flies (Family Tabanidae), and sandflies (Family Ceratopogonidae), the responses were subdivided according to season (summer, autumn, winter, and spring).

Statistical Analyses

Analyses were performed by using Minitab statistical software, version 14 (Minitab Inc., State College, PA, USA). The ages of the case-patients and control participants were compared by using a 2-sample *t* test. Chi-square analysis, Fisher exact test, and logistic regression determined the odds ratio (OR) and 95% confidence interval (CI) for BU after adjusting for age and town of residence (Point Lonsdale, St Leonards, or all other towns on the Bellarine Peninsula). Multivariate models were determined by applying a backward elimination technique to the logistic regression while adjusting for age and town of residence.

In all statistical models, p values ≤0.05 were considered statistically significant.

Ethics

The Barwon Health Research and Ethics Advisory Committee and the Victorian Department of Human Services approved the study. Consent to participate in the study was implied by those who returned the self-completed written questionnaire.

Results

Characteristics of the case-patients and control participants are shown in Table 1. Both sexes were equally affected by BU. Most case-patients were elderly adults, and their median age was higher than that of the control participants (p = 0.01). Among case-patients, ≈69% reported that they were either permanent residents of Point Lonsdale or had visited the town ≈3 months before onset of symptoms. Personal health factors such as cancer, immunosuppressive medication taken within the previous year, or having had an *M. bovis* BCG vaccination did not alter the odds of having BU.

Table 2 displays the participants' behavioral and lifestyle choices on the Bellarine Peninsula. Wearing insect repellent (OR 0.38, 95% CI 0.20–0.71) or wearing long trousers when outdoors (OR 0.51, 95% CI 0.27–0.97) were each found to reduce the odds of having BU. Immediately washing a wound sustained outdoors was also found to decrease the odds for disease (OR 0.47, 95% CI 0.24–0.94). Owning a household pet or using gardening products such as fertilizer, potting mix, and topsoil was not found to be associated with BU. Participants were also assessed on whether they frequented any of 8 prominent lakes or marshes in Point Lonsdale or St Leonards. Case-patients were more likely than control participants to have visited a small ornamental lake at the western edge of Point Lonsdale during autumn (p = 0.04), but associations with all the other water areas surveyed were not statistically significant.

The extent of insect exposure on the Bellarine Peninsula is shown in Table 3. The odds more than doubled if participants reported that they had received mosquito bites on commonly exposed sites of the body, i.e., lower arms (OR 2.56, 95% CI 1.23–5.33) and legs (OR 2.51, 95% CI 1.18–5.31). The same biting pattern was not noted in response to the same questions regarding bites from March flies and sandflies.

A variety of outdoor activities were surveyed, including beach activities, freshwater or salt water swimming and fishing, surfing, sailing, bushwalking, lawn bowling, golf, bird watching, cycling, and gardening. None of these activities was associated with BU.

A backward elimination technique was applied to further investigate all significant factors. In a multivariate model, after adjusting for age and location, insect repellent use (OR 0.37, 95% CI 0.19–0.69) and being bitten by mosquitoes on the lower legs (OR 2.60, 95% CI 1.22–5.53) were found to be independently associated with BU.

Discussion

BU occurs in focal outbreaks in >30 countries worldwide. Although the disease has been long neglected (*21*), substantial progress has been recently made and includes the development of rapid diagnosis by PCR (*19*), the discovery of the lipid toxin that explains the destructive nature of the disease (*22*), and the identification of the virulence plasmid that harbors the genes that produce the toxin (*23*). Also, increasing evidence supports the use of drug therapy for BU (*24–26*), which until recently has been regarded as a disease that would respond only to surgery. However, the ecologic factors driving the increasing incidence of BU in different regions of the world and the mode of transmission to humans who live in BU-endemic areas remain to be determined.

One of the major differences between BU in southeastern Australia and BU in sub-Saharan Africa is the proportion of older persons affected. Of the total number of cases linked to the Bellarine Peninsula, 33 (67%) case-patients were >60 years of age, whereas many studies from Africa report that most BU cases occur in children <15 years of age (*3*,*6*,*7*,*27*). A high proportion of the permanent residents of the Bellarine Peninsula are older Australians who have relocated to

Table 1. Characteristics of 49 Buruli ulcer case-patients and 609 control-participants, southeastern Australia*

Variable	Case-patients, n (%)	Control-participants, n (%)	p value
Female sex	25 (51)	326 (54)	0.90
Towns in which most time on Bellarine Peninsula was spent			<0.01
Point Lonsdale	34 (69)	212 (35)	
St Leonards	8 (16)	202 (33)	
Other	7 (14)	195 (32)	
Health condition			
Diabetes	1 (2)	38 (6)	0.23
Cancer	5 (10)	40 (7)	0.57
Immunosuppressive medication	2 (4)	36 (6)	0.45
Mycobacterium bovis BCG immunization	16 (33)	174 (29)	0.30
Regular tobacco smoker	3 (6)	71 (12)	0.37

*Median age (interquartile range) for case-patients was 70 (58–82) years and for control participants 61 (48–72) years; p = 0.01.

Table 2. Association of Buruli ulcer with behavioral and lifestyle factors, Bellarine Peninsula, southeastern Australia

Variable	Case-patients, n (%)	Control-participants, n (%)	OR (95% CI)*	p value
Outdoor clothing covers				
Arms	18 (38)	247 (41)	0.62 (0.33–1.18)	0.15
Legs	24 (50)	343 (57)	**0.51 (0.27–0.97)**	**0.04**
Immediately washes wounds	12 (24)	225 (37)	**0.47 (0.24–0.94)**	**0.03**
Uses gardening products				
Potting mix	32 (67)	369 (61)	1.28 (0.68–2.40)	0.44
Pesticides	20 (42)	187 (31)	1.51 (0.83–2.77)	0.18
Fertilizers	30 (63)	307 (50)	1.28 (0.68–2.40)	0.44
Had topsoil delivered to home	6 (13)	65 (11)	1.34 (0.54–3.31)	0.53
Construction site is				
At home	5 (10)	64 (11)	1.24 (0.47–3.32)	0.66
Near home	6 (13)	155 (25)	0.48 (0.20–1.17)	0.11
Wears gardening gloves				
Does not garden	2 (4)	64 (11)	0.36 (0.08–1.56)	0.17
Always/usually	20 (42)	223 (37)	0.97 (0.52–1.80)	0.92
Sometimes/never†	26 (54)	321 (53)		
Washes hands after gardening				
Does not garden	2 (4)	65 (11)	0.33 (0.08–1.42)	0.07
Always/usually	43 (90)	515 (85)	0.61 (0.23–1.61)	0.24
Sometimes/never†	3 (6)	24 (4)		
Owns a pet	21 (43)	343 (56)	0.74 (0.39–1.40)	0.35
Wears insect repellent	15 (31)	328 (54)	0.38 (0.20–0.71)	**<0.01**
Ever visits water area on western edge of Point Lonsdale				
Summer	9 (19)	57 (9)	–	0.08‡
Autumn	9 (19)	49 (8)	–	**0.04‡**
Winter	8 (17)	46 (8)	–	0.07‡
Spring	7 (15)	50 (8)	–	0.22‡

*OR, odds ratio; CI, confidence interval. OR (95% CI) adjusted for age and location; **boldface** indicates statistical significance; – indicates that ORs could not be calculated.
†Denotes reference group.
‡Fisher exact test for small numbers.

the coast for their retirement. However, especially during summer, young families visit the area and increase the local population considerably. Although at least 9 children or teenagers have acquired BU linked to the Bellarine Peninsula, advanced age may be an independent risk factor for BU, as was reported recently in Benin (*28*,*29*).

Our results suggest that protecting the body from environmental exposure appears to reduce the odds for disease. These measures include wearing long trousers and immediately washing wounds after sustaining minor skin trauma; such measures have also been identified as protective factors against BU in west Africa (*3*,*13*,*30*). These findings support the established hypothesis of *M. ulcerans* transmission, which postulates that direct contact with the skin is required for transmission to occur. Negative findings with regard to animal exposure factors (domestic pets and wildlife) also make it unlikely that contact with these animals is a risk factor for infection with *M. ulcerans* on the Bellarine Peninsula, although the bacterium has been isolated from skin ulcers in several wild animals near the original Bairnsdale region and other BU-endemic areas in Victoria (*31*,*32*).

Only 1 case-patient in this study reported having had direct physical contact with environmental water (other than the ocean) from a BU-endemic town, and only 14 (2%) of control participants reported having swum in fresh water on the Bellarine Peninsula (data not shown). Case-patients were more likely than control participants to have visited only 1 of our surveyed water areas, a small park reserve (Table 2), although the low numbers of positive responses to this question limited our ability to draw further insight from these data. A small percentage of participants reported having spent time near marshy areas on the Peninsula, which suggests that transmission is most likely to occur indirectly or that *M. ulcerans* has spread to areas outside of its presumed aquatic environment. Previous studies conducted in BU-endemic African countries found that case-patients were more likely to report that they regularly immersed themselves in, or worked along, rivers or marshy areas (*12*). On the Bellarine Peninsula, contact with lakes and rivers is not a large part of the lifestyle because recreational swimming and boating activities are largely based around coastal saltwater areas. This difference in lifestyle could also possibly explain the lower incidence rate of BU in Australia than in the highly affected African regions.

More case-patients than control-participants reported having been bitten by mosquitoes on the distal areas of their arms and legs, the sites most commonly exposed to

Table 3. Insect exposure as risk factors for Buruli ulcer on the Bellarine Peninsula, southeastern Australia

Variable	Case-patients, n (%)	Control-participants, n (%)	OR (95% CI)*	p value
Season when frequently bitten by mosquitoes				
Summer	24 (49)	373 (62)	0.71 (0.39–1.30)	0.27
Autumn	16 (33)	238 (39)	0.94 (0.49–1.79)	0.85
Winter	7 (15)	37 (6)	–	0.07†
Spring	12 (25)	189 (31)	0.88 (0.44–1.75)	0.71
Area most often bitten by mosquitoes				
Head	18 (37)	290 (48)	0.61 (0.33–1.12)	0.11
Upper arms	30 (61)	353 (58)	1.37 (0.74–2.55)	0.31
Forearms	39 (80)	422 (69)	**2.56 (1.23–5.33)**	**0.01**
Hands	28 (57)	381 (63)	1.36 (0.71–2.62)	0.36
Torso	5 (10)	128 (21)	0.48 (0.18–1.23)	0.13
Upper legs	8 (16)	80 (13)	1.67 (0.73–3.82)	0.23
Lower legs	39 (80)	423 (69)	**2.51 (1.18–5.31)**	**0.02**
Feet	11 (22)	175 (29)	0.94 (0.45–1.97)	0.88

*OR, odds ratio; CI, confidence interval. OR (95% CI) adjusted for age and location; **boldface** indicates statistical significance; – indicates that ORs could not be calculated.
†Fisher exact test for small numbers.

the environment. Although the responses to our insect-exposure questions suggest an association between mosquito bites and *M. ulcerans*, this association may be just a nonspecific marker of environmental exposure. However, use of insect repellent remained an independent predictor of reduced risk, which suggests that insects themselves may play a role in transmission. Although no case-control study can establish causation, our findings are consistent with the hypothesis that mosquitoes play a role in transmission of *M. ulcerans* to humans, at least in southeastern Australia. In a previous outbreak of BU in Australia in the 1990s, contaminated aerosolized water droplets that could be inhaled or ingested were suggested as a means of infection for persons who did not have direct contact with the likely point source. Although mosquitoes and other insects were not tested during that outbreak, they also could have played a role in transmission (*1*,*33*).

We acknowledge several limitations in our study. Reliability of patient recall may be biased because some BU cases date back to 1998. However, the median time between the date of completing the questionnaire and the time of diagnosis of BU was 1.51 years (interquartile range 0.90–3.29 years). Avoidance of recall bias is impossible because the incubation period varies by several months, and delay between first noticing a skin lesion and the diagnosis of BU is often substantial (*34*,*35*). Studies with more cases would help validate and refine the protective and risk factors identified in this study.

Despite many differences between the BU-endemic areas in southeastern Australia and western Africa, case-control studies in these diverse environments produce surprisingly concordant results. Direct contact with the environment appears to be a universal risk factor for acquisition of BU, and use of protective clothing appears to reduce this risk. In this study we also established a new risk factor—exposure to mosquitoes. Our data support an association between mosquitoes and *M. ulcerans* on the Bellarine Peninsula during the outbreak and demonstrate that BU case-patients were likely to have been bitten by mosquitoes. We hypothesize that in this BU-endemic region, mosquitoes become colonized or passively contaminated when they come into contact with *M. ulcerans* in the environment and then transmit it to humans living nearby. However, before introducing specific public health interventions, such as mosquito control and campaigns to encourage the use of repellents and protective clothing for those who live in BU-endemic areas, the presence of *M. ulcerans* in mosquitoes (*36*–*38*) from the BU-endemic area should be confirmed and transmissibility under laboratory conditions should be demonstrated.

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Protection and Virus Shedding of Falcons Vaccinated against Highly Pathogenic Avian Influenza A Virus (H5N1)

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Because fatal infections with highly pathogenic avian influenza A (HPAI) virus subtype H5N1 have been reported in birds of prey, we sought to determine detailed information about the birds' susceptibility and protection after vaccination. Ten falcons vaccinated with an inactivated influenza virus (H5N2) vaccine seroconverted. We then challenged 5 vaccinated and 5 nonvaccinated falcons with HPAI (H5N1). All vaccinated birds survived; all unvaccinated birds died within 5 days. For the nonvaccinated birds, histopathologic examination showed tissue degeneration and necrosis, immunohistochemical techniques showed influenza virus antigen in affected tissues, and these birds shed high levels of infectious virus from the oropharynx and cloaca. Vaccinated birds showed no influenza virus antigen in tissues and shed virus at lower titers from the oropharynx only. Vaccination could protect these valuable birds and, through reduced virus shedding, reduce risk for transmission to other avian species and humans.

Highly pathogenic avian influenza A (HPAI) virus poses a major threat to poultry but is also of great concern for other avian species and humans. In particular, HPAI (H5N1) of Asian lineage is known for its potential to be transmitted to mammals, including humans. Susceptibility to this virus and the possible role as vectors or reservoirs vary greatly between different wild bird and poultry species (*1*,*2*). Gallinaceous poultry are considered to be highly susceptible, whereas waterfowl may show variable clinical signs depending on the strain of infecting virus. Birds of prey are at increased risk for infection with HPAI virus because they regularly feed on avian carcasses and diseased avian prey (*3*,*4*). Many species are migratory or cover an extensive territory and may spread the virus within or between countries. In falconry, birds of prey are also regularly kept in captivity and come in close contact with humans. In this respect, birds of prey represent a bridging species and may pose a risk of transmitting the virus to humans or to other captive avian species, including poultry.

In the past, HPAI rarely occurred in birds of prey and only in isolated cases. In 2000, Manvell et al. (*5*) isolated influenza virus (H7N3) from a Peregrine falcon (*Falco peregrinus*) kept as a falconry bird in the United Arab Emirates. In the same year, during an HPAI (H7N7) outbreak in poultry in Italy, an avian influenza virus of H7 subtype was isolated from a Saker falcon (*Falco cherrug*) (*6*). Both birds showed depression and died, but other pathogens (e.g., *Pasteurella* sp.) were detected as well.

During recent influenza (H5N1) outbreaks, increasing numbers of birds of prey were reported to be infected. HPAI virus (H5N1) was isolated from Hodgson's hawk eagles (*Spizaetus nipalensis*) confiscated at an airport (*7*) and from a Saker falcon (*8*) in Saudi Arabia. During the influenza (H5N1) outbreak among wild birds in Germany, 36 (10.5%) birds with positive influenza (H5N1) results were birds of prey, represented by common buzzards (*Buteo buteo*), peregrines (*F. peregrinus*), and kestrels (*Falco*

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tinnunculus), as well as European eagle owls (*Bubo bubo*), which were found dead (*9*). Diseased free-ranging birds of prey infected with influenza (H5N1) were also reported by several other countries. In March 2007, influenza virus (H5N1) was isolated from falcons in Kuwait (www.poultrymed.com).

Although it is obvious that birds of prey can be infected with HPAI viruses, the pathogenic potential in these species remains unclear. Free-ranging birds frequently suffer from other concurrent diseases or starvation, and captive birds undergo stressful periods due to rearing conditions or training. These situations may immunocompromise the birds, leading to increased vulnerability. However, their potential to shed virus after infection, which is important for virus transmission, potentially also to humans, remains unclear. Clinical signs, pathologic and histopathologic alterations, and tissue tropism of the virus after a controlled infection have not been investigated. This knowledge is needed for a better understanding of HPAI in nondomestic birds, especially for subtype H5N1, which poses a higher risk to humans than do other avian influenza viruses (*10–12*).

Collections of birds of prey are of high commercial and species conservation value; therefore, protection from HPAI is important. Vaccination might reduce the risk for virus transmission by reducing virus shedding, as has been shown in chickens (*13*,*14*). Ultimately, an interruption of virus transmission between and within avian collections would be invaluable for controlling disease, especially in populations of rare species as exemplified by many bird of prey species (*15*).

Vaccination with commercially available inactivated vaccines based on avian influenza virus subtype H5 can confer clinical protection and reduce virus shedding after infection (*16*). Implementation of DIVA (Differentiating Infected from Vaccinated Animals) strategies have been attempted (*17*). Response to vaccination of zoo birds with an AI H5N2 (*18*) or H5N9 (*19*) subtype inactivated vaccine varied considerably among species with respect to peak titers and persistence of specific antibodies. Some species mounted antibodies after the first round of vaccination; others had detectable titers only after a second dose or never produced detectable antibody levels (pelicans [*Pelicanus* spp.] and owls [*B. bubo, Tyto alba*]) (*20*). The authors demonstrated peak hemagglutination inhibition (HI) titers of 2,048 within 2–4 weeks after booster vaccination in bar-headed geese (*Anser indicus*); most other species yielded titers of only 64 to 512 during the same time. Several species, such as the Egyptian goose (*Alopochen aegyptiacus*) and peafowl (*Acryllium vulturinum*), still had antibody titers of 32 to 128 by 6 months after vaccination, while spur-winged geese (*Plectropterus gambensis*) failed to show titers after that time. Such a variation between species was also observed after vaccination of different waterfowl and wader species (*21*).

No detailed information is available about antibody responses and protection after vaccination against HPAI in falcons. Therefore, we analyzed the susceptibility of falcons to an influenza (H5N1) field virus under controlled conditions and evaluated the efficacy of vaccination of falcons with an inactivated influenza (H5N2) vaccine and its effect on epidemiologically relevant parameters. The trial was approved under government registration numbers G 0072/06 and LVL M-V/TSD/7221.3-1.1-45/05 (with expansion LVL M-V/TSD/7221.3-1.1-37/06).

Materials and Methods

Animals

Fifteen juvenile female Gyr-Saker *(F. rusticolus* × *F. cherrug*) hybrid falcons were obtained from 1 breeder. The birds received an intensive health evaluation, which included a general examination, radiographs, laparoscopy, blood cell count, blood chemistry analysis, and parasitologic examination; all results were within normal limits. The falcons were perched according to standard falconry techniques during the vaccination trial (*22*). For challenge infection, the animals were kept individually in stainless steel cages located in negatively pressurized isolation rooms within Biosafety Level 3 facilities. Seven 1-day-old chicks obtained from a disease-free stock, were provided to each bird each day as feed. Unconsumed chicks were removed to measure the daily feed intake of each bird.

Vaccination

Ten falcons were vaccinated (nos. 1–5 intramuscularly and nos. 6–10 subcutaneously) with 0.5 mL (hemagglutinating titer >16) of influenza (H5N2) inactivated vaccine (Intervet, Unterschleissheim, Germany) based on strain A/duck/Potsdam/1402/86; they were revaccinated with the same dose and by the same route 4 weeks later. As a negative control, 5 nonvaccinated falcons were kept with the vaccinated birds.

Before the first vaccination and in weekly intervals until 8 weeks after initial vaccination, individual blood samples were collected from the metatarsalis plantaris superficialis medialis vein directly into a serum tube (Sarstedt, Nümbrecht, Germany) by using a 0.7-mm × 30-mm needle (Sterican, Luer-Lock, Braun Melsungen, Germany). The serum was separated and tested for H5-specific antibodies by the HI test, with low pathogenic avian influenza subtype H5N2 (A/duck/Potsdam/619/85) as antigen according to standardized methods (*23*). A cloacal swab was also examined for influenza A virus RNA by using real-time reverse transcription–PCR (RT-PCR) targeting an M gene fragment to exclude a concurrent infection (*24*).

Challenge Infection

Five months after the initial vaccination, 5 falcons randomly selected from the 10 vaccinated birds (nos. 1, 2, 5, 8, 9) and 5 nonvaccinated control birds (nos. 11–15) were challenged with $10^{6.0}$ 50% egg infectious dose (EID_{50}) of influenza strain A/*Cygnus cygnus*/Germany/R65/2006, a highly pathogenic H5N1 strain that was isolated from a dead whooper swan (*Cygnus cygnus*) during an outbreak of HPAI virus (H5N1) among wild birds in Germany (*25*). Each bird received 1 mL cell culture medium by the oculo-oronasal route. The falcons were observed daily for 11 days after challenge. At the end of the trial, surviving birds were humanely killed. A serum sample was obtained by using the above-described method just before challenge and, for surviving birds, on the last day of the trial. The serum was used for the detection of antibodies against H5 by the HI test (see above) by using 2 different antigens (challenge and vaccine strain). Before challenge and at days 1, 2, 4, 7, and 11 after challenge, an oropharyngeal and a cloacal swab were taken for a semiquantitative detection of avian influenza virus–specific RNA by using a real-time RT-PCR targeting an M gene fragment as recommended in the Diagnostic Manual for Avian Influenza issued by the European Commission (*26*) and described by Spackman et al. (*24*). The method has been improved by using an internal control in parallel in a duplex reaction (*27*). In addition, virus isolation in embryonated chicken eggs was attempted as described by Werner et al. (*28*). Isolated viruses were characterized as HPAI virus (H5N1) by subtype-specific real-time RT-PCRs (*26*) and by a pathotype-specific real-time RT-PCR (*29*).

Gross, Histopathologic, and Immunohistochemical Examinations

Necropsies were performed immediately after death. Samples of nasal cavity, trachea, lung, heart, cerebellum, cerebrum, spinal cord, proventriculus, small and large intestine, liver, pancreas, spleen, skin, and kidney were collected and either snap frozen or formalin fixed (48 h) and processed for paraffin embedding according to standardized procedures. For histopathologic examination, paraffin wax sections (3 μm) were dewaxed and stained with hematoxylin and eosin. Immunohistochemical examination for influenza virus A nucleoprotein (NP) was performed according to Klopfleisch et al. (*30*). Briefly, dewaxed sections were incubated with a rabbit anti-NP serum (1:500). As secondary antibody, biotinylated goat anti-rabbit IgG1 (Vector, Burlingame, CA, USA) was applied. By means of the avidin-biotin-peroxidase complex method, a bright red signal was produced. Positive and negative control tissues of chickens that had been infected experimentally with HPAI virus (H5N1) were included. Tissues from the central nervous system (CNS), small intestine, pancreas, trachea, and lung were used for real-time RT-PCR and for virus isolation.

Results

Immune Response

During the entire trial, control birds remained negative for avian influenza virus H5-specific antibodies. In addition, influenza A virus RNA was not detected in any of the cloacal swabs. No adverse clinical effects were detected as a result of application of the 2 vaccine doses.

Nine of the 10 vaccinated birds mounted homologous H5-specific antibodies 3 weeks after the first vaccination; titers increased significantly after the booster vaccination (Table 1). The remaining bird (no. 5) showed a detectable titer of 8 only 6 weeks after initial vaccination (2 weeks after booster vaccination); HI titer for this bird remained at 8. Differences in titer development according to route of vaccination were not detected (Table 1). Clinical signs (i.e., decreased food intake or worsening general condition) were not observed in any of the vaccinated birds.

Table 1. Titers (log_2) of hemagglutination-inhibiting antibodies against homologous influenza H5 antigen in 10 Gyr-Saker hybrid falcons after vaccination*

Falcon no., vaccination route	Titer at 0–8 weeks after vaccination								
	0†	1	2	3	4‡	5	6	7	8
1, IM	0	0	0	2	4	5	6	6	7
2, IM	0	0	0	2	3	4	6	8	9
3, IM	0	0	0	2	5	6	7	8	8
4, IM	0	0	0	3	4	4	6	7	6
5, IM	0	0	0	0	0	0	3	3	3
6, SC	0	0	0	3	4	5	6	8	7
7, SC	0	0	0	2	3	4	6	8	7
8, SC	0	0	0	3	3	4	5	6	7
9, SC	0	0	0	2	4	6	6	7	7
10, SC	0	0	0	3	4	5	6	6	6

*Vaccination with an inactivated influenza (H5N2) vaccine (strain A/duck/Potsdam/1402/86) at a dose of 0.5 mL containing >4 log_2 hemagglutinating units. IM, intramuscular; SC, subcutaneous.
†Time of first vaccination.
‡Time of booster vaccination.

HI titers against the heterologous challenge strain A/*Cygnus cygnus*/Germany/R65/2006 at the time of challenge are shown in Figure 1. The nonvaccinated birds remained seronegative.

Gross, Histopathologic, and Immunohistochemical Response to Challenge

All nonvaccinated birds died after challenge with HPAI virus (H5N1). The first falcon died on day 3 postchallenge, 3 died on day 4, and the rest died at day 5. Of these, 4 had reduced food intake starting from the day of challenge, and 3 had a slightly bloody tracheal exudate detectable the day after exposure. One bird died with no clinical signs.

All vaccinated birds survived. For 2, food intake was slightly reduced 1 day after challenge. No other vaccinated bird exhibited clinical signs. By 11 days after challenge, the titers of the vaccinated birds increased to 2,048 against the antigen used for vaccination and 1,024 against the challenge strain (Figure 1).

Necropsy showed multifocal acute hemorrhagic necrosis in the pancreas of 3 birds that died spontaneously and moderate to severe splenic hyperplasia in 3 birds. Histopathologic examination of the cerebellum, cerebrum, spinal cord, pancreas, spleen, and kidney of the nonvaccinated birds showed multifocal acute cellular degeneration and necrosis associated with minimal to mild infiltration of few heterophils and detection of HPAI virus antigen (Figure 2). Furthermore, antigen was present in the nasal cavity, trachea, bronchial epithelium, and gastrointestinal tract but not in the liver and skin. None of the euthanized vaccinated birds exhibited any gross or histologic lesions or presence of antigen in any of the tissues.

Virus Excretion after Challenge

In the nonvaccinated falcons, after challenge infection viral RNA was detectable in all oropharyngeal swabs. Virus was also isolated from the pooled oropharyngeal swabs of these birds taken on the same days (Figure 3; Table 2). At day 1 postchallenge, viral RNA was detected in the cloacal swabs of 3 birds, although virus isolation failed. From day 2 postchallenge, all falcons demonstrated the presence of viral RNA and infectious virus in cloacal swabs (Figure 3; Table 2).

In the vaccinated falcons, 1 day after challenge viral RNA was detectable in all oropharyngeal swabs. At day 2 postchallenge, 1 falcon became negative for viral RNA, and at days 7 and 11, only 1 bird remained positive for viral RNA (Figure 3; Table 2). Virus isolation from a pool of all oropharyngeal swabs of all 5 vaccinated birds taken from day 1 postchallenge demonstrated a virus titer of 4.4 $\log_{10}$ EID_{50}/mL at day 1 postchallenge and 1.2 $\log_{10}$ EID_{50}/mL at day 2 postchallenge. From day 4 on, virus could no longer be isolated from the pooled oropharyngeal swabs. Viral RNA was only occasionally detected in cloacal swabs and completely absent in 1 bird (Figure 3, Table 2). Virus could not be isolated from any of the pools of the cloacal swabs.

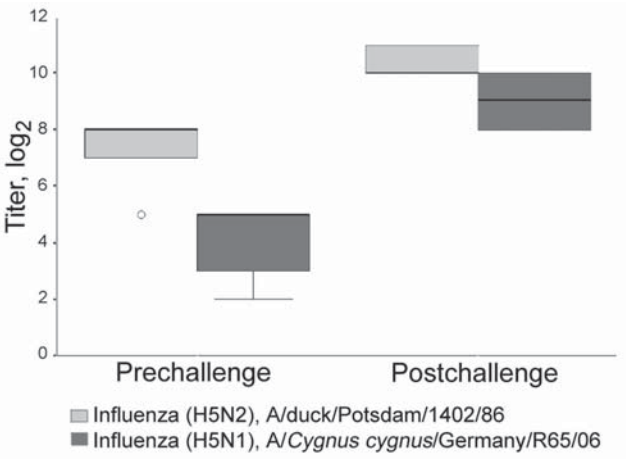

Figure 1. Titers ($\log_2$) of hemagglutination-inhibiting antibodies of 5 vaccinated Gyr-Saker hybrid falcons before and 11 days after challenge with $10^{6.0}$ 50% egg infectious doses of the highly pathogenic avian influenza strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1), tested against antigen of the challenge virus and the low pathogenicity avian influenza vaccine strain A/duck/Potsdam/1402/86 (H5N2). Open circle, individual outlier.

Virus in Tissues

In the nonvaccinated falcons, high loads of viral RNA were detected in the CNS, duodenum, pancreas, trachea, and lung of all control birds that died after challenge (Table 3). Virus isolation from these samples was not attempted.

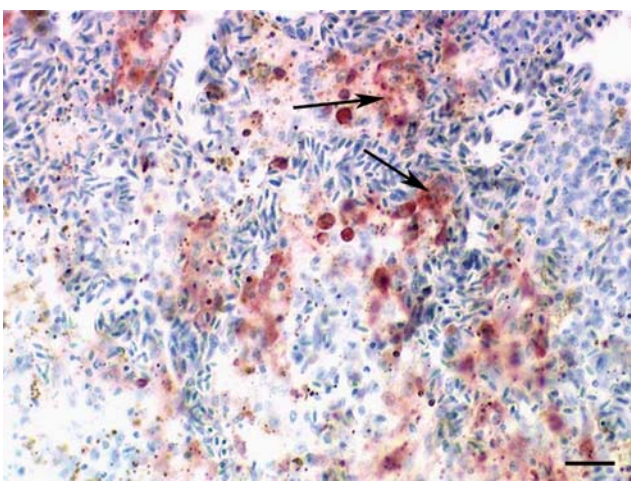

Figure 2. Immunohistochemical demonstration of influenza A virus antigen (red, see arrows) in numerous splenic macrophages of a falcon after challenge with $10^{6.0}$ 50% egg infectious doses of the highly pathogenic avian influenza strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1). Avidin-biotin-peroxidase complex method. Bar = 25 µm.

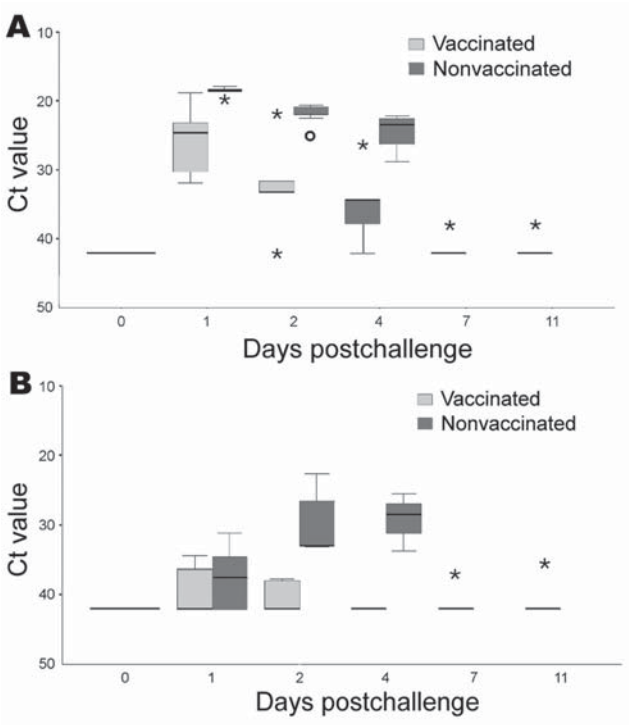

Figure 3. Detection of viral RNA by real-time reverse transcription–PCR (RT-PCR) from oropharyngeal (A) and cloacal (B) swabs of 5 vaccinated and 5 nonvaccinated falcons after challenge with $10^{6.0}$ 50% egg infectious doses of the highly pathogenic avian influenza virus strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1). Y axis shows cycle-of-threshold (Ct) values of real-time RT-PCRs detecting an M gene fragment in individual swab samples of each animal. Asterisks represent extreme values; open circles show individual outliers; black bars within boxes indicate medians.

In the vaccinated falcons, low to moderate loads of viral RNA were demonstrated in the brain and trachea of 3 birds that were euthanized on day 11 postchallenge, in the lung of 2 birds, in the duodenum of 1 bird, and in the pancreas of 1 bird. However, virus was isolated from the trachea of only 2 birds and from the lung of 1 (Table 3). The viral RNA load was as much as 6 $\log_{10}$ lower than that of nonvaccinated animals.

Discussion

Our study is the first, to our knowledge, to demonstrate that falcons are highly susceptible to HPAI virus (H5N1) as exemplified by strain A/*Cygnus cygnus*/Germany/R65/2006; all nonvaccinated birds died within 5 days after challenge. Clinical signs were mild and indicated only by a reduced food intake, which is not considered very obvious because falcons typically do not eat every day. These signs will not be seen in free-ranging birds and may be overlooked in captive animals. However, under natural conditions, more pronounced clinical signs may develop because stress situations and concurrent diseases are more likely than in captivity. Considering virus replication in the CNS, as demonstrated by immunohistochemical examination, CNS disturbances such as ataxia and disorientation might have ensued, although this is difficult to verify when birds are not allowed to fly.

The slightly bloody exudate from the trachea, noted for 3 birds at day 1 postchallenge, may pass unnoticed under field conditions. On the basis of the inconspicuous clinical signs, precisely defining the length of the incubation period is difficult. Gross lesions noted at necropsy were only mild and restricted to the pancreas and, thus, may be overlooked during routine necropsy when influenza is not suspected. The striking alterations of the pancreas are important as they were found macroscopically in 3 of the 5 birds and histopathologically in all 5 birds that died. Such lesions have also been described in mute (*C. olor*) and whooper swans (*C. cygnus*) (*31*), in passerines and budgerigars (*32*), and in emus and geese (*33*). The systemic virus distribution parallels that noted in water fowl during the 2006 outbreak on the Baltic Sea coast (*31*). Nevertheless, carnivorous birds, including buzzards, affected during an outbreak in Germany in 2006 displayed mainly a severe infection of the CNS without systemic virus distribution (unpub. data). The lack of antigen detection in the vaccinated falcons at day 11 postchallenge parallels the minimal virus shedding of the vaccinated falcons. Nevertheless, infection of cells at the site of inoculation can only be excluded by immunohistochemical examination of vaccinated animals during the first days after challenge.

All nonvaccinated falcons shed virus from the oropharynx and cloaca until death. Oropharyngeal shedding peaked at day 1 postchallenge, which might be related to reisolation of inoculum, and decreased toward day 4 postchallenge. The peak of cloacal excretion was at day 2 postchallenge, as reported for chickens (*14*). These findings demonstrate that after infection with influenza A (H5N1) of

Table 2. Excretion of infectious highly pathogenic avian influenza virus (H5N1) in vaccinated and control falcons after challenge

Vaccination status	Excretion route*	Days postchallenge†					
		0	1	2	4	7	11
Vaccinated	Oropharyngeal	<0.5	4.4	1.2	<0.5	<0.5	<0.5
Nonvaccinated	Oropharyngeal	<0.5	5.4	4.4	2.0	No data	No data
Vaccinated	Cloacal	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Nonvaccinated	Cloacal	<0.5	<0.5	3.0	2.7	No data	No data

*Pooled samples of all birds in the group were examined.
†Data represent $\log_{10}$ of 50% egg infectious dose per mL.

Table 3. Viral RNA in tissues of 5 vaccinated and 5 nonvaccinated falcons*

Falcon no.,vaccination route	RNA in tissue, ct value†				
	CNS	Duodenum	Pancreas	Trachea	Lung
Vaccinated					
1, IM	36.12‡	>40.00	38.84‡	>40.00	>40.00
2, IM	38.65‡	>40.00	>40.00	>40.00	>40.00
5, IM	34.22‡	36.14‡	>40.00	33.44‡§	36.94‡
8, SC	>40.00	IH	>40.00	38.71‡	32.85‡§
9, SC	>40.00	IH	IH	29.01§	IH
Nonvaccinated					
11	18.22	25.84	19.70	19.09	20.75
12	23.79	23.37	14.67	19.07	18.95
13	12.85	26.22	20.13	19.82	16.95
14	11.52	21.66	17.14	16.70	15.55
15	14.13	16.31	19.91	14.61	18.04

*Viral RNA detected by real-time reverse transcription–PCR (RT-PCR) in vaccinated falcons euthanized 11 days postchallenge and in nonvaccinated falcons that died after challenge infection with $10^{6.0}$ 50% egg infectious doses of highly pathogenic avian influenza virus strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1). Ct, cycle of threshold; CNS, central nervous system; IM, intramuscular; SC, subcutaneous; IH, inhibited (samples extracted twice with the QIAGEN [Hilden, Germany] Viral RNA kit or Trizol [Invitrogen, Carlsbad, CA, USA]).
†Real-time RT-PCR results are presented as ct values. Ct values >40 are scored as negative.
‡Virus isolation attempted in embryonated chicken eggs.
§Virus was isolated from individual tissue sample.

Asian origin, oropharyngeal swabs may be superior to cloacal swabs for diagnosing infection under field conditions. Duration of virus excretion before death was very short. Therefore, falcons may not play a major role in spreading the pathogen within or between countries, although this possibility cannot be excluded. Moreover, infected birds, like these falcons, may not be able to migrate long distances. However, because they shed a considerable amount of virus for a short time concomitant with virtual absence of overt clinical signs, captive infected falcons may pose a substantial risk for humans and other birds of high commercial and species conservation value. Therefore, measures to reduce this risk are of great importance, especially because depopulation of such birds is not a well-accepted option.

Vaccination of poultry, at least in experimental settings, can reduce virus shedding significantly after challenge, depending on the amount of antigen in the vaccine and the antigenic relationship between vaccine and virulent field virus (*13*,*14*,*34*,*35*). This study shows that vaccination is also an option in falcons. It is safe; no adverse clinical reactions were observed. High titers of specific HI antibodies were induced in most vaccinated animals and persisted for at least 5 months, which indicates that biannual revaccination may suffice. However, as in chickens, sterile immunity could not be induced as shown by continuous detection of virus excretion, particularly from the oropharynx, in vaccinated falcons after challenge infection. However, virus excretion was drastically reduced in vaccinated birds compared with nonvaccinated birds and could be detected only by sensitive real-time RT-PCR. With respect to the marked differences of virus excretion between vaccinated and nonvaccinated falcons, we note that a difference of approximately 3.3 cycle-of-threshold values corresponds to 1 $\log_{10}$ of viral nucleic acid copies (*36*). Figure 3A shows that in oropharyngeal swabs from nonvaccinated falcons, up to 3–4 $\log_{10}$ more viral RNA copies are present than in swabs from vaccinated falcons. The failure to isolate challenge virus from excretions of vaccinated falcons raises the question of the epidemiologic importance of the presence of viral RNA in oropharyngeal swabs (*13*,*14*). Therefore, vaccination is considered to be an important tool to prevent further major outbreaks (*34*). Additionally, the bird-to-human infection route of HPAI seems to require a high amount of excreted virus as well as close contact (*37*), which seems much more difficult to achieve with vaccinated birds. Although residual infectious virus persisted in organs of a few vaccinated birds until day 11 postchallenge, whether viral loads are sufficient for efficient transmission remains unclear. Because no viral RNA could be detected in the oropharyngeal swabs of 2 of these birds, this, however, appears to be unlikely.

In conclusion, we have demonstrated that falcons are highly susceptible to HPAI (H5N1) but can be protected from clinical disease and death by vaccination with a heterologous inactivated vaccine administered intramuscularly or subcutaneously. Virus shedding was grossly reduced after vaccination, thereby decreasing risk for further virus transmission to other avian species as well as to humans. However, use of vaccine will require the establishment of an appropriate surveillance program that includes use of serologic testing, PCR, and sentinel birds.

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Distribution of Eosinophilic Meningitis Cases Attributable to *Angiostrongylus cantonensis*, Hawaii

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During November 2004–January 2005, 5 cases of eosinophilic meningitis (EM) attributable to *Angiostrongylus cantonensis* infection were reported in Hawaii. To determine if this temporal clustering reflected an increased incidence, we ascertained EM and *A. cantonensis* cases by systematic review of statewide laboratory and medical records for January 2001–February 2005 and generalized the data to population estimates. We identified 83 EM cases; 24 (29%) were attributed to *A. cantonensis* infection, which was included in the discharge diagnoses for only 2 cases. Comparison of *A. cantonensis* infection incidence rates (per 100,000 person-years) for the baseline (January 2001–October 2004) and cluster (November 2004–February 2005) periods showed statistically significant increases for the state as a whole (0.3 vs. 2.1), the Big Island of Hawaii (1.1 vs. 7.4), and Maui County (0.4 vs. 4.3). These findings underscore the need to consider the diagnosis of *A. cantonensis* infection, especially in the state of Hawaii.

Eosinophilic meningitis (EM) is a rare clinical entity characterized by meningeal inflammation and eosinophilic pleocytosis in the cerebrospinal fluid (CSF) (*1–7*). Among the infectious causes of EM, *Angiostrongylus cantonensis* is the most common worldwide. *A. cantonensis*, the rat lungworm, was first described in rats in 1935, in Canton, China. The parasite was first postulated to cause human infection in a fatal case in 1944 in Taiwan and was confirmed to be pathogenic for humans through investigations in the early 1960s in Hawaii (*8–12*).

Most of the described cases of symptomatic *A. cantonensis* infection (neurologic angiostrongyliasis) have occurred in regions of Asia and the Pacific Rim (e.g., Taiwan, Thailand, and the Hawaiian and other Pacific Islands) (*4–19*). However, widespread geographic dispersal of *A. cantonensis* is ongoing, facilitated primarily by infected shipborne rats and the diversity of potential intermediate hosts (*9,20–27*). Intercontinental movement of rodent definitive hosts and accidental human hosts translates into the need for worldwide awareness of the association between EM and *A. cantonensis* infection.

Humans become infected by ingesting intermediate hosts, such as snails and slugs, or transport/paratenic hosts, such as freshwater crustaceans, that contain viable third-stage larvae (Figure 1). These larvae can migrate to the central nervous system (CNS) and cause EM (*6–34*). The exposure often is unrecognized and presumptive, such as through ingestion of contaminated produce. The incubation period averages ≈1–3 weeks but has ranged from 1 day to >6 weeks (*5–7,16–20,24,27,32–35*). Common clinical manifestations include headache, meningismus, and hyperesthesia, which usually resolve spontaneously with supportive care; severe cases can be associated with sequelae (e.g., paralysis and blindness) and death (*5,8,11,12,14–19,28,31,33–38*). The utility of anthelminthic and corticosteroid therapy remains controversial and may vary among *A. cantonensis*–endemic areas (*3,7,16–19,24,27–38*).

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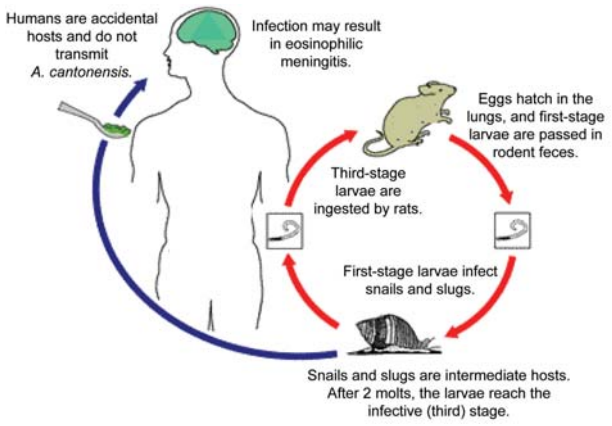

Figure 1. Life cycle of *Angiostrongylus cantonensis*. Source: www.dpd.cdc.gov/dpdx, a website developed and maintained by the Centers for Disease Control and Prevention.

Typically, symptomatic infection is presumptively diagnosed on the basis of epidemiologic and clinical criteria (*4,5,7,13*), as was done in this investigation. Parasitologic confirmation, by detection of larvae or young adult worms in the CSF, is unusual, albeit slightly more common in young children (particularly in Taiwan) (*5,7,13–19,32*). Investigational immunoassays for detection of antibodies to *A. cantonensis* antigens have not been sufficiently characterized or validated to be useful for distinguishing infected and uninfected persons, particularly in epidemiologic investigations (*3,5,27*).

During November 2004–January 2005, 1 parasitologically confirmed and 4 presumptive cases of *A. cantonensis* infection were reported to the Hawaii State Department of Health. The 5 cases included 3 from the Big Island of Hawaii and 2 from Oahu; 1 Oahu case was in a visitor to Hawaii whose lumbar puncture (LP) was performed elsewhere. Recognition of these 5 index cases prompted multifaceted investigations of epidemiologic, clinical, and environmental aspects of EM/*A. cantonensis* infection in Hawaii.

To assess whether the unusual temporal clustering of case reports reflected an increased incidence of EM/*A. cantonensis* infection, we ascertained cases through comprehensive review of statewide laboratory and medical records. Although investigations of EM/*A. cantonensis* infection in various Hawaiian Islands have been described since the 1960s (*4–6,9–13,20,30,35*), to our knowledge, this is the first study to systematically ascertain cases and determine regional incidence rates in this manner.

Methods

Ascertainment and Classification of Cases

Our primary means for ascertaining potential cases of EM and *A. cantonensis* infection was a retrospective review of CSF data provided by clinical laboratories in Hawaii for LPs performed during the study period (January 2001–February 2005). In March 2005, we obtained CSF data for 22 of 24 acute-care hospitals, which encompassed ≈93% of the state's hospital beds; for 1 of the 22 facilities (≈7% of beds), data were unavailable for January 2001–December 2002. The total numbers of patients and LPs during the study period were unavailable (e.g., some laboratories provided CSF data only if particular criteria were met). In January 2005, 1 case of EM/*A. cantonensis* infection in a visitor to Hawaii whose LP was performed elsewhere was ascertained by passive physician reporting to the Hawaii State Department of Health and the Centers for Disease

Table 1. Case definitions for eosinophilic meningitis (EM) and *Angiostrongylus cantonensis* infection, Hawaii, January 2001–February 2005

Diagnosis	Inclusionary criteria	Exclusionary criteria
EM	Had lumbar puncture (LP) during January 2001–February 2005*	Not in Hawaii during exposure period†
	Had cerebrospinal fluid (CSF) with both: ≥6 leukocytes per mm^3 Eosinophil percentage (of leukocyte count) or absolute eosinophil count ≥10	Had any of the following:‡ Leukocytes or eosinophils in CSF below inclusionary levels after adjusting for presence of erythrocytes Grossly bloody CSF Diagnosis or signs (e.g., CSF, radiologic) of intracranial hemorrhage
A. cantonensis infection	Met criteria for EM	Had intracranial hardware when LP was performed
	Met parasitologic or clinical criteria for *A. cantonensis* infection: Parasitologic: *A. cantonensis* larvae or young adult worms in CSF Clinical: manifestations compatible with *A. cantonensis* infection and including ≥2 symptoms/signs§	Was <2 mo of age when LP was performed Had been hospitalized from birth through time of LP Had another possible cause of EM identified

*If a patient had >1 LP, the LP considered in the analyses was the one that met criteria for EM and had the highest absolute eosinophil count.
†The exposure period was defined as the 45-d period before the symptom-onset date (if unknown, the date of the LP).
‡Potential cases of EM were excluded if the eosinophilic pleocytosis was potentially attributable to blood and thus was difficult to evaluate (e.g., traumatic LP, grossly bloody CSF, or intracranial hemorrhage). For CSF specimens with >500 erythrocytes/mm^3, the leukocyte and eosinophil criteria had to be met after using a correction ratio of a decrease of 1 leukocyte for every 500 erythrocytes.
§The symptoms and signs included headache, neck stiffness or nuchal rigidity, visual disturbance, photophobia or hyperacusis, cranial nerve abnormality (e.g., palsy), abnormal skin sensation (e.g., paresthesia, hyperesthesia), sensory deficit, nausea or vomiting, documented fever, increased irritability (if <4 y of age), and bulging fontanelle (if <18 mo of age).

Control and Prevention (CDC); this case was 1 of the 5 index cases that prompted the investigation.

Our case definitions for EM and *A. cantonensis* infection are provided in Table 1. If the inclusionary criteria for EM were met, we reviewed the patient's medical record to obtain additional information regarding the EM and to categorize cases of EM by known or likely cause (e.g., *A. cantonensis* infection). The information collected during chart review included basic demographic data, pertinent dates (e.g., birth, hospitalization, travel, symptom onset, and LP), medical history, medications, clinical manifestations, additional laboratory and radiologic results, and discharge diagnoses. Because the primary focus of the study was *A. cantonensis* infection, if, at the time of the LP, the patient had intracranial hardware (i.e., a well-established cause of EM) or was <2 months of age (i.e., angiostrongyliasis was epidemiologically unlikely), we collected only demographic data and discharge diagnoses.

We attributed cases of EM to *A. cantonensis* infection only if this diagnosis was epidemiologically and clinically plausible and no other possible cause of EM was identified. Examples of possible alternative causes included CNS infection with other microbes, reaction to foreign material in the CNS (e.g., intracranial hardware or myelography dye), medications (e.g., intrathecal vancomycin or gentamicin), neoplasms, multiple sclerosis, and neurosarcoidosis (*1*–*7*). The study neurologist (J.J.S.) facilitated final selection and classification of cases of EM and *A. cantonensis* infection by reviewing the available case data and ensuring that the inclusionary and exclusionary criteria were applied consistently and objectively.

Statistical Analysis and Human Subjects Protection

Data entry was performed with Epi Info version 2002 (CDC, Atlanta, GA, USA), and data analyses were conducted with SAS version 9.1 (SAS Institute, Cary, NC, USA). Two-tailed p values were calculated by using the Fisher exact test for binary variables and the Wilcoxon test for continuous variables. Linear and quadratic regression models were evaluated to assess whether eosinophilic pleocytosis varied with time (i.e., the interval from symptom onset to LP). We calculated incidence rates by generalizing hospital-based frequency data to the population at large for various periods and counties in Hawaii using the US Census Bureau's annual population estimates for 2001–2004 (the estimate for 2004 also was used for January and February 2005) (*39*). We used Poisson regression analyses to compare county-specific annual rates. We defined the 46-month period of January 2001–October 2004 as the baseline period and the 4-month period of November 2004–February 2005 as the cluster period. CDC's policies with regard to human study participants were followed in this investigation.

Results

We identified 83 cases of EM for the 50-month study period (January 2001–February 2005); <1% of the patients whose CSF data were reviewed fulfilled the case criteria (Table 1). The 83 cases included 70 (84%) during the 46-month baseline period (17–21 cases per year) and 13 (16%) during the 4-month cluster period. We attributed 24 (29%) of the 83 EM cases to *A. cantonensis* infection and 59 (71%) to other causes (Table 2). Thirty-five of these 59 cases (42% of 83) were in persons with intracranial hardware, and 9 (11% of 83) were in persons without intracranial hardware who had documented bacterial (n = 5) or viral (n = 4) meningoencephalitis.

The 24 cases of EM attributed to *A. cantonensis* infection included 1 parasitologically confirmed case in an 11-month-old child and 23 clinically defined cases (Table 2). EM was noted in the discharge diagnoses for 11 case-patients (46%). *A. cantonensis* infection, as well as EM, was listed for only 2 cases: the parasitologically confirmed case and 1 other case in January 2005. The 24 case-patients had a median age of 31 years (range 11 months–45 years), and 13 (54%) were male. Of the 13 patients for whom race/ethnicity data were available, 6 (46%) were Caucasian, 3 (23%) Filipino, 3 (23%) Hawaiian/part-Hawaiian, and 1 (8%) Samoan.

For the 22 case-patients with known symptom onset dates, the median interval from onset to LP was 3 days

Table 2. Classification of cases of eosinophilic meningitis (EM; n = 83) and *Angiostrongylus cantonensis* infection by cause or category, Hawaii, January 2001–February 2005

Cause or category	No. (%)
Cases attributed to causes other than *A. cantonensis* infection*	59 (71)
Presence of intracranial hardware	35 (42)
No intracranial hardware	24 (29)
Patient <2 mo of age	
No microbial etiologic agent identified	10
Bacterial meningitis†	3
Enteroviral meningoencephalitis	2
Patient ≥2 mo of age	
Streptococcal meningitis‡	2
Viral meningoencephalitis§	2
Presumptive viral encephalomyelitis	1
Encephalitis not otherwise specified	1
Suspected vertebral artery dissection	1
Cancer	1
Not otherwise specified¶	1
Cases attributed to *A. cantonensis* infection	24 (29)
Clinically defined	23
Parasitologically confirmed	1

*The 59 cases include 35 (42%) in patients with intracranial hardware and 24 (29%) in patients without intracranial hardware. All cases of EM in patients with intracranial hardware when the lumbar puncture was done were attributed to the hardware (Table 1), regardless of the reason for implantation. Two of the 35 such cases were in patients <2 mo of age.
†Etiologic agents were *Escherichia coli*, *Klebsiella* sp., and α-hemolytic *Streptococcus*.
‡Etiologic agents were *S. agalactiae* (group B *Streptococcus*) and *S. pneumoniae*, in 87-y-old and 5-mo-old patients, respectively.
§Etiologic agents were herpes simplex virus and an enterovirus, in 20-y-old and 3-mo-old patients, respectively.
¶Did not meet criteria for *A. cantonensis* infection (Table 1).

(range 0–48 days); the 2 longest intervals were 14 days (2 patients) and 48 days (1 patient). When a linear regression model was applied to data for the intervals ≤14 days, the longer the interval (between symptom onset and LP), the higher the CSF eosinophil percentage and absolute eosinophil count (p = 0.001 and 0.005, respectively). Compared with patients with other causes of EM, *A. cantonensis* case-patients had significantly higher CSF leukocyte counts (median 573/mm^3 vs. 304/mm^3, p = 0.03) and absolute eosinophil counts (median 120/mm^3 vs. 14/mm^3, p<0.001); they also tended to have higher eosinophil percentages (median 15.0% vs. 12.0%), but the difference was not statistically significant (p = 0.08).

The temporal distribution of the 24 cases included 15 (63%) during the baseline period (3–5 cases per year) and 9 (38%) during the cluster period (Figure 2). The mean number of *A. cantonensis* cases per month increased from 0.3 in the baseline period to 2.3 in the cluster period, whereas the mean monthly rates for cases of EM with other causes were essentially unchanged (1.2 and 1.0, respectively). Thus, the proportion of EM cases attributed to *A. cantonensis* increased from 21% (15/70) for the baseline period to 69% (9/13) for the cluster period. The *A. cantonensis* incidence rates for the state as a whole increased from 0.3 per 100,000 person-years in the baseline period to 2.1 in the cluster period (p<0.001) (Figure 2).

The geographic distribution of the 24 cases included 3 counties and 4 islands: Honolulu County (Oahu Island; n = 11 cases, including the case in the visitor), Hawaii County (Big Island of Hawaii; n = 9, including the parasitologically confirmed case), and Maui County (n = 4, including 3 cases associated with Maui Island and 1 with Lanai). Although the absolute number of cases was highest for Honolulu, the county-specific incidence rates (per 100,000 person-years) for the study period as a whole were higher for Hawaii (1.4) and Maui (0.7) than Honolulu (0.3) (Figure 3). The case-patients were significantly more likely to have been in Hawaii County than Honolulu County (risk ratio 4.6, 95% confidence interval 1.9–11.1); the comparison between Hawaii and Maui Counties was not significant (data not shown). The increases in annualized incidence rates (cases/100,000 person-years) from the baseline to the cluster periods were statistically significant for Hawaii County (1.1 vs. 7.4; p<0.001) and Maui County (0.4 vs. 4.3; p = 0.03) but not for Honolulu County (0.2 vs. 1.0; p = 0.07) (Figure 3).

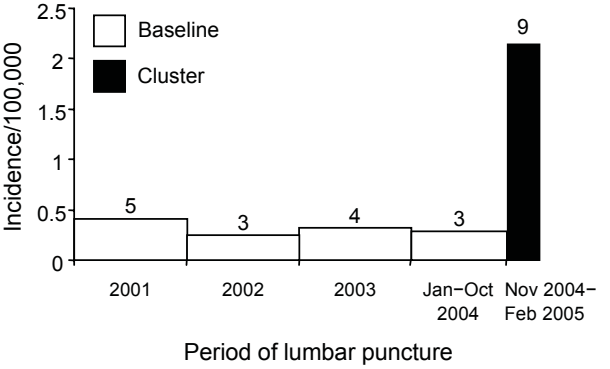

Figure 2. Incidence rates for cases of eosinophilic meningitis attributed to *Angiostrongylus cantonensis* infection, by period, Hawaii, January 2001–February 2005 (n = 24). The number over each bar indicates the number of cases during the period. The incidence rates (per 100,000 person-years) for the entire 50-month study period, the 46-month baseline period (January 2001–October 2004), and the 4-month cluster period were 0.5, 0.3, and 2.1, respectively.

Discussion

This study was prompted by an unusual temporal clustering of 5 reported cases of EM/*A. cantonensis* infection from 2 Hawaiian Islands during November 2004–January 2005. Our primary goal was to assess whether these voluntarily reported cases reflected an increased incidence. To accomplish this, we used a laboratory-and hospital-based approach to ascertain symptomatic cases of EM and *A. cantonensis* infection. To our knowledge, this is the first study to systematically determine incidence rates of EM and *A. cantonensis* infection for the entire state of Hawaii or any angiostrongyliasis-endemic area. We determined that the incidence of angiostrongyliasis was higher during the cluster period (November 2004–February 2005) than the baseline period (January 2001–October 2004). The overall findings of our study support conclusions specific for Hawaii but also highlight general principles about EM and *A. cantonensis* infection. In addition, our study may serve as a useful model in other settings. Surveillance of regional laboratory data, coupled with investigation of medical records of case-patients, may help identify temporal and geographic trends for angiostrongyliasis or other diseases.

Our data underscore that EM is an uncommon entity: <1% of the patients whose CSF data were reviewed fulfilled the laboratory criteria for EM. This diagnosis is commonly missed or dismissed, but the presence of eosinophilic pleocytosis is abnormal and should prompt consideration of both infectious and noninfectious causes. In our study, intracranial hardware was the most frequently identified cause of EM (42% of 83 cases). Although the presence of hardware or other foreign material in the CNS is a well-established cause of EM, the possibility of associated bacterial infection should be considered (*2,6*). In our study, EM also was associated with confirmed cases of bacterial and viral meningoencephalitis, as well as idiopathic cases (no microbial etiology identified) in infants evaluated because of fever or failure to thrive.

We found that a substantial proportion of the EM cases in Hawaii were attributable to *A. cantonensis* infection (29%) and that the proportion was 3-fold higher during the

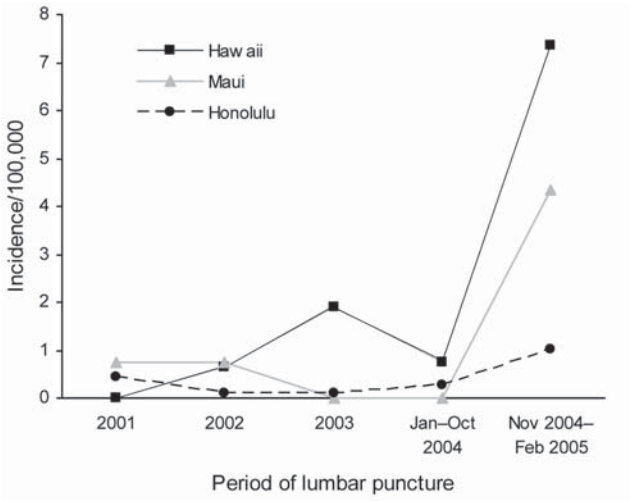

Figure 3. County-specific incidence rates per 100,000 person-years for cases of eosinophilic meningitis attributed to *Angiostrongylus cantonensis* infection, by period, Hawaii, January 2001–February 2005 (n = 24).

cluster than during the baseline period. This rate increase was particularly notable in Hawaii and Maui Counties. Despite the fact that 23 of the 24 cases were clinically defined, the likelihood of misclassification was low. By definition, none of the case-patients had another possible cause of EM identified. In most angiostrongyliasis-endemic areas, parasitologic confirmation is unusual, and a presumptive diagnosis is typical. Furthermore, Hawaii is hyperenzootic for infection with *A. cantonensis* but not *Gnathostoma spinigerum* or *Baylisascaris procyonis*, 2 other parasites commonly associated with EM. Our confidence that the *A. cantonensis* cases were correctly classified as such is further increased by the findings of other components of our multifaceted investigations, which included comprehensive epidemiologic and clinical characterization of patients, with longitudinal evaluation of clinical status and sequelae (N. Hochberg, unpub. data).

One of the limitations of our laboratory/hospital-based study is the likelihood that we underestimated the numbers of cases of EM and *A. cantonensis* infection. By definition, we did not include persons who were asymptomatic, were not medically evaluated, did not have an LP, did not have CSF data that met specified criteria for EM (e.g., if the LP was performed early or late in the course of infection, few eosinophils might have been noted), or did not meet conservative epidemiologic and clinical criteria. In addition, cases of EM/angiostrongyliasis that were associated with exposures in Hawaii but were diagnosed elsewhere were not systematically ascertained. Cases diagnosed after the end of the study period (February 2005) were not included (specifically, 2 cases reported in March and April 2005 that were associated with Hawaii County). Their existence, however, lends even more credence to the temporal clustering of cases in late 2004–early 2005.

A second limitation is that we cannot exclude the possibility that the temporal increases in frequency of cases were artifactual (e.g., reflected heightened awareness of *A. cantonensis* infection or decreased thresholds for performing LPs). However, the investigation was prompted by clustering of 5 voluntary case reports during November 2004–January 2005, when EM and *A. cantonensis* infection were not reportable conditions, and included a parasitologically confirmed case. In addition, for patients who accessed healthcare and had an LP, our methods for case ascertainment were not dependent upon clinicians considering or listing EM or *A. cantonensis* infection in discharge diagnoses. Our methods were systematic, statewide, and unbiased.

We recognize the limitations and the utility of the incidence data. We calculated incidence rates by generalizing relatively small numbers of cases to the population estimates for particular periods in the state and the pertinent counties. Adjusting the frequency data for the sizes of populations and the durations of periods facilitated comparisons between counties, periods, and causes of EM. The cases of EM not attributed to *A. cantonensis* served as a useful internal control for the conclusion that the incidence of angiostrongyliasis increased: the incidence of *A. cantonensis* infection was significantly higher during the cluster period, whereas the incidence of the other EM cases did not increase.

In conclusion, we demonstrated the utility of a comprehensive, laboratory/hospital-based approach for statewide surveillance of EM and *A. cantonensis* infection in Hawaii. We found a cluster of angiostrongyliasis cases between November 2004 through February 2005 primarily centered in Hawaii and Maui Counties. Furthermore, EM and *A. cantonensis* infection were often not included in the discharge diagnoses for the case-patients. Our study therefore underscores the need to educate clinicians in Hawaii and elsewhere about EM and its causes, most notably *A. cantonensis* infection, a potentially severe but preventable infection. Improved detection and reporting may facilitate recognition of clusters of cases and prompt investigations that yield valuable insights about the epidemiologic and clinical characteristics of *A. cantonensis* infection.

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Human *Salmonella* and Concurrent Decreased Susceptibility to Quinolones and Extended-Spectrum Cephalosporins

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The National Antimicrobial Resistance Monitoring System monitors susceptibility among Enterobacteriaceae in humans in the United States. We studied isolates exhibiting decreased susceptibility to quinolones (nalidixic acid MIC ≥32 μg/mL or ciprofloxacin MIC ≥0.12 μg/mL) and extended-spectrum cephalosporins (ceftiofur or ceftriaxone MIC ≥2 μg/mL) during 1996–2004. Of non-Typhi *Salmonella,* 0.19% (27/14,043) met these criteria: 11 Senftenberg; 6 Typhimurium; 3 Newport; 2 Enteridis; and 1 each Agona, Haifa, Mbandaka, Saintpaul, and Uganda. Twenty-six isolates had *gyrA* mutations (11 at codon 83 only, 3 at codon 87 only, 12 at both). All Senftenberg isolates had *parC* mutations (S80I and T57S); 6 others had the T57S mutation. The Mbandaka isolate contained *qnrB2*. Eight isolates contained bla_{CMY-2}; 1 Senftenberg contained bla_{CMY-23}. One Senftenberg and 1Typhimurium isolate contained bla_{SHV-12}; the Mbandaka isolate contained bla_{SHV-30}. Nine Senftenberg isolates contained bla_{OXA-1}; 1 contained bla_{OXA-9}. Further studies should address patient outcomes, risk factors, and resistance dissemination prevention strategies.

Although antimicrobial agents are not indicated for uncomplicated *Salmonella* infections, fluoroquinolones and extended-spectrum cephalosporins are potentially lifesaving treatments for extraintestinal infections (*1*). The National Antimicrobial Resistance Monitoring System (NARMS) has monitored antimicrobial drug resistance among enteric pathogens since 1996. NARMS has documented decreased susceptibility to each of these drug classes, in most instances among separate serotypes (*2*). Historically, decreased susceptibility to fluoroquinolones, which can be monitored by tracking resistance to nalidixic acid, has been noted among *Salmonella* serotypes (ser.) Typhi, Senftenberg, and Virchow (*2*,*3*). More recently, decreased susceptibility to fluoroquinolones has been noted among *Salmonella* ser. Enteritidis (*4*). Decreased fluoroquinolone susceptibility has also been seen among nalidixic acid–susceptible isolates (*5*). Extended-spectrum cephalosporin resistance was noted among 15 non-Typhi *Salmonella* NARMS isolates (including 12 ser. Typhimurium) during 1996–1998 (*6*). In all instances, extended-spectrum cephalosporin resistance was the result of bla_{CMY-2}, a class C plasmid-encoded *ampC* gene (*7*). In addition to conferring resistance or decreased susceptibility to extended-spectrum cephalosporins such as ceftiofur and ceftriaxone, this gene also confers resistance to ampicillin (AMP), amoxillin-clavulanate, cephalothin, and cefoxitin. This AmpC resistance phenotype has been seen in strains of *Salmonella* ser. Newport along with resistance to other drugs including chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. This MDRAmpC strain rose from 1% (1/77) of *Salmonella* ser. Newport submissions in 1998 to 25% (31/124) in 2001 (*4*). CMY β-lactamases are largely re-

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sponsible for extended-spectrum cephalosporin resistance among *Salmonella* ser. Newport, Typhimurium, and others isolated in North America (*6*,*8*).

Coresistance to fluoroquinolones and extended-spectrum cephalosporins would limit therapeutic options for *Salmonella* infections. Decreased susceptibility to both drug classes was identified in Thailand in 1993 (ser. Anatum, Derby, Enteritidis, Typhimurium, Weltevreden, and I 4,5,12:i:-) (*9*), the United Kingdom in 1998 (ser. Senftenberg, Typhimurium, and Virchow) (*10*), Belgium as early as 2001 (ser. Virchow) (*11*), India in 2002 (ser. Typhi) (*12*), the United States in 2002 (ser. Mbandaka) (*13*), France in 2003 (*11*), and Taiwan in 2004 (ser. Choleraesuis, Cairo, and Kaduna) (*14*). In the United States, 27 (4.6%) of 588 *Salmonella* ser. Typhimurium isolates (clinical and slaughter) obtained from food animals in 1999 were resistant to ceftiofur and nalidixic acid: 22 (81%) from turkeys, 4 (15%) from horses, and 1 (4%) from cattle (*15*).

To understand coresistance to both antimicrobial classes among *Salmonella* isolates obtained from humans in the United States, we studied the NARMS human collection from 1996 through 2004, looking for decreased susceptibility to quinolones and extended-spectrum cephalosporins. Information for some of the isolates has been presented elsewhere (*3*,*13*,*16*–*18*). We present the molecular epidemiology of this phenotype and mechanisms responsible for its decreased susceptibility.

Materials and Methods

Isolates and Antimicrobial Drug Susceptibility Testing

NARMS-participating state and local public health laboratories submitted non-Typhi *Salmonella* isolates to the Centers for Disease Control and Prevention (CDC) for antimicrobial susceptibility testing: every 10th isolate from 1996 through 2002 and every 20th isolate from 2003 to present. Serotypes were determined by the submitting laboratory and, for this study, were confirmed by the CDC National *Salmonella* Reference Laboratory according to the Kaufmann-White scheme as described (*19*). MICs were determined by using broth microdilution (Sensititre, Westlake, OH, USA). Isolates exhibiting an amikacin MIC >4 μg/mL were confirmed by Etest (ABBiodisk, Piscataway, NJ, USA). Criteria for decreased susceptibility to quinolones and extended-spectrum cephalosporins were as follows: MIC ≥32 μg/mL for nalidixic acid or ≥0.12 μg/mL for ciprofloxacin and ≥2 μg/mL for ceftiofur or ceftriaxone. Susceptibility testing was performed according to manufacturer's instructions by using control strains *Escherichia coli* ATCC25922 and ATCC35218, and *Klebsiella pneumoniae* ATCC700603 (for extended-spectrum β-lactamase [ESBL] confirmation only). When available, Clinical Laboratory Standards Institute (CLSI) guidelines were used for interpretation (*20*).

Isoelectric Focusing for β-Lactamases

The methods of Rasheed et al. were used with modification (*21*). Three-hour trypticase soy broth cultures (grown at 37°C with shaking at 300 rpm) were pelleted, resuspended in 0.2% sodium acetate to 5% of original culture volume, and freeze-thawed 4 times (dry ice/ethanol bath and 37°C water bath). Preparations were diluted 2-fold with distilled water and swirled occasionally on ice for 30 min. Supernatants were collected after centrifugation (30 min at 20,200× *g*), and 3–5-μL aliquots were resolved for 1.5 h on Ampholine PAGplate polyacrylamide gels, pH 3.5–9.5 (APBiotech**,** Piscataway, NJ, USA). Gels were stained with nitrocefin (500 μg/mL; Becton Dickinson, Franklin Lakes, NJ, USA). Isoelectric points (pIs) were estimated by comparison with the following standard β-lactamases: TEM-12 (pI 5.25), KPC-2 (pI 6.7), SHV-3 (pI 7.0), SHV-18 (pI 7.8), and MIR-1 (pI 8.4).

PCR Detection of Antimicrobial Drug Resistance Genes

Presence of *qnr* genes was determined by using PCR with primers QP1 and QP2 for *qnrA* (*22*), FQ1 and FQ2 for *qnrB* (*23*), and 5′-ATGGAAACCTACAATCATAC-3′ and 5′-AAAAACACCTCGACTTAAGT-3′ for *qnrS*. The *qnrB* allele was determined by amplification and sequencing with primers FQ1 and FQ2. Screening for *aac(6′)-Ib-cr* was performed as described (*24*). Primer pairs used for amplification of β-lactamase genes were: bla_{CMY} (5′-ATGATGAAAAAATCGTTATGC-3′) and (5′-TTGCAGCTTTTCAAGAATGCGC-3′) (*25*); bla_{OXA-1} (5′-AATGGCACCAGATTCAACTT-3′) and 5′-CTTGGCTTTTATGCTTGATG-3′) (*26*); bla_{TEM} (5′-TTC TTGAAGACGAAAGGGC-3′) and (5′-ACGCTCAGTG GAACGAAAAC-3′) (*27*); and bla_{SHV} (5′-GGTTATGCGT TATATTCGCC-3′) and (5′-TTAGCGTTGCCAGTGCTC-3′) (*28*) or at Lahey (5′-GCCGGGTTATTCTTATTTGTC-3′) and (5′-TCTTTCCGATGCCGCCGCCAG-3′) (*29*). bla_{CTX-M} genes were screened by using a multiplex PCR assay (*30*).

DNA Sequencing

Full-length sequences were obtained for β-lactamase genes. A 255-bp region covering the quinolone-resistant determining region (QRDR) of *gyrA* (Met52 to Leu137) was amplified by using primers gyrA1: 5′-CATGAACG-TATTGGGCAATG-3′ and gyrA2: 5′-AGATCGGCCAT-CAGTTCGTG-3′. QRDRs of *gyrB*, *parC*, and *parE* were amplified and sequenced by using previously described primers (*31*), except primers parCF (5′-ATCGTGCGTT GCCGTTTAT-3′) and parCR (5′-GCCGCTTTCGC CACTTC-3′) were used to enhance coverage of *parC*. Amplicons were sequenced by using ABI Big-Dye 3.1 chemistry and ABI 3730XL automated DNA sequencers (PE Bio-

systems, Foster City, CA, USA). Analysis was performed by using BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.htmL) or SeqMan software (DNAStar, Madison, WI, USA). QRDR sequences of *gyrA*, *gyrB*, *parC*, and *parE* were compared with those of *Salmonella* ser. Typhimurium LT2 (GenBank accession nos. AE008801, AE008878, AE008846, and AE008846, respectively).

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed as previously described (*32*). Isolates that produced indistinguishable patterns with *Xba*I (Roche Molecular Biochemicals, Indianapolis, IN, USA) were restricted with *Bln*I. Patterns were analyzed by using the BioNumerics version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) and compared by unweighted pair group method with averages by using the Dice coefficient with a 1.5% band position tolerance window. The DNA sequence and deduced amino acid sequence for the *Salmonella* ser. Senftenberg bla_{CMY-23} gene were assigned GenBank accession no. DQ463751.

Results

Decreased susceptibility to quinolones and extended-spectrum cephalosporins was first noted in NARMS data in 1997 and represented 0.19% (27/14,043) of non-Typhi *Salmonella* from 1996 through 2004 (Table 1). *Salmonella* ser. Senftenberg was the most frequent serotype (n = 11), followed by Typhimurium (n = 6), Newport (n = 3), and Enteritidis (n = 2). The phenotype was found in 9 different serotypes in 13 states (Table 2).

PFGE comparison by *Xba*I and, if applicable, *Bln*I restriction showed that 15/27 *Salmonella* isolates differed by ≥1 band. No indistinguishable patterns among different *Salmonella* serotypes were identified. Of the 3 ser. Newport isolates tested, 2 (AM15201 and AM21465) had indistinguishable *Xba*I patterns but different *Bln*I patterns (87.51% similarity). The 2 Enteritidis isolates (AM09124 and AM15266) were indistinguishable by both enzymes. Of the 11 ser. Senftenberg isolates, 5 exhibited unique *Xba*I PFGE patterns, while the remaining 6 were separated into 2 groups with indistinguishable *Xba*I PFGE patterns (group 1: AM06960, AM08081, AM16094, and AM19422; group 2: AM20227 and AM20256). *Bln*I restriction demonstrated that AM19422 differed from the other group 1 isolates by a single band difference (97.44% similarity). PFGE results for some of the Senftenberg isolates are described elsewhere (*16*,*18*). All Typhimurium isolates exhibited unique *Xba*I PFGE patterns (77%–93% similarity).

Antimicrobial drug susceptibility results are presented in Table 3. For nalidixic acid, 25 isolates exhibited an MIC >32 μg/mL, and 2 (Mbandaka and Newport) had an MIC of 16 μg/mL. For ciprofloxacin, MICs of 0.12–0.5 μg/mL were found for all isolates except the 11 Senftenberg, for which the MIC was >4 μg/mL. For ceftiofur, 14 isolates exhibited resistance (MIC ≥8 μg/mL). For ceftriaxone, 2 isolates exhibited resistance according to the current CLSI breakpoint (64 μg/mL), and 7 exhibited intermediate resistance (MIC 16 or 32 μg/mL). Three isolates (Mbandaka, Senftenberg, and Typhimurium) exhibited an ESBL phenotype according to ceftazidime and cefotaxime MIC alone and with clavulanate. Seven isolates exhibited the MDRAmpC phenotype, including 1 Agona, 2 Newport, 3 Typhimurium, and 1 Uganda. According to current CLSI guidelines, 1 isolate (ser. Senftenberg) was fully resistant to ciprofloxacin, ceftriaxone, ceftazidime, and cefotaxime.

The mechanisms responsible for resistance and decreased susceptibility are shown in Table 4. Some mechanisms for some of the isolates are presented elsewhere (*3*,*17*,*18*). At least 1 *gyrA* mutation was found in 26 of 27 isolates. A *gyrA* mutation at codon 83 only was found for 11 isolates; a mutation at codon 87 only was found for 3; mutations at both codons were found for 12. No functional mutations were detected in *gyrB* or *parE* genes. All Senftenberg isolates had *parC* mutations (S80I and T57S), and 6 other isolates had the T57S mutation. In addition to the T57S mutation in *parC*, the Mbandaka isolate contained a

Table 1. NARMS non-Typhi *Salmonella* serotypes with decreased susceptibility to quinolones and extended-spectrum cephalosporins, United States, 1996–2004*

Year	No. that met MIC criteria/total tested (%)	Serotype				
		Senftenberg	Typhimurium	Newport	Enteritidis	Other (no.)
1996	0/1,324 (0)					
1997	1/1,301 (0.08)		1			
1998	1/1,460 (0.07)			1		
1999	1/1,497 (0.07)	1				
2000	4/1,377 (0.29)	2	1		1	
2001	4/1,419 (0.28)	2	1			Haifa (1)
2002	5/2,008 (0.25)	1	2	1		Mbandaka (1)
2003	4/1,864 (0.21)	2			1	Agona (1)
2004	7/1,793 (0.39)	3	1	1		Saintpaul (1), Uganda (1)
Total	27/14,043 (0.19)	11	6	3	2	5

*NARMS, National Antimicrobial Resistance Monitoring System. Reduced susceptibility to quinolones and extended-spectrum cephalosporins defined as MIC ≥32 μg/mL for nalidixic acid or ≥0.12 μg/mL for ciprofloxacin and ≥2 μg/mL for ceftiofur or ≥2 μg/mL for ceftriaxone.

Table 2. Isolate, year reported, state, and serotype for NARMS non-Typhi *Salmonella* isolates with decreased susceptibility to quinolones and extended-spectrum cephalosporins, United States, 1996–2004*

Isolate	Year	State	Serotype
AM18280	2003	TX	Agona
AM09124	2000	CA	Enteritidis
AM15266	2003	IL	Enteritidis
AM12389	2001	NJ	Haifa
AM15010	2002	NY	Mbandaka
AM03005	1998	NY	Newport
AM15201	2002	ME	Newport
AM21465	2004	GA	Newport
AM20428	2004	GA	Saintpaul
AM06960	1999	FL	Senftenberg
AM08081	2000	FL	Senftenberg
AM08208	2000	GA	Senftenberg
AM09864	2001	FL	Senftenberg
AM11007	2001	MA	Senftenberg
AM14058	2002	TX	Senftenberg
AM16094	2003	FL	Senftenberg
AM18622	2003	FL	Senftenberg
AM19422	2004	FL	Senftenberg
AM20227	2004	GA	Senftenberg
AM20256	2004	FL	Senftenberg
AM02544	1997	MN	Typhimurium
AM08739	2000	KS	Typhimurium
AM11682	2001	NY	Typhimurium
AM14364	2002	WI	Typhimurium
AM14807	2002	NY	Typhimurium
AM20205	2004	PA	Typhimurium
AM19537	2004	CA	Uganda

*NARMS, National Antimicrobial Resistance Monitoring System.

plasmid-mediated *qnrB2* gene and has been described (*13*). Four isolates contained *aac(6')-Ib*, but none contained the ciprofloxacin-modifying *aac(6')-Ib-cr* variant.

Nine AmpC phenotype isolates produced β-lactamase with a pI ≥8.4 (Table 4); 8 contained bla_{CMY-2}, but the Senftenberg strain contained a bla_{CMY-23} gene (GenBank accession no. DQ463751) identical to that found in an *E. coli* isolate (GenBank accession no. DQ438952). This gene differs from bla_{CMY-2} by 1 amino acid. Three of the bla_{CMY}-positive isolates, including the strain positive for bla_{CMY-23}, also contained bla_{TEM-1b}. The Mbandaka isolate was positive for bla_{SHV-30} with pI 7.0 (*33*) and also produced an enzyme with a pI 7.6, the nature of which is still under study. Two isolates (1 Senftenberg and 1 Typhimurium) contained bla_{SHV-12}, and both also contained bla_{OXA} and bla_{TEM-1} genes. Of the 11 Senftenberg isolates, 10 contained bla_{OXA-1} (n = 9) or bla_{OXA-9} (n = 1). No isolates contained bla_{CTX-M} genes.

Discussion

Fluoroquinolone and extended-spectrum cephalosporin coresistance is rare; however, the appearance of this phenotype in 2 commonly isolated serotypes from humans (Typhimurium and Newport) is concerning. Sporadic infections are alarming, but if clonal expansion of an isolate with this phenotype were to take place, as occurred with *Salmonella* ser. Typhimurium DT104 and Newport-MDRAmpC, the clinical consequences could be dramatic. Statistically significant increases in resistance to nalidixic acid (odds ratio [OR] 6.7, 95% confidence interval [CI] CI 2.6–17.7) and ceftiofur (OR 43.2, 95% CI 10.5–177.4) have been documented among non-Typhi *Salmonella* of human origin submitted to NARMS during 1996–2003 (*4*). Of 202 nalidixic acid–resistant non-Typhi *Salmonella* collected by NARMS during 1996–2003, most were ser. Enteritidis (31%) or Typhimurium (10%). Most of the 324 ceftiofur-resistant non-Typhi *Salmonella* collected by NARMS during the same time period were ser. Newport (56%) or Typhimurium (23%). A slightly broader geographic representation can be found in the SENTRY surveillance project, which analyzed 786 *Salmonella* isolates (blood and stool) from medical facilities in Latin America and North America (including Canada) during 2001–2003 (*8*). Of these, 11% were resistant to nalidixic acid, and 2% exhibited decreased susceptibility to ceftazidime, ceftriaxone, or aztreonam.

Extended-spectrum cephalosporin-resistant Newport and Typhimurium isolates are typically obtained from community-acquired infections. Newport-MDRAmpC infections have been associated with consumption of contaminated beef and unpasteurized dairy products (*34*). *Salmonella* containing bla_{CMY} genes have been isolated from ground chicken (Typhimurium DT208), turkey (Agona), and beef (Agona) purchased from retail outlets in the Washington DC area (*35*). In addition, cattle, chickens, turkeys, pigs, horses, and dogs have all been sources of bla_{CMY}-containing *Salmonella,* including common serotypes such as Typhimurium, Newport, and Heidelberg (*26*,*36*,*37*). Decreased susceptibility to fluoroquinolones among *Salmonella* serotypes that typically carry bla_{CMY} genes warrants exploration of factors that could select for decreased susceptibility to fluoroquinolones in animal reservoirs and in the human host.

PFGE showed diversity within some serotypes and indistinguishable strains within others. PFGE diversity among 2 serotypes commonly associated with extended-spectrum cephalosporin resistance (Newport and Typhimurium) is not surprising, given that CMY-producing strains have been seen at least since the late 1990s. Isolates of ser. Enteritidis are highly clonal; therefore, PFGE-indistinguishable patterns among isolates with no apparent epidemiologic link are not unusual. All PFGE-indistinguishable Senftenberg isolates from group 1 were isolated in the same state. Results for the Florida Senftenberg isolates are described elsewhere (*16*,*18*).

Salmonella ser. Senftenberg exhibiting decreased susceptibility to fluoroquinolones has been associated with

nosocomial infections in healthcare facilities in the United States (*18*). All 11 isolates contained identical *gyrA* mutations (S83Y and D87G) and *parC* mutations (T57S and S80I). These *parC* mutations have been identified in several *Salmonella* serotypes including Senftenberg (*38*). Ten Senftenberg isolates included in this study contained bla_{OXA} genes; the bla_{OXA}-negative Senftenberg strain contained a bla_{CMY-23} mechanism of extended-spectrum cephalosporin resistance. Acquisition of a bla_{CMY} gene by a traditionally nalidixic acid–resistant serotype warrants further epidemiologic and laboratory investigation. The bla_{OXA-1} gene has been identified in *Salmonella* ser. Typhimurium and is

Table 3. Susceptibility results for NARMS non-Typhi *Salmonella* isolates with decreased susceptibility to quinolones and extended-spectrum cephalosporins, United States, 1996–2004*

	Resistance								
Isolate	NAL	CIP	XNL	CRO	TAZ	TAZ/CLAV	FOT	FOT/CLAV	Other†
AM18280	>32	0.25	>8	16	32	16/4	16	8/4	AMP, AMC, CHL, FOX, KAN, STR, SUL, SXT, TET
AM09124	>32	0.5	2	≤0.25	0.5	0.25/4	0.25	0.12/4	ND
AM15266	>32	0.5	2	≤0.25	0.5	0.25/4	0.25	0.12/4	(CHL)
AM12389	>32	0.5	2	≤0.25	0.25	0.25/4	0.1	0.12/4	(CHL), SUL, SXT, TET
AM15010	16	0.25	8	8	64	0.5/4	4	0.25/4	AMP, (CHL), SUL, SXT
AM03005	16	0.25	2	0.5	0.25	0.12/4	0.12	≤0.06/4	AMP, AMC, CHL, FOX, (GEN), KAN, STR, SUL, SXT
AM15201	>32	0.12	>8	8	16	16/4	16	8/4	AMP, AMC, CHL, FOX, STR, SUL, TET
AM21465	>32	0.12	>8	16	16	16/4	8	8/4	AMP, AMC, CHL, FOX, STR, SUL, TET
AM20428	>32	0.5	2	≤0.25	0.5	0.5/4	0.5	0.12/4	(CHL), (FOX)
AM06960	>32	>4	8	8	0.5	0.25/4	1	0.12/4	AMP, AMC, (CHL), GEN, KAN, STR, SUL, SXT
AM08081	>32	>4	4	0.5	0.5	0.25/4	1	0.12/4	AMP, AMC, CHL, FOX, (GEN), KAN, STR, SUL, SXT
AM08208	>32	>4	2	≤0.25	0.5	0.25/4	0.5	0.25/4	AMP, (AMC) CHL, GEN, KAN, STR, SUL, SXT, TET
AM09864	>32	>4	8	8	64	0.25/4	8	0.25/4	AMP, (CHL), (FOX), GEN, KAN
AM11007	>32	>4	4	0.5	1	0.5/4	1	0.5/4	AMP, AMC, CHL, (FOX), KAN, SUL
AM14058	>32	>4	>8	>64	64	64/4	128	>64/4	(AMI), AMP, AMC, CHL, FOX, KAN, SUL
AM16094	>32	>4	4	≤0.25	0.5	0.25/4	1	0.25/4	AMP, (AMC), CHL, (FOX), (GEN), KAN, SUL, SXT
AM18622	>32	>4	8	1	2	0.5/4	4	0.5/4	AMP, AMC, CHL, KAN, STR, SUL, SXT
AM19422	>32	>4	4	≤0.25	0.5	0.5/4	2	0.25/4	AMP, AMC, (GEN), KAN, STR, SUL, SXT
AM20227	>32	>4	2	≤0.25	1	2/4	1	0.5/4	AMP, AMC, (CHL), (FOX), GEN, KAN, STR, SUL, SXT
AM20256	>32	>4	4	≤0.25	0.5	0.5/4	1	0.25/4	AMP, (AMC), (CHL), (GEN), KAN, SUL, SXT
AM02544	256	0.25	>16	64	128	0.5/4	32	0.12/4	AMP, (AMC), KAN, STR, SUL, TET
AM08739	>32	0.25	>8	32	16	16/4	16	8/4	AMP, AMC, CHL, FOX, GEN, KAN, STR, SUL, TET
AM11682	>32	0.25	>8	16	16	8/4	16	8/4	AMP, AMC, FOX
AM14364	>32	0.25	>8	32	32	16/4	32	16/4	AMP, AMC, CHL, FOX, GEN, KAN, STR, SUL, TET
AM14807	>32	0.25	>8	16	32	16/4	16	32/4	AMP, AMC, CHL, FOX, STR, SUL, TET
AM20205	>32	0.25	2	≤0.25	0.5	0.5/4	0.25	0.25/4	AMP, KAN, STR, SUL, TET
AM19537	>32	0.12	>8	16	16	16/4	8	8/4	AMP, AMC, CHL, FOX, (GEN), (KAN), STR, SUL TET

*NARMS, National Antimicrobial Resistance Monitoring System; NAL, nalidixic acid; CIP, ciprofloxacin; XNL, ceftiofur; CRO, ceftriaxone; TAZ, ceftazidime; TAZ/CLAV, ceftazidime/clavulanate; FOT, cefotaxime, FOT/CLAV, cefotaxime/clavulanate; AMP, ampicillin; AMC, amoxicillin/clavulanate; CHL, chloramphenicol; FOX, cefoxitin; KAN, kanamycin; STR, streptomycin; SUL, sulfamethoxazole or sulfisoxazole; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; AMI, amikacin; GEN, gentamicin.
†Drugs in parentheses had intermediate results.

Table 4. Resistance mechanisms among NARMS non-Typhi *Salmonella* with decreased susceptibility to quinolones and extended-spectrum cephalosporins, United States, 1996–2004*

Isolate	*gyrA* codon 83 change	*gyrA* codon 87 change	*parC* codon 57 change	*parC* codon 80 change	β-Lactamase isoelectric points	β-Lactamase genes
AM18280	S83Y	WT	T57S	WT	≥8.4	bla_{CMY-2}
AM09124	S83F	WT	WT	WT	ND	ND
AM15266	WT	D87Y	WT	WT	ND	ND
AM12389	S83Y	WT	WT	WT	ND	ND
AM15010	WT	WT	T57S	WT	7.0, 7.6†	bla_{SHV-30}
AM03005	S83Y	D87G	T57S	WT	ND	ND
AM15201	S83F	WT	T57S	WT	≥8.4	bla_{CMY-2}
AM21465	S83F	WT	T57S	WT	≥8.4	bla_{CMY-2}
AM20428	S83F	WT	WT	WT	5.4	bla_{TEM-1b}
AM06960	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM08081	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM08208	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM09864	S83Y	D87G	T57S	S80I	5.3, 6.9, 8.0	bla_{TEM-1}, bla_{OXA-9}, bla_{SHV-12}
AM11007	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM14058	S83Y	D87G	T57S	S80I	5.4, ≥8.4	bla_{TEM-1b}, bla_{CMY-23}
AM16094	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM18622	S83Y	D87G	T57S	S80I	7.4, 7.8	bla_{OXA-1}
AM19422	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM20227	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM20256	S83Y	D87G	T57S	S80I	7.4	bla_{OXA-1}
AM02544	WT	D87N	WT	WT	5.4, 8.0	bla_{TEM-1}, bla_{OXA-1}, bla_{SHV-12}
AM08739	S83Y	WT	WT	WT	5.4, ≥8.4	bla_{TEM-1b}, bla_{CMY-2}
AM11682	S83F	WT	WT	WT	≥8.4	bla_{CMY-2}
AM14364	S83Y	WT	WT	WT	5.4, ≥8.4	bla_{TEM-1b}, bla_{CMY-2}
AM14807	S83Y	WT	WT	WT	≥8.4	bla_{CMY-2}
AM20205	WT	D87N	WT	WT	5.4	bla_{TEM-1b}
AM19537	S83Y	WT	T57S	WT	≥8.4	bla_{CMY-2}

*NARMS, National Antimicrobial Resistance Monitoring System; WT, wild type; ND, none detected.
†Gene responsible not yet identified.

reported to be carried by an integron (*39*); bla_{OXA-9} has been associated with Tn1331 (*40*).

The epidemiology of *Salmonella* with decreased susceptibility to fluoroquinolones is relatively well characterized, as is that of *Salmonella* with bla_{CMY}-mediated extended-spectrum cephalosporin resistance. Conversely, little is known about the events leading to quinolone and extended-spectrum cephalosporin coresistance and the epidemiology of these infections in humans. Patients with *Salmonella* infections who exhibit decreased susceptibility to both antimicrobial drug classes should be interviewed to determine risk factors and the effects of antimicrobial drugs and other potential selective factors on this phenomenon.

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Dr Whichard is a researcher with NARMS at CDC. Her interests include β-lactamases, multidrug-resistant *Salmonella* isolates, bacteriophages, and other mobile genetic elements.

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Non-A Hepatitis B Virus Genotypes in Antenatal Clinics, United Kingdom

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In the United Kingdom, the National Screening Programme for identification of hepatitits B virus (HBV) infection in pregnant women uses HBV e antigen (HBeAg) and antibody to HBeAg (anti-HBe) as markers of infectivity to determine use of immunoglobulin for hepatitis B. Serum samples from 114 HBV-infected women were analyzed. Viral loads correlated with HBeAg/anti-HBe status and viral genotypes. Among 95 mothers whose serum contained anti-HBe, viral loads ranged between undetectable and 8.6×10^6 IU/mL (median 228 IU/mL). Ten (10.5%) of these mothers had plasma viral loads $>10^4$ IU/mL; 6 were infected with genotype E and one each with genotypes A, B, C, and D. All viruses had precore stop codon or basal core promoter mutations. Preponderance of genotypes other than A among antenatal mothers in the United Kingdom reflects increasing globalization and trends in immigration. HBeAg serostatus is no longer sufficiently accurate for inferring potential infectivity of pregnant HBV carriers.

Hepatitis B virus (HBV) infection remains a major health problem worldwide and mother-to-infant transmission represents one of the most efficient ways of maintaining hepatitis B carriage in any population. Intervention to prevent this route of infection is a key part of the global program of hepatitis B control. Although there are 3 routes of transmission of HBV from infected mothers to their infants, including transplacental and postnatal, most transmission is likely to occur perinatally at the time of labor and delivery (*1*). HBV e antigen (HBeAg) in maternal serum is associated with high infectivity; in the absence of intervention after delivery, including both passive and active immunization, 90% of babies born to carrier mothers whose serum contains HBeAg will become chronically infected with HBV (*2*,*3*). Babies born to mothers whose serum contains antibody to HBeAg (anti-HBe) become infected far less frequently (*4*). However, babies who are infected may be at risk of developing fulminant hepatitis B (*2*).

The prevalence of HBV infection in the United Kingdom is low (0.4%) (*5*). In the late 1990s, the World Health Organization (WHO) recommended introduction of global universal hepatitis B immunization programs (*6*); by March 2002, a total of 151 countries, including 34 in Europe, had introduced HBV vaccine within their national immunization programs. However, current control of mother-to-infant HBV transmission in the United Kingdom is based on selective hepatitis B immunization of infants at risk. A recent WHO survey in Europe indicated that 8 other countries also used this approach (*7*). This requires routine antenatal screening for HBV infection (*8*,*9*), offered by 34 countries in Europe, with infants born to all hepatitis B–infected mothers being offered immediate postnatal active immunization with hepatitis B vaccine. In the United Kingdom, babies at highest risk for infection, those born to mothers whose serum does not contain anti-HBe, are offered additional passive immunization prophylaxis (*10*) with 200 IU of hepatitis B immunoglobulin (HBIg) within 24 hours of delivery. In this protocol, detection of anti-HBe is used to infer low infectivity.

Despite full prophylaxis for neonates, a small proportion of infants still become persistently infected (*11*–*13*) and are at risk of developing sequelae of chronic HBV infection and increasing the HBV reservoir. Although the causes for these failures could be many, we noted that in management of HBV–infected healthcare workers, inference of infectivity is now based upon plasma viral load for HBV rather than HBe markers. Until 2001 in the United Kingdom, fitness of an HBV-infected healthcare worker to undertake in-

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vasive procedures was predicated upon absence of HBeAg, a protocol that was found to enable transmission to patients (*14*). All transmission involved infections by viruses with the pre-core premature stop codons, which reflected changes in viral genotypes caused by increased migration in UK healthcare workers. To investigate potential inappropriate categorization of infection risk through continued use of HBe markers in the antenatal setting, we undertook a study to relate HBe markers to HBV DNA levels and genotypes as predictors of potential infectivity.

Patients and Methods

Patients

As part of routine antenatal care, screening for HBV infection is offered to all pregnant mothers at the University College London Hospital. Pregnant HBV carriers who came to the hospital from September 1989 through September 2004 were identified. Serum samples from 114 HBV-infected mothers were available for further testing. Ethnic origin of mothers was not recorded.

Serologic Tests

Serum was separated and stored at –20°C in the Department of Virology, University College London Hospital, in accordance with laboratory policy to archive samples from carriers because of the long incubation time to clinical expression of HBV-related chronic liver disease. Samples would have been tested at initial collection for HBsAg by using a range of commercial assays and had reactivity confirmed by neutralization tests. Further testing for HBeAg, anti-HBe, antibody to hepatitis B virus core antigen (anti-HBc), and immunoglobulin M to HBc would have been performed routinely to determine the need for HBIg and confirm carrier status.

Quantitative PCR and Sequencing

Viral load for HBV DNA was measured as described (*15*). Briefly, HBV DNA was extracted from serum by using the QIAamp Virus BioRobot 9604 and QIAamp96 Virus Kit reagents (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. Twenty microliters of extract was used for input into a Taqman-based assay for HBV DNA in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Serum samples containing >100 IU/mL of viral DNA were selected for sequencing. Five microliters of extract was used for nested amplification of the entire virus surface antigen gene as described (*16*). We amplified precore and basal core promoter (BCP) regions of HBV DNA from anti-HBe–positive serum samples that contained $\geq 10^4$ IU/mL of HBV DNA. Briefly, 5 μL of extracted HBV DNA was amplified by using primers H4072, 5′-TCTTGCCCAAGGTCTTACAT-3′, and C outer (outer antisense), 5′-TCCCACCTTATGAGTCCAAG-3′, in the first round and primers H4072 (primer sequence as above) and C inner, 5′-CAGCGAGGCGAGGGAGTTCTTCTT-3′, in the second round. Conditions for amplification were the same for both rounds: 94°C for 4 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. Amplicons were sequenced with CEQ 8000 Genetic Analysis Systems (Beckman Coulter, Fullerton, CA, USA) in accordance with the manufacturer's instructions.

Generated nucleotide sequences were assembled and analyzed by using the SeqMan program (DNASTAR Inc., Madison, WI, USA). Alignments of nucleotide sequences were conducted to determine phylogenetic relationships between different isolates of HBV by using the MegAlign program (DNASTAR, Inc.). Data were used to construct a phylogenetic tree. Further analysis was also conducted with HBV STAR analysis, which assigns HBV genotypes by using a position-specific scoring matrix (www.vgb.ucl.ac.uk/star.shtml). Statistical significance was determined by using the Fisher exact test in the Arcus Quickstat package (www.camcode.com/arcus.htm).

Results

Thirteen (11.4%) of 114 HBsAg-positive serum samples contained detectable HBeAg, 95 (83.3%) contained anti-HBe, and 6 (5.3%) did not contain HBeAg or anti-HBe. HBIg had been recommended only for babies born to 13 mothers whose serum contained HBeAg and to 6 mothers whose serum did not contain HBeAg or anti-HBe.

HBV DNA, HBeAg, Anti-HBe, and Genotypes

HBV DNA was detected in 96 (84%) serum samples (Figure). The 13 HBeAg-positive serum samples had viral loads that ranged from 7.8×10^5 to 1×10^8 IU/mL (median 2.2×10^7 IU/mL). In 95 samples positive for anti-HBe, viral loads ranged from undetectable to 8.6×10^6 IU/mL (median 228 IU/mL). In 6 serum samples with neither e markers detected, viral loads ranged from undetectable to 750 IU/mL (median 120 IU/mL). Ten (10.5%) of 95 anti-HBe–positive samples had viral loads $>10^4$ IU/mL, ranging up to 8.6×10^6 IU/mL.

HBV DNA extracts from 40 serum samples were successfully sequenced and genotyped. Genotypes E (13/40, 32.5%) and B (10/40, 25%) predominated. Genotypes A (6/40, 15%), C (9/40, 22.5%), and D (2/40, 5%) accounted for the remaining genotypes. In 10 serum samples with viral loads $>10^4$ IU/mL and anti-HBe, the distribution of genotypes was significantly different, with an excess of genotype E ($p = 0.05$ by Fisher exact test; Table). Of the 10 viruses infecting these mothers, 5 had precore changes, 3 had precore and BCP changes, and 2 had BCP changes.

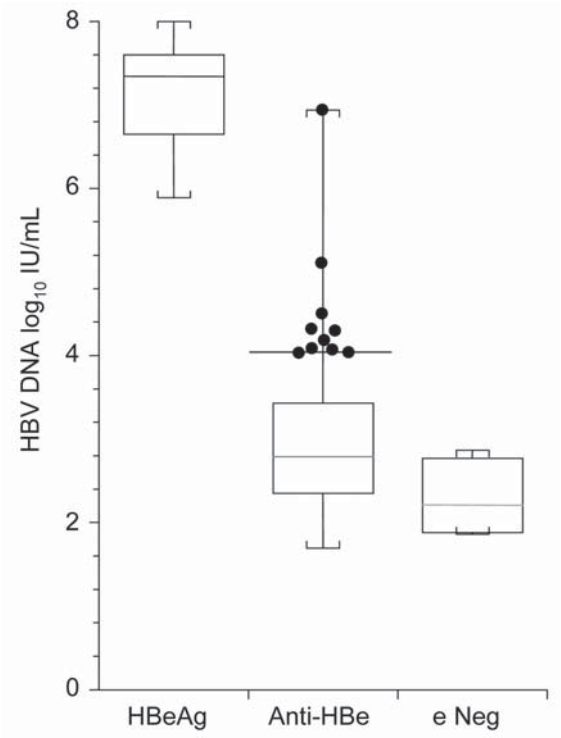

Figure. Box and whisker plots of hepatitis B virus (HBV) load in 3 groups of mothers whose serum contained hepatitis B virus e antigen (HBeAg), antibody to hepatitis B virus e antigen (anti-HBe), or neither of these markers (e Neg). Boxes are middle quartiles, horizontal lines are medians, whiskers are ranges, and dots represent 10 anti-HBe–seropositive mothers whose serum contained >10^4 IU/mL HBV DNA. Thirty-three anti-HBe–seropositive mothers and 1 mother whose serum did not contain either marker did not have detectable HBV DNA (<50 IU/mL).

Discussion

This study investigated the continuing use in the United Kingdom of maternal HBeAg markers as predicators for enhanced neonatal HBIg prophylaxis in addition to neonatal vaccine. Among 51 countries in Europe, the United Kingdom, along with 14 others, has elected to not introduce routine neonatal HBV immunization at this time (*7*), rather opting for selective screening in the antenatal clinics and targeted prophylaxis to infants born to infected mothers. This policy requires efficient HBV screening in clinics. We recognize that resources required for implementing this policy are not available in many countries. This policy has the advantage of enabling the addition of HBIg to prophylaxis for infants born to mothers with high infectivity, although how widespread this practice is in Europe is not known. HBIg is a costly intervention and is limited by availability. It is also a blood product that has the risk for transmission of prion disease through inclusion of donations from persons with variant Creutzfeldt-Jakob disease in the plasma pool.

Serum samples from 114 hepatitis B carrier mothers were examined. Thirteen (11.4%) contained HBeAg, with concentrations of HBV DNA ranging from 7.8 × 10^5 to 1 × 10^8 IU/mL. All infants born to these mothers would have been at high risk of acquiring HBV and should have been offered active immunization with the HBV vaccine, as well as passive prophylaxis with HBIg. Six serum samples did not contain detectable HBeAg or anti-HBe. Although HBV DNA levels were low in all samples, infants of these mothers would still have been given HBIg in accordance with guidelines, probably unnecessarily. Eighty-five of 95 serum samples with anti-HBe had HBV DNA levels <10^4 IU/mL and infants of these mothers would have received only active immunization. Ten (10.5%) of 95 serum samples had HBV DNA concentrations ≥10^4 IU/mL, and 2 (2.1%) of these had high viral loads >10^5 IU/mL (110,000 IU/mL and 8,690,000 IU/mL, respectively). The infants of these mothers would not have been offered HBIg on the basis of maternal anti-HBe as a marker of low infectivity. It is not known whether such infants are more likely to become infected as they had received only vaccine prophylaxis.

In the late 1970s in Japan, use of anti-HBe as a marker for low infectivity had been based on the observation (*17*) that anti-HBe–seropositive carriers were unlikely to transmit hepatitis B sexually or to their infants. This belief was verified by observations in genitourinary medicine clinics (*18*) and included in Department of Health policy in the United Kingdom that allowed hepatitis B carriers to conduct exposure-prone procedures if their serum did not contain HBeAg. In retrospect, it seems likely that at the time of promulgation of these guidelines, most infections with hepatitis B virus in the UK workforce would have been with genotype A. This Department of Health policy continued until description of several surgical transmissions from HBV-infected healthcare workers (*14*) and the recognition that some carriers whose serum contained anti-HBe had high viral loads. After this episode, estimation of plasma

Table. Characteristics of HBV in 10 infected mothers seropositive for antibodies to HBV e antigen and with serum HBV DNA levels ≥10^4 IU/mL*

Carrier mother	Genotype	HBV DNA, IU/mL	Basal core promoter	Precore†
1	D	2.5 × 10^4	W	W28
2	E	1 × 10^4	W	W28
3	E	1.1 × 10^4	130K/131I	W28 G29D
4	C	1.1 × 10^5	130K/131I	W
5	B	1.7 × 10^4	130K/131I	W28
6	E	8.6 × 10^6	W	W28
7	A	3.4 × 10^4	130K/131I	W
8	E	1.9 × 10^4	W	W28
9	E	2.2 × 10^4	130K/131I	W28
10	E	1.0 × 10^4	W	W28

*HBV, hepatitis B virus; W, wild-type sequence.
†W28, premature stop at codon 28.

HBV DNA load was introduced to manage infected healthcare workers (*19*). Most of the surgeons involved had been born in HBV-endemic countries outside Europe and would have been infected by a genotype other than genotype A. All viruses transmitted had premature stop codons in the precore region, which are changes not commonly seen in genotype A infections.

Dominance of nongenotype A infections among antenatal women in the United Kingdom, with genotype A accounting for only 15%, is explained by the recent observation that a net of ≈6,000 HBV carriers immigrate annually to the United Kingdom (*5*) from areas such as eastern Europe, where non-A viruses predominate. This immigration will undoubtedly change clinical expression of HBV carriage in the United Kingdom and provides an example of reemergence of an old virus disease with different characteristics. Flaring (increase in alanine aminotransferase levels caused by immune-mediated destruction of hepatocytes) and late escape (elevated levels of viral DNA) of virus from host-dependent modulation (innate or adaptive immune responses to infection with HBV) is seen more frequently with non-A viruses than with European genotype A HBV. All but 1 of the viruses in serum samples from 10 anti-HBe carrier mothers who had high viral loads were non-A, and all carried changes associated with enhanced virus replication. Five had changes in the precore region, 2 had changes in the BCP, and 3 had changes in both regions.

BCP mutations at nucleotide positions 1762/1764 and precore mutation G1896A, which results in a premature stop at codon 28, reduce or prevent expression of HBeAg. Both mutations are likely the result of virus evolution and selection of the fittest strains (*20*) during host immune responses. BCP changes result in decreased transcription of precore/core mRNA, reduced secretion of HBeAg (*21*), and enhanced virus production in vitro (*22*,*23*). These changes have been detected more often in viruses with genotypes A and C than in those with genotypes B, D, and E (*24*). However, in our study, BCP mutations were seen in viruses with genotypes A, C, D, and E. These mutations are thought to arise before precore changes (*25*). The premature stop precore mutation is restricted to HBV genotypes containing a thymidine at nucleotide position 1858, which is required for stabilizing the stem loop structure (*26*). This mutation, which is found in viruses with genotypes B, D, E, G and some strains with genotypes C and A (*27*), explains the high prevalence of premature stop variants in Asia and the Mediterranean region, where predominant genotypes are B, C, and D and their previous low prevalence in the United Kingdom. Our study demonstrates changing phenotypes of virus infections caused by population movement. These changes are unlikely to be limited to the United Kingdom and have wider implications for infectious diseases globally.

Our study demonstrates that reliance on only HBV serologic markers leads to misclassification of HBV carrier mothers. A proportion of low-infectivity carriers had high levels of virus in plasma but their infants would not have received optimal enhanced prophylaxis with postnatal HBIg. This policy could allow avoidable breakthrough infections in infants. In view of the influx of immigrant HBV carriers into the United Kingdom, a new HBV antenatal screening strategy is needed to identify and offer adequate protection to infants at risk of acquiring HBV infection. Quantification of HBV DNA is a more objective direct measure of potential infectivity and brings this procedure in line with management of HBV-infected healthcare workers (*19*). However, the cut-off level of HBV DNA needed to define potential activity has yet to be established. Finally, given the emerging pattern of an overall increase in HBV carriage in the United Kingdom, consideration should once again be given to a national program of immunization of infants.

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Epidemiology of *Streptococcus dysgalactiae* subsp. *equisimilis* in Tropical Communities, Northern Australia

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Streptococcus dysgalactiae subsp. *equisimilis* (groups C and G streptococci [GCS/GGS]) is an increasingly recognized human pathogen, although it may follow indirect pathways. Prospective surveillance of selected households in 3 remote Aboriginal communities in Australia provided 337 GCS/GGS isolates that were *emm* sequence-typed. Lancefield group C isolates (GCS) were localized to specific households and group G isolates (GGS) were more evenly distributed. GCS/GGS was more frequently recovered from the throat than group A streptococci (GAS [*S. pyogenes*]) but rarely recovered from skin sores, and then only with *Staphylococcus aureus* or GAS. Symptomatic GGS/GGC pharyngitis was also rare. Specific *emm* sequence types of GCS/GGS did not appear to cycle through the communities (sequential strain replacement) in a manner suggesting acquisition of type-specific immunity. These communities already have high levels of streptococcal and poststreptococcal disease. GCS/GGS may increase in importance as it acquires key virulence factors from GAS by lateral gene transfer.

In 1933, Rebecca Lancefield described a precipitin reaction that differentiated β-hemolytic streptococci into several groups according to the group-specific carbohydrate; these included groups A to E and unclassified strains (*1*). The isolates of group C streptococci (GCS) she investigated were of animal origin. Group G streptococci (GGS) were subsequently recognized in vaginal swabs from parturient women (*2*) and, for much of the next 50 years, GCS and GGS were considered to be nonpathogenic flora of the throat, gut, and vagina. When it became apparent that GCS and GGS could be human pathogens, it also emerged that they were a diverse group of streptococci consisting of at least 4 species, *Streptococcus anginosus*, *S. equi*, *S. equisimilis*, *and S. zooepidemicus* (*3*). In contrast, with few exceptions, group A streptococci (GAS) belong to 1 species, *S. pyogenes*.

Certain strains of GCS and GGS have been increasingly reported to cause infections similar to those caused by GAS such as pharyngitis, sepsis, skin and soft tissue infection, toxic shock, reactive arthritis, and postinfectious glomerulonephritis (*3*). Similar to GAS, human strains of GCS and GGS tend to have large colonies and a hyaluronic acid capsule; they also produce M protein that has structural, immunochemical, and biologic features similar to the M protein of GAS (*4*). Subsequent studies of the bacterial genome, including multilocus sequence typing of housekeeping genes, has demonstrated that large colony–forming human GCS and GGS are members of 1 species, *S. dysgalactiae* subsp. *equisimilis* (GCS/GGS) (*5*).

As with GAS, the M protein of GCS/GGS is responsible for resistance to phagocytosis (*4*). There is extensive sequence homology between gene sequences of GCS/GGS M protein and the *emm* gene of GAS; sequence heterogeneity at the 5′ end results in distinct *emm* sequence types (*6*). More than 35 years ago, Widdowson et al. recognized 2 M protein–related antigenic groups (I and II) in GAS that matched known skin and throat M serotypes (*7*). Skin types were subsequently found to have a cell surface lipoproteinase that binds fibronectin and causes opacity in horse serum (serum opacity factor [SOF]) (*8*). SOF is absent from identified rheumatogenic M types.

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Using molecular techniques to differentiate M protein classes, Bessen et al. found that class I strains show a correlation with SOF-negative strains and contain serotypes associated with acute rheumatic fever (ARF) (*9*), whereas class II strains are associated with skin tropism. GCS/GGS only possesses class I M protein with a surface-exposed conserved region similar to M protein of known rheumatogenic GAS strains (*4*). Most human GCS/GGS appear to be SOF negative, although SOF-positive *emm* types (*stG166b.0* and *stG480.0*) have been reported (*10*). Although there are no published cases of ARF proven to have been caused by GCS/GGS, M protein characteristics of GCS/GGS probably play a role in clinical disease and tissue tropism, and suggest the potential for rheumatogenicity (*11*).

We conducted this study in Aboriginal communities of tropical Australia in which rates of ARF and rheumatic heart disease (RHD) are among the highest reported; however, in this region streptococcal pharyngitis is apparently rare and pyoderma is common (*12*). Outbreaks of acute poststreptococcal glomerulonephritis (APSGN) are also common (*13*). The primary aim of the study was to investigate the epidemiology of β-hemolytic streptococci and to determine whether there are unique aspects applicable to the pathogenesis of ARF/RHD. We also used molecular typing to specifically characterize the epidemiology of GCS/GGS throat carriage, pharyngitis, and skin infection in these communities and to examine their relationship to GAS epidemiology and ARF.

Methods

Community Surveillance

The study was conducted in 3 remote Aboriginal communities located in the northern part of Northern Territory in Australia in which the prevalence of RHD was >25 per 1,000 population compared with <1 per 1,000 in the non-Aboriginal population. Community consultation, ethical approval (Human Research and Ethics Committee of the Northern Territory Department of Health and Community Services and Menzies School of Health Research, Darwin, Australia), household enrollment, data collection, and surveillance for ARF have been reported in detail (*12*). Surveillance was conducted in community 1 from July 2003 through June 2005, community 2 from July 2003 through June 2004, and in community 3 from July 2004 through June 2005. Local logistic problems restricted data collection in community 2. Community 1 is ≈500 km from community 2 and 700 km from community 3. The communities' names have not been used at their request.

A high degree of day-to-day population mobility prevented regular follow-up of persons over an extended period. Households were representative of family groupings and were studied as distinct epidemiologic units. Study households were selected on the basis that at least 1 occupant had a known history of ARF or RHD; this was done to increase chances of encountering additional cases of ARF. A household was defined as a family group that lived in 1 house or 2 adjacent houses. Persons were considered to belong to a household if they said they belonged at enrollment and were present on at least 2 subsequent visits. Crowding was based on the number of occupants per bedroom.

We attempted to visit each household on a monthly basis. At each visit, all children and adults present were questioned about sore throat and skin sores. All throats were examined and swabbed for culture, limbs and exposed areas were examined, and pyoderma lesions were also swabbed. Each personal contact was called a consultation.

Laboratory Methods

Specimen transportation, culture methods, and species identification have been described (*14*). Only large colony–forming β-hemolytic streptococci were selected and Lancefield grouped by using a Streptococcal Grouping Kit (Oxoid Diagnostic Reagents, Basingstoke, United Kingdom); suspected GAS isolates were tested for pyrrolidonyl arylamidase. Care was taken to exclude groups A, C, and G isolates of *S. anginosus* (*15*). Occasionally, *S. anginosus* morphology and β-hemolysis resembled that of GAS, but when colonies were streaked out and incubated overnight, the plates had a distinctive caramel odor. *S. anginosis* also failed to provide a PCR product for *emm* sequence typing.

The procedures for *emm* sequence typing followed those of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) (*10*) with minor modifications. Seqman software (DNASTAR Inc., Madison, WI, USA) was used for sequence analysis and results were compared with the CDC *emm* sequence database. New *emm* sequence subtypes were assigned by the moderator. In this article, an *emm*ST refers to an *emm* sequence subtype. We also examined the translated *emm* sequences for plasminogen binding A repeats to identify *emm*STs of the plasminogen binding M-like protein (PAM) phenotype (*16*).

Data Analysis

Epidemiologic data were analyzed by using Stata 8 (Stata Corporation, College Station, TX, USA). Confidence intervals were calculated by using standard methods. Correlation of household crowding and carriage was done by using Pearson correlation coefficient. Because of variability in household visits and the number of persons present at each visit, recovery rates were expressed per 100 consultations.

Results

We enrolled 49 households and made 531 household visits. These households provided 4,841 throat swabs and

484 skin sore swabs from 420 episodes of pyoderma. Limited data were obtained from community 2, and most of the comparative analysis was done between communities 1 (population ≈2,500) and 3 (population ≈1,800). These communities are truly remote, being accessible only by air for much of the wet season (December to April).

We identified and *emm* sequence-typed 350 isolates of GAS, 80 isolates of GCS, and 257 of GGS (Table 1). Four new GCS/GGS nucleotide sequence subtypes were described (GenBank accession nos. in parentheses): *stC839.2* (AM403090), *stC1400.3* (AM403091) *stC1400.4* (AM403092), and *stG480.3* (AM403093). GGS was isolated from only 1 child with a sore throat; this isolate was *stG6792.0*, one of the most common types. GGS/GCS was recovered from persons with 9 episodes of pyoderma, but always with *Staphylococcus aureus*, and in 2 persons with GAS. There were 7 different *emm*STs identified in skin swabs; most belonged to common types.

GCS were distributed unevenly (Table 1). The recovery rate for community 1 was 10 times that for community 3. Recovery rates for GGS and GAS across the communities were more even (*17*), which suggested that that the difference observed for GCS may be real. Throat swab samples from 154 (23.9%) children (those <15 years of age) had GCS/GGS, and samples from 126 (19.5%) children had GAS. Although GGS was more prevalent in community 1, there was greater diversity of *emm*STs in community 3, with 15 different *emm*STs compared with 9 different *emm*STs in community 1. Month-to-month recovery rates varied widely with medians of 3.3 (interquartile range [IQR] 1.2–3.3) per 100 consultations for GCS and 5.0 (IQR 3.4–7.5) for GGS in community 1, and 3.6 (IQR 1.6–5.9) for GGS in community 3. There was no apparent seasonal variation, although recovery rates of GCS, GGS, and GAS from throat swab samples peaked together in communities 1 and 3 during May 2005. A region-wide APSGN outbreak at that time was attributed to GAS *emm*55.0 (*18*), but recovery rates of GCS *stG643.0*, GGS *stC1400.0*, and GGS *stC74a.0* also increased sharply (Figure 1). This finding went largely unnoticed until *emm* typing of GCS/GGS was completed >12 months later.

The age distribution of GCS/GGS throat carriage in these communities was similar to that of GAS, with the highest recovery rates in 5- to 9-year-old children and 10- to 14-year-old children (*12*). However, different *emm*STs of GCS/GGS did not appear to cycle through the commu-

Table 1. Streptococcal *emm* sequence subypes (STs) of study isolates by Lancefield type in 3 communities, Northern Territory, Australia*

	Community 1		Community 2		Community 3			
Subtype	No.	Rate†	No.	Rate†	No.	Rate†	Total	Total rate†
GCS *emm*ST								
stC839.0	29	0.96					29	0.60
stG643.0	26	0.86					26	0.54
stGrobn.0	9	0.30					9	0.19
stC6979.0	8	0.26			1	0.07	9	0.19
stC839.2	4	0.16					4	0.08
stC6746.0	1	0.03			1	0.07	2	0.04
stC2sk.1					1	0.07	1	0.02
Total	77	2.54	–	–	3	0.20	80	1.65
GGS *emm*ST								
stC1400.0	34	1.13	5	1.69	27	1.76	66	1.36
stG4831.0	34	1.13			7	0.46	41	0.85
stG480.0	28	0.93			10	0.65	38	0.78
stG6792.0	35	1.16	1	0.34	1	0.07	37	0.76
stC74a.0	25	0.83	1	0.34	3	0.20	29	0.60
stC6979.0	8	0.26			2	0.13	10	0.21
stC5344.1	9	0.30					9	0.19
stG6.0					6	0.39	6	0.12
stG10.0					5	0.33	5	0.10
stG2078.0					4	0.26	4	0.08
stC.NSRT2.0	1	0.03			2	0.13	3	0.06
stC36.0					3	0.20	3	0.06
stC1400.4					2	0.13	2	0.04
stC1400.3					1	0.07	1	0.02
stG166b.0					1	0.07	1	0.02
stG480.3					1	0.07	1	0.02
stG652.0	1	0.03					1	0.02
Total	175	5.76	7	2.37	75	4.90	257	5.30

*GCS, group C streptococci; GGS, group G streptococci. Lancefield type and *emm*STs do not always match and *stC6979* can be either GCC or GGC.
†Per 100 consultations.

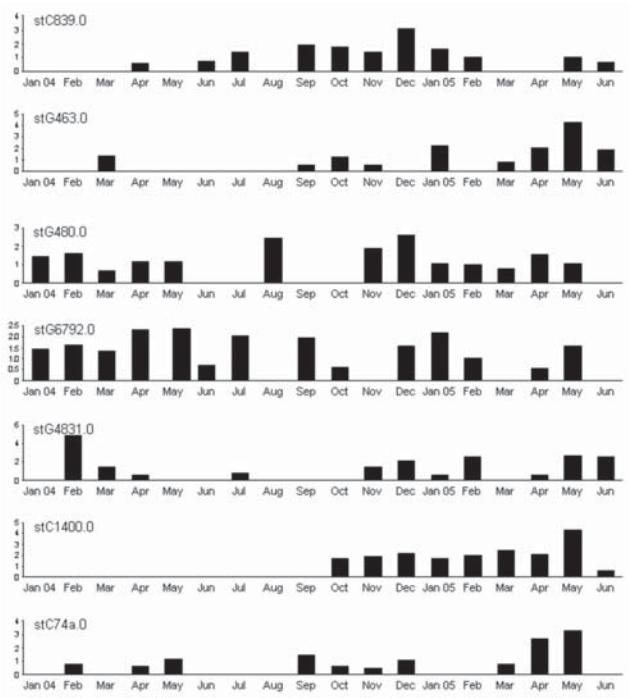

Figure 1. Monthly recovery rates of most common *Streptococcus dysgalactiae* subsp. *equisimilis* (group C and group G streptococci) *emm* sequence subtypes (STs) in community 1, Northern Territory, Australia. Values along the y-axes are no. bacterial isolates per 100 consultations. No obvious pattern of sequential strain replacement was seen as with *Streptococcus pyogenes* (group A streptococci) (*17*).

nity in the same way as GAS (Figure 1) (*17*). There was no evidence of sequential *emm*ST replacement. It was difficult to follow persons month by month, although we did identify a child who had GCS *stC839.0* in throat samples on 11 occasions over 14 months and a household that harbored GGS *stG4831.0* for at least 18 months. GCS appeared to be highly prevalent (>5.0/100 consultations) in 3 households in community 1 yet was absent from 3 others in the same community. There was poor correlation between recovery rates of both GCS and GGS and household crowding (r = 0.24).

When GCS/GGS throat isolates were compared with GAS throat isolates, they appeared to be almost mutually exclusive (Figure 2); on only 14 occasions were GAS and GCS or GGS recovered together. The *emm*STs of the 15 isolates (1 child had GAS, GCS, and GGS) were representative of the whole GCS/GGS population (Table 2) and no *emm*ST was dominant. These results are consistent with the assumption that throat carriage of GAS and carriage of GCS/GGS are independent of each another. However, on further investigation, this did not appear to be true. Persons with a positive throat culture for GAS during the study were more likely to have a positive culture for GCS/GGS than those who never had GAS recovered from the throat (65 [36%] of 180, 95% confidence interval [CI] 22%–33% compared with 168 [17%] of 993, 95% CI 15%–19%, relative risk 2.3, 95% CI 1.7–3.0). The relative risk for persons who had ≥6 throat swabs taken over the course of the study and for those who had <6 swabs taken was the same. We observed a poor correlation between household recovery rates of GAS and GCS/GGS (r = 0.39 for community 1 and r = 0.16 for community 2).

There was a marked discrepancy between β-hemolytic streptococcal recovery rates from the throat and the skin. GCS/GGS comprised 328 (60%) of 548 throat isolates (95% CI 56%–64%) and 9 (6.9%) of 131 skin isolates (95% CI 3.2%–12.6%) skin isolates. Sequence analysis of GCS/GGS *emm* showed that all isolates from throat and skin samples were negative for the skin-tropic determinant PAM.

Table 1 shows that 2 *emm*STs with group C carbohydrate by Lancefield typing had group G *emm* sequences (*stG643.0* and *stGrobn.0*) as determined by using the CDC database (*10*). Likewise, 8 GGS (*stC1400.0*, *stC74a.0*, *stC6979.0*, *stC5344.1*, *stCNSRT2.0*, *stC36.0*, *stC1400.3*, and *stC1400.4*) belonged to *emm*ST with GCS characteristics. Of 19 *stC6979.0* isolates, 9 were GCS and 10 were GGS. In May 2004, GCS *stC6979.0* and GGS *stC6979.0* were isolated from 2 persons in the same household. Initial investigation of the distribution of virulence genes in these isolates suggests that the GCS and GGS *stC6979.0* are 2 distinct strains. Further studies to differentiate them are under way.

Discussion

The reported prevalence of GCS/GGS carriage and incidence of related disease varies greatly worldwide. Most studies originate from temporal climate regions of the Northern Hemisphere and limited data are available from tropical regions. In the minds of most researchers and clinicians, the contributory role of GCS/GGS to acute pharyngitis is consistent with supporting evidence from numer-

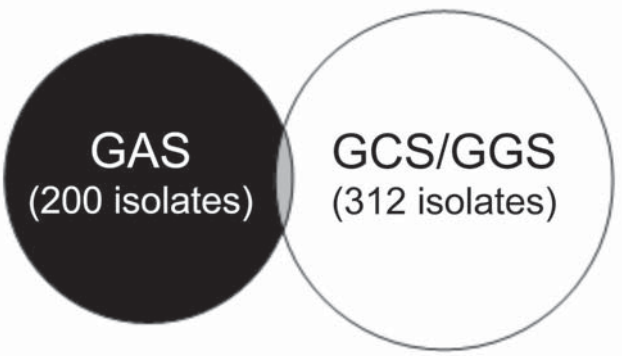

Figure 2. Venn diagram of positive throat swabs, Northern Territory, Australia, showing that group A streptococci (GAS) and *Streptococcus dysgalactiae* subsp. *equisimilis* (GCS/GGS) appear almost mutually exclusive. Thirteen persons had GAS and GCS or GGS, and 1 child had GAS, GCS, and GGS.

Table 2. *Streptococcus dysgalactiae* subsp. *equisimilis emm* sequence subtypes (STs) of 15 isolates from 3 communities, Northern Territory, Australia*

Subtype	No. isolates
GCS *emm*ST	
stC2sk.1	1
stC839.0	2
stGrobn.0	1
GGS *emm*ST	
stC-NSRT2.0	1
stC1400.0	3
stC1400.3	1
stC74a.0	3
stG480.0	1
st4831.0	1
stG6.0	1

*Isolates obtained from throat swab samples that also contained *S. pyogenes*. GCS, group C streptococci; GGS, group G streptococci.

ous studies, albeit of varying quality (*19, 20*). Outbreaks of GCS/GGS-related disease have also been reported (*21*). However, several studies, some of good quality, are less supportive of this view (*22,23*). Our surveillance failed to produce convincing evidence of GAS pharyngitis in children of these communities (*12*) and GCS/GGS pharyngitis. We did show that GCS/GGS is more commonly found in the throat than GAS. Moreover, study participants who carried GAS at any time were more likely at some stage to carry GCS/GGS. The link is probably household environmental factors, but there may be a streptococcal carrier phenotype (*24*). There is no evidence from our data that GCS/GGS displaces GAS from the throat.

Reported rates for throat carriage of GCS ranged from 0% to 12% in 1 Finnish community (*25*) and from 0% to 9.3% in Indian schoolchildren (*26*). GGS throat carriage was more common than GAS in this study (*12*); this was also true for Indian and Bangladeshi schoolchildren (*26,27*) and a Nigerian community (*28*). However, results of studies conducted >10 years ago should be interpreted with caution because of previous taxonomic confusion with failure to distinguish small and large colony forms of GCG and GGS.

Throat carriage of GCS/GGS, as distinct from carriage of GAS, was quite uneven. GCS was concentrated in a few households in community 1 where long-term carriage was common, but GCS was nearly absent from community 3. The reason for this absence is unknown. GGS was more evenly distributed across the communities, but more highly concentrated in specific households. The community pattern of sequential strain replacement seen with GAS (*24,29*) was absent, which suggests that acquisition of M protein type–specific immunity against GCS/GGS may not play a role in these communities. There is no evidence that type-specific immunity is protective against GCS/GGS (*5,30*). In addition, we observed no seasonal variation of GAS carriage (*12*).

Although these isolates were recovered from persons in remote communities of Australia and new subtypes of established *emm* types were found, no new *emm* STs were found. Until now, there has been little published information regarding the existing scope of *emm* types of GCS/GGS. The findings of this study suggest that there may not be a huge diversity, at least not to the extent that is seen with GAS.

The degree of throat tropism of GCS/GGS and lack of independent skin pathogenicity was a conspicuous finding. GCS/GGS causes many diseases similar to GAS, including skin and soft tissue infection (*3*), but reports of childhood pyoderma are few. GGS was found in 3% of pyoderma lesions in an Indian study, but always with *S. aureus* (*31*). Similar rates were reported from children in Trinidad (*32*). A West African study reported a 16% recovery rate of GGS from pyoderma (*28*), but this study was conducted >35 years ago. APSGN has been associated with GCS/GGS pyoderma in Trinidad (*32*), although the evidence for causation is tenuous. The Top End outbreak of APSGN in May 2005 in the Northern Territory of Australia was not associated with an increase in GCS/GGS skin infection in study communities. However, an apparent but unexplained increase in throat carriage of GCS/GGS occurred during this period, concomitant with increased throat carriage and skin recovery of GAS (*17*).

Although GCS/GGS has class I M protein, other factors could account for the observed tropism. In GAS, PAM is associated with skin tropism and *emm* pattern type D (*33*). An animal model of pyoderma suggests that skin infection with these strains requires streptokinase and PAM-bound plasminogen (*34*). However, other mechanisms must be involved because pyoderma can also be caused by non-PAM pattern D types and other *emm* pattern types. The GCG/GGS isolates in this study, as elsewhere, lack PAM. The critical gene for streptokinase activity in GAS, *ska* (the subcluster 2b β-domain), may have been acquired from GCS/GGS by lateral gene transfer (*33*).

There is additional evidence for lateral gene transfer with *rofA* and related genes. The gene encoding the key determinant for GAS binding to skin fibroblast fibronectin, SfbI, is located in a highly recombinatorial region of the GAS genome (*35*). The s*fbI* gene has a homolog, g*fbA*, in GCS/GGS that is likely a product of horizontal gene transfer and recombination (*36*). The role of fibronectin binding in skin and soft tissue infection has yet to be elucidated. The *rofA* gene is a positive regulator of s*fbI* and is present in GAS *emm* patterns types A–C and E, but is less common in pattern type D. GAS *rofA* is another gene that was possibly acquired from GCS/GGS by horizontal gene transfer (*37*). GAS *emm* patterns A–C are more phylogenetically primitive and less genetically diverse (*38*) than *emm* pattern types D and E. The throat may have been the original

niche for human colonization with GAS, and the ability to cause skin infection may be a more recently acquired trait.

Studies of bacterial housekeeping genes indicate that most gene traffic is toward GCS/GGS from GAS (*5*) and importation of GAS alleles into GCS/GGS is a relatively recent event. GAS is a completely human-adapted organism and human strains of GCS/GCS are more likely to be related to their animal flora origins. Humans may have acquired specific strains of GCS/GCS through animal domestication and these bacteria are now becoming human-adapted; 1 mechanism appears to be through phage-mediated acquisition of GAS alleles (*5*). There is some evidence that this process is more intense where the community streptococcal burden is high and strain turnover is rapid, such as in remote Aboriginal communities of the Northern Territory (*39*). If this is true, we could witness a regional increase in virulence of GCS/GGS over time, possibly including acquisition of rheumatogenic determinants.

Evidence is lacking that GCS/GGS causes ARF. Nonetheless, mouse antibodies to GCS/GGS M protein react with human cardiac myosin (*11*), and levels of antibodies to streptolysin O and hyaluronic acid increase after infection with GCS/GGS (*3*). ARF is driven by an exaggerated immune response to as-yet-undefined streptococcal epitopes, with possible immune priming from sequential streptococcal infections (*40*). An immune response to GCS/GGS, whether or not it is protective, may contribute to the priming process even if subsequent GAS infection is an absolute requirement for ARF.

We did not find a comparable study that examined the community and household dynamics of GCS/GGS carriage. Our study was originally intended as a longitudinal cohort study. However, the high population mobility, household turnover, and disruption of local community events compromised longitudinal surveillance (*12,17*). As such, the study became a series of point prevalence observations with accompanying data limitations. The study focused on households rather than persons, given the transience of the population, and we looked actively for throat and skin infection rather than waiting for presentation at the community health center. There were potential problems with variability of specimen collection and processing, and the lack of data from community 2 necessitated a move to community 3. We investigated selected study households, which may not have been representative of the whole community.

Because there is a relatively poor correlation between *emm* sequence type and GCS/GGS clone than with GAS (*5*), epidemiologic studies of GCS/GGS based on *emm* typing may need to be supplemented by techniques such as multilocus sequence typing, which define clonal type. Likewise, Lancefield grouping provides useful information, but is an unreliable epidemiologic tool unless supplemented by other methods. Nevertheless, the prospective nature of this study, and its size, make it likely that its findings provide a reasonable representation of the true epidemiology of GCS/GGS in this population. GCS/GGS was common in remote Aboriginal communities with high rates of streptococcal disease. Its contribution to illness, and even death, may manifest through indirect pathways, some of which have yet to be determined.

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Genetic Diversity among Clonal Lineages within *Escherichia coli* O157:H7 Stepwise Evolutionary Model

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Escherichia coli O157:H7 variants were examined for trait mutations and by molecular subtyping to better define clonal complexes postulated on the O157:H7 evolution model. Strains of β-glucuronidase–positive, sorbitol-negative O157:H7 isolated in United States and Japan were identical to A5 clonal strain and shared sequence type (ST)–65 by multilocus sequence typing (MLST); thus, they belong in A5. However, these strains exhibited pulsed-field gel electrophoresis (PFGE) profile differences that suggested genomic divergence between populations. Sorbitol-fermenting O157 (SFO157) strains from Finland, Scotland, and Germany were identical to A4 clonal strain and belong in A4. Some SFO157 strains, isolated years apart and from different countries, had identical PFGE profiles, suggesting a common origin. Despite similarities, some Finnish and Scottish and all of the German strains have ST-75 ("German clone"), whereas others have ST-76, a new variant ("Scottish clone"). MLST of strains in other clonal complexes also discriminated strains thought to be identical and showed that genetic differences will further distinguish clonal populations into subclones.

An evolutionary model postulates that *Escherichia coli* O157:H7 evolved from ancestral *E. coli* by stepwise acquisition or loss of virulence and phenotypic traits (*1*). At the center of the model is a hypothetical "intermediate" strain (ancestor A3), which evolved from the A2 clonal complex of O55:H7 strains that are closely related but ancestral to O157:H7 (*2*). It is hypothesized that the A3 intermediate strain is a missing link, a primitive O157:H7 that ferments sorbitol (SOR), is β-glucuronidase positive (GUD+), and has the Shiga toxin 2 gene (stx_2), and from which evolved 2 distinct pathways. The loss of SOR phenotype and the acquisition of stx_1 gene led to the emergence of the A5 clonal complex of SOR–, GUD+ O157:H7 strains, which then lost GUD expression, resulting in the prototypic O157:H7 clonal complex (A6). Also from A3 emerged a divergent lineage caused in part by the loss of motility giving rise to the A4 clonal complex of SOR+, GUD+, nonmotile strains that are designated as SFO157. These clonal complexes on the model were predicted on the basis of phenotypes, multilocus enzyme electrophoresis (MLEE), and the presence of the +93 single nucleotide polymorphisms (SNP) in the *uid*A gene that encode for GUD (*1*). However, except for the A6 clonal complex (O157:H7), from which strains were readily available, only a few strains existed for the other clonal complexes. As a result, these clonal complexes were not well defined because of the limited characterization criteria used and the lack of strains.

Molecular typing methods have improved the ability to characterize and discriminate closely related strains. Genetic studies have also elucidated some of the mutations that occurred in the stepwise emergence of clonal complexes. For example, in the transition from A5 to A6, the loss of GUD expression was found to be due to a frame-shift mutation caused by G-G insertion at +686 in the *uid*A gene (*3*). Similarly, in the divergence of A4 from A3, the loss of

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motility was caused by a 12-bp deletion in the *flhC* flagella master regulon gene (*4*). These mutations provide unique markers for tracing the model's evolutionary events and, coupled with better typing methods, have provided more discriminatory means to reexamine genetic relatedness among O157:H7 clonal complexes.

In addition, new or previously rare strains have been isolated more frequently. The A5 clonal complex of GUD+ O157:H7 was represented solely by strain G5101, isolated from a hemorrhagic colitis patient in the United States in 1994 (*5*). Similarly, the A4 clone complex of SFO157 was represented by 493–89 and a few other German strains isolated from hemolytic uremic syndrome (HUS) patients in Bavaria in 1988 (*6*). Recently, however, GUD+ O157:H7 strains have been isolated in the United States and from outbreaks and sporadic infections in Hokkaido, Japan (*7*). Likewise, SFO157 strains, which have also been isolated from cattle (*8*), are increasingly causing sporadic infections and outbreaks of HUS (*9*,*10*) and have been isolated in the Czech Republic (*11*), Finland (*12*,*13*), Scotland (*14*), and other countries (*6*).

In this study, we examined strains from various clonal complexes, including GUD+ O157:H7 strains from the United States and Japan and SFO157 strains from Finland, Scotland, and Germany, for unique mutational markers. We also used molecular typing to better define the various clonal complexes in the O157:H7 evolution model.

Materials and Methods

Bacterial Strains

The 3 GUD+ O157:H7 strains from the United States were originally isolated from clinical samples, and the 22 strains from Japan were isolated from outbreak patients with diarrhea or no symptoms and sporadic cases in Hokkaido (*7*). The SFO157 strains included 8 from Germany (*15*), 8 from Finland (*13*), and 5 from Scotland (*14*). Most of these were isolated from patients with symptoms of hemorrhagic colitis or HUS. However, 3 Scottish strains (designated H1085 and variations thereof) were isolated from cat feces at a farm where a hemorrhagic colitis infection had occurred. The other strains examined are from the Food and Drug Administration (FDA) and the Shiga Toxin-Producing *Escherichia coli* Center (Michigan State University, East Lansing, MI, USA), except for LSU-61, an O157:H7 strain isolated from deer (*16*).

Characterization

To reconfirm their phenotypic traits, strains were plated on cefixime-tellurite sorbitol MacConkey agar (Bacteriological Analytical Manual, www.cfsan.fda.gov/~ebam/bam-4a.html) to test for SOR fermentation and resistance to 2.5 μg/mL potassium tellurite. The plates also had a Colicomplete disc (BioControl, Bellevue, WA, USA) to test for GUD activity. All isolates were serotyped for O157 and H7 antigens (RIM O157:H7, Remel, Lenexa, KS, USA) and tested by several PCR assays for trait virulence genes, mutational markers, and SNPs. A multiplex PCR (*17*) was used to test for stx_1, stx_2, *γ-eae* for intimin, *ehxA* for enterohemolysin, and the +93 SNP in the *uidA*. The GUD+ O157:H7 strains were tested for the +776 SNP and for the +686 G-G insertion in *uid*A (*3*); the SFO157 strains were tested for the presence of the H7 *fliC* gene and for the 12-bp *flhC* deletion (*4*). Shiga toxin (Stx) production was verified serologically with the Verotoxin-producing *E. coli*–Reversed Passive Latex Agglutination Test (Denka Seiken, Japan), and enterohemolysin activity was tested on Ca^{++} blood agar plates (*18*).

Pulsed-Field Gel Electrophoresis (PFGE)

*Xba*I-digested genomic DNA was analyzed in 1% agarose gel in 0.5× Tris-boric acid-EDTA TBE buffer at 14°C by using CHEF MAPPER (BioRad, Hercules, CA, USA) (*19*). The runtime was 18 h at 6V/cm, with initial and final switch times of 2.16 and 54.17 s, respectively. The gel was stained with ethidium bromide (1 μg/mL), observed on the Gel Doc 2000 system (BioRad), and analyzed with the BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium).

Multilocus Sequence Typing (MLST)

The MLST protocol (www.shigatox.net/cgi-bin/mlst7/index) uses PCR primers to amplify internal segments of 7 specific housekeeping genes (aspartate aminotransferase [*aspC*], caseinolytic protease [*clpX*], acyl-CoA synthetase [*fadD*], isocitrate dehydrogenase [*icdA*], lysine permease [*lysP*], malate dehydrogenase [*mdh*] and *uidA*), which are purified and sequenced. Each unique sequence is given an allele number, and the combinations of alleles from the 7 genes are used to compile the organism's allelic profile. Each unique profile is designated as a sequence type (ST), which is then compared with those of other pathogenic *E. coli* strains in the *Ec*MLST database (*20*).

Results

Strain Characterizations

All the GUD+ O157:H7 strains examined had traits identical to the A5 type strain (G5101); were SOR–, GUD+, and tellurite resistant; and produced both O157 and H7 antigens (Table 1). Except for 2 US strains (TW06289 and TW06290) that did not have stx_1, all carried stx_1, stx_2, *γ-eae*, *ehxA*, and the +93 *uidA* SNP. Serologic analysis confirmed the production of both Stx, and all strains also had enterohemolysin activity. All GUD+ O157:H7 strains had the +776 SNP and, consistent with their GUD+ phenotype,

Table 1. Characteristics of *Escherichia coli* O157 strains*

Source	No.	SOR	GUD	Te	O157	H7	*fliC*	*stx*$_1$	*stx*$_2$	*γeae*	*ehx*A	+93	+776	+686	BAP	Δ*flhC*
G5101		–	+	R	+	+	+	+	+	+	+	+	+	–	+	–
USA	1	–	+	R	+	+	ND	+	+	+	+	+	+	–	+	NA
	2	–	+	R	+	+	ND	–	+	+	+	+	+	–	+	NA
Japan	22	–	+	R	+	+	ND	+	+	+	+	+	+	–	+	NA
498/89		+	+	S	+	–	+	–	+	+	+	+	–	NA	–	+
Germany	5	+	+	S	+	–	+	–	+	+	+	+	–	NA	–	+
	2	+	+	S	+	–	+	–	–	+	+	+	–	NA	–	+
Scotland	4	+	+	S	+	–	+	–	+	+	+	+	–	NA	–	+
	1	+	+	S	+	–	+	–	+	+	–	+	–	NA	–	+
Finland	6	+	+	S	+	–	+	–	+	+	+	+	–	NA	–	+
	2	+	+	S	+	–	+	–	–	+	+	+	–	NA	–	+
LSU-61		+	+	R	+	+	+	–	–	+	+	+	–	NA	+	–

*SOR, sorbitol fermentation; GUD, β-glucuronidase activity; Te, tellurite resistant (R) or sensitive (S); O157, antigen; H7, antigen; *fliC,* H7 flagella gene; *stx*$_1$, shiga toxin 1 gene; *stx*$_2$, shiga toxin 2 gene; *γeae*, γ-intimin; *ehxA*, enterohemolysin gene; +93, *uid*A single nucleotide polymorphisms (SNP); +776, *uid*A SNP; +686, *uid*A G-G insertion; BAP, enterohemolysin activity on blood agar plates; Δ*flhC*, 12-bp deletion; ND, not done; NA, not applicable.

did not have the +686 *uidA* G-G insertion. Because these strains expressed H7, they were not tested for the H7 *fliC* gene or for the *flhC* deletion.

All SFO157 strains examined were SOR+, GUD+, and tellurite sensitive, and expressed the O157 but not the H7 antigen (Table 1). Despite the absence of H7, all strains carried the H7 *fliC* gene. Except for 2 German (210–89, CB1009) and 2 Finnish (IH56929, IH56776) strains that did not have *stx*, all SFO157 carried only *stx*$_2$, *γ-eae*, +93 *uidA* SNP, and the 12-bp *flhC* deletion, but not the +776 *uid*A SNP. Since SFO157 strains are GUD+, they were not tested for the +686 insertion in *uidA*. Also, except for strain H1085 1a/1, all SFO157 strains carried *ehxA*, but none had enterohemolysin activity. All these traits are consistent with those of the A4 type strain (493–89).

Analysis of LSU-61 showed that it had a mix of traits from various clonal complexes. It is SOR+, GUD+, tellurite resistant, and had both O157 and H7 antigens. It had the +93 SNP but not the +776 SNP in *uidA*; had no *stx*; had *γ-eae* and *ehxA*; and showed enterohemolytic activity on blood agar plates (Table 1).

Molecular Subtyping

PFGE profiles of the US and 11/22 Japanese GUD+ O157:H7 strains are shown in Figure 1. The Japanese strains had nearly identical profiles, showing >95% similarity. Among the US strains, TW09099 and G5101 shared ≈90% similarity, but the other 2 strains were only 75% similar to G5101. The profiles of the US and Japanese strains shared only ≈70% similarity overall. MLST showed that all GUD+ O157:H7, including G5101, had ST-65 (Table 2).

The PFGE profiles of some SFO157 strains isolated within Germany (1782/88 and 4326/93) and Finland (IH57086 and IH57225) were identical (Figure 2). But profile identity was also observed among strains from Finland (IH56906) and Scotland (H1085c and H2687) and strains from Finland (IH56929) and Germany (5412/89) (Figure 2). MLST analysis showed that all the German, 4 Finnish, and 1 Scottish strain had ST-75 but that the other 4 Scottish strains and the rest of the Finnish strains had a distinct *mdh* allele and were genotyped as ST-76 (Table 2).

Discussion

The stepwise evolutionary model postulates that ancestral O157 clonal group (A3) split into 1 lineage, leading to the common GUD–, SOR– O157:H7 (NSF O157) clonal complex (A6) and a second branch of SFO157 that retained many primitive traits (A4) (Figure 3). The A4 and A5 clonal complexes on the evolution model are closely related to O157:H7 (A6 clonal complex) (Figure 3) and share many traits, including the +93 *uid*A SNP, which is found only in O157:H7 and its nonmotile variants (*1*). Another common trait is the γ-*eae* allele, which is also found in few other serotypes (*21*), including the O55:H7 strains in the A2 clonal complex that is ancestral to O157:H7 (*2*) (Figure 3). The A5 clonal complex of GUD+ O157:H7 strains is postulated to have emerged from the A3 intermediate strain. Analysis of A5 type strain (G5101) showed that it carried a +776 *uid*A SNP, which appears to have been acquired before the emergence of A5, because it is found only in A5 strains and A6 clonal complex of O157:H7 strains (*3*). All the GUD+ O157:H7 strains tested had identical traits as G5101, including the unique *uidA* markers (+93 and +776 SNP and absence of +686 G-G insertion) that are consistent with the mutational events postulated for the emergence of A5 and confirm that these strains also belong in A5. The fact that all these GUD+ O157:H7 strains have ST-65 supports that conclusion (Table 2). Despite similarities, however, there were differences in PFGE profiles. The Japanese strains had nearly identical PFGE profiles, which is consistent with the results by Nagano et al. (*7*), who also observed profile identity among clinical and environmental GUD+ O157:H7 isolates in Japan (*22*). In contrast, the US strains showed more diversity and shared only 70% similarity with

Table 2. Sequence type, serotype, and isolation information of strains of *Escherichia coli*

Sequence type	Strain	Source	Year	Serotype
65*	G5101	USA	1995	O157:H7
	EC96038	Japan	1996	O157:H7
	EC96024	Japan	1996	O157:H7
	EC97144	Japan	1997	O157:H7
	TW09099	USA	2003	O157:H7
	TW06290	USA	1997	O157:H7
	TW06289	USA	1997	O157:H7
66	86–24	USA	1986	O157:H7
	93–111	USA	1993	O157:H7
	Sakai	Japan	1996	O157:H7
69	EDL-933	USA	1982	O157:H7
73	C586–65	Sri Lanka	1965	O55:H7
	TB182A	USA	1991	O55:H7
	5905	USA	1994	O55:H7
	3256–97	USA	1997	O55:H7
75	493/89	Germany	1989	O157:H–
	1782/88	Germany	1988	O157:H–
	5412/89	Germany	1989	O157:H–
	CB569	Germany	1987	O157:H–
	210/89	Germany	1989	O157:H–
	CB1009	Germany	1990	O157:H–
	514/91	Germany	1991	O157:H–
	4326/93	Germany	1993	O157:H–
	IH 53440	Finland	1997	O157:H–
	IH 56776	Finland	1998	O157:H–
	IH 57086	Finland	1999	O157:H–
	H1410	Scotland	2002	O157:H–
	IH 57225	Finland	1990s	O157:H–
76	IH 57201	Finland	1999	O157:H–
	IH 56909	Finland	1999	O157:H–
	IH 56969	Finland	1999	O157:H–
	IH 56929	Finland	1999	O157:H–
	H2687	Scotland	2003	O157:H–
	H1085C	Scotland	2003	O157:H–
	H1085 3a	Scotland	2003	O157:H–
	H1085 1a/1	Scotland	2003	O157:H–
77	ECOR-37†	USA	1970s	Ont:Hnt
237	LSU-61	USA	2001	O157:H7

*Only selected β-glucuronidase positive O157:H7 strains from Japan are shown.
†Not typeable.

the Japanese strains, which suggests the occurrence of recent genomic divergences among the US populations of GUD+ O157:H7 strains.

The SFO157 strains of A4 clonal complex were also postulated to have evolved from the A3 intermediate strain (Figure 3). Analysis of the A4 type strain 493–89 showed that a key mutation that led to the emergence of A4 was the loss of motility caused by the 12-bp *flh*C deletion (*4*). Other A4 traits included tellurite sensitivity and the carriage of both *ehxA* and the H7 *fliC*, neither of which is expressed. All the SFO157 strains tested had these markers, which were postulated to have been acquired in the emergence of A4, which confirmed that these SFO157 strains also belong in the A4 clonal complex. The genomic similarities of the SFO157 strains are also reflected in their PFGE profiles, as 2 German strains (1782/88 and 4326/93) isolated 5 years apart had identical profiles; a Finnish strain (IH56909) isolated in 1999 was identical to 2 Scottish strains (H1085c, H2687) that were isolated in 2003; and a Finnish strain (IH56929) was identical to a German strain (5412/89) that was isolated 10 years earlier. These results are consistent with similarities observed among SFO157 strains from Germany (*15*), Finland (*12*,*13*), and the Czech Republic (*11*). The fact that there are profile identities among strains isolated years apart and from different geographic areas suggests that these SFO157 strains isolated from various European countries may have a common origin. Despite PFGE profile similarities, MLST showed genetic differences within the SFO157 populations (Table 2). The German, 4 Finnish, and 1 Scottish strain (H1410) had ST-75, which we designated as the "German clone," while the remaining Scottish and Finnish strains had ST-76 ("Scottish clone") (Figure 3). This genetic difference existed among strains with identical PFGE profiles, as German strain 5412/89 and Finnish strain IH56929 had ST-75 and ST-76, respectively.

Preliminary studies show that MLST (Table 2) may also better define other clonal complexes. In the A6 complex of O157:H7 strains, the Sakai strain that caused outbreaks in Japan and 2 US strains had identical *lysP* alleles (ST-66) but were distinct from the commonly used EDL933 reference strain (ST-69), a finding that suggests that genetic differences may also further distinguish the A6 clonal complex into subclones (Figure 3).

The A1 clonal complex, which consists of O55:H7 strains that are SOR+, GUD+, and carry *γ-eae*, was postulated to have given rise to A2 by the acquisition of the *stx*$_2$

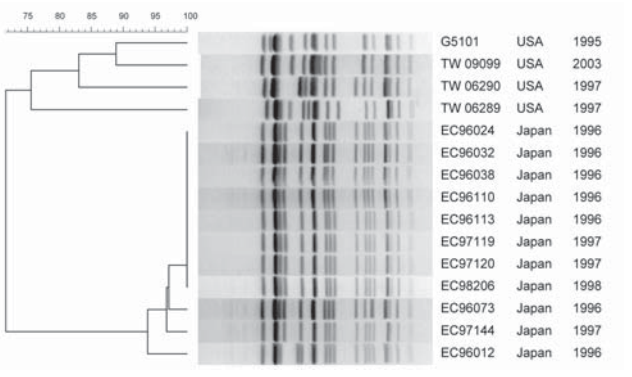

Figure 1. Pulsed-field gel electrophoresis of *Xba*I-digested DNA from GUD+ O157:H7 strains. Strain designation, source and year of isolation are shown at right. This unweighted pair-group method with arithmetic mean dendrogram was generated in BioNumerics software (Applied Maths, St-Martens-Latem, Belgium) by using Die coefficient with a 1.0% lane optimization and 1.0% band position tolerance. The scale above the dendrogram indicates percent similarity.

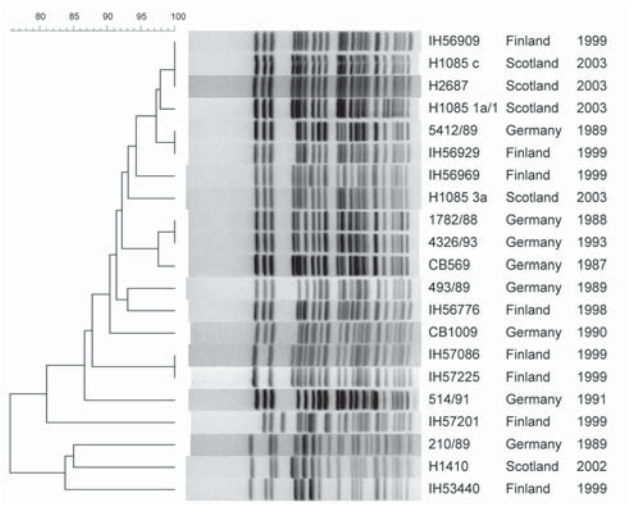

Figure 2. Pulsed-field gel electrophoresis of *Xba*I-digested DNA from SFO157 strains. Strain designation, source, and year of isolation are shown at right. This unweighted pair-group method with arithmetic mean dendrogram was generated in BioNumerics software (Applied Maths, St-Martens-Latem, Belgium) by using Die coefficient with a 1.0% lane optimization and 1.0% band position tolerance. The scale above the dendrogram indicates percent similarity.

phage (*1*). Based on phenotypes, 2 O55:H7 strains (C586–65 and TB182A) that did not have *stx* were previously thought to be A1 strains, but MLST showed both strains to have ST-73, identical to the Stx_2-producing O55:H7 strains (5905 and 3256–97) in the A2 clonal complex (Table 2). Analogous to the data obtained with the GUD+ O157:H7 strains, these O55:H7 strains had the same ST, but the PFGE profiles of C586–65 and TB182A shared only 60% similarity to 5905 and 3256–97 (data not shown). The genetic data on the transition from A1 to A2 is limited, so it is uncertain whether C586–65 and TB182A are derivatives of A1 or, perhaps, are A2 strains that have lost the stx_2 phage (see below).

Similarly, a marmoset isolate of *E. coli* (ECOR37) was previously shown to be closely related to A2 by MLEE. This strain had A2-like traits, including *γ-eae* (*23*), but its O and H antigens were serologically untypeable. PCR testing showed that ECOR37 had both the O55 *wzx* gene, required for export of O lipopolysaccharide, and the H7 *fliC* gene (data not shown) and, therefore, is an O55:H7 strain. However, ECOR37 shared only 60% similarity in PFGE profiles with A2 strains (data not shown). MLST also showed ECOR37 to have distinct *mdh* and *clpX* alleles (ST-77) (Table 2) and so this strain does not appear to belong in the A2 clonal complex (ST-73). We can only speculate that ECOR37 is an ancestral strain of A1 and A2, but its position in the model is uncertain (Figure 3).

LSU-61 is an O157:H7 that is SOR+; GUD+; tellurite resistant; carried *γ-eae*, *ehxA*, the +93, but not the +776 *uidA* SNP; and had no *stx*. Except for the absence of stx_2, LSU-61 had many of the traits proposed for the A3 intermediate, which has not yet been isolated. If LSU-61 is the A3 intermediate strain, we would expect it to be genetically related to the other clones because A3 is thought to have evolved from A2 and to be the intermediate for both A4 and A5 (Figure 3). However, the PFGE profile of LSU-61 showed only 60% similarity to stains in the A2, A4, A5, and A6 clonal complexes (data not shown), and it had a distinct *fadD* allele (ST-237) (Table 2). Despite these dissimilarities, the fact that LSU-61 has both O157 and H7 antigens and carries traits of neighboring clonal complexes (especially +93 *uidA* SNP and *γ-eae*) is compelling evidence that it belongs in the O157:H7 complex. The absence of the +776 *uidA* SNP in LSU-61 indicates that it is ancestral to A5; however, the exact position of LSU-61 on the evolutionary model remains to be determined (Figure 3).

In our study, we encountered various strains that had identical STs and traits as the clonal type strains, except for *stx*. Both stx_1 and stx_2 are phage encoded, and there is great diversity in *stx*-phage insertion sites among strains (*24*). Sometimes, these phages may be induced, resulting in strains that have lost the ability to produce Stx (*25*) or conversely, strains may acquire the ability to produce Stx by phage infection. This acquisition or loss of *stx* phages shows that this trait may not be a stable marker. Thus, the association of *stx* genotypes with clonal complexes should be interpreted with caution.

In summary, the use of unique trait markers and molecular typing methods better defined some of the clonal complexes postulated on the O157:H7 evolution model. The GUD+ O157:H7 and the SFO157 strains obtained

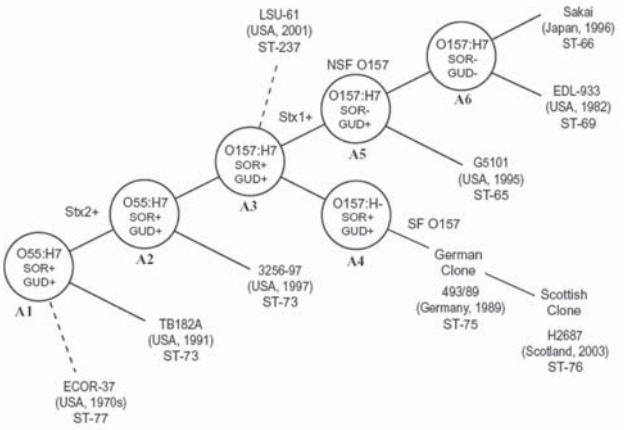

Figure 3. Evolution model for *Escherichia coli* O157:H7. Figure modified and updated from (*1*) to include the sequence type (ST) data showing subclones within clonal complexes. Some strains, whose position on the model remains to be determined, are shown with dashed lines.

worldwide had the unique mutation markers postulated for the emergence of the A5 and A4 clonal types and, therefore, belonged in these respective clonal complexes. Molecular subtyping showed genetic similarities and identities among strains within clonal complexes, but MLST identified genetic differences that further segregated these strains into subclones within a clonal complex.

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Dr Feng is a research microbiologist with the Center for Food Safety and Applied Nutrition, FDA. His research interests are molecular characterization and evolutionary analysis of enterohemorrhagic *E. coli* O157:H7 and its atypical variants as well as detection of these pathogens in foods.

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Methamphetamine Use and Methicillin-Resistant *Staphylococcus aureus* Skin Infections

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infections and methamphetamine use are emerging public health problems. We conducted a case–control investigation to determine risk factors for MRSA skin and soft tissue infections (SSTIs) in residents of a largely rural southeastern community in the United States. Case-patients were persons >12 years old who had culturable SSTIs; controls had no SSTIs. Of 119 SSTIs identified, 81 (68.1%) were caused by MRSA. Methamphetamine use was reported in 9.9% of case-patients and 1.8% of controls. After we adjusted for age, sex, and race, patients with MRSA SSTIs were more likely than controls to have recently used methamphetamine (odds ratio 5.10, 95% confidence interval 1.55–16.79). MRSA caused most SSTIs in this population. Transmission of MRSA may be occurring among methamphetamine users in this community.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing public health problem for urban and rural communities in the United States (*1,2*). Skin and soft tissue are the most common sites of MRSA infection, comprising >75% of MRSA disease (*3,4*). Skin and soft tissue infections (SSTIs), commonly caused by *S. aureus*, annually account for an estimated 11.6 million visits to hospital outpatient departments and emergency departments in the United States (*5*), and the percentage of SSTIs caused by MRSA in urban emergency departments increased from 29% in 2001 and 2002 to 64% in 2003 and 2004 (*6*). Some of the first reports of MRSA were in injection drug users in urban Detroit during the early 1980s (*7,8*).

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Illegal methamphetamine use in the United States led to a rising number of methamphetamine-related hospital admissions from the early 1980s through the early 2000s (*9*). In 2004, 0.2% of the national population ≥12 years of age reported using methamphetamine in the previous month; 0.6% reported using it in the previous year (*10*). The prevalence of methamphetamine use has been reported to be >5% in at-risk populations such as young men from low-income, urban neighborhoods (*11*) and urban HIV-positive men who have sex with men (*12*).

On August 2, 2005, the Georgia Division of Public Health invited the Centers for Disease Control and Prevention (CDC) to assist in an on-site investigation of increased SSTIs among patients of a low-cost, fee-for-service clinic in rural Georgia. The clinic's nurse practitioner had noted a history of methamphetamine use in multiple patients with SSTIs. Methamphetamine use has been associated with MRSA skin infections among urban HIV-positive men who have sex with men (*12*), but no study has evaluated the association of methamphetamine use and MRSA infection in a community with a large rural population. The objectives of this investigation were to define the public health effects and to determine risk factors, including methamphetamine use, for MRSA SSTI among residents of a community in the southeastern United States.

Methods

Epidemiologic Investigation

We conducted a prospectively enrolled case–control investigation at 3 emergency departments and 3 urgent care clinics in Georgia from September 6 through October 31, 2005. Two low-cost urgent care clinics that serve primarily

low-income populations and all emergency departments in a 3-county area were included in an attempt to capture sites where methamphetamine users might seek medical care for SSTI. The third urgent care clinic was affiliated with one of the participating hospitals but was located in a neighboring county. According to the 2000 US Census, 43.9% of the population of these 3 counties lives in rural areas (*13*).

We defined a case-patient as a person >12 years of age with a laboratory culture–confirmed SSTI who came to a participating emergency department or clinic for treatment during the investigation period. Clinicians at participating institutions identified patients with culturable SSTIs and were asked to incise, drain, and culture all infected skin and soft tissue. Patients with SSTIs that were not culturable, such as simple cellulitis, were not included. Patients whose primary language was not English were enrolled if they could speak English fluently enough to answer survey questions. Patients with new or recurrent SSTI could also be enrolled; however, we excluded patients who had previously enrolled in the investigation.

Controls were patients >12 years of age with no current skin infection who were frequency matched by investigation site at a rate of 3 controls to 1 case-patient with MRSA infection. Controls were excluded if they reported a current skin infection or if infection was identified on physical examination. Persons could be enrolled as control patients if illness was minor and comparable in severity to an SSTI. For example, patients with major trauma and critically ill patients were excluded from control selection.

Upon seeking treatment, patients voluntarily consented to be interviewed by trained staff of the participating healthcare facilities, local public health departments, or CDC to identify SSTI case-patients. To ensure as much privacy as possible, the interviews were usually conducted in the patient's room with no family or friends present. The interview survey contained questions about demographics, clinical history, and potential risk factors for SSTI. Each patient was asked a specific question about methamphetamine use: "In the past 3 months, have you used methamphetamine (crystal meth or meth)?" If the patient answered yes, 2 follow-up questions were asked: 1) "How did you take methamphetamine?" with the choices "smoked or inhaled," "injected," or "swallowed or took pills," and 2) "Have you shared drug equipment or rinse water with anyone else, including a significant other?" To identify healthcare exposure, patients were asked whether they had had surgery or dialysis or if they had stayed overnight in a hospital within the previous 3 months. All patients, and their parents if the patients were <18 years of age, were given a letter explaining the investigation and asked to give verbal informed consent to enroll in the investigation.

We examined trends in *S. aureus* skin infections and cultures at one of the main emergency departments in our investigation by reviewing billing codes and laboratory microbiology reports from January 2004 through September 2005, the start of the case–control survey investigation. This investigation was deemed exempt from review by the CDC Institutional Review Board because it was part of a public health response by CDC and the Georgia Division of Public Health.

Laboratory Investigation

Specimens were obtained from at least 1 infection site in all case-patients. Staff at all 3 hospital emergency departments and the urgent care clinic affiliated with 1 of the hospitals collected cultures and performed antimicrobial drug susceptibility testing at their facility. Two low-cost, urgent care clinics sent all cultures to CDC for culture and antimicrobial drug susceptibility testing. All 6 investigation sites sent both MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates to CDC for further characterization.

All available isolates from methamphetamine users and a random sample of isolates not related to methamphetamine use from each of the 6 investigation sites were tested at CDC for antimicrobial susceptibility by the Clinical and Laboratory Standards Institute broth microdilution method (*14*). We tested for susceptibility to chloramphenicol, clindamycin, daptomycin, doxycycline, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, penicillin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin. In addition, we performed the cefoxitin disk diffusion test to predict *mec*A-mediated resistance to oxacillin (*14*) and the D-zone test for inducible clindamycin resistance (*15*). Isolates were also tested by using PCR for genes encoding the staphylococcal cassette chromosome *mec* (SCC*mec*) resistance complex, Panton-Valentine leukocidin (PVL) cytotoxin, and toxic shock syndrome toxin (*16*). Chromosomal DNA was analyzed by pulsed-field gel electrophoresis (PFGE) after digestion with *Sma*I restriction endonuclease (*17*). The relatedness of PFGE patterns in different isolates was defined by using Dice coefficients and 80% relatedness by the unweighted pair-group method with arithmetic averages (Applied Maths, BioNumerics, Austin, TX, USA) (*18*).

Statistical Methods

We conducted univariate analysis of the data to describe patient demographics and compared binary and categorical variables with the χ^2 test; continuous variables were compared by using the *t* test with unequal variances. We evaluated risk factors for MRSA SSTIs by using conditional logistic regression with stratification by investigation site. Risk estimates were adjusted for age (categorized as ≤18 years, 19–34 years, 35–64 years, and ≥65 years), sex, and race (categorized as white and nonwhite) because they were potential confounding variables.

Results

Epidemiologic Investigation

We identified 119 case-patients with skin infections in the investigation. MRSA was isolated from 81 (68.1%) of the skin and soft tissue cultures, MSSA from 20 (16.8%), and bacteria other than *S. aureus* from 18 (15.1%) (Table 1). Compared with controls with no skin infection, a higher percentage of patients with MRSA SSTIs were male ($p<0.001$). The proportion of patients that were male did not differ significantly between controls and patients with either MSSA or non–*S. aureus* SSTIs ($p = 0.67$ for MSSA, $p = 0.12$ for non–*S. aureus*) or between patients with MRSA and MSSA SSTIs ($p = 0.16$).

Fifteen patients who reported recently using methamphetamine were identified: 8 with MRSA SSTIs, 2 with MSSA SSTIs, and 5 controls. Half (8 [53.3%]) of the methamphetamine users were male. Ten percent of patients with MRSA skin infections (8/81) reported using methamphetamine in the past 3 months, significantly more than the 2% of controls (5/283) who reported this behavior ($p<0.001$). After adjusting for age, sex, and race, we determined that patients with MRSA SSTI were significantly more likely to have recently used methamphetamine than were controls (adjusted odds ratio [AOR] 5.10, 95% confidence interval [CI] 1.55–16.79) (Table 2). Of the 8 methamphetamine users with MRSA SSTIs, most (5 [62.5%]) smoked or inhaled the drug. Only 1 (12.5%) injected the drug, and 1 (12.5%) took the drug orally. For 1 methamphetamine user with MRSA SSTI, we could not determine the route of drug administration. Of the 8 methamphetamine users with MRSA SSTIs in our investigation, 2 (25.0%) reported sharing drug equipment or rinse water with other persons; we did not have information on drug-sharing behavior for 1 methamphetamine user with a MRSA SSTI.

In our study population, having had a skin infection within the previous 3 months was the factor most strongly associated with current MRSA skin infection (AOR 7.92, 95% CI 4.10–15.28) (Table 2). Recent sexual contact with someone with a skin infection was also a significant risk factor for MRSA skin disease (AOR 5.42, 95% CI 1.68–17.50), when compared with recent sexual contact with a person without a skin infection. Frequent skin-picking behavior was independently associated with MRSA SSTI (AOR 2.53, 95% CI 1.22–5.23). Crowded living conditions, defined as >1 person per bedroom, had a small but significant association with MRSA SSTI (AOR 1.78, 95% CI 1.004–3.15).

Only 10% of MRSA case-patients had healthcare-associated risk factors traditionally associated with MRSA infection, namely, recent hospitalization, surgery, or dialysis. Additional factors not significantly associated with MRSA SSTI in our study population included use of antimicrobial agents in the previous 6 months, recent stays in a jail or prison, bathing less than daily, history of diabetes or liver disease, recent tattoo or body piercing, and participation in contact sports in the previous 3 months. In addition, very few or no patients were HIV positive (2 [0.5%]), homeless (0), or recently had sex with someone of the same sex (7 [1.6%]), suggesting that none of these were significant risk factors for MRSA SSTI in this population.

The number of visits for *S. aureus* skin infections at one of the main emergency departments in our investigation increased from ≈1 per 1,000 emergency department visits to 12 per 1,000 visits over the 20 months leading up to the investigation (Figure 1). This emergency department accounted for 46.2% of all study participants in our investigation. Over the same period, MRSA infections increased

Table 1. Demographic characteristics of study participants with (case-patients) and without (controls) skin and soft tissue infections (SSTIs)*

Characteristic	Patients with SSTIs			Patients without SSTIs (N = 284), no. (%)
	MRSA (N = 81), no. (%)	MSSA (N = 20), no. (%)	Other† (N = 18), no. (%)	
Age, y				
≤18	12 (14.8)	0	2 (11.1)	18 (6.3)
19–34	30 (37.0)	13 (65.0)	8 (44.4)	102 (35.9)
35–64	35 (43.2)	6 (30.0)	7 (38.9)	135 (47.5)
≥65	4 (4.9)	1 (5.0)	1 (5.6)	29 (10.2)
Male sex‡	48 (59.3)§	8 (40.0)	10 (55.6)	104 (36.6)
Race¶				
White	73 (90.1)	18 (90.0)	16 (88.9)	244 (85.9)
Black	5 (6.2)	2 (10.0)	2 (11.1)	36 (12.7)
Other	3 (3.7)	0	0	3 (1.1)
Hispanic ethnicity#	2 (2.5)	0	0	4 (1.4)

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*.
†Bacteria other than *S. aureus* isolated from SSTI in our investigation included other *Staphylococcus* spp., *viridans group streptococci*, Group B *Streptococcus*, *Enterobacter cloacae*, *Stenotrophomonas maltophilia*, and mixed flora.
‡6 records did not indicate sex (1 MRSA case, 1 MSSA case, and 4 controls).
§$p<0.0001$, when compared with controls.
¶For 1 control, race was not indicated.
#3 records did not indicate ethnicity (2 MRSA cases, 1 other skin infection).

Table 2. Risk factors for MRSA skin and soft tissue infection*

Risk factors	Case-patients, no. (%)	Controls, no. (%)	Crude OR (95% CI)	Adjusted OR† (95% CI)
Drug use and medical history				
Recent skin infection‡	34 (42.0)	22 (7.8)	8.41 (4.54–15.59)	7.92 (4.10–15.28)
Recent methamphetamine use‡	8 (9.9)	5 (1.8)	5.64 (1.80–17.69)	5.10 (1.55–16.79)
Antimicrobial agents within 6 months	40 (49.4)	114 (40.1)	1.43 (0.87–2.34)	1.52 (0.89–2.60)
Recent hospitalization, surgery, or dialysis‡	8 (9.9)	27 (9.5)	1.06 (0.46–2.44)	1.24 (0.51–2.97)
Diabetes	10 (12.4)	23 (8.1)	1.61 (0.73–3.57)	2.03 (0.83–4.98)
Liver disease	1 (1.2)	9 (3.2)	0.38 (0.05–3.07)	0.59 (0.70–4.91)
Contact exposure				
Household contact with someone with skin infection	21 (25.9)	27 (9.5)	3.26 (1.72–6.17)	3.19 (1.58–6.48)
Crowding (>1 person/bedroom)	44 (54.3)	111 (39.1)	2.06 (1.22–3.45)	1.78 (1.004–3.15)§
Recent sexual contact‡	48 (59.3)	182 (64.1)	0.85 (0.51–1.42)	0.68 (0.38–1.22)
Recent sexual contact with someone with skin infection‡	7 (8.6)	6 (2.1)	4.28 (1.40–13.08)	5.42 (1.68–17.50)
Recent contact sports‡	9 (11.1)	11 (3.9)	2.92 (1.17–7.31)	1.37 (0.47–4.03)
Recent jail‡	4 (4.9)	9 (3.2)	1.46 (0.44–4.90)	1.75 (0.48–6.42)
Hygiene practices				
Frequent skin picking	17 (20.1)	24 (8.5)	2.77 (1.40–5.47)	2.53 (1.22–5.23)
Bathe less than daily	5 (6.2)	31 (10.9)	0.50 (0.19–1.34)	0.56 (0.19–1.67)

*MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odds ratio; CI, confidence interval.
†All models are adjusted for age, sex, race, and methamphetamine use, except the model for methamphetamine use, which is adjusted only for age, sex, and race.
‡Recent = within the 3 months prior to survey.
§p = 0.048.

from 2 to 38 per month in the same emergency department. Most emergency department *S. aureus* cultures for both SSTIs and non-SSTIs were resistant to methicillin, with the prevalence of methicillin-resistance remaining stable over the same 20-month period (median 82%, range 50–100%).

Laboratory Investigation

MRSA (n = 32) and MSSA (n = 13) isolates tested were commonly susceptible to clindamycin, daptomycin, doxycycline, gentamicin, levofloxacin, linezolid, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin (Table 3). None of the MRSA isolates and only 1 (7.7%) of the MSSA isolates had inducible clindamycin resistance. MRSA susceptibility patterns of isolates from methamphetamine users and nonusers were similar, except that both MRSA isolates susceptible to erythromycin were found in those who did not use methamphetamine. The MSSA isolate from a methamphetamine user was susceptible to all but penicillin.

We detected genes for PVL in all MRSA isolates and 5 (41.7%) MSSA isolates; however, the MSSA isolate from a methamphetamine user did not carry the PVL locus. All available MRSA isolates from 6 methamphetamine users and 21 nonusers of methamphetamine had type IV SCC*mec* resistance complex and were PFGE type USA300. Most of the MRSA isolates were a single strain, PFGE type USA300-0114 (4 [66.7%] were methamphetamine users, 15 [71.4%] were non-methamphetamine users) (Figure 2). One third (33.3%) of MRSA isolates from methamphetamine users and one fifth (19.0%) of MRSA isolates from non-methamphetamine users were variants of USA300-0114, such as USA300-0047.

Discussion

MRSA caused over two thirds of all skin infections in the Georgia community we investigated, which is among the highest reported rates of MRSA in SSTI nationwide (*16*). We found that many previously known risk factors for MRSA skin infection, such as recent skin infection and household contact with someone with a skin infection (*19*), were common in this population. However, we also identified a novel association between MRSA skin infections and methamphetamine use in a community with a large rural population. Methamphetamine use was reported in nearly 1 in 10 patients with MRSA SSTI and was more common in patients with MRSA skin infections than in patients without skin infections. While most community-associated MRSA SSTI occur in persons without defined risk factors (*16*), some settings such as prisons and military training facilities appear to facilitate and amplify MRSA transmission (*20,21*). A similar amplification of transmission may be occurring among methamphetamine users in this community.

Methamphetamine use is associated with a number of socioeconomic and behavioral risk factors that may predispose persons to MRSA SSTI. We found that MRSA SSTI was associated with living with someone with a skin infection, which may increase skin contact with infected persons. Skin-picking was also associated with MRSA SSTI. Methamphetamine use causes formication, a sensa-

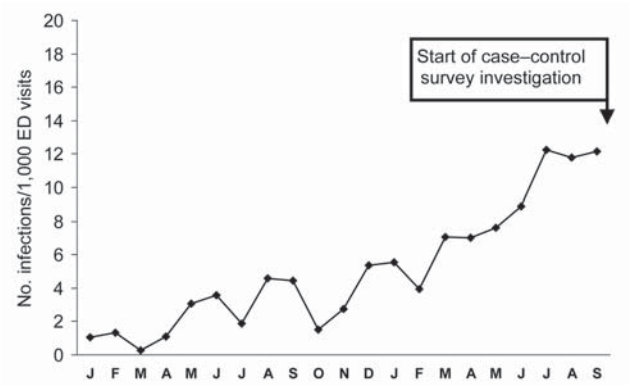

Figure 1. Number of *Staphylococcus aureus* skin infections at a southeastern United States emergency department, January 2004–September 2005.

tion of something crawling on the body or under the skin, which can lead to skin-picking behavior, skin breakdown, and portals of infection. Other poor hygiene habits that can break the skin, such as fingernail biting, have been associated with MRSA SSTI (*12*). Methamphetamine use may be associated with limited access to medical care, stays in correctional facilities, and homelessness, all of which have been associated with MRSA SSTI in previous studies (*20,22*). However, our investigation did not find these to be significant risk factors for MRSA SSTI in this population.

Methamphetamine use has been associated with HIV (*23*) and sexually transmitted bacterial infections (*24*), purportedly from increased unprotected sex related to the sexually stimulating property of the drug. A study among urban HIV-positive men who have sex with men found that, in addition to methamphetamine use, use of other sexually stimulating drugs such as nitrates ("poppers") and sildenafil (e.g., Viagra) was associated with MRSA SSTI (*12*). These previous findings and the results of the current investigation suggest that the use of methamphetamine and other sexually stimulating drugs may increase direct skin-to-skin sexual contact and transmission of MRSA, which can be transmitted through sexual contact (*25*). We found an increased risk for MRSA SSTI in case-patients who had recently had sex with someone with a skin infection.

Injection of the drug may act as a method of introducing the bacteria into the skin if users fail to clean injection sites or share drug paraphernalia and other potentially contaminated items (*26*). Injection of methamphetamine can lead to transmission of bloodborne pathogens when injection equipment is shared, as demonstrated in an outbreak of hepatitis B among methamphetamine users in Wyoming (*27*). A recent case series of 14 patients with MRSA necrotizing fasciitis found that 43% of the patients had current or past injection drug use (*28*). In contrast to early reports of MRSA in urban injection drug users, our investigation suggested that MRSA skin infections in methamphetamine users are not necessarily due to unclean drug injection. Few methamphetamine users in our population injected the drug or shared equipment; rather, the methamphetamine users in this community commonly smoked or inhaled the drug.

The absolute number of SSTIs at 1 emergency department in this investigation increased during the 18 months preceding the investigation, but the percentage of MRSA isolates was stable over that period. This increase in SSTIs led to a concomitant increase in MRSA SSTIs, which were more common among men, and echoes repeated reports of MRSA SSTI outbreaks in male populations (*20,29*). This sex difference was not due to increased methamphetamine use in men in our population, since our population of sur-

Table 3. Antimicrobial susceptibility patterns and toxin gene presence of selected MRSA and MSSA isolates*

Antimicrobial agent or toxin	MRSA isolates† (N = 32), no. (%)	MSSA isolates (N = 13), no. (%)
Antimicrobial susceptibility		
Chloramphenicol	32 (100.0)	10 (76.9)‡
Clindamycin	32 (100.0)	12 (92.3)
Inducible resistance (D-zone test)	0	1 (7.7)
Daptomycin	32 (100.0)	13 (100.0)
Doxycycline	32 (100.0)	13 (100.0)
Erythromycin	2 (6.5)	6 (46.2)
Gentamicin	32 (100.0)	13 (100.0)
Levofloxacin	27 (84.4)	12 (92.3)
Linezolid	32 (100.0)	13 (100.0)
Penicillin	0	2 (15.4)
Rifampin	32 (100.0)	13 (100.0)
Trimethoprim-sulfamethoxazole	32 (100.0)	13 (100.0)
Vancomycin	32 (100.0)	13 (100.0)
Toxin gene presence		
Panton-Valentine leukocidin	32 (100.0)	5 (38.5)
TSST–1	0	0

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; TSST, toxic shock syndrome toxin.
†Methicillin resistance was determined by the oxacillin MIC and disk diffusion using a 30-μg cefoxitin disk (*14*).
‡Three (23.1%) isolates had intermediate resistance to chloramphenicol.

Figure 2. Dendrogram of pulsed-field types for methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolated from methamphetamine users.

veyed methamphetamine users was evenly divided between the sexes. We also did not find many MRSA infections in nonwhite patients. This finding contrasts with previous reports of higher incidence of MRSA SSTIs in African Americans in urban centers compared to other races (*4*) and likely reflects the predominantly white racial distribution (98.9%) in these 3 rural southeastern US counties (*13*).

The laboratory investigation found that the most common MRSA strain causing community SSTI was PFGE type USA300-0114, a highly conserved strain that has been implicated in multiple community outbreaks (*19*). The second most common MRSA strain in this community, and the only other strain found among methamphetamine users, was USA300-0047, which has only a 1-band difference from USA300-0114. MRSA SSTIs in methamphetamine users were not due to a novel or unusual strain of MRSA but rather the most common strain of MRSA in community settings across the United States.

Our investigation is subject to some limitations. First, we did not identify nor do we have data on every SSTI patient who came to the participating clinics and emergency departments for treatment; not every patient with SSTI provided specimens for culture or participated in our survey. Second, we relied on patient report of methamphetamine use and did not conduct drug screens for confirmation. Third, we excluded patients who could only speak Spanish, which may have added to the low number of Hispanic study participants and affected the generalizability of the results. However, Hispanic, foreign-born, and non-English primary speakers each comprise only 5%–10% of the population of these 3 counties (*13*). Fourth, we were unable to test for other physiologic theories of why methamphetamine may be associated with MRSA, which include weakening the immune system and predisposing users to MRSA carriage by changing the nasal environment. Fifth, we were unable to test whether methamphetamine itself or drug paraphernalia were contaminated with MRSA. Lastly, transmission of MRSA in this population may have occurred in either the community or in the healthcare setting; for some cases, we were unable to determine the origin of the community strains.

Our investigation has direct implications for clinicians. Most clinicians in the participating emergency departments and urgent care clinics did not routinely drain or culture SSTIs. Incision and drainage is a primary therapy for SSTI, and empiric antimicrobial drug therapy may be given in addition to incision and drainage (*30*). Because of the growing and changing resistance patterns in the community, clinicians should consider culturing SSTI (*30*). In this population, antimicrobial agents currently recommended for treatment of MRSA (e.g., clindamycin, doxycycline, and trimethoprim-sulfamethoxazole) would be appropriate choices for empiric treatment of outpatient SSTI because of low prevalence of resistance (*30*.) Patients should also be educated about the risks for transmission through household and sexual skin-to-skin contact. Transmission of MRSA in this community is likely due to various factors, and some of these community strains may have been transmitted through healthcare exposure.

Patients with MRSA SSTIs who seek treatment may help clinicians identify a vulnerable population of methamphetamine users. Prevention measures, such as improved hygiene and correct care for wounds, may be helpful when directed at methamphetamine users. However, MRSA SSTIs in methamphetamine users may also impact family and community members who do not use methamphetamine. The same strains of MRSA were circulating among both users and nonusers in our investigation. Public health officials and clinicians should be aware of proper identification, appropriate treatment, prevention of MRSA SSTIs, and the link between methamphetamine use and these SSTIs.

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Pandemic Influenza and Hospital Resources

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Using estimates from the Centers for Disease Control and Prevention, the World Health Organization, and published models of the expected evolution of pandemic influenza, we modeled the surge capacity of healthcare facility and intensive care unit (ICU) requirements over time in northern Netherlands (≈1.7 million population). We compared the demands of various scenarios with estimates of maximum ICU capacity, factoring in healthcare worker absenteeism as well as reported and realistic estimates derived from semistructured telephone interviews with key management in ICUs in the study area. We show that even during the peak of the pandemic, most patients requiring ICU admission may be served, even those who have non–influenza-related conditions, provided that strong indications and decision-making rules are maintained for admission as well as for continuation (or discontinuation) of life support. Such a model should be integral to a preparedness plan for a pandemic with a new human-transmissible agent.

The threat of an avian influenza A (e.g., subtypes H5N1, H7N7) pandemic has forced healthcare authorities and health services to draft and discuss preparedness plans (*1*–*5*). The responsibility for management of the national and regional risks due to pandemic influenza was underscored by the outbreak of avian influenza (H7N7) in 2003 in the Netherlands, which led to culling one third of domestic poultry (including 30 million chickens), with 1 human casualty, a veterinary surgeon who died from acute lung injury after infection with the virus (*6*,*7*). The increasing pandemic threat of influenza A (H5N1) is reflected by 291 cases of human disease reported to the World Health Organization (WHO) as of April 11, 2007, with 172 human deaths (*8*). Because the question is not whether a pandemic will occur but, rather, when (*9*), policymakers have been urged to take action in preparedness planning.

Preparing for an influenza pandemic is difficult for healthcare systems because of many uncertainties. Strikingly little knowledge has been obtained from the scattered cases of avian influenza in humans (*10*).

In influenza patients admitted to an intensive care unit (ICU), severe disease may develop with a sepsis-like pattern with a proinflammatory cytokine storm (*11*), but it is unknown what percentage of patients fall ill after acquiring the virus (attack rate) and what percentage require hospital admission and, subsequently, ICU admission. Attack rate, hospital and ICU length of stay, and death rate can only accurately be factored in after a new virus has emerged (*3*). Therefore, almost all assumptions in the models published to date have drawn on the knowledge obtained from the large 20th-century pandemics (*12*–*14*). In summary, a model for preparedness of the healthcare system should be highly adaptable and flexible to factor in new information emerging in the early stages of the pandemic.

The University Medical Center Groningen (UMCG) is a large tertiary care university hospital covering ≈12% of the total Dutch population and ≈30% of the total surface area of the Netherlands. Under Dutch law, UMCG has an important role in the event of an avian influenza pandemic, not only for the patient population that it serves but also as a regional coordinating center (*15*). Training courses that emphasized the need to enhance collaboration and communication for pandemic influenza were held with regional and municipal health authorities, general practitioners, and representatives of all hospitals in the northern region. We present a model, similar to models by Anderson et al. (*16*) for Australia and New Zealand and Menon et al. for England (*14*). We show that increased hospitalization in combination with healthcare worker (HCW) absenteeism

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will have a substantial, but in our model manageable, effect on hospital and ICU bed occupancy. Furthermore, we discuss the choices to be made for ongoing, non–influenza-related emergencies during an influenza pandemic and the effect of enhancing the contingency plans already in place. Although surge capacity of hospital resources is typically limited (*1*), we explored whether, under specified assumptions and appropriate planning and training, a pandemic is manageable.

Methods

We used FluSurge 2.0 (*17*) and a computer model in an Excel file developed by one of the authors to calculate the impact of an influenza pandemic in the Netherlands on hospital admission and occupancy rate of all ICU beds (i.e., those with facilities for mechanical ventilation). Data on population (≈1.7 million) and age distribution (Table 1) were obtained from publicly available sources. The age distribution in the Dutch population data were provided in 5-year groupings, and we therefore converted these data to an even distribution to allow for calculations with the FluSurge program (*14*). Data on total hospital beds, ICU beds, and number of nurses and their full-time equivalents were obtained from publicly available sources (*18*). ICU capacity was also obtained from reports from hospital administrators during training sessions for pandemic influenza in May 2006, organized by the public health authorities in the region. These data on reported ICU capacity were discussed during a semistructured telephone interview with ICU medical staff in August 2006. Using these data, we estimated the regular bed capacity and maximal surge capacity. Data on the impact of a pandemic influenza on healthcare services were adopted from the National Institute for Public Health and the Environment (RIVM) (*19*,*20*). RIVM presented tables for 25% and 50% disease attack rates, representing best and worst case scenarios. From these tables we calculated the 30% attack rate (percentage of the population that becomes ill) by linear transformation. A 30% attack rate is the most likely scenario, according to the Centers for Disease Control and Prevention, and is defined as the most likely scenario by RIVM.

We also calculated, within the model, the total number of patients admitted to the hospitals at each point in time during the pandemic. We defined the first day (day 0) as the moment that WHO declares human-to-human transmission (phase IV or V in the current WHO phase of pandemic alert). We took into account the time each patient occupies a hospital or ICU bed (range 8–15 days), on the basis of experience with patients admitted to ICU with a diagnosis of pneumonia or sepsis. Finally, we incorporated estimated risk of death per patient, reducing the number of admitted patients at any one time. Because the data of the RIVM are in week blocks, we evenly distributed the number of hospital admissions and the proportion of deaths across the week days.

In our calculations, we also factored the effect of treatment (within 48 hours of infection) with antiviral medication on the spread and the impact of the pandemic, although the exact effect size is still uncertain (*14*,*21*). Antiviral medication is assumed to reduce the total number of hospital admissions by 50% and death rate by ≈30%.

In addition, we incorporated in the model the probable absenteeism of HCWs either due to illness or to care duties at home or in individual social environments. We assumed that HCWs will become ill at a rate similar to that of the general population. We extrapolated national population data of illness and deaths to the total number of HCWs in our HCW database.

Finally, we incorporated the effect of strict treatment decisions at the patient level on the peak occupancy rate of ICU beds. We applied a 48-hour restriction of treatment time at the ICU for patients occupying an ICU bed. We focused our preparedness plan on adults, assuming an outbreak pattern similar to that of Spanish flu (*22*) and severe acute respiratory syndrome (SARS), in which adolescents and adults accounted for most cases.

Results

We present the impact of a pandemic with new human-transmissible influenza on hospital resources in the northern part of the Netherlands. Using the figures of the RIVM, and assuming a 30% cumulative disease attack rate, we estimated that ≈12% of the population will consult a general practitioner (Table 2). The percentage of persons triaged for hospital admission is 0.3%. We assumed excess deaths among these selected patients, some 50% of whom may require mechanical ventilation (Figure 1). In the northern part of the Netherlands 5,629 regular hospital beds are available. The hospitals in this region have a total of 30% (non–influenza-related) acute care, which would leave 3,940 regular hospital beds that could be made available for influenza-related hospital admissions. If the attack rate

Table 1. Age distribution of inhabitants of 3 northern provinces in the study, the Netherlands

	Age range, y					
Province	0–15	16–24	25–44	45–64	≥65	Total, all ages
Groningen	99,065	72,714	164,371	151,590	86,818	574,558
Friesland	125,174	70,397	174,768	172,600	99,665	642,604
Drenthe	92,241	45,885	127,674	136,915	81,212	483,927
Total	316,480	188,996	466,813	461,105	267,695	1,701,089

Table 2. Avian influenza impact for 3 northern provinces in the study, the Netherlands*

Week	Days	No. patients	General practitioner consultations	Hospital admissions	Deaths
0	1–7	0	0	0	0
1	8–14	105	11	0	0
2	15–21	4,694	515	11	0
3	22–28	145,898	16,559	315	84
4	29–35	347,288	44,699	977	420
5	36–42	25,935	3,696	95	74
6	43–49	578	84	0	0
7	50–56	11	0	0	0
8	57–63	0	0	0	0
9	64–70	0	0	0	0
Total		524,507	65,562	1,397	578

*30% attack rate, pandemic period 9 weeks.

reaches a maximum of 50% with a mean length of stay of 15 hospital days per patient, without any intervention, this would lead to a peak of 1,227 occupied regular hospital beds, which would suffice for influenza-related acute care. Therefore, we centered our calculations around the peak occupancy of intensive care beds. We calculated the number of hospital admissions per week, spread evenly across 7 days in the respective week, and we subtracted the number of deaths, also evenly spread across the week. We assumed that 25%–50% of total hospital admission patients would require some form of mechanical ventilator support, and we provide calculations for the extremes of our estimates. On the basis of results from a semistructured telephone interview with ICU medical staff of the hospitals in the 3 northern provinces, a maximum of 136 (of a total of 200) ICU beds could be dedicated to influenza-related acute-care patients. We estimate that 90 ICU beds will be made available in a short period. In the scenario of no additional intervention, if the full capacity of all 136 ICU beds is used, with an attack rate of 30%, 25% ICU admissions, and a mean length of stay of 8 days, we would have a shortage of 3 ICU beds at day 28 after onset, when we expect the pandemic to peak. This shortage in ICU capacity is exacerbated with any increase in hospital length of stay or ICU length of stay.

HCWs would become ill in the pandemic in proportion to the attack rate in the general population, and we illustrated the impact of HCW absenteeism on loss of ICU bed capacity for all presented scenarios (Figures 1, 2). Furthermore, we visualized the effect of intensified treatment decisions on the occupancy of ICU beds (Figure 2). For this situation, we used the representative case scenario estimate data, i.e., 30% attack rate and a mean length of stay of 8 days, and show the effect of intensified treatment decision resulting in reduction of ICU occupancy by 5% and 20%. Intensified treatment decision was defined as discon-

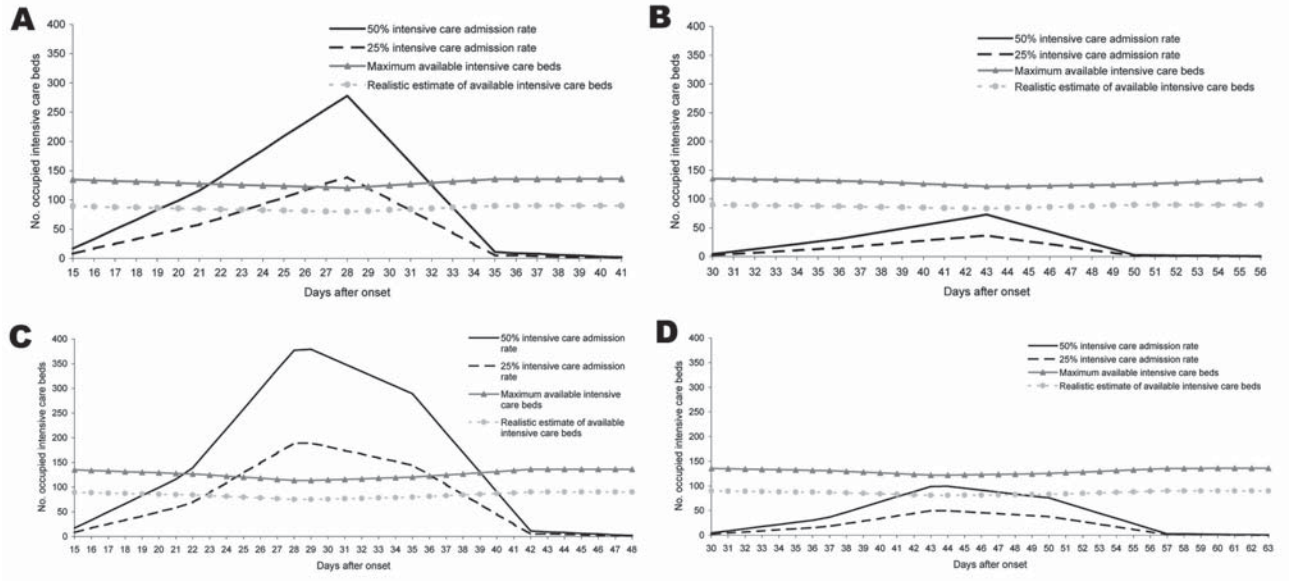

Figure 1. A) 30% attack rate and mean length of stay of 8 days without antiviral medication, pandemic period 9 weeks; B) 30% attack rate and mean length of stay of 8 days with antiviral medication, pandemic period 14 weeks; C) 30% attack rate and mean length of stay of 15 days without antiviral medication, pandemic period 9 weeks; D) 30% attack rate and mean length of stay of 15 days with antiviral medication, pandemic period 14 weeks.

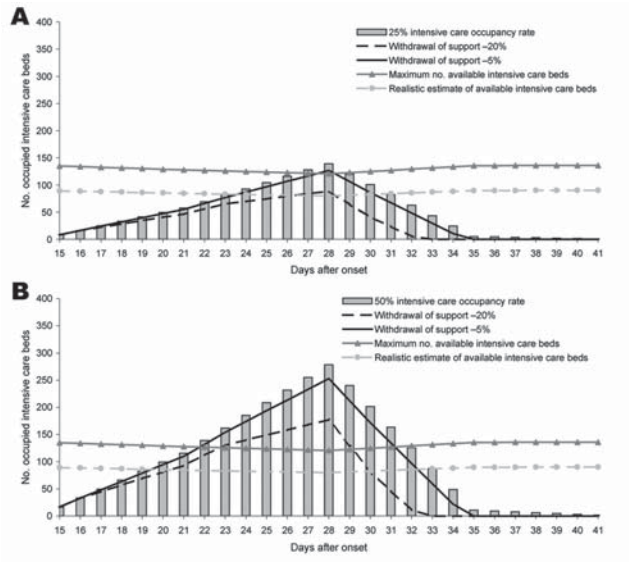

Figure 2. A) Effect of intensified treatment decision (25% intensive care unit [ICU] admission rate, mean length of stay of 8 days) without antiviral medication, pandemic period 9 weeks; B) effect of intensified treatment decision (50% ICU admission rate, mean length of stay of 8 days) without antiviral medication, pandemic period 9 weeks.

tinuation of mechanical ventilation after 48 hours, based on ample consultations within ICU teams and with partners and next of kin of patients that the patients are deemed to have no realistic hope for recovery. Finally, we made sensitivity analyses, with changing assumptions within the model; this additional material is presented in an online Technical Appendix (available from www.cdc.gov/EID/content/13/11/1714-Techapp.pdf).

Discussion

We provide calculations for hospital bed and ICU capacity for an influenza pandemic made for 1 region in the Netherlands showing that even during the peak of the pandemic, hospital facilities can continue to provide adequate healthcare service to the public. As a novel element we include calculations for HCW absenteeism. We have not considered potential erosion of professionalism with increased absenteeism due to fear and panic among staff or due to staff members' caring for sick family members. Although morale was high during the SARS outbreak in Singapore and Toronto (*23*), some examples of strained professional behavior have been reported (*24*). We believe that erosion of professionalism and morale may be partly preventable by implementing effective protection for HCWs (*25*,*26*), with appropriate training to comply with protocols for personal protection. For a new pandemic, the important issues to factor in are magnitude and duration, calculation of staff shortages, and the limited capacity to call in external resources.

We show that an influenza pandemic can be managed, even allowing emergency care for non–influenza-related acute cases, especially when firm decision-making rules are followed and antiviral therapy is used. Without withdrawing or withholding life support to those deemed to have no realistic chance of survival, the system is bound to collapse (Figure 2). With appropriate patient management, however, adequate healthcare can be provided even during the peak of the pandemic. We recognize the ethical impact this has on the clinicians and nurses who have to make these decisions. Many clinicians now realize that end-of-life decisions are an integral part of healthcare (*27*) and can be considered independent of any specific religious background or culture (*28*). ICU staff in the Netherlands have been trained to take charge of decision processes about foregoing life support in the ICU (*27*). They are aware of potential difficulties in communicating with members of the ICU team, including medical, nursing, and technical staff in decisions at the end of life. The challenge during an outbreak of pandemic influenza will be in orchestrating and implementing these decisions under extreme time pressure. Relatives of patients as well as team members may need more time than available to accept that some patients on life support who are not responding to treatment will not recover. Some may insist on continuation of support, although it would be unwise and possibly disrespectful to these patients to continue futile treatment and unfair to others who might have been saved if those resources had been available. A generous and time-consuming approach may not apply under the anticipated extreme conditions of pandemic influenza (*27*).

Decision-making rules have to be adapted to real-time information updates obtained during the course of the pandemic, and briefings and exchange of information throughout the pandemic crisis are pivotal. Existing guidelines and protocols such as the Pneumonia Severity Index or its modification recommended by the American Thoracic Society or the British CURB-65, propagated by the British Thoracic Society, may not apply fully but can be used initially to guide management of patient treatment (*29*). Our overall assessment that an influenza pandemic with assumptions described here can be managed at the level of healthcare institutions clearly contrasts with the sobering and daunting analysis presented for ICU capacity in the United Kingdom or Australasia (*14*,*16*).

There are limitations to our analysis. We based our model on incomplete and sometimes conflicting or inconsistent information on the impact of an influenza pandemic. We assume that more reliable data will only become available when the pandemic is in progress. The effect of antiviral medications, vaccination campaigns, and, for instance,

closure of schools and airports may alter the key characteristics of the pandemic, all having the effect that onset is delayed and that the course is more protracted, with a much lower peak (*12*). Even a less-than-perfect vaccine might have a tremendous impact on the course of the pandemic. Stockpiling of influenza A (H5N1) virus is now being considered in order to produce vast quantities of vaccine despite the limited protection capacity against the new virus.

The need for surge capacity of hospital resources is more dependent on the combination of excess hospital admissions and length of stay than on the mere number of hospital admissions. In the Netherlands, stockpiling of oseltamivir has been implemented, both for the public at large and for healthcare facilities and HCWs working on the frontlines during the influenza pandemic. Stockpiling of antimicrobial agents to combat secondary bacterial pneumonia is yet another important logistic challenge (*30*). The small percentage of patients admitted to hospital in our model (based on past experiences) implies that relatively small increases in admittance rate will have a huge impact on hospital resources requirement.

Extensive exposure may lead to seroconversion to avian influenza viruses, as has been shown for influenza A (H11N9) virus among waterfowl hunters and wildlife professionals (*31*). The policy in the Netherlands since this was discovered has been that all persons involved in culling should wear respiratory masks, gowns, gloves, and eye protection. Although the effectiveness of these precautions has not been prospectively tested, they might protect persons from contracting respiratory viral disease. In our hospital protocol for management of patients of new pandemic influenza and of other high-risk respiratory pathogens, we have included extensive measures to separate these patients from other patients and focus on the protection of staff (*1*). Adherence to similar protocols has been shown to protect HCWs caring for patients with SARS (*26*). In summary, we recommend using and updating the model presented here, or similar models, as an integral part of a preparedness plan and as a management tool for contingency of pandemic influenza.

Mr Nap is pursuing a PhD degree in hospital and intensive care capacity planning, including infectious diseases surge capacity planning. His interests include infectious diseases epidemiology, disasters, and application of mathematical modeling to hospital and intensive care resource planning.

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Role of Terrestrial Wild Birds in Ecology of Influenza A Virus (H5N1)

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House sparrows, European starlings, and Carneux pigeons were inoculated with 4 influenza A (H5N1) viruses isolated from different avian species. We monitored viral replication, death after infection, and transmission to uninfected contact birds of the same species. Sparrows were susceptible to severe infection; 66%–100% of birds died within 4–7 days. High levels of virus were detected from oropharyngeal and cloacal swabs and in organs of deceased sparrows. Inoculation of starlings caused no deaths, despite high levels of virus shedding evident in oropharyngeal swabs. Least susceptible were pigeons, which had no deaths and very low levels of virus in oropharyngeal and cloacal swabs. Transmission to contact birds did not occur frequently: only A/common magpie/Hong Kong/645/2006 virus was shown to transmit to 1 starling. In summary, recent influenza (H5N1) viruses are pathogenic for small terrestrial bird species but the rate of intraspecies transmission in these hosts is very low.

Highly pathogenic avian influenza viruses of subtype H5N1 were identified in Southeast Asia in 1996 and have spread in recent years across broad regions of Eurasia and Africa. These viruses have shown high lethality in chickens and other poultry species (*1*–*3*). Outbreaks of avian influenza, H5N1 subtype and others, have caused massive losses to commercial poultry flocks in recent years (*4*). Direct transmission of H5N1 subtype from infected poultry is thought to be responsible for virtually all of the human influenza (H5N1) infections since 1997. Because of the effects of influenza (H5N1) on human health and agriculture and its potential to mutate and cause a global pandemic, epidemiologic studies of the viruses' host range and their means of dispersal are urgently needed (*5*).

Highly pathogenic poultry isolates from the 1997 and 2001 influenza (H5N1) outbreaks typically cause few disease signs in experimentally infected ducks (*6*,*7*). These viruses' low pathogenicity in waterfowl presumably facilitated efficient carriage to the highly susceptible hosts. Some influenza (H5N1) strains isolated during subsequent outbreaks are highly pathogenic in waterfowl (*7*,*8*), and some are shed by infected ducks for prolonged periods (*9*). Together with the commercial transportation of poultry and poultry products, migratory waterfowl are likely to have played a role in the wide dispersal of highly pathogenic influenza (H5N1) viruses.

Land-based wild bird populations may also be vulnerable to lethal influenza (H5N1) infection and could contribute to the spread and interspecies transmission of the viruses. Small terrestrial birds are potentially important hosts in influenza (H5N1) ecology because many of them intermingle freely with wild and domestic populations of waterfowl and poultry. However, data describing their susceptibility to influenza virus (H5N1) infection or their potential to transmit the viruses are limited.

A study investigating the host range of A/chicken/Hong Kong/220/97 showed that it causes lethal infection in budgerigars and finches (*10*). In contrast, the same virus replicated poorly in sparrows, causing no deaths, and, when pigeons were inoculated, replication of this virus was not evident. A more recent chicken influenza (H5N1) isolate (A/chicken/Yamaguchi/7/2004), highly lethal to chickens and quail, also replicates extensively and causes high mortality rates in budgerigars (*11*). Since 2002, influenza (H5N1) viruses have been isolated from dead birds of

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several wild terrestrial species, including magpie, tree sparrow, pigeon, and large-billed crow (*8*,*12*,*13*). Viruses of a novel influenza (H5N1) genotype were isolated during a survey of live tree sparrows (*Passer montanus)*; these isolates were highly pathogenic to chickens (*14*). Together, these reports indicate that some small, land-based bird species are susceptible to infection, sometimes fatal, with highly pathogenic influenza (H5N1) viruses.

We inoculated sparrows, starlings, and pigeons with several recent highly pathogenic influenza (H5N1) viruses isolated from a variety of avian hosts. The primary aims of the study were to test the susceptibility of different species to infection, investigate the duration and routes of viral shedding from the birds, and assess the possibility of intraspecies viral transmission in these hosts.

Materials and Methods

Influenza A Viruses

Four influenza A (H5N1) virus strains were studied, 2 from previously known susceptible hosts (duck and quail) and 2 from previously unknown hosts (common magpie and Japanese white-eye). The A/duck/Thailand/144/2005 (A/DK/TH/144/05) and A/quail/Thailand/551/2005 (A/Q/TH/551/05) viruses were isolated from western Thailand and tested for their pathogenicity in ducks (*15*). The other 2 viruses, A/common magpie/Hong Kong/645/2006 (A/CM/HK/645/06) and A/Japanese white-eye/Hong Kong/1038/2006 (A/JW/HK/1038/06) were provided to us by K.C. Dyrting and C.W.W. Wong (Agriculture, Fisheries and Conservation Department in Hong Kong). They were isolated from dead wild birds collected in January and February 2006 during the heightened Hong Kong territory-wide avian influenza surveillance of dead wild birds that started in October 2005.Upon arrival at St Jude Children's Research Hospital, the viruses were propagated in 10-day-old embryonated chicken eggs.

Animal Studies

Wild house sparrows (*Passer domesticus*) and European starlings (*Sturnus vulgaris*), both members of the order Passeriformes, were captured. Six-week-old white Carneux pigeons (*Colomba* spp.), members of the order Columbiformes, were purchased from Palmetto Pigeon Plant (Sumter, SC, USA) and Double T farms (Glenwood, IA, USA). Birds were housed in cages in the St Jude Children's Research Hospital Animal Biosafety Level 3+ containment facility, food and water were provided ad libitum, and general care was provided as required by the Institutional Animal Care and Use Committee. Before inoculation with virus, oropharyngeal and cloacal swabs were collected to exclude preexisting influenza A virus infection.

Three sparrows and pigeons were inoculated intranasally with 1 million 50% egg infectious doses (EID_{50}) in 50 μL or 500 μL phosphate-buffered saline, respectively, for each virus. Because of their limited availability, starlings were inoculated with 3 viruses (1 million EID_{50} in 150 μL), and group sizes were reduced (1 bird for A/DK/TH/144/05, 3 birds for A/CM/HK/645/06, and 2 birds for A/JW/HK/1038/06). One day after inoculation, uninfected contact birds, at a ratio of 1:1 for sparrows and starlings or 2:3 for pigeons, were housed together with inoculated animals to study intraspecies transmission. Birds were monitored daily for death and illness for a 14-day period. After inoculation, oropharyngeal and cloacal swabs were collected on days 2, 4, 6, 8, and 11 for sparrows and starlings and days 3, 5, and 7 for pigeons. Influenza virus was detected by using 10-day-old embryonated chicken eggs as previously described (*7*). EID_{50} virus titers were determined in positive swabs by using the method of Reed and Muench (*15*). The lower limit of quantitation of the assay is $10^{0.75}$ EID_{50}/mL, and average virus titers in organs and swabs were calculated by using the $\log_{10}$ value of each sample.

Serology

Fourteen days after inoculation with virus, serum specimens were collected from inoculated and contact birds, and hemagglutination-inhibition (HI) titers were determined according to standard methods (*16*,*17*) by using chicken erythrocytes and 4 hemagglutinating units of virus. An HI titer >10 suggested a recent influenza virus infection; an HI titer <10 was considered negative.

Results

Infection of Different Bird Species with Influenza (H5N1) Virus

The ability of 4 different influenza A (H5N1) viruses to infect and cause disease in house sparrows, European starlings, and white Carneux pigeons was determined. Infection of sparrows caused death in 66%–100% of the infected animals, depending on the inoculated virus (Table 1). The average time to death varied from 4.2 days for A/DK/TH/144/05 to 6.3 days for A/Q/TH/551/05 virus (data not shown). High viral loads were detected in brain and lung tissues of deceased sparrows (Figure, panel C). In contrast, none of the starlings or pigeons died after inoculation with these viruses.

Re-isolation of virus from oropharyngeal and cloacal swabs obtained at various time points after inoculation indicated that all the sparrows and starlings were infected by all viruses tested. In contrast, the frequency of virus re-isolation from inoculated pigeons varied widely among viruses. Of the 4 different H5N1 subytpes, A/CM/HK/645/06 demonstrated the broadest host range, infecting not only spar-

Table 1. Influenza A virus titers in oropharyngeal and cloacae swabs of infected birds*

Species	Virus	Mortality rate, %	Virus titer (EID_{50}/mL) in swabs from infected animals					
			Day 2		Day 4		Day 6	
			T	C	T	C	T	C
Sparrow	A/DK/TH/144/05	100	2.4	1.7	4.7	4.1	–†	–
	A/Q/TH/551/05	100	1.0	<1	1.5	1.3	3.1	1.0
	A/CM/HK/645/06	66	2.6	0.8	2.3	2.1	1.6	1.1
	A/JW/HK/1038/06	100	2.1	<1	2.7	3.3	–	–
			Day 2		Day 4		Day 6	
			T	C	T	C	T	C
Starling	A/DK/TH/144/05	0	3.8	0.8	3.3	<1	2	<1
	A/Q/TH/551/05	0	ND	ND	ND	ND	ND	ND
	A/CM/HK/645/06	0	3.3	0.8	3.6	1	1.7	1.5
	A/JW/HK/1038/06	0	2.5	1	2	<1	1.8	<1
			Day 3		Day 5		Day 7	
			T	C	T	C	T	C
Pigeon	A/DK/TH/144/05	0	<1	<1	<1	<1	<1	<1
	A/Q/TH/551/05	0	<1	0.5	0.8	<1	<1	<1
	A/CM/HK/645/06	0	1.9	<1	1.4	<1	<1	<1
	A/JW/HK/1038/06‡	0	0.5	<1	0.4	<1	<1	<1

*EID_{50}, 50% egg infectious dose; T, oropharyngeal swab; C, cloacal swab; ND, not done.
†Birds did not survive the infection.
‡Swabs were taken on days 3, 6, and 10 after infection.

rows and starlings but also all of the inoculated pigeons. The A/DK/TH/144/05 virus, which caused 100% mortality in sparrows within 4.2 days after inoculation, was not re-isolated from inoculated pigeons.

Quantification of the virus titer in the swabs demonstrated that sparrows and starlings shed similar amounts of virus in oropharyngeal swabs. However, virus titers in the cloacal swabs of sparrows were higher than in those obtained from infected starlings (Table 1). Comparison of peak virus titers in oropharyngeal swabs confirmed the similarity in oral shedding between sparrows and starlings. In contrast, peak virus titers in the cloacal swabs of starlings were lower (Figure, panels A and B). The 2005–2006 influenza (H5N1) viruses replicated relatively poorly in pigeons, as shown by average oropharyngeal and cloacal shedding on days 3 and 5 (Table 1) and by peak virus titers in oropharyngeal and cloacal swabs (Figure, panels A and B).

Intraspecies Transmission of Influenza (H5N1) Viruses

The capacity of current influenza (H5N1) viruses to transmit from infected birds to same-species uninfected birds was assessed for these 4 viruses. No evidence of transmission in sparrows and pigeons was found, as attempts to isolate the virus from contact birds failed (Table 2). Also, no virus-specific antibodies were detected by HI in the contact birds (data not shown). In starlings, transmission of virus to contact birds was observed once for A/CM/HK/645/06 virus, but this was not seen in 2 further experiments.

Discussion

The susceptibility of 3 species of wild terrestrial birds to influenza A (H5N1) virus and their ability to transmit to contact birds were assessed. Our studies show that major differences in susceptibility to influenza (H5N1) virus infection exist among these bird species and that, under

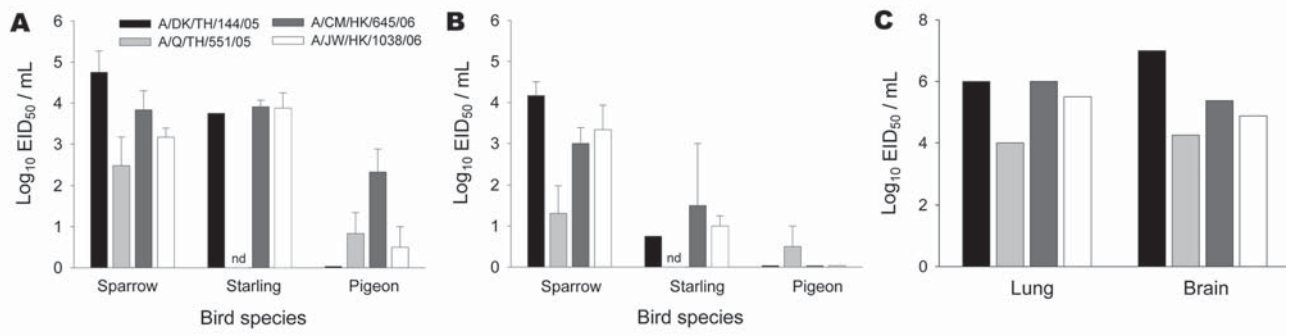

Figure. Average peak influenza A virus titers in oropharyngeal (A) and cloacal (B) swabs during the course of influenza (H5N1) infection in 3 terrestrial bird species. C, influenza A virus titers in lungs and brains of deceased sparrows. Data are presented as log_{10} 50% egg infectious doses per milliliter (log_{10} EID_{50}/mL). ND, no data available.

Table 2. Transmission of influenza (H5N1) virus from infected to contact birds of the same species

Species	Influenza A virus			
	A/DK/TH/144/05	A/Q/TH/551/05	A/CM/HK/645/06	A/JW/HK/1038/06
Sparrow	0*	0	0	0
Starling	0	ND†	33	0
Pigeon	0	0	0	0

*Percentage of contact birds from which virus was isolated.
†Experiment not done.

our conditions, transmission occurred infrequently. Pigeons, starlings, and sparrows were more susceptible to experimental infection with the recent (H5N1) isolates than they were to A/chicken/Hong Kong/220/97 (H5N1) virus (*6*,*10*,*18*). Although drawing conclusions on the basis of a single 1997 isolate is inappropriate, these data are consistent with studies that have demonstrated increased virulence or host range for recent influenza (H5N1) viruses in mammalian species, including mice, ferrets, and domestic and wild cats (*19*–*22*). Whereas a previous study showed that a 2003 chicken influenza (H5N1) isolate can cause severe neurologic disease in pigeons, we observed no signs of disease in influenza (H5N1)–infected pigeons (*23*). Such a difference in pathogenicity between our study and others may be due to subspecies differences or a change in inoculum size.

A critical question concerning these small avian species is whether they can serve as intermediate hosts or reservoirs for influenza (H5N1) viruses and transmit them to poultry and mammals. Sparrows were highly susceptible to influenza (H5N1) infection; however, they did not transmit to sentinel contact birds, despite a relatively low infectious dose (≈500 EID_{50} for A/DK/TH/144/05 virus, data not shown) and the fact that virus was common in drinking water and fecal samples. Although it is possible that the high pathogenicity of these viruses prevented bird-to-bird transmission, the data suggest that this species can act as an intermediate host and potentially transmit to both poultry and mammals but not serve as a reservoir for prolonged shedding of highly pathogenic influenza (H5N1) viruses. In contrast, the characteristics of influenza (H5N1) infection in starlings, i.e., nonfatal with longer-term shedding, suggest that starlings could act as an intermediate host and a reservoir for influenza (H5N1) virus. However, evidence of transmission to contact starlings was limited, which implies that these strains are unsustainable in a starling population. Because pigeons shed only low amounts of virus upon infection and they did not transmit to contact birds, their role in the ecology of influenza (H5N1) virus may be minor.

Our results indicate that there are considerable differences in susceptibility to influenza (H5N1) virus among various small terrestrial wild bird species. The high virulence of several recent isolates in sparrows suggests that this and other populations of small terrestrial birds may have substantial losses during current and future outbreaks. Further mutation of circulating influenza (H5N1) viruses might enhance their adaptation to hosts such as starlings and sparrows, further increasing virulence or allowing these species to become efficient intermediate hosts in the ecology of influenza (H5N1) viruses.

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etymologia

tularemia

[tü-lə-rē-mē-ə]

An infectious, plaguelike, zoonotic disease caused by the bacillus *Francisella tularensis*. The agent was named after Tulare County, California, where the agent was first isolated in 1910, and Edward Francis, an Officer of the US Public Health Service, who investigated the disease. Dr. Francis first contracted "deer fly fever" from a patient he visited in Utah in the early 1900s. He kept a careful record of his 3-month illness and later discovered that a single attack confers permanent immunity. He was exposed to the bacterium for 16 years and even deliberately reinfected himself 4 times.

Tularemia occurs throughout North America, many parts of Europe, the former Soviet Union, the People's Republic of China, and Japan, primarily in rabbits, rodents, and humans. The disease is transmitted by the bites of deerflies, fleas, and ticks; by contact with contaminated animals; and by ingestion of contaminated food or water.

Clinical manifestations vary depending on the route of introduction and the virulence of the agent. Most often, an ulcer is exhibited at the site of introduction, together with swelling of the regional lymph nodes and abrupt onset of fever, chills, weakness, headache, backache, and malaise.

Source: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; Benenson AS, editor. Control of communicable diseases manual. Washington: American Public Health Association; 1995; www.whonamedit.com

Canonical Insertion-Deletion Markers for Rapid DNA Typing of *Francisella tularensis*

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To develop effective and accurate typing of strains of *Francisella tularensis,* a potent human pathogen and a putative bioterrorist agent, we combined analysis of insertion-deletion (indel) markers with multiple-locus variable-number tandem repeat analysis (MLVA). From 5 representative *F. tularensis* genome sequences, 38 indel markers with canonical properties, i.e., capable of sorting strains into major genetic groups, were selected. To avoid markers with a propensity for homoplasy, we used only those indels with 2 allelic variants and devoid of substantial sequence repeats. MLVA included sequences with much diversity in copy number of tandem repeats. The combined procedure allowed subspecies division, delineation of clades A.I and A.II of subspecies *tularensis*, differentiation of Japanese strains from other strains of subspecies *holarctica,* and high-resolution strain typing. The procedure uses limited amounts of killed bacterial preparations and, because only 1 single analytic method is needed, is time- and cost-effective.

Francisella tularensis is a highly infectious, facultative intracellular pathogen and the causative agent of the zoonotic disease tularemia. Based on virulence tests and biochemical assays, *F. tularensis* is divided into 4 subspecies, a division that has recently been corroborated by genetic typing (*1*,*2*). Each subspecies shows a discrete natural geographic distribution and also varying degrees of virulence (*3*). Human disease caused by *F. tularensis* subsp. *tularensis* may be fulminate or even lethal, whereas disease caused by other subspecies is less severe, although often incapacitating and protracted (*4*). In addition, recent molecular and epidemiologic analyses of natural isolates of *F. tularensis* subsp. *tularensis* suggest a population split of the subspecies into 2 major groups of isolates, which differ in virulence and geographic distribution (*5*–*7*).

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Robust and rapid typing schemes for *F. tularensis* are needed, not only because of their use in clinical and public health work but also because of a rising concern associated with risks for bioterrorism (*4*,*8*). Because of its virulence, *F. tularensis* is included among the top 6 "category A" potential bioterrorism agents believed to have the greatest potential for adverse public health effect with mass casualties. If deliberate release of the organism is suspected, the need to understand the pathogenic potency of an isolate and also its putative origin will be urgent.

In standard medical practice, subspecies determination of *F. tularensis* typically involves biochemical fermentations. Such analyses are labor-intensive, hampered by the fastidious growth characteristics of the organism on artificial media, and associated with a substantial risk for laboratory-acquired infections (*2*,*9*).

Several DNA-based methods have been found useful for typing of *F. tularensis* at the subspecies level (*1*,*10*–*13*). Among these, pulsed-field gel electrophoresis (PFGE) is more widely adopted and was recently proposed for diagnostic and epidemiologic work on *F. tularensis* by PulseNet laboratories throughout the United States (*7*). PFGE typing is, however, far from ideal for the purpose. It involves making concentration-adjusted suspensions of live bacteria, which has the potential for creating infectious aerosols, is time-consuming, produces complex banding pattern data, and has a restrictive discriminatory capacity when applied to *F. tularensis* (*7*,*14*–*17*).

High-resolution typing of *F. tularensis* is currently attainable only by the use of multilocus variable-number tandem repeat analysis (MLVA). The method capitalizes on differences among strains in copy numbers of sequence repeats at multiple genomic loci. MLVA has been successfully applied in epidemiologic studies on tularemia (*5*,*6*,*18*,*19*). Killed bacterial preparations can be used in

the assay and, in contrast to PFGE, MLVA produces discrete-character numeric data, which are well suited for easy transfer among laboratories. For discrimination of strains of *F. tularensis*, MLVA is the obvious choice.

A limitation inherent in MLVA is the risk for erroneous estimates of relationships among strains at larger genetic distances. The high rates at which MLVA markers mutate (*20*,*21*), and possible functional constraints on these sequences, may cause homoplasy effects, i.e., share of mutational changes for reasons other than common ancestry (*22*,*23*), implicating a risk for spurious strain affiliation. In work on *Bacillus anthracis*, the issue was addressed by analysis of single-nucleotide polymorphisms (SNPs), which exhibited canonical properties for resolving major genetic lineages (*24*). In a hierarchical typing approach, which conformed with concepts of traditional bacterial taxonomy, a 2-step procedure was suggested, including assay of canonical SNPs for resolution of major genetic clades and MLVA for high-resolution typing (*24*). A limitation of the procedure is that it involves 2 assays, thus increasing time and cost.

When aiming to construct an improved typing strategy for *F. tularensis*, we focused on insertion-deletion (indel) markers. By definition, indels are caused by insertion or deletion of ≥1 base pairs of a DNA molecule. Among indels, the evolutionary rates diverge widely. When used as a complement to MLVA, more slowly evolving indels, i.e., loci displaying a relatively low degree of variability, would be preferable. A practical reason to use canonical indel markers was that fragment analysis can by used for simultaneous assay of both indel and MLVA markers, thereby minimizing time and cost.

We identified indel markers with canonical properties in *F. tularensis* and used them to resolve major genetic lineages of the species. We also developed a strategy that combines indel analysis with MLVA for rapid and accurate discrimination of isolates of the species.

Material and Methods

Genome Sequences, Strains, and DNA Preparations

We used genome sequences for the 5 strains, U112 (aka FSC040, ATCC 15482), FSC147 (GIEM 543), SCHU S4 (FSC237), OSU18, and LVS (FSC155) (online Appendix Table 1, available from www.cdc.gov/EID/content/13/11/1725-appT1.htm), for in silico work, and in total, 23 isolates (online Appendix Table 2, available from www.cdc.gov/EID/content/13/11/1725-appT2.htm, and online Appendix Table 3, available from www.cdc.gov/EID/content/13/11/1725-appT3.htm) were selected for the experimental work. These were chosen to represent each of the 4 currently recognized *F. tularensis* subspecies and were selected from the *Francisella* Strain Collection (FSC) maintained at the Swedish Defence Research Agency, Umeå, Sweden. Bacteria were grown on modified Thayer-Martin agar (*25*), suspended in phosphate-buffered saline, and immediately heat killed. DNA was prepared by using silica and guanidine isothiocyanate buffer (*26*). Extended information on strains and, when appropriate, GenBank accession numbers, are available in online Appendix Tables 1–3.

Identification and Selection of Indel Markers

Multiple alignment of genomic sequences for *F. tularensis* strains U112, FSC147, SCHU S4, OSU18, and LVS was performed by using Mauve 2.0 β multiple alignment software (*27*) and the progressive alignment option. The output file produced by Mauve was parsed by using a custom Perl script to retrieve multiple aligned sequences for indel loci that fulfilled the following criteria: 1) the loci should exist in all compared strains, 2) only 2 allelic variants should exist, 3) at least 25 bp of sequences lacking other indels should flank identified loci, 4) indels should be 5- to 200-bp long, and 5) direct repeated sequences of substantial length should not be present at indel loci because such sequences may increase the risk for homoplastic mutation.

Primer Design and PCRs

Oligonucleotide primers for PCR amplification were designed by using the Primer3 tool (*28*) and a Perl script to supply aligned sequences and required coordinate information. To reduce experimental cost, the forward primer of each primer pair was synthesized with an additional 17-bp M13 tail added to the 5′ end of the primer (Table). This enabled the use of fluorescently labeled M13 PCR primers to simultaneously amplify marker loci and label the PCR amplicons. The M13 primers were labeled terminally with D2-PA, D3-PA, or D4-PA dyes at the 5′ end (Proligo Primers and Probes, Hamburg, Germany).

PCR amplification was performed in 96-well microtiter plates. Each reaction mixture contained 0.15 mmol/L dNTP, 0.6 U DyNAzymeII polymerase (F-501L, Finnzymes, Espoo, Finland), 1 μL PCR buffer for DyNAzyme DNA polymerase (Finnzymes), 2 μL of template DNA (20 ng/μL), 0.3 pmol/L forward primer, 0.8 pmol/L reverse primer, and 0.8 pmol/L labeled M13 primer. Filtered sterile water was added to a final volume of 25 μL. The PCR reactions were performed in a MyCycler thermal cycler (BioRad, Hercules, CA, USA) with the following program: 95°C for 2 min; 15 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 45 s; 20 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 45s; and then a 7-min final extension step at 72°C. MLVA was performed as previously described, except modified to use fluorescence-labeled forward primers (*6*). The physical distribution of 38 selected indel markers identified in this

Table. Insertion-deletion loci, genomic locations, and primers

Ftind locus*	Positions†	Pattern	Forward primer sequence (5′→3′)‡	Reverse primer sequence (5′→3′)
1	1152573–1152844	12222	TCTCGTGACAGAGCTTTACAA	GGGAGAATTGATTATGGCTTAC
2	895732–896067	12222	AGCAGCGTATCGAAGAGATAG	TAAATCTAGTTGGCTGAGTAATAAAGTC
3	769704–770059	12222	CAAACCTAATTGCTCCAGAAC	GCAGCATATCTTTGGTCATCTAT
4	520340–520556	12222	TTTGAAAAGCTAGAAAAAGATGC	ACCAAGAATATTAAAAGCCAAATC
5	1628363–1628558	12222	AACTAAGTTGTTTTAGTGGGTTCC	CAATTTTATACCCCAGTTAATATTTGA
6	562346–562675	12222	CAACAATCTCACCATTACCTAAAA	GCTAGGCAAGCCATTATATTTATC
7	688418–688771	12222	CCAAAATATACCAAAATATCCTATCA	ATTTATGCAATATCACAAGTTCCA
8	198167–198521	12222	GTGACCTAATCAAAGAGCAACTAA	ATCTGCATACTTGAGTAAATGCTT
9	1830520–1830768	11211	CTCAAGAAATTAAAGGGATGAGTT	ATTTGCTCAGTACCTGCTAATGTA
10	1113820–1114081	11211	CATTCCTAGTRATAGCTCCTGCT	ATTAAGCTTCAACACTATCATCATCT
11	1238526–1238784	11211	TACTTTTAATGCTTCAGCGACA	AATCACCAATAACCCAGACAAC
12	725006–725258	11211	GCCTATGCTGGTAAAGTTGG	TCACCAATAGCTTCCATAACAC
13	1490938–1491179	12211	AACTCCTGGTTTCCCACAC	GCTACAAAACTCACTATGTTCAGAC
14	625186–625399	12211	GACTGAACAACAACTGGATTATCAC	TGTAGTCCATTAGGGCAGTAATCTT
15	573074–573303	12111	GGTTTTGTTGCTAAATCTGC	ACGCTGATCATCAATCATTC
16	1628145–1628393	12111	TCCTTTAAAGAAACGGCATA	TCTGTACGGAACCCACTAAA
17	239966–240157	12111	CATGAAAACTTGGTTATAGCTGA	GCGCAAGATCAGCTTAGTT
18	439229–439434	12111	AGAGTTAACCCATTCAACAAGA	GGCAAGGTTTCTGGATAGAC
19	408363–408515	12111	TTTGATAGCTCAAATGCAAGA	AGCTAGCTTGCCTCTTTTCT
20	602863–603177	11122	AAATCATTTAACAATTGGTATCTTT	TAGCTCTGAGTTAGAAAAACTCG
21	271531–271863	11122	TCTTCTTGTATAAGATGCGCTAAA	GGTTAAGTTAGGGCAATGTAAGAT
22	5648–5976	11122	TGACAAAGAAGACTAAGCACAAAT	GGTTTGATAAATGCAAACTATATGAT
23	1062332–1062553	11122	TCAACCGGCTTTATGAGAGTA	TATTACGAGACCGAAAATACGATA
24	1641399–1641720	11122	AATTCAAAAAGCGATAAGTAACCT	GCCAGCAACATACTCTTTTGT
25	267938–268267	11122	AAATTAAAGCAAGGACAGGTTTAT	TCCATAGTTATTTCAACTTGGTTT
26	1828819–1829145	11122	AGCTGCTAAATCTAAACTCTTTGC	GCTCCCTCAACTAGATCTATCATC
27	960872–961191	11122	AATCGCATACATTTCTGCTGTA	GCTTTTCCAAATGAGGATATTAAA
28	1136267–1136582	11122	AAAAGTAGCTGCAGAAGTATACCC	TTCTCAAAATGTAAACATGCTTCT
29	1190422–1190738	11122	CTTGAGCTTACGCCCTTTTAT	ATGTCCGCAATATTGTCCTAAC
30	871284–871614	11112	CTGCATTTTCAACATTACTCAGAT	ATTCATAAAGATCATCCATTCCTC
31	518787–519092	11112	AGCTGTAGTGATATAAAGAAAAGTTACAT	CTATTTCGTAGCGAGTAAGAATTT
32	1709427–1709741	11112	TTATGCAAATAACTATCCAAGTGTT	TTACCATTAGCTTCAAAAGTCTGT
33	511958–512251	11112	TACAAGCGTACCATCTAAGTCA	CATATTGGGATGTCAAGCA
34	99015–99303	11112	TTGATATAACCAACATAAACACTGC	TGAGTATAGAAATACAAAGCTACGC
35	772225–772590	11121	TGTGTAGTAACCCAGGAACTTTAT	AATTTGATGCCATATGAGAGAAT
36	282847–283070	11121	TTTGGTATGAGTATTCTGGTCCTA	GTATTTTGGTTTAGCTTACGGATT
37	1486225–1486603	11121	AATATTTGCAACCAATGATGATAC	CAGTATCTTTGATGTTAGGGACAA
38	95621–95874	11121	GCTACGACAGGTCTATCTTTCTC	CAACTTATGATTGGTGATGATGT

*Ftind, *F. tularensis* insertion-deletion marker.
†Location of the DNA amplified by PCR in the chromosome of *Francisella tularensis* strain SCHU S4.
‡Sequences given for forward primers represent the target-specific parts of the primers used. For inexpensive fluorescent labeling, each forward primer was synthesized with a 17-bp extension at the 5′-end, corresponding to an M13 sequence (5′-GTAAAACGACGGCCAGT-3′).

study and 25 MLVA markers throughout the genome of strain SCHU S4 (*29*) is illustrated in Figure 1.

PCR Amplicon Separation

PCR reaction mixtures, 2 μL from each, were pooled and diluted 15-fold. One μL of diluted sample was added to 40 μL of sample loading solution, containing DNA Size Standard-600 (Beckman Coulter Inc., Fullerton CA, USA), and sealed with a drop of mineral oil. Finally, PCR amplicons were separated and detected by using a CEQ 8800 Genetic Analysis System (Beckman Coulter Inc.). Binning of indel fragment size-calls was straightforward because of highly precise size determinations (online Appendix Table 2). Maximum size divergence between size-call and genome sequence data was 3 bp among 38 selected indel markers for strains U112, FSC147, SCHU S4, or LVS.

Statistical Analysis

Simpson's index of diversity (1 – D) (*30*) was determined for each investigated marker as a measure of both richness and evenness, calculated as

$$1-\left[\frac{1}{N(N-1)}\right]\sum_{j=1}^{s} nj(nj-1)$$

where N is the number of strains, s is the number of recorded states for a marker, and nj is the number of strains

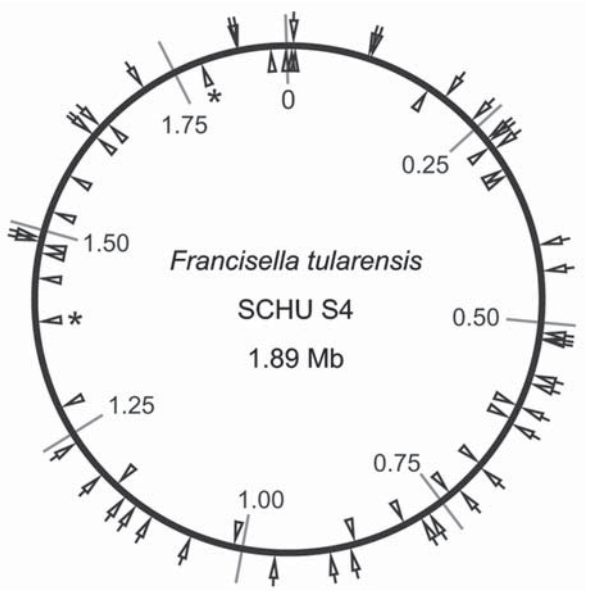

Figure 1. Locations of 38 insertion-deletion and 25 multilocus variable-number tandem repeat analysis (MLVA) markers on the physical genome map of *Francisella tularensis* subsp. *tularensis* strain SCHU S4. Positions are given with reference to the predicted origin of replication set at position 0. Indel and MLVA marker locations are depicted by wedges on the outside and inside of the circle, respectively. Two asterisks indicate the duplicate occurrence of the MLVA loci Ft-M14 at 2 different locations because it is part of a large sized genome duplication *(1,25)*.

belonging to the *j*th marker state. Both distance-based clustering, by using Hamming distance (*31*) and the neighbor-joining method, and maximum parsimony (MP) were performed with PAUP* version 4c10 (*32*). MP analyses were performed by using 50 replicates without branch swapping and 10,000 bootstrap pseudoreplicates. Nodes supported by <50% bootstrap pseudoreplicates were collapsed in depictions of the obtained consensus topologies. Indel size and distribution of repeat size frequency were analyzed by using the R statistical package (*33*).

Results

Identification and Selection of Indel Loci

In the genomic sequences of each of 5 *F. tularensis* strains (online Appendix Table 1), a total of 280 indel loci were identified, all exhibiting only 2 allelic variants and a size range of 5–200 bp. Small-sized indels predominated; 70% were shorter than 20 bp (Figure 2, panel A). To enable the selection of loci free from such repeat nucleotide sequences, which may have a propensity to initiate deletion or insertion mutations, indels were analyzed with regard to the size of associated repeats. Two repeat size peaks were identified, 1 at 10 bp ± 1 bp and another ≤3 bp (Figure 2, panel B). In 62 loci, no repeats were found. After exclusion

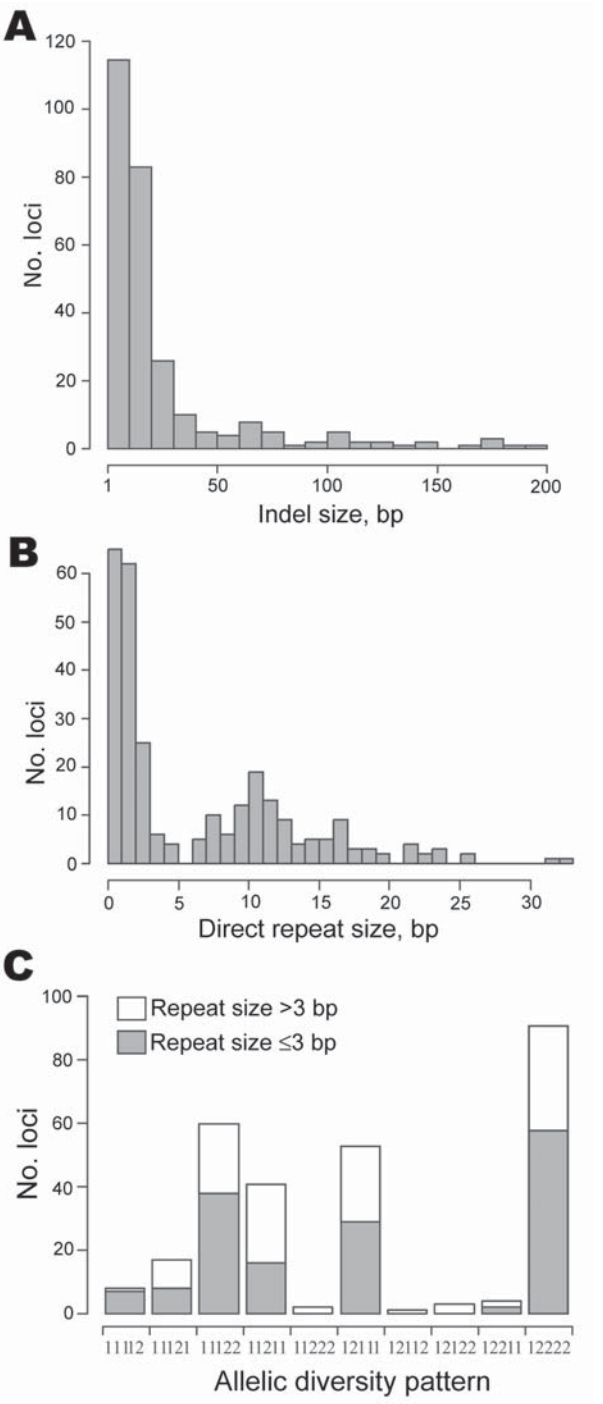

Figure 2. Properties of 280 insertion-deletion (indel) loci identified by analysis of 5 *Francisella tularensis* genome sequences. The diagrams show distributions of indel sizes (A), repeat sizes detected at these loci (B), and10 allelic diversity patterns (C); the number 1 or 2 represents each of the 2 allelic variants. A string of numbers includes, in order, strain U112 (subsp. *novicida*), FSC147 (subsp. *mediasiatica*), SCHU S4 (subsp. *tularensis*), OSU18 (subsp. *holarctica*), and LVS (subsp. *holarctica*). Empty and filled bars correspond to the presence or absence of repeats >3 bp long, respectively.

of loci associated with repeats >3 bp in length, 158 loci were retained for typing purposes.

To facilitate selection of indel loci represented in various strains, we analyzed the diversity of the 280 allelic variants among the 5 *F. tularensis* genomes included. Among the genomes, only 10 discrete allelic diversity patterns were found, depicted in Figure 2, panel C, as allelic variant 1 or 2 in each of the genomes in order of strains U112, FSC147, SCHU S4, OSU18, and LVS (e.g., 1,2,1,1,1 denotes that a deletion was present in the genome sequence of strain FSC147, but not in any of the others). After loci associated with repeats >3 bp in length were excluded, 7 allelic patterns were retained and used as a basis for selecting indel loci for the assay (Figure 2, panel C).

By these measures, a subset of 38 loci was selected (Table; online Appendix Table 2). These loci showed maximum diversity, represented each allelic pattern among the 5 genomes, and also exhibited a physical separation on the SCHU S4 chromosome (Figure 1).

Analysis by the Combined Procedure of 24 Strains of *F. tularensis*

Twenty-four strains, representing all 4 subspecies of *F. tularensis* and clades A.I and A.II of *F. tularensis* subsp. *tularensis*, underwent indel analysis and MLVA (online Appendix Tables 2, 3). Of these, 23 yielded indel PCR amplicons in the range of 145–399 bp, representing an allele of each of 38 loci analyzed. In the remaining strain, isolate FSC454, PCR amplification failed for 7 indel loci tested. FSC454 is an atypical *Francisella* isolate of uncertain taxonomic status recently isolated in Spain (R. Escudero, pers. comm.). FSC454 was excluded from further analyses.

Another atypical strain, ATCC 6223, yielded aberrant amplification results. This strain has lost virulence for mammals, a key characteristic of *F. tularensis*. It exhibits unusual colony morphologic features and a slow growth rate. When subjected to PCR amplification, the genome of strain ATCC 6223 yielded 2 DNA amplicons for an indel locus denoted Ftind-32. Ftind-32 and ATCC 6223 were retained for further analysis, and both alleles were considered.

A graphic representation of the observed amplification patterns at indel and MLVA loci is shown in Figure 3. A difference in mutational stability was apparent between indel and MLVA loci. Indel loci showed a binary pattern that grouped *F. tularensis* in agreement with traditional taxonomy based on phenotype. In accordance with previous genetic typing by MLVA, PFGE, or sequencing of 7 housekeeping genes, the indel analysis distinguished 2 major subpopulations of type A strains (denoted A.I and A.II) and also showed Japan-derived *F. tularensis* strains to be distinct from strains of *F. tularensis* subsp. *holarctica* isolated in other parts of the Northern Hemisphere. Furthermore, indel analysis identified additional subpopulations among *F. tularensis* subsp. *holarctica* strains. Geographic origins of these subpopulations suggest dispersal over large distances. Two strains from the United States, OSU18 (represented by genome sequence data only) and FSC035, were identical at all indel loci and constitute a distinct genetic entity. Strains FSC012 from the United States and FSC519 from Sweden formed another entity. Finally, 6 strains originating in Sweden or Russia represented a third subpopulation. Compared with indel analysis, MLVA showed much more extensive polymorphisms, which was helpful for characterizing individual strains. Simpson's index of diversity ranged between 0.17 and 0.97 for the MLVA loci and between 0.09 and 0.52 for the indel loci, which reflects the fact that only 2 allele states were present for the indel loci while the MLVA loci were more diverse, with up to 16 alleles (for MLVA marker Ft-M3).

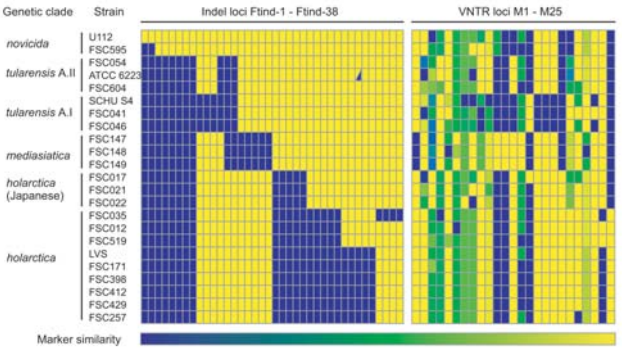

Figure 3. Heat map of marker states for 38 insertion-deletion (indel) and 25 multilocus variable-number tandem repeat analysis (MLVA) loci examined. Each *Francisella tularensis* strain is represented by a single row of colored boxes and each DNA loci by a single column. Relative genetic similarity is represented by the similarity of the colors on the gradient scale ranging from blue to yellow. For the binary indel markers, the state of each marker in the genome of strain *F. tularensis* subsp. *novicida* U112 represents the index and is depicted in yellow. Blue indicates the amplification of an allelic variant distinct from that of the index genome. For strain ATCC 6223, both alleles were amplified at loci Ftind-32, and the corresponding box is thus divided into a yellow and a blue part. For MLVA loci, blue represents the largest allele size for each multistate marker; yellow represents the smallest.

Phylogenetic Inferences Based on MLVA and Indel Data

Genetic relationships among *F. tularensis* strains were inferred by MP analysis of the MLVA data, indel data, or both indel and MLVA data (Figure 4). The use of MLVA data alone resulted in weak support for delineation of deeper branching patterns, few nodes having >50% support in bootstrap analysis (Figure 4, panel A). For such purposes, indel data alone were more valuable (Figure 4, panel B). The use of combined indel and MLVA data resulted in well-supported deep nodes and discrimination of the strains included in this study (Figure 4, panel C).

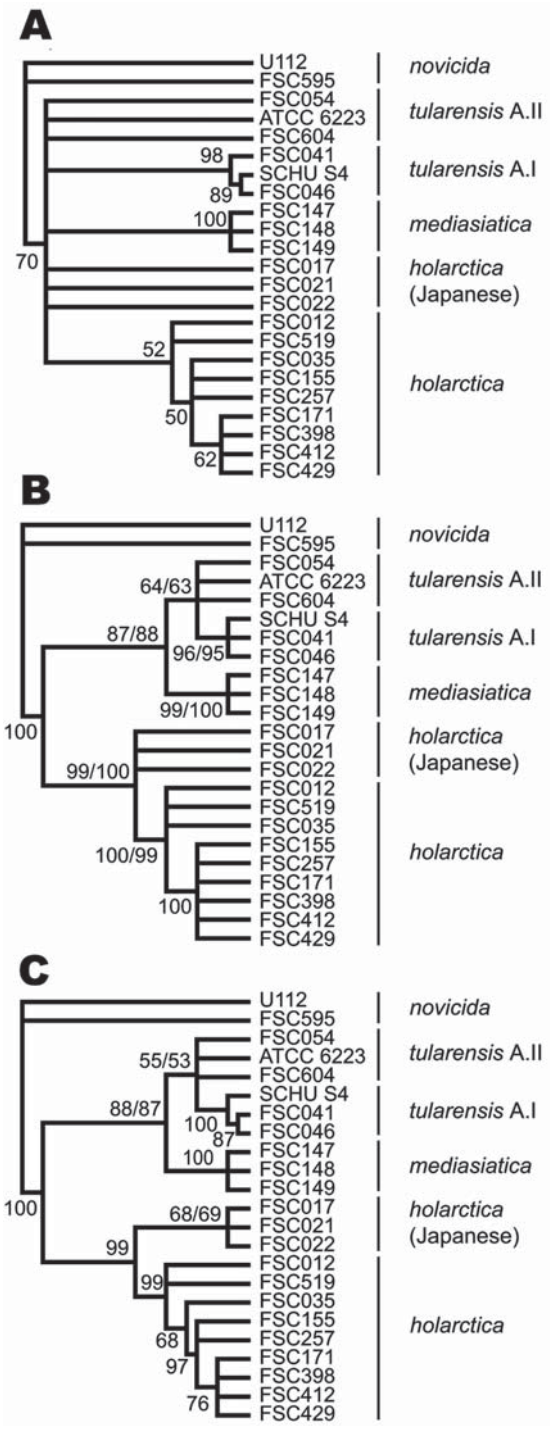

Figure 4. Cladograms depicting relationships among *Francisella tularensis* strains obtained by maximum parsimony and bootstrap analysis that used indel, multilocus variable-number tandem repeat analysis (MLVA), or combined data. Nodes supported by <50% of bootstrap pseudoreplicates were collapsed. A) Cladogram obtained solely from the use of MLVA data. B) Cladogram from the use of indel data. C) Cladogram from the combined use of indel and MLVA data. The dual bootstrap support values presented represent the use of each of 2 alleles, found at locus Ftind-32 of strain ATCC 6223.

In strain ATCC 6223, dual bootstrap support values (Figure 4, panels B, C) represent values obtained by using each of the 2 alleles amplified for locus Ftind-32. The same topology was obtained regardless of which allele was included, and the allele used had minor effect on bootstrap support values. Results were highly similar when using inference by neighbor joining (data not shown).

Discussion

By combining canonical indels with MLVA, robust subspecies and major clade typing of *F. tularensis* was successfully combined with high-resolution typing among strains. By the use of killed bacterial preparations, the 2 marker sets were rapidly assayed by fragment analysis.

The present canonical indel/MLVA typing concept adapts well to the principles of diagnostic work inherent in public health laboratories. The concept generates portable straight numeric data and, similar to the tests of biochemical reactions, 2 alternative states are determined at multiple indels (Figure 3). The MLVA output consists of multistate discrete numbers and has proven superior to PFGE for reliable resolving of discrete strains of the species (*6*,*7*).

Typing of *F. tularensis* provides useful public health information. This is especially relevant to North America, where subpopulations varying in virulence occur naturally in the same geographic region. According to a recent report, major genetic subpopulations within the type A tularemia population (A.I and A.II) seem connected with different mortality rates in humans (*7*). Potential clinical correlates to type B subpopulations remain to be studied. Ongoing work shows that >90 European isolates all fall within the subpopulations described here (unpub. data).

A most conspicuous need for rapid and reliable characterization of isolates of *F. tularensis* relates to bioterrorism. Whenever tularemia appears in an area believed to be free from the agent, characterization of isolates will become urgent. Such characterization abilities may also prove useful in understanding how *F. tularensis* may spread under peaceful circumstances. Reminders of the agent's potential for infection include the unexplained introduction of the disease on Martha's Vineyard in 1937 and more recently in northern Spain in 1997–1998, along with the highly publicized 2004 laboratory infections with respiratory type A tularemia at Boston University (*5*,*17*,*34*).

In public health laboratories, indel/MLVA typing may replace more risky and time-consuming biochemical characterization, which is based on growth of *F. tularensis*. After initial culture of the agent, noninfectious DNA is rapidly analyzed by PCR and fragment analysis for determination of indel and MLVA data.

A major achievement of the present study was the identification of canonical indels for combined use with MLVA. From studies of *Bacillus* spp., only SNPs have

been predicted to exhibit mutation rates sufficiently slow to be useful for unambiguous assignment of bacteria at deeper taxonomic levels (*24*). SNPs with canonical properties are not yet recognized in *F. tularensis,* and their combined use with MLVA has thus not yet been evaluated. An SNP-based approach does conform with well-developed evolutionary models to support data analysis (*35*), models that do not exist for indel mutations. A drawback is, however, that the involvement of 2 different analytic methods in a combined MLVA/SNP-based analysis makes it more complicated. By use of fragment analysis for both steps, the indel/MLVA approach is more effective. This study indicates that canonical indels can be integrated into evolutionary analyses for measuring large genetic distances while MLVA provides a detailed examination at short distances.

When selecting indels for the presented typing procedure, we took precautions to avoid DNA-marker discovery bias and homoplastic markers, problems that had been carefully addressed in work on other bacterial pathogens (*36*,*37*). To minimize discovery bias, we used *F. tularensis* genomes classified as being distantly related by independent methods. Genomes selected represented all 4 subspecies of *F. tularensis* that also form major genetic clades, according to MLVA, PFGE, microarray, and various arbitrarily primed–PCR analyses (*1*). To avoid homoplasy, including gene conversion, we excluded indels associated with repeat sequences. Our genome sequence data and the overall tree structure obtained from analysis of indel data lent support to a paucity of homoplasy effects. Except for locus Ftind-32 in the type strain ATCC 6223, which exhibited 2 PCR amplicons, only 4 of 280 identified loci showed incongruent evolutionary allele patterns. These 4 loci were all found among those repeat-containing loci that were excluded according to our selection criteria.

The reason behind a deviant result of strain ATCC 6223 at 1 locus is unknown but may be related to laboratory-induced mutations. ATCC 6223 was originally isolated in 1920 from a human lymph node in Utah, became avirulent by laboratory passage in the early years, but still retained properties that made it useful for antigen production. Recent microarray studies showed that it lacks portions of the genetic repertoire shared by all other *F. tularensis* strains (*10*).

MLVA discriminates among individual isolates within subspecies but may cause false estimates of relationships at deeper phylogenetic levels. Although in a previous study that used the present 25-marker MLVA scheme, discrimination of *F. tularensis* subspecies and major genetic clades was achieved, bootstrap support at these deeper levels was weak (*6*). Also in the present study, deep structural relationships among strains inferred by MP analysis of MLVA data were found to be weakly resolved. Conversely, strong support was shown for deep-level nodes obtained by using indel data. A combined analysis with both MLVA and indel data retained the deep-level support and yielded the most resolved topology. Furthermore, despite the inability of the indel or MLVA data to provide support for a separate clade of Japanese strains, such separation was supported by the combined analysis. This demonstrates that topologic constraints imposed by canonical indel data reduced the number of alternative positions of a combined tree and consequently increased the support for a clade.

When the present approach is used for routine purposes, the number of DNA markers might well be reduced yet retain a high level of discrimination and robustness. However, such a reduction needs to be evaluated to ensure proper marker selection. The inclusion by international collaboration of large numbers of geographically distributed strains will be facilitated by the unambiguous nature of data collected and the use of low quantities of killed bacteria. For ordinary clinical purposes, only a few indel markers may be required to rapidly receive relevant information, i.e., whether an isolate belongs to a subspecies or major genetic clade. A reference laboratory may wish to add more markers for tracing outbreaks and for forensic applications. Tailored combinations of these markers can be easily integrated into multiplex assays with 4–8 markers per PCR amplification and subsequent multicolor fragment analysis to decrease analytical time and cost.

In essence, we used 5 genome sequences representative of the species *F. tularensis* to identify 158 canonical indel DNA-markers, of which 38 were selected to provide robust information specific to each major genetic clade. By combining analysis of these indel markers with MLVA, discrimination of individual strains was achieved. The usefulness of indels with canonical properties may not be restricted to *F. tularensis*. The current availability of multiple genome sequences should allow testing this typing strategy for other clinically relevant pathogens.

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Epidemiologic and Virologic Investigation of Hand, Foot, and Mouth Disease, Southern Vietnam, 2005

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During 2005, 764 children were brought to a large children's hospital in Ho Chi Minh City, Vietnam, with a diagnosis of hand, foot, and mouth disease. All enrolled children had specimens (vesicle fluid, stool, throat swab) collected for enterovirus isolation by cell culture. An enterovirus was isolated from 411 (53.8%) of the specimens: 173 (42.1%) isolates were identified as human enterovirus 71 (HEV71) and 214 (52.1%) as coxsackievirus A16. Of the identified HEV71 infections, 51 (29.5%) were complicated by acute neurologic disease and 3 (1.7%) were fatal. HEV71 was isolated throughout the year, with a period of higher prevalence in October–November. Phylogenetic analysis of 23 HEV71 isolates showed that during the first half of 2005, viruses belonging to 3 subgenogroups, C1, C4, and a previously undescribed subgenogroup, C5, cocirculated in southern Vietnam. In the second half of the year, viruses belonging to subgenogroup C5 predominated during a period of higher HEV71 activity.

Hand, foot, and mouth disease (HFMD) is a common febrile illness of early childhood, characterized by 3–4 days of fever and the development of a vesicular enanthem on the buccal mucosa, gums, and palate and a papulovesicular exanthem on the hands, feet, and buttocks (*1*). HFMD is caused by acute enterovirus infections, particularly by viruses belonging to the human enterovirus A (HEVA) species (*1*).

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The genus *Enterovirus* of the family *Picornaviridae* is divided into 9 species, 5 of which infect humans. These viruses include the prototype species poliovirus, as well as HEVA, HEVB, HEVC, and HEVD. Viruses belonging to the HEVA species include 11 serotypes of coxsackievirus A (CVA; serotypes 2–8, 10, 12, 14, and 16), and human enterovirus 71 (HEV71) (*2*,*3*).

Although all HEVA viruses can cause HFMD, infection with HEV71 is also associated with a high prevalence of acute neurologic disease (*4*). Despite their close genetic relationship to HEV71, the HEVA CVA viruses rarely cause acute neurologic disease. HEV71 infection is associated with a wide spectrum of acute central nervous system syndromes, including aseptic meningitis, poliomyelitis-like paralysis, brainstem encephalitis, and acute neurogenic pulmonary edema (*4*). Children <5 years of age are particularly susceptible to HEV71-associated acute neurologic disease, which may occasionally cause permanent neurologic disability or death (*4*).

Since the discovery of HEV71 in 1969 (*5*), numerous outbreaks of this infection have occurred throughout the world (*4*). The prevalence of HEV71 infection in the Asia-Pacific region has greatly increased since 1997, concurrent with an increase in the prevalence of HFMD and acute neurologic disease (*6*–*11*). Outbreaks have been recorded in Japan (*12*), Malaysia (*7*), Singapore (*4*), South Korea (*6*), the People's Republic of China (*13*), and Australia (*14*–*16*). The most extensive epidemic of HEV71 occurred in Taiwan in 1998, with $\approx 1.3 \times 10^5$ cases of HFMD, 405 cases of severe neurologic disease, and 78 deaths. The deaths were due primarily to the development of brainstem encephalitis and neurogenic pulmonary edema (*8*,*17*).

Before 1999, most cases of encephalitis in southern Vietnam occurred in children >5 years of age, of which ≈60% were identified as Japanese encephalitis (diagnostic records of the Pasteur Institute, Ho Chi Minh City, Vietnam). Since 2002, however, viral encephalitis has increasingly been observed in younger children, particularly in those <4 years. Furthermore, since 2002 <27% of encephalitis cases have been confirmed as Japanese encephalitis, which indicates that the epidemiology of viral encephalitis in southern Vietnam may be changing. This situation led us to consider other possible causes for viral encephalitis.

In 2003, we isolated HEV71 (at the Pasteur Institute, Ho Chi Minh City, Vietnam) from 12 patients with encephalitis, who sought treatment at the hospital during an HFMD outbreak in southern Vietnam. To our knowledge, this was the first identification of HEV71 in Vietnam. Although laboratory surveillance has been shown to provide adequate warning of impending outbreaks of HEV71-associated acute neurologic disease (*18*), laboratory surveillance for HEV71 has not yet been established in Vietnam.

Materials and Methods

Study Participants and Specimen Collection

Children <15 years of age were admitted to a large pediatric hospital in Ho Chi Minh City, Vietnam. This hospital serves ≈70% of the city's pediatric population; 764 children with HFMD were enrolled in the study. HFMD was defined as a febrile illness (>37.5°C), accompanied by a papulovesicular rash in a characteristic distribution (oral mucosa, extremities of limbs, buttocks). A total of 1,928 specimens were collected from the children on the day of admission. Each child had at least 1 specimen collected from vesicle fluid, throat swab, or stool. Children who also exhibited acute neurologic disease had a cerebrospinal fluid specimen collected. All specimens were extracted with chloroform (1:10 in phosphate-buffered saline) before virus isolation in cell culture.

Virus Isolation

Virus isolation was undertaken in cell culture by using both human rhabdomyosarcoma (RD) (ATCC CCL136) and African green monkey kidney (Vero) (ATCC CCL81) cell lines. Each specimen underwent at least 2 cell culture passages in RD and Vero cells before being reported as negative. Samples demonstrating viral cytopathic effect (CPE) were screened for enterovirus RNA by reverse transcription–PCR (RT-PCR), as outlined in the following section.

RNA Extraction from Cell Culture Supernatants

Total cellular RNA was extracted from cell culture supernatants that demonstrated CPE; Tri-reagent (Ambion, Austin, TX, USA) was used. The RNA obtained from 250 μL of infected cell culture supernatant was suspended in 30 μL RNase-free water and stored at –80°C before use.

Enterovirus Screening Assays

Cell cultures showing CPE were screened for enterovirus RNA. Two "pan enterovirus" and 1 HEV71-specific RT-PCR assays were used, as described (*19–22).*

Pan Enterovirus RT-PCR Assay, 5′ Untranslated Region (UTR)

Briefly, cDNA was prepared in a 10-μL reaction mixture containing 6 μL RNA template, 0.5 mmol/L dNTP, 200 U Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) (Promega, Madison WI, USA), and M-MuLV RT buffer (Promega). cDNA synthesis was performed for 1 h at 42°C. In the PCR step, the 5′UTR was amplified by using 2 μL of cDNA in a 20-μL reaction volume, as described by Romero and Rotbart (*19*). The PCR products were examined by gel electrophoresis. Oligonucleotide primers for this assay (forward primer MD90, reverse primer MD91) flank a conserved nucleotide sequence in the 5′UTR of the enterovirus genome and amplify an expected product size of 154 bp.

Pan Enterovirus RT-PCR Assay, VP4

Enterovirus VP4 gene RT-PCR was performed by using primers OL68–1 and MD91, as described (*20*). Briefly, cDNA was prepared from a 10-μL reaction mixture containing 5.5 μL RNA, 0.5 mmol/L dNTP, 200 U M-MuLV RT (Fermentas, Burlington, Ontario, Canada), M-MuLV RT buffer (Fermentas), and the antisense primer OL68-1. cDNA synthesis was performed for 1 h at 37°C. In the PCR step, the VP4 gene was amplified by using 2 μL of cDNA in a 20-μL reaction volume with previously described cycling conditions (*20*).

HEV71-specific RT-PCR Assay

The HEV71-specific RT-PCR was performed as described (*21*,*22*) to provide rapid identification of HEV71 in cell culture supernatants that were positive in the screening RT-PCR assay. First, strand cDNA was prepared as outlined above. In the PCR step, the VP1 gene was amplified by using 2 μL of cDNA in a 20-μL reaction volume, as described (*22*). The PCR products were examined by gel electrophoresis. Oligonucleotide primers for this assay (forward primer MAS01S, reverse primer MAS02A) flank a region within the VP1 gene unique to HEV71 and amplify an expected product size of 376 bp.

RT-PCR for Confirmation and Sequencing

HEV71 Complete VP1 RT-PCR Assay

The VP1 gene of 23 HEV71 strains isolated in this study was amplified by RT-PCR by using in-house oli-

gonucleotide primers that flank the entire VP1 gene region, HEV71-VP1-F2 (5′-ATAATAGCAYTRGCG GCAGCCCA-3′; forward) and HEV71-VP1-R1 (5′-TGR GCRGTGGTAGAYGAYAC-3′; reverse). First-strand cDNA synthesis was performed as above, except the reaction was primed with HEV71-VP1-R1. For the PCR step, 2 μL of first-strand cDNA was added to a 50-μL reaction volume containing 1.5 mmol/L $MgCl_2$, 1 mmol/L each of primers HEV71-VP1-F2 and HEV71-VP1-R1, 0.3 mM dNTP, 2.5 U Taq DNA polymerase (Fermentas), and Taq polymerase buffer (Fermentas). PCR cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 1 min. This cycling was followed by a final extension at 72°C for 5 min. PCR products (≈1.1 kb) were examined by gel electrophoresis and purified by using the GENECLEAN III kit (Qbiogene, Irvine, CA, USA).

Partial VP1 RT-PCR Assay

To identify HEV viruses that were not detected by the VP4 RT-PCR screening assay, a molecular serotyping method based on RT-PCR amplification and sequencing of a portion of the VP1 gene was performed as described (*23*). An ≈340-bp fragment was amplified by RT-PCR by using the forward primer 292 (5′-MIGCIGYIGARACNGG-3′) and reverse primer 222 (5′-CICCIGGIGGIAYRWACAT-3′), under conditions exactly as described by Oberste et al. (*23*). PCR products were examined by gel electrophoresis and purified by using the GENECLEAN III kit (Qbiogene).

Nucleotide Sequencing of HEV71 VP4 and VP1 Gene Amplicons

Enterovirus VP4 gene amplicons were sequenced on both strands by using the PCR primers. HEV71 VP1 gene amplicons were sequenced on both strands by using the PCR primers and internal VP1 primers 161 and 162, described by Brown et al. (*24*). Sequencing was performed by using the Big Dye Cycle Sequencing kit version 3.0 and an ABI377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The SeqMan software module in the Lasergene suite of programs (DNASTAR, Madison, WI, USA) was used to format the nucleotide sequences. Partial VP1 and VP4 sequences for 173 HEV71 strains and 214 CVA16 strains have been submitted to the European Molecular Biology Laboratory database (partial VP1 gene accession nos. EU072122-EU072195; VP4 gene accession nos. EU051005-EU051317).

HEV71 VP1 Gene Nucleotide Sequence Data from GenBank

In addition to 23 VP1 gene sequences from HEV71 strains isolated in Vietnam, 26 VP1 gene nucleotide sequences of HEV71 strains available in the GenBank database were included in this analysis, allowing the generation of a dendrogram containing 49 strains isolated between 1970 and 2005 (Table 1). The strains used to reproduce the

Table 1. HEV71 VP1 gene nucleotide sequences used in reconstruction of the HEV71 dendrograms*

Isolate	Source	GenBank accession no.
CVA16-G10	GenBank	NC_001612
BrCr-CA/USA/70	GenBank	U22521
shzh02–62	GenBank	AY895136
shzh04–12	GenBank	AY895144
shzh01–3	GenBank	AY895132
shzh04–3	GenBank	AY895142
shzh01–4	GenBank	AY895134
SB12007-SAR-03	GenBank	AY905548
SB12282-SAR-03	GenBank	AY905546
SB9465-SAR-03	GenBank	AY258302
SB9508-SAR-03	GenBank	AY258301
S10822/SAR/98	GenBank	AF376079
2037-MD/USA/95	GenBank	AF009556
S18191/SAR/02	GenBank	AY189154
1M/AUS/12/00	GenBank	AF376098
13/KOR/00	GenBank	AY125976
06/KOR/00	GenBank	AY125970
8M/AUS/6//99	GenBank	AF376109
1245a/TWN/98	GenBank	AF176044
3799/SIN/98	GenBank	AF376117
MY104–9/SAR/97	GenBank	AF376072
2027/SIN/01	GenBank	AF376111
SB2864/SAR/00	GenBank	AF376066
8102-WA/USA/87	GenBank	AF009526
7423-MS/USA/87	GenBank	U22522
2229-NY/USA/76	GenBank	AF135868
1011-ND/USA/79	GenBank	AF135864
1001V/VNM/05	This study	AM490141
1089T/VNM/05	This study	AM490142
1091S/VNM/05	This study	AM490143
1129V/VNM/05	This study	AM490144
1135T/VNM/05	This study	AM490145
1177T/VNM/05	This study	AM490146
1192S/VNM/05	This study	AM490147
1277S/VNM/05	This study	AM490148
1301V/VNM/05	This study	AM490149
1303S/VNM/05	This study	AM490150
540V/VNM/05	This study	AM490151
559S/VNM/05	This study	AM490152
666T/VNM/05	This study	AM490153
707V/VNM/05	This study	AM490154
718T/VNM/05	This study	AM490155
730T/VNM/05	This study	AM490156
777T/VNM/05	This study	AM490157
784S/VNM/05	This study	AM490158
900S/VNM/05	This study	AM490159
926V/VNM/05	This study	AM490160
933V/VNM/05	This study	AM490161
962T/VNM/05	This study	AM490162
999T/VNM/05	This study	AM490163

*HEV71, human enterovirus 71; CVA16, coxsackievirus A16.

HEV71 tripartite genogroup structure identified by Brown et al. (*24*) were isolated in the United States, Japan, Australia, Malaysia, Singapore, Taiwan, the People's Republic of China, Hungary, South Korea, and the United Kingdom.

Phylogenetic Analysis

VP1 and VP4 gene sequences were subjected to nucleotide-nucleotide BLAST analysis (blastn) by using the online server at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast). Alignment of the 23 HEV71 complete VP1 gene sequences was undertaken by using the ClustalW program (*25*). A dendrogram was constructed by using the neighbor-joining method with PHYLIP version 3.5 (*26*) and drawn by using TreeView (*27*). Bootstrap analysis with 1,000 pseudoreplicates was performed by using the program Seqboot (*28*). Coxsackievirus A16 (CVA16), strain G10 (*29*), was used as an outgroup in the analysis.

Statistical Methods

Differences between proportions were tested by using the χ^2 test with Yates correction or Fisher exact test. Epi Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for the analysis.

Results

Virus Isolation from HFMD Patients

An enterovirus was isolated from 411 (53.8%) of the 764 HFMD patients enrolled in the study. The number of CVA16, HEV71, and other enterovirus serotypes isolated from HFMD patients is presented in Table 2. CVA16 was identified in 214 (52.1%) and HEV71 in 173 (42.1%) of the enterovirus-positive HFMD patients. Twenty-four (5.8%) enteroviruses of another serotype were also isolated from HFMD patients (Table 2).

Procedures for the isolation and identification of enterovirus strains obtained in the study are presented in a flowchart (Figure 1). Of the 411 enteroviruses isolated in this study, 170 were identified by using HEV71-specific primers. Another 3 were identified as HEV71 when the VP4 and partial VP1 RT-PCR products were sequenced. We used the RT-PCR assay and sequencing of the VP4 gene as a screening tool because a single set of primers allowed us to obtain a preliminary identification of HEV71 or CVA16. In our laboratory, 256 enterovirus isolates were sequenced in both VP1 and VP4, and 100% concordance was found between the VP1 and VP4 results for HEV71 (130 isolates) and CVA16 (61 isolates); only 28 (43%) of 65 other enteroviruses had concordant results in both the VP1 and VP4 sequences (unpub. data). Thus, 24 non-HEV71, non-CVA16 isolates were identified as other enteroviruses.

Table 2. Total number of enterovirus serotypes isolated from hand, foot, and mouth disease cases, southern Vietnam, 2005

Virus serotype*	No. cases
CVA16	214
HEV71	173
Other enterovirus	24
Negative	352
Total	763

*CVA16, coxsackievirus A16; HEV71, human enterovirus 71.

Clinical Features of HFMD

The clinical features observed in HFMD patients enrolled in the study are presented in Figure 2, panel A. By definition, children enrolled in the study all displayed the characteristic papulovesicular rash of HFMD; 214 cases of HFMD were associated with CVA16 infection, and 173 cases were associated with HEV71 infection. Notably, the formation of ulcers on the oral cavity was observed less frequently with HEV71 infection than CVA16 infection (102 [58.9%] of 173 HEV71 patients vs.178 of 214 CVA16 patients [83.2%]; p<0.0001, odds ratio [OR] 0.29, 95% confidence interval [CI] 0.18–0.48). Cough was also observed more frequently with HEV71 infection than CVA16 infection (70 of 173 [40.5%] vs. 59 [27.6%] of 214; OR 1.79, 95% CI 1.14–2.8). Altered sensorium was experienced by 10 (5.8%) of the 173 HEV71 patients and, as expected, by none of the CVA16 patients. This finding was significant (p = 0.0003), but due to the small numbers, the OR could not be calculated.

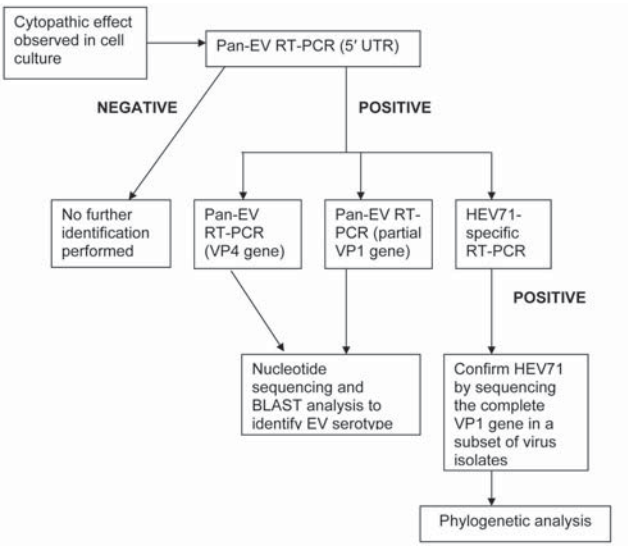

Figure 1. Flowchart showing the procedures used for isolating and identifying enterovirus strains cultured from clinical specimens obtained from children admitted to a large pediatric hospital in Ho Chi Minh City, Vietnam, with a diagnosis of hand, foot, and mouth disease (HFMD) during 2005 and enrolled in this study. EV, enterovirus; RT-PCR, reverse transcription–PCR; 5′ UTR, 5′ untranslated region; HEV71, human enterovirus 71.

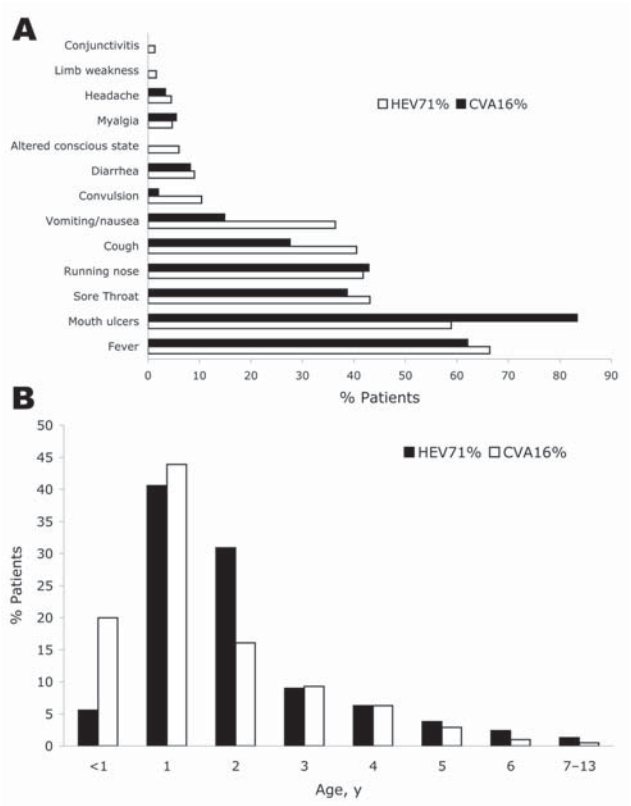

Figure 2. Clinical features of hand, foot, and mouth disease (HFMD) in children admitted to hospital in southern Vietnam during 2005. Features were associated with the isolation of coxsackievirus A16 (CVA16) (214 cases) or human enterovirus 71 (HEV71) (173 cases) from vesicle, throat swab, or stool specimens. A) Percentage distribution of clinical signs and symptoms among identified cases of HFMD. B) Percentage age distribution of patients with identified cases of HFMD.

Clinical signs of neurologic infection were observed primarily with HEV71-associated HFMD. Convulsions were observed for 18 (10.4%) of 173 HEV71 patients and 4 of 214 (1.9%) CVA16 patients, respectively ($p = 0.0007$, OR 6.10, 95% CI 1.95–25.15). Vomiting was also significantly more frequent for HEV71 patients (63 [36.4%] of 173) than for CVA16 patients (30 [14.0%] of 214; p value <0.0001, OR 3.51, 95% CI 2.08–5.94). Only patients with HEV71-associated HFMD had alteration of consciousness (10 [5.8%] of 173, $p = 0.0003$) or limb weakness (3 of 173 [1.7%]). In all, acute neurologic disease accounted for 29.5% (51/173) of identified cases of HEV71-associated HFMD. The case-fatality rate for HEV71-associated acute neurologic disease was 5.9% (3/51) and for all HEV71-associated HFMD was 1.7% (3/173). No fatal cases of CVA16-associated HFMD were identified.

Other clinical signs and symptoms did not differ significantly between HEV71 and CVA16 patients. Sore throat (43.1% of HEV71 patients and 38.7% of CVA16 patients) and runny nose (41.8% of HEV71 patients and 42.9% of CVA16 patients) were observed in approximately half of the HFMD patients. A smaller number of HFMD patients exhibited symptoms of gastrointestinal disorder, such as diarrhea (9.0% of HEV71 patients and 8.2% of CVA16 patients). Myalgia (4.7% of HEV71 patients and 5.5% of CVA16 patients) and headache (4.5% of HEV71 and 3.4% of CVA16 patients) were less common symptoms.

The HFMD cases observed in southern Vietnam occurred primarily in children <5 years of age (Figure 2, panel B). Most HEV71 (136/173 patients) and CVA16 (171/214 patients, 79.9%) infections were identified in children <3 years of age; the peak age-specific incidence of HEV71 (71/173 patients, 40.5%) and CVA16 (94/214 patients [43.9%) infections were identified in children 1–2 years of age.

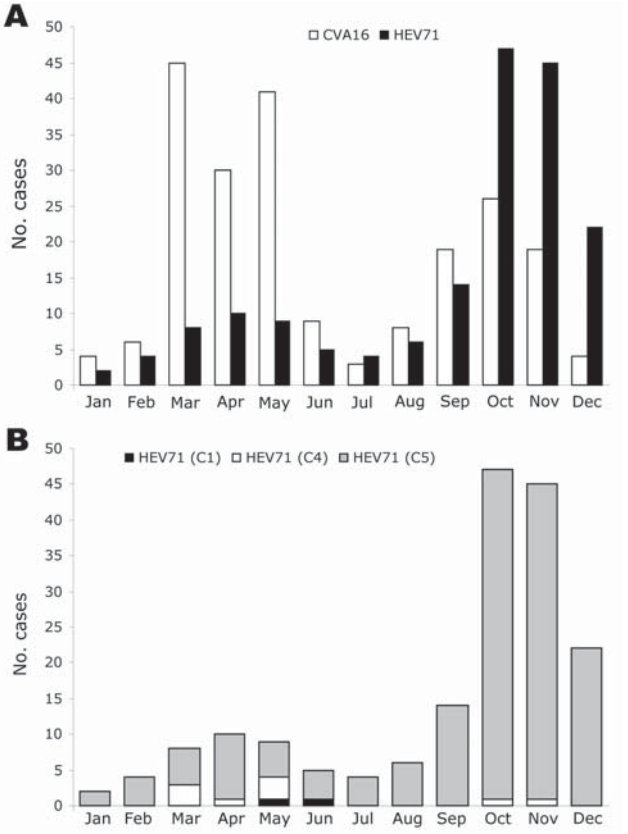

Figure 3. Monthly distribution of 387 cases of hand, foot, and mouth disease (HFMD) associated with isolation of either coxsackievirus A16 (CVA16) (214 cases) or human enterovirus 71 (HEV71) (173 cases), southern Vietnam, 2005. RNA was extracted from cells inoculated with vesicle, throat swab, or stool specimens. Partial VP4 gene sequences were amplified by reverse transcription–PCR (RT-PCR) by using specific primers (*22*), the amplified cDNA sequenced, and the serotype and/or genogroup specificity determined by BLAST analysis. A) Monthly distribution of CVA16 and HEV71-associated HFMD cases. B) Monthly distribution of 173 HFMD cases associated with HEV71 infection with strains belonging to subgenogroups C1, C4, or C5.

Epidemiology of HFMD

The distribution of CVA16- and HEV71-associated HFMD cases by month during 2005 is presented in Figure 3, panel A. HFMD was identified in southern Vietnam throughout the year; HEV71 and CVA16 were also isolated throughout the year. Two peaks of HFMD activity were observed during 2005. The first peak occurred from March through May. CVA16 was the predominant virus during this time, accounting for 81.1% (116 cases) of HFMD compared to 18.9% (27 cases) for HEV71 (Figure 2, panel A). The second peak of HFMD activity occurred from September through December. HEV71 was the predominant virus during this time, accounting for 65.3% (128 cases) of HFMD compared to 34.7% (68 cases) for CVA16 (Figure 3, panel A).

Figure 4 depicts the geographic distribution of HFMD cases due to HEV71 (Figure 4, panel A) and CVA16 (Figure 4, panel B) who were brought for treatment to a major children's hospital in Ho Chi Minh City. Children admitted to this hospital are predominantly drawn from the urban area but were also referred from provinces surrounding Ho Chi Minh City.

Molecular Epidemiology of HEV71

The HEV71 isolates were further analyzed to determine the monthly distribution of viral subgenogroups in southern Vietnam during 2005 (Figure 3, panel B). This analysis was achieved by RT-PCR amplification of complete VP4 and partial VP1 gene sequences, nucleotide sequencing, and BLAST analysis (*20*). Using these methods, we identified 3 HEV71 subgenogroups, C1, C4, and a previously undescribed subgenogroup, C5. Two virus isolates (1.2%) belonging to subgenogroup C1 were identified, 1 each in May and June. A total of 9 (5.2%) subgenogroup C4 strains were identified; 7 were isolated from March through May and 1 each in October and November. Strains belonging to the new subgenogroup C5 (162 [93.6%]/173) were the predominant genetic lineage identified in southern Vietnam during 2005. Subgenogroup C5 viruses were identified in each month and were the primary cause of the large increase in HFMD from September through December.

Because we had identified a putative new subgenogroup of HEV71 (C5) by analysis of complete VP4 and partial VP1 gene sequences (Figure 3, panel B), we conducted further nucleotide sequence analysis of the complete VP1 gene of 23 HEV71 isolates whose VP4 sequences were representative of all clusters observed in dendrograms generated from the screening data (*9*,*24*). Complete VP1 gene sequence analysis is considered the most rigorous method for determining the molecular phylogeny of HEV71 strains (*6*,*24*), and our analysis needed to be confirmed with a subset of all the isolates (Figure 5). We used previously published VP1 gene cDNA sequences to reconstruct the subgenogroup lineage structure of HEV71, first identified by Brown et al. (*24*) (Table 2).

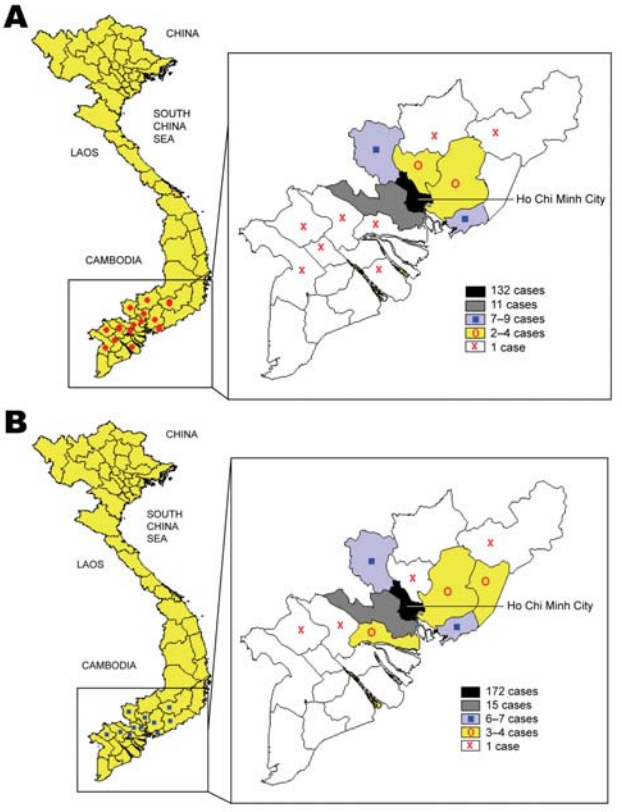

Figure 4. Geographic distribution of hand, foot, and mouth disease cases associated with human enterovirus 71 (A) or coxsackievirus A16 (B) infection, southern Vietnam, 2005.

Two of the Vietnamese HEV71 isolates clustered within subgenogroup C1; 5, within subgenogroup C4; and 16, within the new subgenogroup C5 (Figure 5). The subgenogroup clustering of the HEV71 Vietnamese isolates is strongly supported by bootstrap analysis, which indicates that 3 independent genetic HEV71 lineages (C1, C4, and C5) circulated in southern Vietnam during 2005. This, together with the year-round isolation of CVA16 and HEV71 from HFMD patients (Figure 3, panels A, B), suggests that both viruses circulate endemically in southern Vietnam.

A comparison of the percentage identity of the complete VP1 gene nucleotide sequences of HEV71 subgenogroup C1–4 viruses with that of 16 Vietnamese subgenogroup C5 strains is presented in Table 3. Viruses belonging to subgenogroup C5 shared 89.1%–91.0%, 88.8%–90.1%, 88.8%–89.8%, and 87.7%–90.2% similarity to viruses belonging to subgenogroups C1, C2, C3, and C4, respectively. The consistent 9%–12.3% difference in nucleotide sequence identity between putative subgenogroup C5 strains

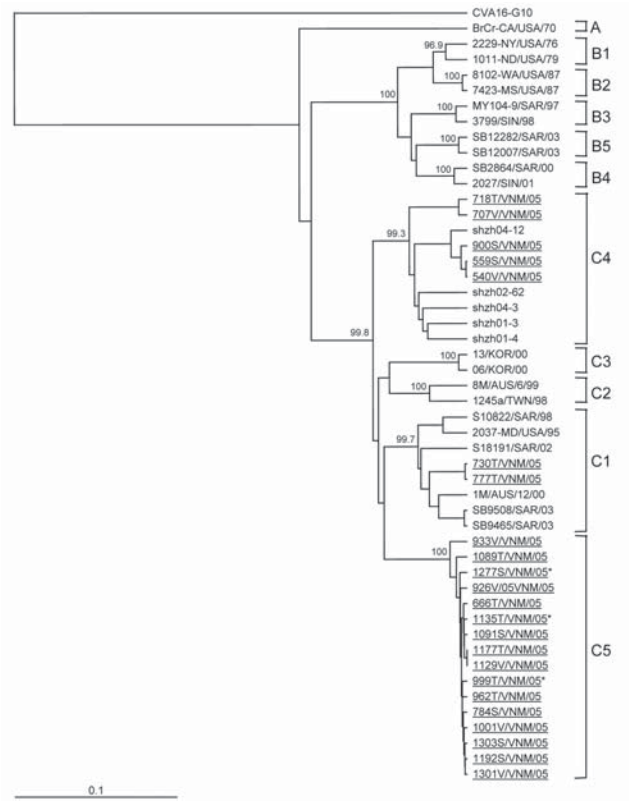

Figure 5. Dendrogram constructed by using the neighbor-joining method *(25)* showing the genetic relationships between 23 human enterovirus 71 (HEV71) strains isolated in southern Vietnam during 2005 (underlined), based on the alignment of complete VP1 gene sequences. Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the tree are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. Strain names indicate a unique numerical abbreviation of country and year of isolation. Asterisks (*) denote HEV71 isolates obtained from fatal cases. The prototype coxsackievirus 16 (CVA16)–G10 strain (*28*) was used as an outgroup. The dendrogram shows genogroups A, B, and C as identified by Brown et al. (*24*). Details of the strains used to prepare the dendrogram are shown in Table 1.

and those belonging to subgenogroups C1–C4 provides strong evidence for the classification of C5 as a new and separate subgenogroup of HEV71.

Discussion

To our knowledge, this study provides the first comprehensive epidemiologic and virologic survey of HFMD, CVA16, and HEV71 infection in Vietnam. Similar to the situation in other countries, HEV71 infection was associated with a subset of HFMD cases in which acute neurologic disease developed. Our epidemiologic and phylogenetic data suggest that both CVA16 and HEV71 circulate endemically in southern Vietnam.

Nearly one third of the HEV71-associated HFMD cases identified in our study were complicated by acute neurologic disease. The case-fatality rates of 1.7% in all identified HEV71 infections and 5.9% in HEV71 acute neurologic disease cases are higher than those observed in other studies (*7*,*30*,*31*). However, the case-fatality rates calculated in our study may overestimate the true values because only HFMD patients who were brought for treatment at a major children's hospital were included in the study. The best estimates of case-fatality rates for HEV71 infection have come from a large seroepidemiologic study of the 1998 HFMD epidemic in Taiwan (*32*); the authors estimated a case-fatality rate of 96.96 per 100,000 population in infants <1 year of age, declining to 6.64 per 100,000 population in children >5 years of age. To rigorously determine the incidence and case-fatality rate of HEV71 infection in southern Vietnam, a similar population-based seroepidemiologic study should be undertaken.

Although cases of HFMD were identified throughout the year, 2 periods of increased prevalence were identified—from March through May and from September through December. In southern Vietnam, these months are interim periods between the dry and wet seasons. CVA16 was the predominant virus isolated in the first period, and HEV71 infection was the predominant virus isolated in the second period. Ongoing epidemiologic surveillance will be necessary to determine whether this pattern of HFMD and enterovirus activity recurs in a regular annual cycle.

Phylogenetic analysis based on nucleotide sequence alignment of the complete VP1 gene of 23 representative strains of HEV71 from southern Vietnam showed that they belonged to 3 subgenogroups, C1, C4, and to the previously undescribed subgenogroup C5. Since 1997, 2 genetically distinct major lineages (B, C) of HEV71 have circulated in different parts of the Asia-Pacific region (*6*,*9*). Viruses

Table 3. Percentage identity of complete VP1 gene nucleotide sequences of HEV71 genogroup C viruses*

	% Nucleotide identity				
Subgenogroup	C1	C2	C3	C4	C5
C1	–	88.4–90.9	89.7–91.2	87.3–91.9	89.1–91.0
C2		–	90.2–91.4	88.7–91.0	88.8–90.1
C3			–	89.3–90.3	88.8–89.8
C4				–	87.7–90.2
C5					–

*HEV71, human enterovirus 71.

belonging to genogroup B have predominated in Southeast Asia, whereas viruses belonging to genogroup C have predominated in northern Asia (*6*,*9*,*11*,*33*). Before 1997, HEV71 strains belonging to subgenogroup C1 were identified in several small outbreaks around the world (*15*,*24*). Since 1997, subgenogroup C1 viruses have circulated endemically in the Asia-Pacific region and have been found to cocirculate as a minor subgenogroup together with a predominant HEV71 subgenogroup during several outbreaks (*6*,*11*,*34*). In this study, subgenogroup C1 viruses comprised only 1.1% of HEV71 strains isolated, indicating low-level circulation. Viruses belonging to subgenogroup C2 have circulated widely in the Asia-Pacific region between 1998 and 2000 (*9*,*11*,*16*) and were responsible for the large outbreak in Taiwan in 1998 (*6*,*8*,*9*,*33*). Two new genetic lineages of genogroup C, subgenogroups C3 and C4, have emerged recently in northern Asia. Viruses belonging to subgenogroup C3 first appeared in the People's Republic of China in 1998 (*6*) and reemerged in South Korea in 2000 (*6*,*9*). Viruses belonging to subgenogroup C4 were first identified in the People's Republic of China in 1998 and again in 2000 (*35*) before their identification in southern Vietnam during 2005. Furthermore, a new subgenogroup, C5, circulated widely in southern Vietnam throughout 2005 and became the predominant virus strain identified during the second half of the year.

Our data indicate that the molecular epidemiology of HEV71 in southern Vietnam conforms to the northern Asian epidemiologic pattern of endemic circulation of genogroup C virus strains, with evidence of the ongoing evolution of new subgenogroups, similar to that observed for genogroup B HEV71 strains in Southeast Asia (*6*,*9*,*33*). Furthermore, the year-round isolation and circulation of multiple independent genetic lineages of HEV71 (*36*) suggest that this virus circulates endemically within the human population of southern Vietnam.

In conclusion, this study has established that HEV71 circulates endemically in southern Vietnam and thus represents a substantial threat to the health of children in this region. Improvements in public sanitation and personal hygiene alone are unlikely to prevent HEV71 transmission within the community. A vaccine is necessary to prevent HEV71-induced neurologic disease in susceptible children. However, until such a vaccine is available, virus activity in the community must be monitored through the establishment and maintenance of sentinel surveillance.

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Severe Spotted Fever Group Rickettsiosis, Australia

William J.H. McBride,* Joshua P. Hanson,† Robert Miller,‡ and Drew Wenck§

We report 3 cases of spotted fever group rickettsial infection (presumed Queensland tick typhus) in residents of northern Queensland, Australia, who had unusually severe clinical manifestations. Complications included renal failure, purpura fulminans, and severe pneumonia. Clinical illness caused by *Rickettsia australis* may not be as benign as previously described.

Queensland tick typhus (QTT) is caused by *Rickettsia australis*, an obligate intracellular organism that is transmitted to humans through the bite of 1 of 2 known tick vectors, *Ixodes holocyclus* or *I. tasmani* (*1*). The disease occurs along the eastern coast of Australia, including Queensland. QTT is considered to be a mild illness. Clinical features are fever, headache, and myalgia. An eschar may be seen at the site of the tick bite, and a maculopapular or vesicular skin rash is usually noted. Patients usually make an uncomplicated recovery. We report 3 cases of presumed QTT in Australia that were unusually severe and showed manifestations that, to our knowledge, were previously unreported with this disease.

The Cases

Case 1

The first case occurred in a 40-year-old woman from Kuranda, Queensland, with a 1-week history of fever, myalgia, headache, and dry cough. She had sustained tick bites to the back and leg while planting trees. She was febrile (temperature 38.1°C). A widespread maculopapular rash with a minor petechial component was present. No eschars were found. Renal dysfunction was evident by blood tests (Table). A provisional diagnosis of leptospirosis was made, and she was treated with intravenous penicillin. Urea and creatinine levels continued to increase over 3 days to 14.8 mmol/L and 300 μmol/L, respectively, despite administration of intravenous fluids. She showed no dehydration or hypotension. Urinalysis showed 1+ proteinuria only. The patient's clinical condition improved slowly over 7 days. The rash had a more noticeable petechial component at this stage of the disease. The patient was discharged on day 7 and given a 5-day course of oral doxycycline, 100 mg, twice a day. She had fully recovered on subsequent follow-up. A diagnosis of QTT was made retrospectively on the basis of serologic results.

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Case 2

The second case occurred in a 69-year-old woman from Innisfail, Queensland, who was hospitalized with a 2-week history of fever, myalgia, neck pain, and confusion. She was febrile (temperature 39.2°C) and had tachycardia (140 beats/min). Multiorgan failure, purpura fulminans, and digital necrosis developed over a 2-day period, and she was transferred to an intensive care unit. Intubation and ventilation were required. She had widespread cutaneous and digital necrosis. There were no eschars or lymphadenopathy. Prothrombin time was 22 s (normal range 8–14 s), activated partial thromboplastin time was 53 s (normal range 25–38 s), and fibrinogen level was 1.6 g/L (normal range 1.5–4.0 g/L). The latex D dimer titer was 8 (normal <1). Results of a PCR for *Neisseria meningitidis* in blood and an extensive screen for primary vasculitides and prothrombotic disorders were negative. A skin biopsy specimen showed vessel thrombosis and no evidence of vasculitis, which is consistent with purpura fulminans.

Treatment included broad-spectrum antimicrobial drugs and doxycycline. Clinical recovery was prolonged, and she required temporary renal dialysis. Fourteen digital amputations were performed on her hands and feet (Figure 1). There was serologic evidence of *R. australis* infection. She lived in a house that bordered bushland and had received tick bites. However, she could not recall her last exposure. Tissue from the skin biopsy specimen was tested by PCR with primers against the rickettsial 17-kDa gene (*2*), and immunohistochemical analysis was performed with polyclonal rabbit antisera against spotted fever group (SFG) rickettsiae. Both tests showed negative results.

Case 3

The third case occurred in a 45-year-old man from Deeral, Queensland, who was seen with headache, malaise, and vomiting 10 days after a tick bite. He also had a cough, dyspnea, and insulin-dependent diabetes. He was employed working on rural roads. He was febrile (temperature 38.2°C) and had tachycardia (145 beats/min) and tachypnea (40 breaths/min). He required 15 L/min oxygen by mask to maintain oxygenation. There was a fine petechial rash and left inguinal lymphadenopathy. A chest radiograph showed bilateral interstitial infiltrates (Figure 2). Over the ensuing hours, respiratory failure developed, and he required intu-

Table. Laboratory test results in 3 severe cases of infection with *Rickettsia australis*, Australia

Test	Case 1	Case 2	Case 3	Reference range
Hemoglobin, g/L	124	130	142	115–160
Neutrophil count, cells/L	6.19	11.4	8.8	2.00–8.00 × 10^9
Lymphocyte count, cells/L	0.39	0.4	1.58	1.00–4.00 × 10^9
Platelet count, cells/L	63	30	167	140–400 × 10^9
Sodium, mmol/L	131	135	139	135–145
Urea, mmol/L	9.8	27.8	5.0	3.0–8.0
Creatinine, μmol/L	190	430	100	70–120
Bilirubin, μmol/L	28	105	41	<20
Alkaline phosphatase, U/L	295	250	587	30–120
Gamma-glutamyl transferase, U/L	205	121	877	<50
Alanine aminotransferase, U/L	131	47	404	<40
Aspartate aminotransferase, U/L	178	152	417	<35
Leptospirosis immunoglobulin M	Negative	Negative	Negative	
Duration of symptoms before doxycycline treatment, d	17	14	10	
R. australis antibody titer*	Negative to 2,048 over 9 d	64–1,024 over 8 d	Negative to >1,024 over 9 d	Negative <64 Positive ≥64

*By immunofluorescence.

bation and ventilation. Twelve hours after admission, his arterial blood gas results were pH 7.32, pO_2 59 mmHg, and pCO_2 55 mmHg on 100% oxygen while ventilated. His creatinine level increased to 180 μmol/L over the first 2 days and then slowly returned to normal. He was given broad-spectrum antimicrobial drugs, including doxycycline, on the day of admission. Treatment with doxycycline continued for 7 days. His recovery was marked by gross generalized edema. Serologic results were diagnostic for infection with *R. australis*. He was ventilated for 16 days and spent 3 weeks in the intensive care unit. He returned to work 2 months after admission.

Conclusions

The clinical features of QTT have been described in 2 reviews (*1*,*3*). One review of 62 cases included patients from Flinders Island in the Bass Strait, an area now known to be endemic for a new rickettsia in the SFG, *Rickettsia honei (4)*. These reviews describe an illness marked by malaise, headache, and myalgia. A maculopapular rash appears in most patients. The rash may become petechial or vesicular in some cases. An eschar is seen in up to half the cases and lymphadenopathy in ≤70%. Less common clinical manifestations include joint pain, splenomegaly, cough, conjunctivitis, sore throat, nausea, abdominal pain, and photophobia. One patient had minor renal dysfunction (*1*). Mild-to-moderate elevation of hepatic transaminase levels is common.

One fatal case of QTT has been described in a 68-year-old man from northern Queensland (*5*). His illness was clinically marked by progressive renal failure, bilateral pulmonary infiltrates, acidosis, abnormal liver function test results, thrombocytopenia, and hypoprothrombinemia.

The first patient described in this report had moderate renal impairment, which is commonly associated with leptospirosis. Her renal dysfunction did not improve with rehydration but had fully resolved when her condition was reevaluated 3 weeks after hospital discharge. Renal failure is a feature of other spotted fever rickettsial illnesses. Rocky Mountain spotted fever (RMSF), which is caused by *R. rickettsii,* is associated with a multifocal perivascular interstitial nephritis. Renal dysfunction is believed to be a consequence of hypovolemia secondary to rickettsial disease–induced alterations in capillary permeability (*6*,*7*). Renal failure has also been recorded as a complication of infection with *R. conorii* (Mediterranean spotted fever) (*8*).

The second patient had a clinical picture usually associated with overwhelming bacterial infection. *N. meningitidis* was considered a possible cause of her illness. However, her 2-week illness before deterioration was not typical.

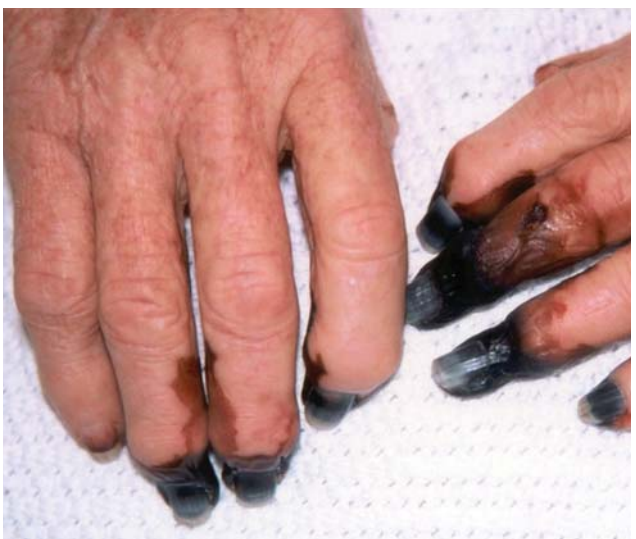

Figure 1. Digital gangrene in a patient (case 2) with *Rickettsia australis* infection.

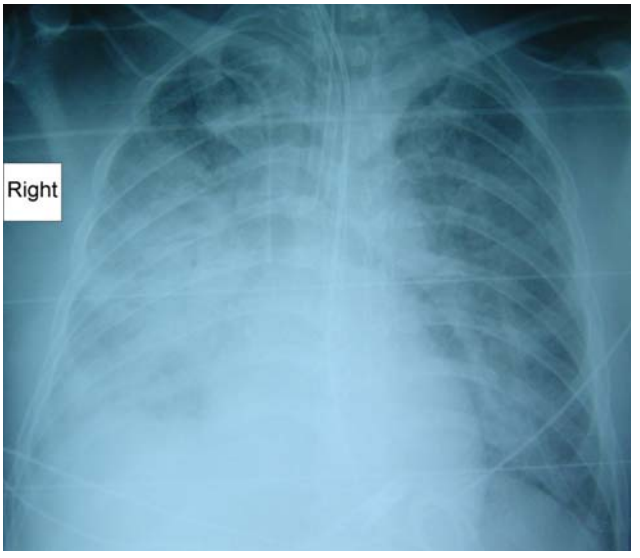

Figure 2. Radiograph showing pneumonia in a patient (case 3) with *Rickettsia australis* infection.

Serologic results were diagnostic for infection with *R. australis*. Purpura fulminans has been described in RMSF. In a review of cases of gangrene complicating this infection, 6 cases with remarkable similarities to our second case were described (*9*).

The predominant clinical complication of the third patient was pulmonary involvement. This characteristic has only been described once in QTT (*5*). Pulmonary involvement has been described in RMSF, in which the pathology changes are thought to be related to noncardiogenic pulmonary edema consequent to capillary endothelial damage (*10*,*11*).

There are 12 described rickettsiae of the spotted group (*12*). RMSF is associated with a mortality rate of 7%, even with treatment (*5*), and *R. conorii* has been associated with severe disease and fatal cases. Other rickettsiae are considered to cause mild illness.

The 3 cases described here were seen over a 4-year period at Cairns Base Hospital in northern Queensland. We are aware of 2 other cases seen at other hospitals, 1 complicated by renal failure, confusion, abnormal coagulation test results, and impaired gas exchange, and the other with severe pneumonia (P. Marshall, R. Miller, pers. comm.).

Although QTT is a mild disease in most patients, its diagnosis should now be considered in patients who reside in or visit the rickettsial disease–endemic area of eastern coastal Australia and are hospitalized with renal failure or impaired pulmonary function. Delays in seeking treatment may have contributed to illness severity in 2 of our patients. Given the serologic cross-reactivity between members of the SFG rickettsia, it cannot be assumed that all cases described were caused by *R. australis*. Another rickettsia of the SFG has been recently described in northern Queensland (*13*), and distinguishing between species will be important in future studies.

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Novel Human Herpesvirus 8 Subtype D Strains in Vanuatu, Melanesia

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We show human herpesvirus 8 with diverse molecular subtype D variants to be highly endemic among the Ni-Vanuatu population. Most K1 genes were nearly identical to Polynesian strains, although a few clustered with Australian or Taiwanese strains. These results suggest diverse origins of the Ni-Vanuatu population and raise questions about the ancient human population movements in Melanesia.

Human herpesvirus 8 (HHV-8), or Kaposi Sarcoma Associated Herpesvirus (KSHV), is the etiologic agent of Kaposi sarcoma (KS). HHV-8 is not a widespread ubiquitous virus; its presence is mainly restricted to areas where classic or endemic KS is highly prevalent, i.e., estimates of HHV-8 seroprevalence in the general adult population range from 5% to >50% (*1*).

Exploiting the highly genetic variability of the HHV-8 K1 gene, molecular epidemiology led to the identification of 5 major K1 subtypes (A–E), some of which appear to be strongly linked to the geographic origin of the samples. Thus, the few known subtype D strains have been reported only in inhabitants from the Western Pacific region (*2*).

For people of Oceanian ancestry (including Melanesian, Polynesian, and Micronesian), very little data are available on the clinical and molecular epidemiology of HHV-8 and its associated diseases (*3*–*9*). Thus, we studied HHV-8 in the Vanuatu, an archipelago in the Southwest Pacific region, formerly named New Hebrides, which contains >80 islands (6 provinces) (Figure 1). Indigenous Melanesians, also called Ni-Vanuatu, constitute 98% of the current population of ≈210,000. A recent study suggested that HHV-8 was rare in the Ni-Vanuatu population (*10*).

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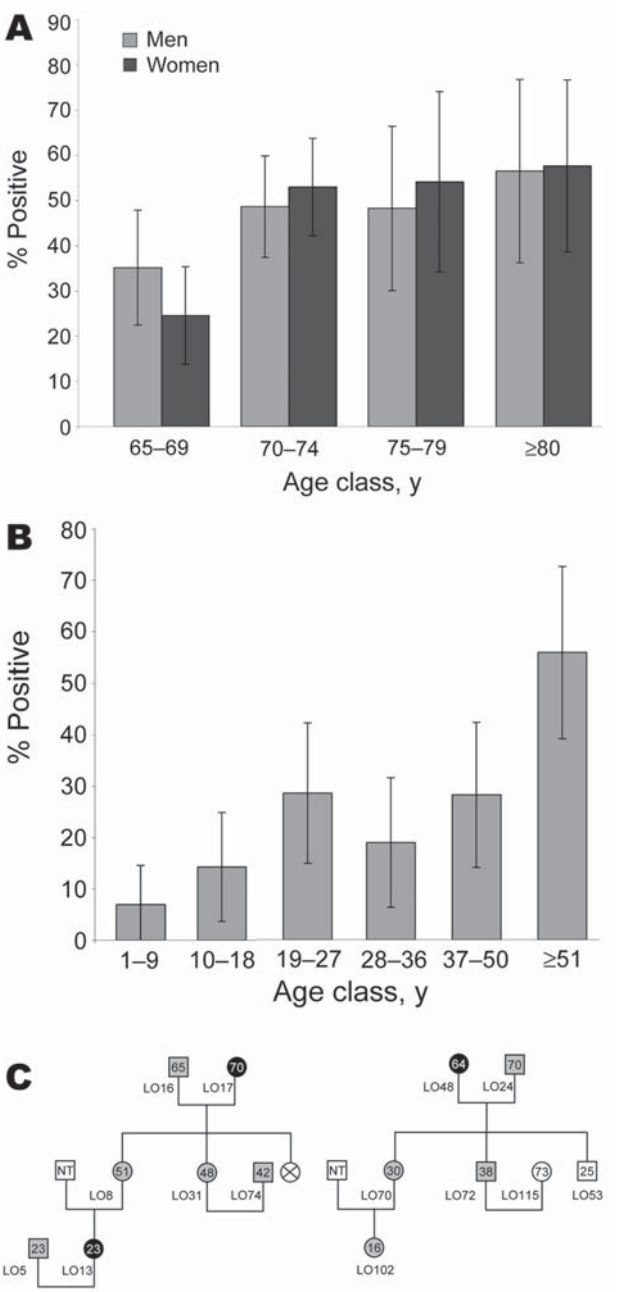

Figure 1. A) Age-dependent herpesvirus 8 (HHV-8) seroprevalence rates in 376 Ni-Vanuatu persons >65 years and living in 18 islands representative of the 6 provinces of the Vanuatu Archipelago. Seropositivity was based on strict criteria, and only samples clearly reactive at a dilution >1:160 were considered HHV-8 positive. B) Age-dependent HHV-8 seroprevalence rate in 283 Ni-Vanuatu persons from 13 families originating from 4 islands (3 from Loh, 2 from Tanna, 4 from Ambae, and 4 from Esperitu Santo) of the Vanuatu archipelago. Error bars indicate 95% confidence intervals C) Pedigrees of 2 families from Loh Island in which the presence of HHV-8 was examined in members of 3 generations. Gray circles and squares denote infected women and men, respectively. Black circles denote infected women for whom sequence of K1 gene fragment was obtained. Numbers within circles and squares indicate ages of the patients; NT, not tested.

Our goal for this cross-sectional study was to evaluate the prevalence of HHV-8 in the Vanuatu archipelago by using stringent serologic criteria and to characterize its genetic diversity.

The Study

Our work was performed on a large collection of ≈4,500 plasma and peripheral blood buffy coat (PBBC) samples from different islands of the archipelago, obtained in the framework of our previous studies on human T-cell lymphotropic virus (HTLV-1) (*11,12*). The field survey, carried out from April 2003 through August 2005, has been extensively described (*11*).

To detect plasma HHV-8 antibodies, an inhouse immunofluorescence assay (IFA) using BC-3 cells expressing only latent-associated nuclear antigens encoded by ORF73, was performed to detect plasma HHV-8 antibodies (*13*). Because HHV-8 seroprevalence increases with age in a virus-endemic population, we first tested a series of 376 samples, from persons >65 years of age (mean 72, median 70, range 65–96 years; 182 men and 194 women) originating from the 6 provinces of the archipelago (online Appendix Figure, available from www.cdc.gov/EID/content/13/11/1745-appG.htm). Among these 376 plasma samples, 170 (45.2%) were IFA positive at a 1:160 dilution, showing a clear typical nuclear spotted seroreactivity. The HHV-8 seroprevalence was similar between men (45.6%) and women (44.8%). The prevalence of HHV-8 increased with age, rising from 29.6% (65–69 years) to 57.1% (≥80 years) (Figure 1, panel A) ($p = 0.0005$ trend χ^2 test). This high level of HHV-8 seroprevalence was present in all 6 provinces (online Appendix Figure).

A second serologic survey that used 237 plasma samples taken from 13 families with genealogic trees was performed (Figure 1, panels B and C). Among these 237 samples, 12 originated from elderly persons included in the 376 samples tested before. The HHV-8 seroprevalence was clearly age-dependent, rising from 6.9% among children 1–9 years of age to 28.2% in adults 50 years of age, followed by a new increase in persons ≥51 years of age (55.9%) ($p<10^{-4}$ trend χ^2 test). These results demonstrate that HHV-8 infection is endemic, widespread, and circulates in the Ni-Vanuatu population.

We then characterized these HHV-8 strains molecularly. All DNA samples (1 μg), extracted from the PBBC, were first amplified by PCR for human β-globin sequences to control amplifiability. HHV-8 infection was determined by a nested PCR to obtain a 737-bp fragment of the open reading frame of the K1 gene (ORFK1). The first PCR was performed with the primer set K1AG75S/K1AG1200AS

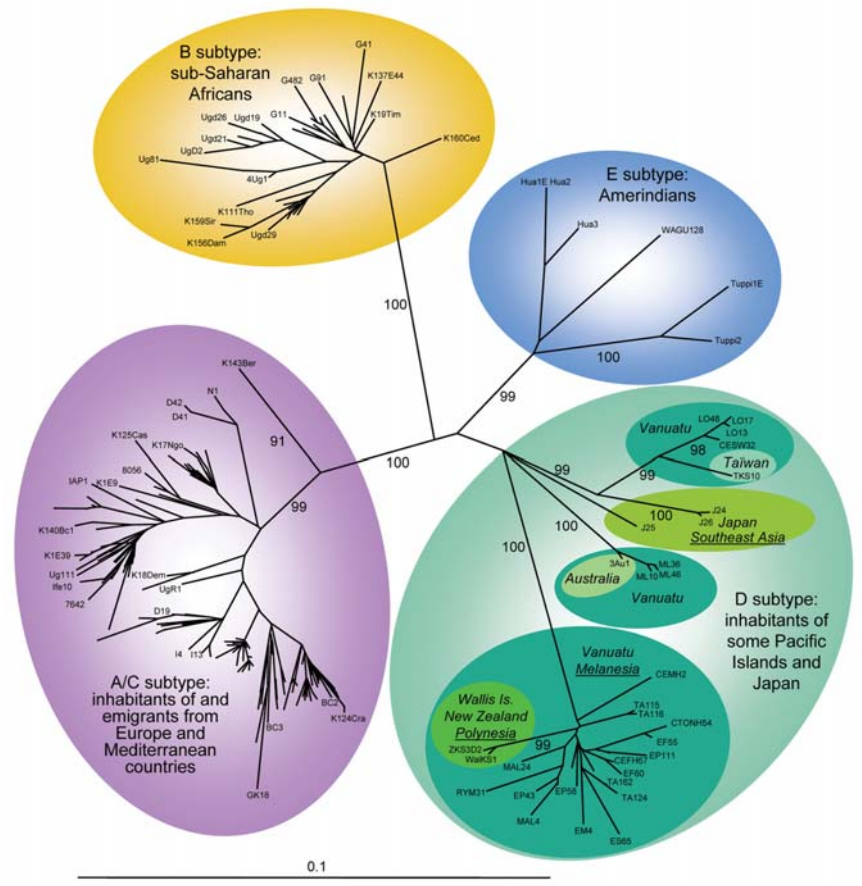

Figure 2. Unrooted phylogenetic tree generated by the neighbor joining (NJ) method with a 624-bp fragment of the K1 gene. The phylogeny was derived by the NJ method by using the GTR model in the PAUP program version 4.0b10 (Sinauer Associates, Sunderland, MA, USA). Reliability of the inferred tree was evaluated by bootstrap analysis on 1,000 replicates. Branch lengths are drawn to scale, with the bar indicating 0.1-nt replacement per site. Numbers on each node indicate the percentage of bootstrap samples (1,000) in which the cluster is supported. Only bootstrap values ≥75 are given. Not all samples have been labeled because of space constraints. The 30 new ORFK1 HHV-8 sequences (GenBank accession nos. EF589742–EF589771) were analyzed with 195 HHV-8 available sequences from the GenBank database.

(*14*) and followed by a nested PCR with a second set of primers VR1S/VR2AS1 (*15*). All PCR products were purified from gel, cloned, and sequenced. Sequences were verified on both DNA strands. ORFK1 amplification was obtained from 32 (21.6%) of the 148 HHV-8–seropositive samples tested but in none of the 26 HHV-8–seronegative samples. Sequences were obtained for only 30 of the 32 ORFK1-positive PCRs (Table).

Comparative sequence analysis indicates that the 30 new sequences differed from each other. Furthermore, among them, 3 groups can be clearly identified. The first group comprises most strains (23/30) and corresponds to the sequences found in persons from the south central islands of the archipelago (Mallicolo, Ambrym, Epi, Tongoa, Emae, and Tanna); the second group comprises 4 sequences from persons living in the northern islands of Loh (LO13, LO17, LO48) and Santo (CESW32); and the third group involves only 3 sequences (ML10, ML36, ML46) from persons living in the northern island of Motalava.

Phylogenetic analyses were performed on the 30 novel sequences obtained in this study, on all subtype D available K1 sequences, and on representatives of the different HHV-8 subtypes/subgroups, as described (*4*). The phylogenetic analyses were performed with all of the sequences available. These sequences include 3 strains from Japan (J24, J25, and J26), 1 from Australia (3Au1), 1 from Taiwan (TKS10), 1 from New Zealand (ZKS3), and 1 from Wallis (WalKS1) (*2,4, 6–8*). Our results demonstrate that the Ni-Vanuatu HHV-8 clustered in 3 different genotype D subclades, which are highly supported phylogenetically with high bootstrap values of 99% or 100% (Figure 2). The first one comprising most strains corresponds to sequences closely related to each other and to the 2 Polynesian strains WalKS1 and ZKS3 (*4,8*). The second group (Loh/Santo), with only 4 sequences, was closely related to the Taiwanese strain TKS10 (*8*). The last 3 sequences from Motalava were nearly identical to the only strain from Australia (*6*).

Table. Demographic, geographic, and serologic data of HHV-8 seropositive persons from the Vanuatu Archipelago, confirmed by molecular analysis*

Virus strain	Age, y	Sex	Island of origin	Province	IFA titers (LANA)	PCR K1	GenBank accession no.
LO 17	70	F	Loh	TORBA	10.240	+	EF589758
LO 13	23	F	Loh	TORBA	160	+	EF589757
LO 48	64	F	Loh	TORBA	640	+	EF589759
ML 10	60	F	Mota Lava	TORBA	640	+	EF589763
ML 36	60	M	Mota Lava	TORBA	640	+	EF589764
ML 46	61	M	Mota Lava	TORBA	1.280	+	EF589765
ES 65	82	F	Santo	SANMA	160	+	EF589756
CES W32	60	F	Santo	SANMA	320	+	EF589746
MA 55	60	F	Maewo	PENAMA	2,560	+	EF589760
MAL 4	74	M	Mallicolo	MALAMPA	320	+	EF589761
MAL 24	70	M	Mallicolo	MALAMPA	1.280	+	EF589762
RYM 31	80	F	Ambrym	MALAMPA	40	+	EF589766
RYM 42	74	F	Ambrym	MALAMPA	80	+	EF589767
EP 43	80	F	Epi	SHEFA	20.480	+	EF589753
EP 58	79	F	Epi	SHEFA	20.480	+	EF589754
EP 111	74	F	Epi	SHEFA	1,280	+	EF589755
TON 72	75	M	Tongoa	SHEFA	5.120	+	EF589772
CTON H54	71	M	Tongoa	SHEFA	2.560	+	EF589747
EM 1	70	M	Emae	SHEFA	640	+†	NA
EM 4	75	F	Emae	SHEFA	2.560	+	EF589752
CEM H2	73	M	Emae	SHEFA	1.280	+	EF589744
EF 43	73	F	Efate	SHEFA	640	+	EF589748
EF 52	74	M	Efate	SHEFA	1.280	+	EF589749
EF 55	81	M	Efate	SHEFA	640	+	EF589750
EF 56	75	F	Efate	SHEFA	5.120	+†	NA
EF 60	73	F	Efate	SHEFA	640	+	EF589751
CEF H56	72	M	Efate	SHEFA	80	+	EF589742
CEF H67	70	M	Efate	SHEFA	640	+	EF589743
TA 115	77	M	Tanna	TAFEA	1.280	+	EF589768
TA 116	70	F	Tanna	TAFEA	320	+	EF589769
TA 124	70	F	Tanna	TAFEA	5.120	+	EF589770
TA 162	70	M	Tanna	TAFEA	5.120	+	EF589771

*IFA, immunofluorescence assay; LANA, HHV-8 specific antibody directed against latent nuclear antigen; PCR K1, PCR for amplification of a 737-bp fragment of the ORFK1 genomic region of HHV-8; NA, not available.
†Weak PCR signal.

Furthermore, phylogenetic analysis showed a star-like tree with a long branch for the Polynesian clade, which includes most Ni-Vanuatu strains. This finding strongly suggests a common origin or ancestor for these strains, with a possible founder effect (Figure 2). Based on stringent serologic and molecular analyses, our study demonstrates for the first time, to our knowledge, that HHV-8 infection is endemic in a Melanesian population.

Our serologic findings are consistent with those found in some remote villages of Papua New Guinea (*5*,*9*). On the basis of these studies, it is tempting to suggest that intrafamilial HHV-8 transmission occurs in such populations, as previously demonstrated in highly HHV-8 endemic populations of African origin (*13*).

From the molecular point of view, finding such a high molecular diversity of HHV-8 subtype D with some Polynesian-, Taiwanese- and Australian-like strains was surprising. These heterogeneous findings contrast with the more homogenous situation found for HTLV-1 genotypes in the same population (*11*).

Conclusions

Our molecular findings suggest that HHV-8 has been introduced in the Ni-Vanuatu populations by different migrations of infected persons. This conclusion is strengthened by the clustering of the Australian- and Taiwanese-like strains in the northern islands of Loh and Motalava. A variety of scenarios have been proposed to explain the peopling of near and remote Oceania, and our data highlight the possible multiple origins of Ni-Vanuatu ancestors. Ongoing molecular studies on both viral and mitochondrial/nuclear DNA will contribute to this debate through analyses of the variations observed. Indeed, these variations are intimately linked with the dispersal of early human settlers; analyses of the genetic variability of HHV-8 can help us reconstruct the patterns of human dispersal into Oceania (*11*).

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Mr Cassar is a PhD candidate with primary research interest in the clinical and molecular epidemiology and physiopathology of dengue viruses. He is working on the epidemiology of HTLV-1 and HHV-8 in Melanesian populations.

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Onchocerca jakutensis Filariasis in Humans

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We identified *Onchocerca jakutensis* as the causative agent of an unusual human filariasis in a patient with lupus erythematosus. To our knowledge, this is the first case of human infection with *O. jakutensis* and the first human case of zoonotic onchocercosis involving >1 worm.

Zoonotic filarial infestations occur worldwide, and in most reported cases the involved species are members of the genus *Dirofilaria*. However, zoonotic *Onchocerca* infections are rare and to date only 13 cases (originating from Europe, Russia, the United States, Canada, and Japan) have been described. In all of these cases only 1 immature worm was found, and the causative species was identified as *O. gutturosa*, *O. cervicalis*, *O. reticulata*, or *O. dewittei japonica* on the basis of morphologic and in some cases serologic parameters (*1–4*). *O. cervicalis* and *O. reticulata* are found in the ligaments of the neck and extremities of horses, *O. gutturosa* is typically found in the nuchal ligaments of cattle, and *O. dewittei japonica* is found in the distal parts of the limbs and adipose tissue of footpads of wild boars.

We identified the causative agent of a zoonotic *Onchocerca* infection with multiple nodules in a patient with systemic lupus erythematosus (SLE) who had been receiving hemodialysis. The parasite was identified in paraffin-embedded tissue samples by PCR and DNA sequence analysis.

The Study

The patient was a 59-year-old woman with SLE who had developed multiple nodules on the neck and face over several years. Because of major renal insufficiency, she also had been receiving hemodialysis 3 times per week (3.5 hours) for >10 years. The first clinical differential diagnoses were cutaneous SLE, nephrogenous dermatopathy, calciphylaxis, and calcinosis. The clinical picture was obscured by secondary inflammations and ulcerations caused by self-inflicted trauma. Multiple sampling attempts by cutaneous core biopsies resulted in histologic diagnosis of unspecific, secondary inflammatory changes. Deep surgical excision of 1 subcutaneous nodule on the scalp indicated subcutaneous helminthosis (Figure). The patient was treated with ivermectin and subjected to 2 plastic surgeries for facial reconstruction, after which she recovered.

At this point, species identification of the causative agent was still pending. A history of travel anamnesis and location of the nodules indicated a possible *Dirofilaria* infection, but a specific PCR showed negative results. Morphologic features of the few available sections suggested *Onchocerca* spp. To our knowledge, multiple nodules had never been reported in cases of infection with zoonotic *Onchocerca*. Because a definitive morphologic identification of the causative nematode was not possible, molecular identification from DNA isolated from the only available material (formalin-fixed and paraffin-embedded tissue) was conducted.

To evaluate the causative genus, universal filarial primers were constructed on the basis of filarial sequences available in GenBank (primer FILfw 5′-CGGTGATATTGGTTGGTCTC-3′ for the first internal transcribed spacer region and primer FILrev 5′-CTAGCTGCGTTCTTCATCGATC-3′ for the 5.8S rRNA gene). PCR and sequencing were performed and a similarity matrix was calculated after multiple sequence alignment (*5*).

The DNA fragment obtained was 226 bp and showed greatest similarities to *Onchocerca* sequences, ranging from 87% to 95%. Similarities to *Wuchereria*, *Brugia*, *Mansonella*, *Dirofilaria*, and *Acanthocheilonema* were lower, ranging from 75% to 80%. Assignment to the genus *Onchocerca* was obvious, but species identification still posed a problem because published *O. volvulus* sequences showed higher similarities among each other (98.8%–100%) than

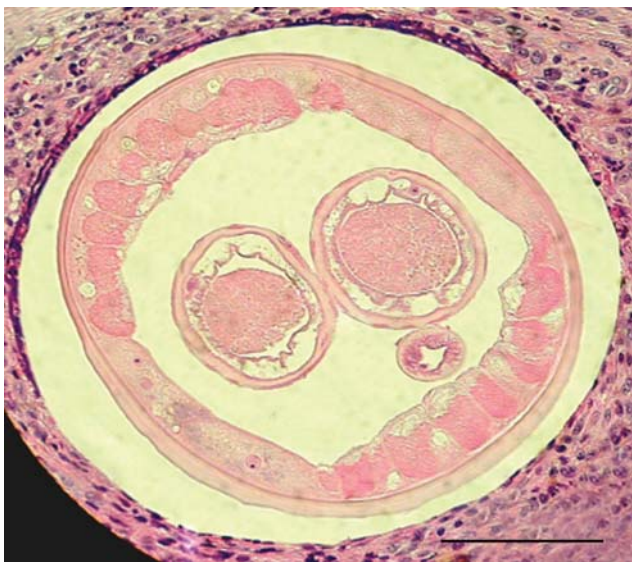

Figure. Transverse section of a female worm and surrounding tissue isolated from the patient (hematoxylin and eosin stained). Scale bar = 100 μm.

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with our sequence. The only exception was a clinical *O. volvulus* strain (OvNod1–3) from Bolo, Cameroon, which showed 94.8% sequence similarity. However, the authors of that report indicated that their strain might be a zoonotic *Onchocerca* sp. (*6*).

An identical thymine mononucleotide repeat motif in our strain and strain OvNod1–3, which was shorter in all *O. volvulus* sequences, indicated that both strains were not *O. volvulus* because repeat motifs have been reported to occur in species-specific patterns. The negative results with an *O. volvulus*–specific PCR (*6*) corroborated this assumption. Therefore, 2 additional primer pairs for *Onchocerca* spp. identification were constructed, 1 for the mitochondrial NADH dehydrogenase subunit 5 gene (OND5fw 5′-CTCCTGTTAGTTGTTTGGTTC-3′, OND5rev 5′-GCAAACCCCTACCAATAGC-3′) and 1 for the 16S mitochondrial rRNA gene (O16fw 5′-GCGTGATGGCATAAAAGTAGC-3′, O16rev 5′-CAACCCTGTTAACTCCGGAG-3′), on the basis of available *Onchocerca* spp. sequences (*7*,*8*). PCR products were sequenced and similarity matrices were calculated (Tables 1, 2). The NADH amplicon was 201 bp and the 16S rRNA amplicon was 225 bp. Both amplicons unambiguously identified our strain as *O. jakutensis* with 100% and 99.55% sequence similarities, respectively. Sequence data were deposited in GenBank and are available under the following accession nos.: EF202184, EF202185, and EF202186.

Conclusions

The limiting factor in identifying the causative agent in our patient was the nature of the sample material. Because only a few formalin-fixed and paraffin-embedded sections were available, morphologic identification was not possible. PCR-based identification was restricted because DNA has a tendency to degrade when stored in formalin, which limits the length of the target sequence to ≈300 bp and limits its discriminatory power (*9*). A different approach with 3 PCRs, 1 for genus identification and 2 for species identification, and primers for highly variable multicopy targets enabled us to accurately identify the causative agent as *O. jakutensis*.

To our knowledge, *O. jakutensis* has never been identified as an agent of human filariasis. It has been identified as a rare parasite of red deer in Germany, Poland, and Russia, and may also be found in other northern European countries (*10*). Our patient came from the United States and had traveled all over Europe. She could thus have acquired the infection in several different locations.

Two findings for this patient were particularly unusual and obscured the identification of the parasite. The first finding was that she had, in contrast to all previous human cases of zoonotic onchocercosis, multiple nodules. The second finding was that her face (periorbital and buccal), neck, and scalp were affected, although zoonotic filariae are typically found in similar or identical tissues as in their

Table 1. Sequence similarities in the NADH dehydrogenase subunit 5 gene in the *Onchocerca* sp. isolated in this study and other *Onchocerca* spp.*

	% Sequence similarity											
Species	*arm*	*duk*	*fas*	*fle*	*gib*	*gut*	*jak*	*lie*	*och*	*ram*	*vol*	This study
arm	100	94.03	91.54	91.54	92.04–92.53	86.07–92.54	92.04	91.54–92.54	89.55–93.03	81.59	93.03–94.03	92.04
duk		100	94.53	92.54	96.51–97.01	92.04–96.51	94.53	97.51–98.51	95.02–97.01	82.59	98.01–99.00	92.02
fas			100	89.05	92.04–92.53	88.56–94.03	94.53	95.02–96.02	92.04–95.52	82.59	93.53–94.53	94.53
fle				100	93.03–95.03	86.57–92.04	91.04	91.04–92.04	90.55–92.04	82.09	92.53–93.53	91.04
gib					99.5	90.05–95.02	92.04–92.54	94.03–95.52	94.03–95.52	84.08–84.58	95.52–97.01	92.04–93.54
gut						90.55–97.51	89.05–94.53	91.54–96.02	91.04–95.52	79.10–82.59	91.04–96.52	89.05–94.53
jak							100	94.53–95.52	92.54–95.02	82.09	94.03–95.02	100
lie								98.51–100	94.53–98.51	83.08–84.08	96.52–98.51	94.53–95.52
ochi									94.53–100	81.59–84.58	94.03–98.01	92.54–95.02
ram										100	83.58–84.08	82.09
vol											98.51–100	94.03–95.02
This study												100

**arm, armillata; duk, dukei; fas, fasciata; fle, flexuosa; gib, gibsoni; gut, gutturosa; jak, jakutensis; lie, lienalis; och, ochengi; ram, ramachandrini; vol, volvulus.*

Table 2. Sequence similarities in the mitochondrial 16S rRNA gene in the *Onchocera* sp. isolated in this study and other *Onchocerca* spp.*

	% Sequence similarity											
Species	*arm*	*duk*	*fas*	*fle*	*gib*	*gut*	*jak*	*lie*	*och*	*ram*	*vol*	This study
arm	100	91.56	96.00	96.00	94.67	93.78–94.22	94.22	93.33-93.78	93.33–93.78	93.33	91.55–92.00	93.78
duki		100	95.56	92.00	95.56	94.67–95.11	94.67	95.11	96.89–97.33	91.11	96.89–97.33	91.11
fas			100	95.56	96.89	96.00–96.44	96.44	96.44–96.88	95.56–96.00	93.33	94.66–95.11	96.00
fle				100	94.22	94.22–94.66	95.56	93.78–94.22	92.40–92.88	93.78	92.44–92.89	95.11
gib					100	97.78–98.22	97.78	96.89–97.33	94.67	93.78	93.77–94.22	97.33
gut						99.10–100	96.89–97.33	96.00–96.89	93.33–93.77	91.56–92.00	93.33–93.78	96.89–97.33
jak							100	98.22–98.67	92.88–93.33	92.44	92.00–92.44	99.56
lie								99.56–100	93.33–93.78	92.44–92.88	92.44–92.88	97.78–98.22
och									99.56–100	91.11–91.55	97.33–98.22	93.33–93.78
ram										100	90.22–90.67	92.00
vol											99.56–100	92.44–92.89
This study												100

**arm, armillata; duk, dukei; fas, fasciata; fle, flexuosa; gib, gibsoni; gut, gutturosa; jak, jakutensis; lie, lienalis; och, ochengi; ram, ramachandrini; vol, volvulus.*

natural hosts (*11*). *O. jakutensis* is usually found in tissues of the outer thigh and caudal part of the back; >2 nodules per infected host are rare (*12*,*13*).

It is unlikely that these findings are associated with greater virulence of *O. jakutensis* than of other zoonotic *Onchocerca* spp. However, parasite virulence might be related to the patient having had autoimmune disease since childhood and as a result having received long-term immunosuppressive therapy. The immune status of the patient was further impaired by renal insufficiency for >10 years. However, no data exist on the immune status of patients in any of the previously reported cases of infection with zoonotic *Onchocerca* spp. For other nematodes, e.g., *Strongyloides stercoralis*, a correlation between immune status of the patient and severity of disease is well established. One report describes more severe skin manifestations caused by *O. volvulus* in HIV patients (*14*).

We have identified a zoonotic infestation with an *Onchocerca* sp. that can cause disease in humans. The combination of impaired immunity and unusually progressing infestation highlights a new aspect of zoonotic filariasis.

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Methicillin-Resistant *Staphylococcus aureus* in Meat Products, the Netherlands

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A new methicillin-resistant *Staphylococcus aureus* (MRSA) clone related to pig and cattle farming was detected in the Netherlands. We investigated the extent of S. aureus presence in meat and found 36 *S. aureus* strains in 79 samples. Two strains were MRSA; 1 was multilocus sequence type 398, the clone related to farming.

In 2003 a new clone of methicillin-resistant *Staphylococcus aureus* (MRSA) related to pig and cattle farming emerged in the Netherlands (*1*,*2*). A survey of pigs showed that nearly 40% carried this clone (*3*). Detecting this strain was relatively easy with pulsed-field gel electrophoresis (PFGE) since it is nontypable (NT), the method used for surveillance of MRSA at the National Reference Centre for MRSA (National Institute of Public Health and the Environment, Bilthoven, the Netherlands). Further typing of NT-MRSA showed that almost all strains belonged to 1 multilocus sequence typing cluster, ST 398 (*2*). We undertook this study to determine to what extent *S. aureus*, and more specifically, MRSA, was present in Dutch meat products.

The Study

Samples of various meat products from pigs and cattle, obtained from local supermarkets and butcher shops, were examined for contamination with methicillin-susceptible *S. aureus* (MSSA) and MRSA. A total of 79 raw meat products (pork, n = 64; beef, n = 15) were collected from 31 different shops (butcher shops, n = 5; supermarkets, n = 26) from February through May 2006. Table 1 shows how many samples were investigated per shop. A small portion of the meat products (mean 7.9 g, SD 3.97) was plated directly onto chromogenic agar for the detection of MRSA (MRSA ID; bioMérieux, La-Balme-les-Grottes, France). All sides of the meat portion were streaked over a part of the agar plate, and from this inoculated area, the material was spread by using a sterile loop. The piece of meat was then put into 5-mL enrichment broth containing Mueller-Hinton broth and 6.5% NaCl. After 24-h incubation at 35°C, the enrichment broth was subcultured on Columbia agar plates with 5% sheep's blood (CA), a MRSA-ID plate, and 1 mL of the enrichment broth was put into a second enrichment broth containing phenol-red mannitol broth with ceftizoxime (5 μg/mL) and aztreonam (7.5 μg/mL) (Regional Public Health Laboratory, Groningen, the Netherlands). The second enrichment broth was subcultured on CA and MRSA-ID. All plates were incubated for 48 h at 35°C. Presumptive *S. aureus* colonies were confirmed with a latex agglutination test (Staphaurex Plus; Murex Diagnostics Ltd, Dartford, UK), a tube coagulase test with rabbit plasma, and DNase (DNase agar; Oxoid Ltd, Basingstoke, UK). Confirmation of methicillin resistance and *S. aureus* species identification was performed by an in-house–developed, validated duplex real-time PCR for the *mecA* gene and the *S. aureus*–specific 442-bp fragment described by Martineau et al. (*4*; P.H.M. Savelkoul and A.M.C. Bergmans, pers. comm.). Susceptibility to cefoxitin and doxycycline was determined by using disk diffusion according to the Clinical Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) standards (*5*). All isolated *S. aureus* strains (MSSA and MRSA) were genotyped by amplified fragment gel electrophoresis (AFLP) (*6*). *Spa* types were defined according to the procedure previously described by Harmsen et al. (*7*).

Table 1. *Staphylococcus aureus* in meat samples, the Netherlands, 2006

No. samples/shop	No. shops	No. *S. aureus*–positive samples
1	14	7
2	6	3
3	4	3
4	3	3
5	2	2
9	1	1
10	1	0

Direct inoculation of plates yielded no MRSA-positive isolates (Table 2). The first enrichment broth yielded *S. aureus* from 30 positive meat samples, 25 pork and 5 beef. In 1 pork sample, 2 phenotypically different *S. aureus* isolates were found. One *S. aureus* isolate in pork meat was identified as MRSA. When the double-enrichment broth culture system was added, another 6 samples were *S. aureus* positive, 1 of which contained MRSA. Combining the results of both enrichment broth culture procedures, 34 samples were

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Table 2. Number of MSSA and MRSA strains in pork and beef, by culture system, the Netherlands, 2006*†

Meat	Total no. samples	Single enrichment broth			Single and double enrichment broth		
		MSSA strains	MRSA strains	No. positive samples	MSSA strains	MRSA strains	No. positive samples
Pork	64	24	1	24	29	2	29
Beef	15	5	0	5	5	0	5
Total	79	29	1	29	34	2	34

*MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*.
†No *S. aureus* was found by direct culture.

positive, harboring 36 phenotypically different *S. aureus* isolates (Table 2). Twenty-seven (42.2%) pork samples and 5 (33.3%) beef samples harbored *S. aureus*. Two pork samples yielded 2 phenotypically different *S. aureus* isolates. Two isolates from pork (2.5% of total samples) were found to be methicillin resistant. A total of 19 shops (61.3%) had at least 1 positive meat sample.

AFLP typing showed 8 genetic lineages, covering 72.2% (26/36) of the isolated strains and a smaller number of unique sporadic isolates 27.8% (10/36) (Figure). *Spa* typing showed that in 6 of these genetic lineages, 1 *spa* type was identified, and in 1 lineage, 2 closely related *spa* types were identified (Figure).

From the 2 samples that contained 2 phenotypically different strains, the 2 strains from 1 sample (TY4376 and TY4378) belonged to the same lineage, and the other sample contained 2 strains (TY4367 and TY4368) belonging to 2 different genetic lineages. In 5 (83.3%) of 6 shops in which >1 *S. aureus* isolate was found, typing showed clonal relationship among strains originating from the same shops (Figure).

PFGE typing of the 2 MRSA isolates showed that 1 MRSA isolate (TY4390) was nontypable by *Sma*I digestion and identical to isolates found in pigs (TY4400 and TY4433). This strain harbored *spa* type 108, which resembled the *spa* types of the pig and farmer strains (034 and 011, respectively) (*1*–*3*). These strains belong to a separate cluster in the AFLP analysis (Figure). The other MRSA isolate was identical to the US300 clone (TY 4381) and harbored *spa* type 024.

Conclusions

To our knowledge, this is the first survey investigating the presence of MSSA and MRSA in meat products in the Netherlands. Two meat samples (2.5%) contained MRSA. Furthermore, *S. aureus* is found regularly in low amounts in meat sold to consumers. The prevalence of *S. aureus* in meat products was found to be 4%, 22.7%, and 65% in 3 other studies performed in Egypt, Switzerland, and Japan, respectively (*8*–*10*).

Contamination of the meat products could be traced back to certain abattoirs in Switzerland and poor hygienic and sanitary conditions in Egypt (*10*,*11*). The high rate of clonal relatedness of different strains within particular shops indicates cross-contamination of the meat at some point during processing. Therefore, the strain in the sample is not necessarily indicative of the strain that was carried by the animal at the source.

This study demonstrates that MRSA has entered the food chain. As the amounts were very low, the pathogen

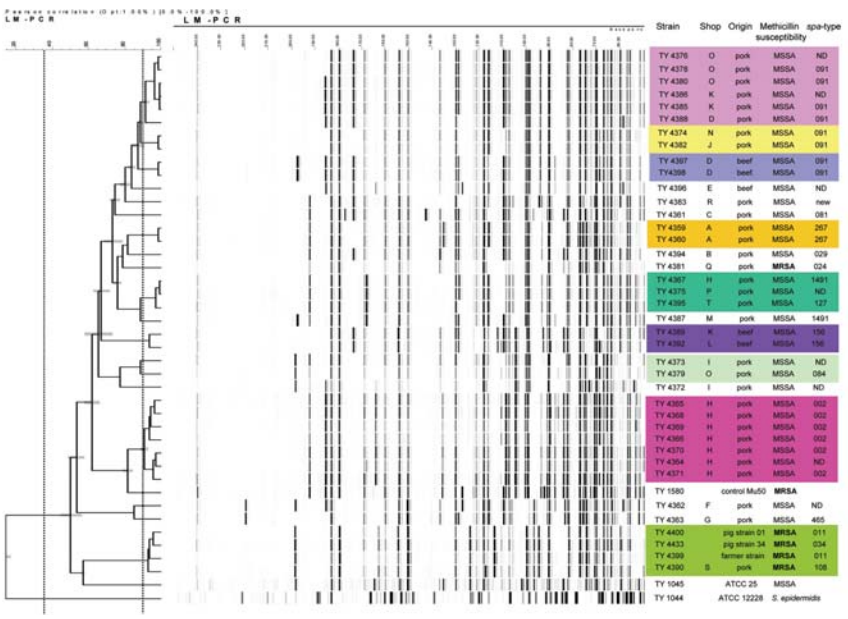

Figure. Amplified fragment gel electrophoresis typing and *spa* typing results of the *Staphylococcus aureus* isolates, methicillin susceptible (MSSA) and methicillin resistant (MRSA), in pork and beef. The boxes indicate clonally related strains. The columns indicate the strain number, the shop where the sample was bought, the origin of the sample, methicillin susceptibility, and *spa* type. ATCC, American Type Culture Collection; ND, not determined.

is not likely to cause disease, especially if meat is properly prepared before consumption. However, contamination of food products may be a potential threat for the acquisition of MRSA by those who handle the food. Also, a large hospital outbreak with MRSA due to contamination of food products has been described (*11*). This occurred in a hospital ward in Erasmus Medical Center in Rotterdam, the Netherlands. In this outbreak, an immunocompromised patient was probably infected by ingestion of MRSA-contaminated food, and subsequently, severe sepsis developed and the patient died. Also, an outbreak of foodborne illness caused by MRSA has been described (*12*). However, this exotoxin-mediated disease is not dependent on the methicillin susceptibility of the causative *S. aureus* strain.

All reports of MRSA in meat products described previously dealt with MRSA of human origin that was contaminating the meat. In this report, the NT-MRSA in the meat was associated with farming and is most likely of animal origin. Although the pig-related MRSA strain was found in only 1 product and in very low amounts, this finding does show that MRSA has made its way into the food chain.

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Human Bocavirus Infection in Children with Gastroenteritis, Brazil

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Human bocavirus (HBoV) was detected in 14 (2%) of 705 fecal specimens from Brazilian children with gastroenteritis. Coinfection with rotavirus, adenovirus, or norovirus was found in 3 (21.4%) HBoV-positive specimens. None of the HBoV-positive patients had respiratory symptoms.

Human bocavirus (HBoV) was first identified in pooled human respiratory tract specimens from Swedish children in 2005 and was provisionally classified within the genus *Bocavirus* of the family *Parvoviridae* (*1*). Previously, the only parvovirus known to be pathogenic in humans was B19 virus, which is responsible for Fifth disease in children (*2*). Because HBoV was first found in respiratory specimens, most epidemiologic studies have focused on such specimens. Shortly after its first description in Sweden, HBoV was detected in respiratory tract specimens from patients with respiratory illness in several parts of the world (*3*–*8*).

Other members of the family *Parvoviridae* that infect animals cause diseases such as leukopenia/enteritis syndrome, seen most commonly in dogs 8–12 weeks of age, with clinical features of vomiting, anorexia, lethargy, and diarrhea that lead to rapid dehydration (*9*). For this reason, we hypothesized that HBoV may play a role in human gastrointestinal disease. In this study, we retrospectively tested stool specimens collected from 2003 through 2005 from Brazilian children with acute diarrhea to investigate whether this virus can infect the human gastrointestinal tract and be detected in feces, and to assess the frequency of such infections.

The Study

A total of 705 stool specimens from Brazilian children <15 years of age (median 3.5 years) with acute diarrhea were obtained from January 2003 through December 2005 and screened by PCR for HBoV DNA. Of these specimens, 285 (40.4%) were collected from hospitalized patients and 420 (59.6%) from outpatients: 142 (20.2%) from the emergency department and 278 (39.4%) from walk-in clinics. Only 1 specimen was obtained per patient. A total of 314 (44.5%) patients were <2 years of age, 190 (27%) were 2–5 years of age, 120 (17%) were 6–10 years of age, and 61 (8.6%) were 11–15 years of age. Age was not known for 21 patients. Relevant clinical information was collected on a standard questionnaire. This information included hospitalization status, age, sex, and clinical symptoms.

Specimens were collected at university hospitals in 3 different cities in Brazil located in areas with distinct sanitation conditions and socioeconomic backgrounds. The specimens were previously tested for other enteric viruses and bacteria, including rotavirus, norovirus, astrovirus, adenovirus, *Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter* spp., and *Shigella* spp. (*10*,*11*). The study protocol was reviewed and approved by the Ethics Committee of the Instituto de Puericultura e Pediatria Martagão Gesteira of the Federal University of Rio de Janeiro.

Stool suspensions were prepared as 10% (w/v) in phosphate-buffered saline (pH 7.2), clarified by centrifugation at 2,500× *g* for 5 min. Two hundred microliters of each suspension was used for DNA extraction with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCRs were performed as described (*7*) by using forward primer HBoV 01.2 (5′-TATGGCCAAGGCAATCGTCCAAG-3′) and reverse primer HBoV 02.2 (5′-GCCGCGTGAACATGAGAAACAGA-3′) for the nonstructural protein 1 gene. A 291-bp amplicon was generated. DNA samples were subjected to 1 cycle at 95°C for 15 min, followed by 45 cycles at 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were detected by agarose gel electrophoresis and staining with ethidium bromide.

To confirm the presence of HBoV, amplified DNAs of PCR-positive samples were purified by using the Wizard SV gel and PCR Clean-Up system kit (Promega). Sequences were determined by using the BigDye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and analyzed with the SeqMan, EditSeq, and MegAlign programs in the Lasergene software package (DNASTAR, Madison, WI, USA). Nucleotide sequences obtained in this study were deposited in GenBank under accession nos. EF560205–EF560216.

Fourteen (2%) of 705 diarrhea stool samples were positive for HBoV by PCR. Rotavirus was detected in 84 (11.9%) samples, adenovirus in 34 (4.8%) samples, noro-

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virus in 24 (3.4%) samples, and astrovirus in 2 (0.3%) samples. Enteropathogenic bacteria were found in 57 (8.1%) samples (*10*,*11*). The frequency of enteric pathogens identified in epidemiologic studies is variable (45%–54%) and dependent on several parameters such as country and type of method used for diagnosis (*12*–*14*). In our study, a potential pathogen was found in 215 (30.5%) samples (including HBoV-positive samples). No bacterial or virus pathogen was found in 499 (69.5%) samples. Samples were not tested for intestinal parasites, which in general account for ≈11% of the diarrhea etiology in developing countries (*12*,*14*).

There was no obvious temporal clustering of the HBoV-positive patients. The median age of HBoV-infected children was 1.9 years; 11 children (78.6%) were <2 years of age, 1 child was 35 months of age, 1 child was 11 years of age, and 1 child was 15 years of age. A total of 57% were boys and 43% were girls. Three patients were coinfected with other enteric viruses (1 with adenovirus, 1 with rotavirus, and 1 with norovirus). All HBoV-positive patients had diarrhea but none reported concomitant respiratory symptoms. Fever was reported in 2 patients, vomiting in 1, and bloody diarrhea in 2. One hospitalized boy (the oldest study participant) was reported to be positive for HIV and cytomegalovirus, and 1 hospitalized girl was undergoing dialysis. Ten (71.2%) HBoV-positive children were hospitalized because of diarrhea; 4 were outpatients (3 from walk-in clinics and 1 from an emergency department).

A semiquantitative PCR of HBoV in stool specimens was performed by using dilutions of DNA extracted from stool samples. We detected DNA up to a dilution of 10^{-3} in 3 samples. In the remaining samples, DNA was detected only in undiluted samples. Sequence analysis showed high nucleotide similarity between Brazilian samples and the Chinese respiratory HBoV WLL-3 strain (GenBank accession no. EF584447) from the People's Republic of China (91.8%–99.6%) and among the Brazilian samples (96.4%–100%) (Figure). We could not compare our enteric strains with a Spanish enteric strain (*8*) because we sequenced a different portion of the virus genome. Strain MC-8 showed the lowest homology with the WLL-3 strain (91.8%) and with the other Brazilian strains (96.4%). We are conducting additional sequencing to characterize the complete genome of this strain to confirm that it represents a new variant of the virus.

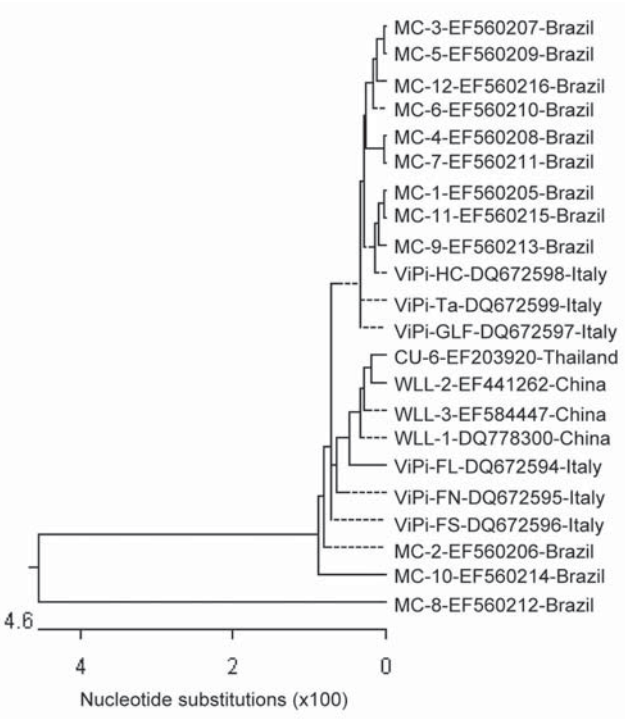

Figure. Phylogenetic analysis of nucleotide sequences of the nonstructural protein 1 gene of enteric strains of human bocavirus from Brazil. The dendrogram was constructed with the Clustal W algorithm of the MegAlign program in the Lasegene software package (DNASTAR, Madison, WI, USA). The length of each pair of branches represents the distance between sequence pairs. Dashed lines on a phenogram indicate a negative branch length. GenBank accession nos. are shown with strains.

Conclusions

HBoV has been isolated from respiratory specimens from patients with acute respiratory illness, and increasing evidence suggests a causal relationship with this disease (*3*–*8*). The presence of HBoV in the human gastrointestinal tract was demonstrated by Vicente et al. (*8*), as well as in our study. In the first study, virus was isolated from feces of children with gastroenteritis with or without symptoms of respiratory infection. Coinfection with other intestinal pathogens was found in 28 (58.3%) of 48 HBoV-positve samples. In our study, none of the HBoV-positive patients reported respiratory symptoms. Coinfection with other enteric viruses was found in 3 (21.4%) of 14 HBoV-positive samples. High titers of DNA in some specimens suggest that the virus replicates in the human gut. However, additional studies that include control groups are needed to demonstrate an association between HBoV infection and gastroenteritis.

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Drug-Resistant Malaria Parasites Introduced into Madagascar from Comoros Islands

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To determine risk for drug-resistant malaria parasites entering Madagascar from Comoros Islands, we screened travelers. For the 141 *Plasmodium falciparum* isolates detected by real-time PCR, frequency of mutant alleles of genes associated with resistance to chloroquine and pyrimethamine was high. International-level antimalarial policy and a regional antimalarial forum are needed.

In the southwestern Indian Ocean, the epidemiologic features of malaria and antimalarial drug resistance differ considerably between islands that are very close geographically. Malaria remains a major public health problem in Madagascar and the Comoros Islands, whereas the situation is different on other nearby islands. Chloroquine resistance ranges from moderate in Madagascar (*1*–*3*) to high in the Comoros Islands (*4*,*5*), whereas pyrimethamine resistance is absent in Madagascar (*3*,*6*) but present at high levels in the Comoros Islands (*5*). The paradoxical situation of resistance to antimalarial drugs and prevalence of mutant-type parasites (for the *Plasmodium falciparum* chloroquine resistance transporter [*pfcrt*] gene, 90% of 76T alleles in the Comoros Islands vs. 3% in Madagascar; for the *P. falciparum* dihydrofolate reductase [*dhfr*] gene, 69% of 108N alleles in the Comoros Islands vs. 0% in Madagascar) (*2*,*5*,7) in such close geographic proximity led us to perform this study.

Historically, the Comoros Islands and Madagascar have been linked by human travel, and the importation of pathogens has already been documented, in the cholera epidemic of 1998–1999 (*8*) and in more recent outbreaks of arbovirus infection (*9*,*10*). Most travel between the Comoros Islands and Madagascar occurs through the seaport and airport of Mahajanga, the main city on the northwestern coast of Madagascar. To improve the monitoring of antimalarial drug resistance in Madagascar, we assessed the frequency of *P. falciparum* mutant alleles of genes associated with resistance to chloroquine (*pfcrt* and *P. falciparum* multidrug resistance 1 [*pfmdr-1*] gene) and pyrimethamine (*dhfr*) among travelers entering Madagascar from the Comoros Islands.

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The Study

The study was performed from March to July 2006, in the seaport and the airport of Mahajanga, on the northwest coast of Madagascar (Figure 1). These sites are the main communications crossroads between the Comoros Islands and Madagascar. The study was approved by the National Ethics Committee of the Ministry of Health and Family Planning of Madagascar.

All travelers from the Comoros Islands who consented to participate on arrival in Madagascar, regardless of their age, sex, nationality, and presence or absence of symptoms, were enrolled in the study. For each participant, , a questionnaire was filled out and a finger-prick blood sample was collected. Rapid diagnostic tests (OptiMAL-IT, DiaMed AG, Cressier sur Morat, Switzerland) and thick/thin blood smears were performed in the field. Patients with a positive rapid test result were promptly treated with an artesunate and amodiaquine combination (Arsucam, Sanofi-Aventis, Paris, France), according to Madagascar's national malaria policy.

Thick/thin blood smears were stained and analyzed by an experienced technician, without reference to rapid test results. A minimum of 200 consecutive fields were counted for each thick blood film before a slide was classified as negative. The number of parasites in thick blood films was determined per 200 or 500 leukocytes, assuming 8,000 leukocytes/μL of blood. Thin blood smears were also examined for other *Plasmodium* spp.

Parasite DNA was extracted from blood samples by using the phenol/chloroform method. *P. falciparum* carriers were detected by real-time PCR in a RotorGene 3000 thermocycler (Corbett Life Science, Sydney, New South Wales, Australia), as described by Mangold et al. (*11*). PCR and restriction fragment length polymorphism analyses were performed for 3 genes (codon 108 of *dhfr*, codon 76 of *pfcrt*, and codon 86 of *pfmd-1*) for the detection of mutant alleles. (Detailed descriptions of these methods are available from http://medschool.umaryland.edu/cvd/plowe.html.) Laboratory strains of *P. falciparum* were used as controls (positive and negative) and included in all PCR and enzyme digestion procedures (DNA from the W2, HB3, and 3D7 reference strains from the Malaria Research and Reference Reagent Resource Center, Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Disease, National Institutes

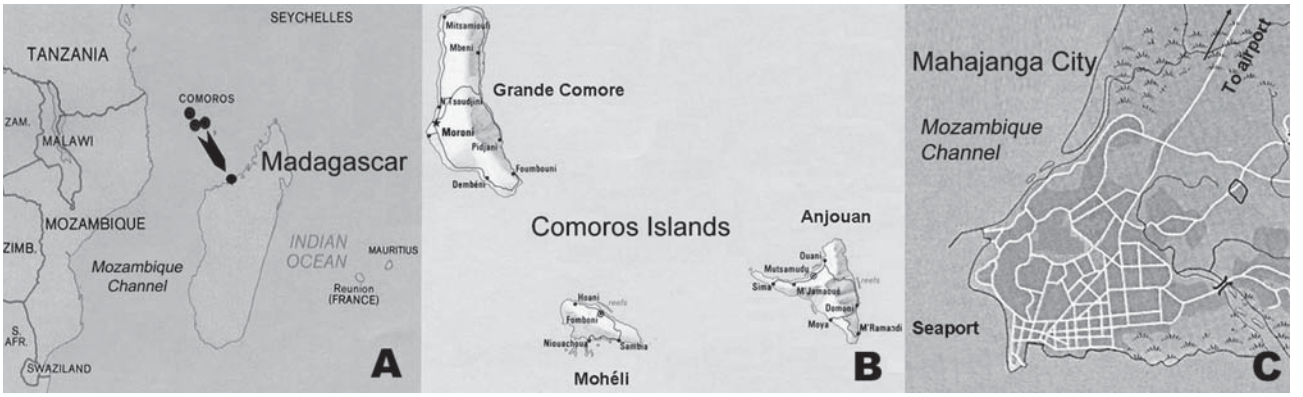

Figure 1. Regional map of the Indian Ocean, showing A) location of Comoros Islands and Madagascar; B) Comoros Islands; and C) location of Mahajanga seaport and airport, Madagascar.

of Health, Manassas, VA, USA). Statistical analyses were performed by using SPSS software (SPSS Inc., Chicago, USA). Odds ratios were calculated from logistic regression parameter estimates, and p values were determined with the significance level set at $p \leq 0.05$.

Among the 1,130 travelers registered on arrival in Mahajanga, 947 agreed to participate in the study (crude participation rate 83.8%). The baseline characteristics of the enrolled travelers are given as a function of nationality (Comorian or Malagasy) in the Table. The frequency of *P. falciparum* carriers was 0.5% (5/947) according to rapid diagnostic test, 3.2% (30/947) according to microscopy, and 14.9% (141/947) according to real-time PCR.

For the 141 *P. falciparum* isolates detected by real-time PCR, the frequency of the mutant alleles of genes associated with resistance to chloroquine (*pfcrt* and *pfmdr-1*) and pyrimethamine (*dhfr*) was 80.1% (113/141) for the 76T mutant allele of the *pfcrt* gene, 99.3% (140/141) for the 86Y mutant allele of the *pfmdr-1* gene, and 95.0% (134/141) for the 108N allele of the *dhfr* gene. More detail is provided in the Table. Univariate analysis of risk factors associated with the carriage of *P. falciparum* mutant alleles showed that for Comorian travelers, only a history of travel in Africa in the past 3 months was identified as significant (odds ratio 2.29, 95% confidence interval 1.27–4.13, $p<0.01$). We used these data to generate a map assessing the potential risk for the spread of *P. falciparum* mutant-type alleles in Madagascar (Figure 2).

Conclusions

Despite some methodologic limitations (limited study period, limited number of passengers screened, taking into account only registered travelers), this study provides the first, to our knowledge, direct measurement of parasite movement between the Comoros Islands and Madagascar. This study thus enables an assessment of the potential threat of *P. falciparum* mutant allele parasites being introduced into Madagascar.

First, we noted that for detection of *P. falciparum* carriers, real-time PCR was 4.6 times more sensitive than microscopy and 30 times more sensitive than rapid diagnostic

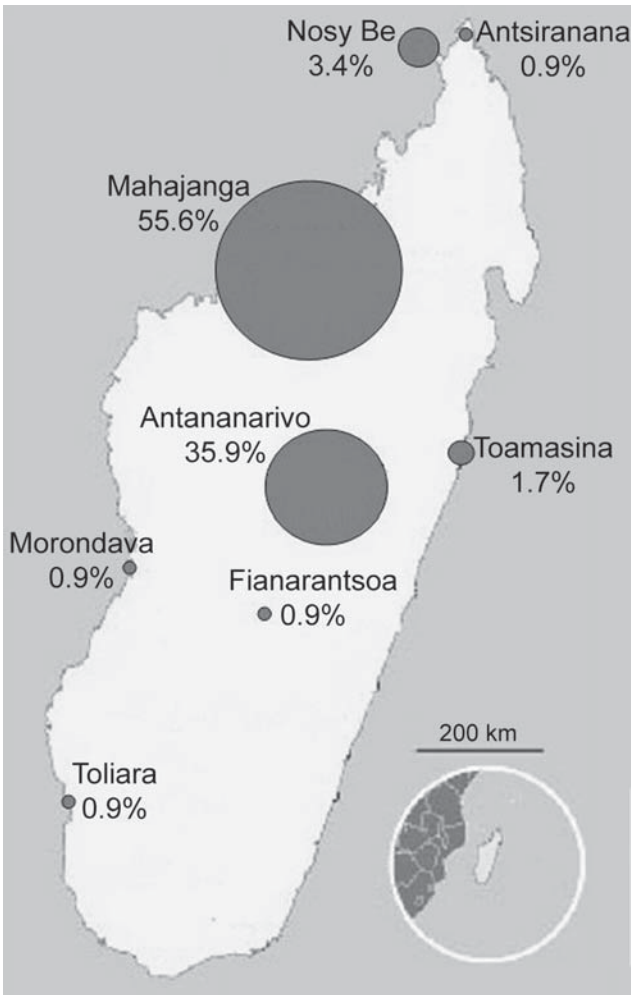

Figure 2. Map assessing the potential risk for spread of *Plasmodium falciparum* mutant-type alleles associated with resistance to chloroquine and pyrimethamine from the Comoros Islands to Madagascar, Mahajanga, Madagascar, 2006.

testing based on parasite lactate dehydrogenase detection, according to the threshold detection level of the techniques used (*11*,*12*). The results suggest that most of the *P. falciparum* carriers had low-level parasitemia. Second, most of the imported parasites carried resistance-associated mutations, consistent with the frequency of mutant forms of *P. falciparum* circulating in the Comoros Islands (*4*,*5*). Third, according to the places in which the Comorian travelers stayed and the places in which the Malagasy travelers lived, the potential area of antimalarial drug–resistant parasite spread was located in the northwestern area, which has a transmission season of >6 months per year (*13*).

On the basis of our findings, we suggest that antimalarial drug policy should be formulated at an international,

Table. Baseline characteristics of enrolled travelers arriving in Mahajanga, Madagascar, from Comoros Islands, 2006*

Characteristic	Comorian travelers, n = 662	Malagasy travelers, n = 285	p value
Place of arrival, n (%)			
Airport	553 (83.5)	148 (51.9)	$<10^{-6}$
Seaport	109 (16.5)	137 (48.1)	
Female	41%	29%	$<10^{-3}$
Age			
Mean age in years (SD)	35.4 (14.7)	35.7 (12.5)	NS
<5 y, %	3.7	1.5	NS
Declared site of residence, %			
Grande Comore	65.2	NA	
Anjouan	31.2	NA	
Mohéli	3.6	NA	
Northwestern Madagascar	NA	64.1	
Central Highlands Madagascar	NA	23.8	
North Madagascar	NA	7.1	
East Madagascar	NA	3.6	
Southwestern Madagascar	NA	1.4	
Northeastern Madagascar	NA	0.4	
Mean duration of stay in days (SD)	63.5 (146.8)	111.8 (300)	NS
Place of stay in Madagascar, %			
Northwestern Madagascar	65.1	NA	
Central Highlands Madagascar	32.0	NA	
North Madagascar	1.9	NA	
East Madagascar	0.5	NA	
Southwestern Madagascar	0.5	NA	
Place of stay in Comoros Islands, %			
Grande Comore	NA	79.3	
Anjouan	NA	20.3	
Mohéli	NA	0.4	
Malaria symptoms at arrival, %†	3.3	3.9	NS
Medical history declared by travellers in the 3 previous months, %			
Suspected malaria	4.8	7.4	NS
Confirmed malaria	1.7	2.5	NS
Treated with antimalarial drugs	4.5	7.4	NS
History of travel in past 3 months			
In Africa, %	3.5%	4.9%	NS
In Asia, %	0.2%	3.9%	NS
No. malaria-positive samples (%)	105 (74.5)	36 (25.5)	
Frequency of mutant alleles,‡ %			
76T	82.0	75.0	NS
86Y	100.0	97.2	NS
108N	96.2	91.7	NS
Triple mutant type, **76T-86Y-108N**	82.2	71.4	NS
Double mutant type 1, 76K-**86Y-108N**	14.4	17.8	NS
Double mutant type 2, **76T-86Y**-108S	2.2	3.6	NS
Single mutant type, 76K-**86Y**-108S	1.1	3.6	NS
Wild type, 76T-86Y-108N	0	3.6	NS

*NS, not significant; NA, not applicable; **boldface** indicates mutant types.
†Fever, headache, diarrhea, shivering, vomiting.
‡Associated with resistance to chloroquine (*pfcr*t and *pfmdr-1*) and pyrimethamine (*dhfr*).

rather than a national, level. Given that the Malagasy government's goal is moving toward malaria elimination, international considerations in antimalarial policy would avoid the possibility of a coherent national policy being annulled by mutations originating in or spreading through neighboring countries. For example, the introduction of high-level chloroquine or pyrimethamine resistance from the Comoros Islands could compromise the use of the artesunate and amodiaquine combination as a first-line treatment for uncomplicated falciparum malaria or the use of sulfadoxine-pyrimethamine for intermittent preventive treatment in pregnant women and render these strategies useless.

In conclusion, we suggest the creation, as soon as possible, of a regional antimalarial forum in the Indian Ocean, similar to the East African Network for Monitoring Antimalarial Treatment (www.eanmat.org). Such a forum would enable the countries of the region to share national information on antimalarial drug efficacy, such as the prevalence of drug resistance molecular markers, and to debate proposed changes in national policy.

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Rocky Mountain Spotted Fever, Panama

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We describe a fatal pediatric case of Rocky Mountain spotted fever in Panama, the first, to our knowledge, since the 1950s. Diagnosis was established by immunohistochemistry, PCR, and isolation of *Rickettsia rickettsii* from postmortem tissues. Molecular typing demonstrated strong relatedness of the isolate to strains of *R. rickettsii* from Central and South America.

Rocky Mountain spotted fever (RMSF) is a febrile exanthematic disease caused by *Rickettsia rickettsii*, a gram-negative, obligately intracellular pathogen. *R. rickettsii* primarily invades the endothelium of small and medium-sized blood vessels of all major tissues and organ systems, causing systemic vasculitis. Typical symptoms include headache, fever, rash, and myalgia; meningoencephalitis and renal failure may also occur. The case-fatality rate of untreated RMSF in different areas is 10%–80%.

RMSF is endemic in much of the Western Hemisphere, including Central and South America. In Panama, 5 cases of RMSF, 2 fatal, were reported in the 1950s from the vicinity of Ollas Arriba, Trans-Istmus Highway, and Panama City (*1*–*4*).

In 1952, *R. rickettsii* was isolated from 2 of the patients who died and from a pool of *Amblyomma cajennense* ticks from the Ollas Arriba area, findings that suggest that this tick is a vector of RMSF in Panama (*2*). In 1961, *R. rickettsii* was isolated from a pool of 20 immature *Amblyomma* ticks collected from an opossum and a lizard from the Canal Zone (*5*). Serosurveillance of 1,400 samples tested by complement fixation with *R. rickettsii* antigen in 1951–1952 detected 5.4%–15.2% seropositivity in 9 provinces of Panama (*6*). We describe here a recent fatal pediatric case of RMSF, the first report, to our knowledge, of this disease in Panama since the 1950s.

The Patient

On December 18, 2004, a 4-year-old female patient from a rural area west of the Panama Canal was admitted to Hospital del Niño, Panama City, Panama, with an 8-day history of intense headache, fever, malaise, myalgia, and arthralgia of the lower extremities and 3 days of generalized petechial rash. Ceftriaxone was administered empirically for suspected meningococcemia. Laboratory tests showed hyponatremia (126 mEq/L), hypoalbuminemia (2 g/dL), thrombocytopenia (48×10^9 cells/L), increased immature neutrophils (26%), and an elevated level of liver enzymes (aspartate aminotransferase 325 U/L and alanine aminotransferase 137 U/L). Results of routine blood cultures were negative. Cerebrospinal fluid analysis showed only protein elevation. Serologic test results for equine encephalitis virus, dengue, hantavirus, and calicivirus as well as bacterial cultures were all negative. On December 18, the patient had seizures, which required intensive care management. Despite intense medical efforts, she died soon thereafter. The main autopsy findings included myocarditis, interstitial nephritis, interstitial pneumonitis, encephalitis, and generalized lymphadenitis. Postmortem frozen unfixed and formalin-fixed tissues were sent to the US Centers for Disease Control and Prevention for etiologic assessment.

Immunohistochemical evaluation of formalin-fixed, paraffin-embedded tissues that used an immunoalkaline phosphatase technique demonstrated spotted fever group rickettsial antigens associated with rickettsia-like cells in vascular endothelium of multiple tissues, including heart (Figure 1), lung, adrenal gland, and kidney. Serum collected on the day of death had immunoglobulin (Ig) G and IgM microimmunofluoresence antibody titers of 2,048 to *R. rickettsii* antigen. DNA samples prepared from frozen brain, liver, lymph node, and spleen autopsy specimens were tested by PCR as described previously (*7*). When amplified, a 208-bp fragment of the conserved 17-kDa *Rickettsia* antigen gene showed spotted fever rickettsial DNA was present in all autopsy tissues. An OmpA gene fragment (70–602 nt) was amplified from brain and lymph node tissues (GenBank accession nos. DQ002503 and DQ002504). DNA sequencing of the *omp*A amplicons demonstrated that their nucleotide sequences were identical to each other and had 100% sequence similarity to the homologous *omp*A fragment of *R. rickettsii* strain Sheila Smith, isolated from a patient in Montana. An isolate of *R. rickettsii* (designated Panama 2004) was obtained from brain tissue in Vero E6 cells; its OmpA gene fragment (DQ164838) had 100% nucleotide sequence similarity to the reference sequence of *R. rickettsii* type strain Sheila Smith. *R. rickettsii* from Panama is similar to the other *R. rickettsii* strains circulating in Central and South America but differs from strain Sheila Smith in at least 1 locus containing tandemly repeated sequences (Figure 2).

The clinical characteristics of this patient, e.g., fever, headache, myalgia, petechial rash, and neurologic signs, initially aroused clinical suspicion for a viral fever caused by Venezuelan equine encephalitis (VEE) virus, particularly because there was a VEE outbreak in this area at that

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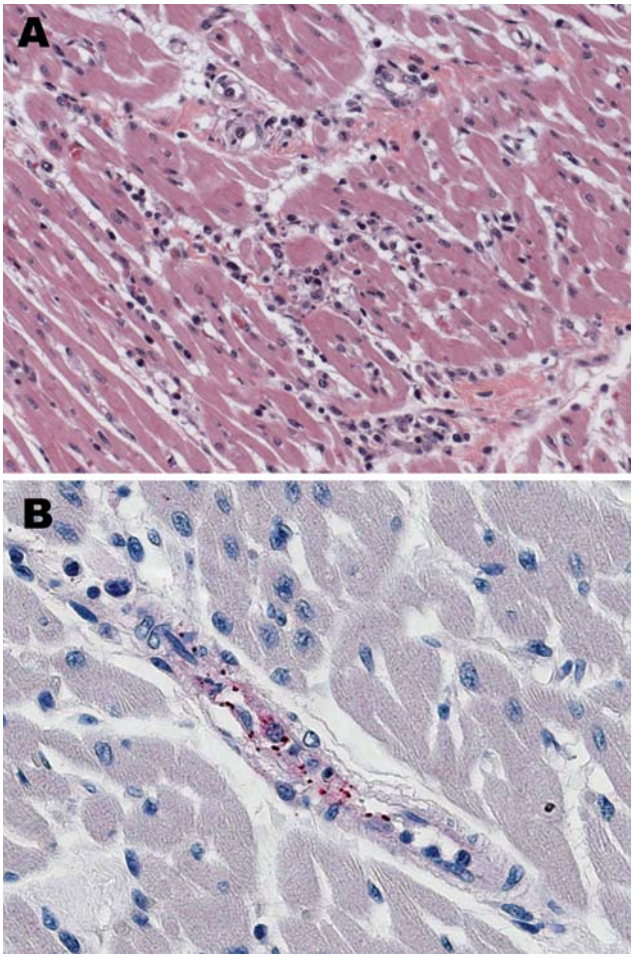

Figure 1. Histologic and immunohistochemical evaluation of heart tissue. A) Lymphohistiocytic inflammatory cell infiltrates in the myocardium (hematoxylin and eosin stain; original magnification ×25). B) Immunohistochemical detection of spotted fever group rickettsiae (red) in perivascular infiltrates of heart (immunoalkaline phosphatase with naphthol-fast red substrate and hematoxylin counterstain; original magnification ×250).

time. The failure to diagnose a spotted fever rickettsiosis was partially the result of diminished awareness of RMSF among local physicians and absence of adequate specific diagnostic tests in Panama. Detection of hyponatremia, hypoalbuminemia, and thrombocytopenia, all observed in this patient, indicates endothelial vascular damage and increased vascular permeability and can aid in making a presumptive diagnosis (*8*). Death secondary to RMSF is associated with delays in diagnosis and delays in initiating appropriate antimicrobial therapy within the first 5 days of the clinical disease (*9*). To prevent fatal outcomes, treatment of suspected case-patients should be initiated before the results of diagnostic tests are received, and treatment should be administered for 7–10 days or until the patient has been without fever for at least 3 days (*9*).

R. rickettsii had been isolated previously in Panama from a few patients during the 1950s and from pools of *A. cajennense* ticks during the 1950s and 1960s (*1*,*2*,*5*). As occurred in this instance, cases of RMSF have likely been missed in Panama during the intervening decades; however, the low frequency of recognized cases of RMSF does not correspond to the relatively high seroprevalence of complement-fixing antibodies to spotted fever group rickettsiae in residents of Panama (*6*). Indeed, inoculation of other pools from *Amblyomma* larvae and *A. cajennense* adults caused seroconversion to spotted fever group antigens in guinea pigs, but *R. rickettsii* was not isolated (*5*). Recent studies of *Amblyomma* ticks in Brazil have detected both *R. bellii* and an agent associated with *A. americanum* in the United States known as "*Rickettsia amblyommii*" (*10*). *R. amblyommii* has been implicated as a cause of a mild, self-limiting rickettsial illness in which seroconversion to *R. rickettsii* antigens occurs (*11*,*12*). *R. parkeri*, found in *A. maculatum* and *A. triste*, has been also identified as a cause of disease in the United States and has been presumptively associated with infections of humans in Uruguay (*13*). Since the characterization of rickettsial agents associated with *Amblyomma* species that bite humans is a newly emergent field in the Americas (*13*–*15*), other typhus group and spotted fever group agents may be found in Panama.

Conclusions

This case confirms that *R. rickettsii* is still present in Panama. A high index of suspicion is necessary for an early diagnosis and empiric treatment of RMSF. Proposed widening of the Panama Canal and current construction of a new portion of the Trans-Istmus Highway disrupts the adjacent forest areas and would likely increase the frequency of human–tick contacts. Thus, RMSF should be considered

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Montana      GAGCATTCAAAAACTACTATAAGGCCTTATGCCTTATGCCTTATTCCT
Brazil       GAGCATTCAAAAACTACTATAAGGCCTTAT-------GCCTTATTCCT
Colombia     GAGCATTCAAAAACTACTATAAGGCCTTAT-------GCCTTATTCCT
Costa Rica   GAGCATTCAAAAACTACTATAAGGCCTTAT-------GCCTTATTCCT
Panama       GAGCATTCAAAAACTACTATAAGGCCTTAT-------GCCTTATTCCT
             ****************************......**********
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Figure 2. Differentiation of *Rickettsia rickettsii* type strain Sheila Smith from Montana from *R. rickettsii* strains from Central and South America. A tandem repeat region corresponding to 563048–563028 nt of the strain Sheila Smith genome and flanking sequences were amplified with AF (5′-GTGATTGCTATATTTCGCTTT-3′) and AR (5′- CTAAGATTTGTTCCGTATAGG-3′) primers as described elsewhere (*7*). Repeat sequence (GCCTTAT, indicated with brackets) present in 3 copies in strain Sheila Smith, whereas only 2 copies were present in *R. rickettsii* isolates from Brazil, Colombia, Costa Rica, and Panama. Homologous sequences of these strains are deposited to GenBank under the following accession nos.: DQ666020, *R. rickettsii* strain Panama 2004; DQ666021, *R. rickettsii* strain Brazil; DQ666022, *R. rickettsii* strain Colombia; DQ666023, and *R. rickettsii* strain Costa Rica.

early in the differential diagnosis of febrile infections in tick-exposed persons in Panama. Further study of endemic rickettsioses, rickettsial agents, and possible tick vectors in Panama is warranted.

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WU Polyomavirus in Children with Acute Lower Respiratory Tract Infections, South Korea

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In South Korea, WU polyomavirus (WUPyV) was detected in 34 (7%) of 486 children with acute lower respiratory tract infections, 3 (4.2%) of 72 asymptomatic children, and as coinfection with other respiratory viruses in 23 (67.6%) children. Although WUPyV was frequently detected, its clinical role has not been distinguished from that of coinfecting viruses.

The polyomaviruses JC virus and BK virus usually produce asymptomatic infections, but in immunocompromised patients, they can become oncogenic or induce disease (*1*–*3*). In 2007, new polyoma viruses such as WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) were identified in respiratory specimens from children with acute respiratory tract infections (*4*,*5*). Gaynor et al. (*4*) reported WUPyV prevalence of 3.0% in Australia and 0.6% in the United States. Allander et al. (*5*) reported 1.0% prevalence of KIPyV in nasopharyngeal aspirates from children with mainly respiratory tract diseases. However, the clinical roles of WUPyV and KIPyV during acute respiratory tract infection needed to be clarified because of the high frequency of their codetection with other respiratory viruses (*4*,*5*). The purpose of our study was to determine prevalence of recently identified WUPyV and KIPyV in children who were asymptomatic and children who had acute lower respiratory tract infection.

The Study

At Sanggyepaik Hospital, Seoul, South Korea, from September 2006 through June 2007, nasopharyngeal aspirates were collected from 558 children <6 years of age: 486 were hospitalized with acute lower respiratory tract infection, and 72 were asymptomatic (those who visited the well-being clinic or were being admitted for elective surgery). Informed consent was obtained from the children's parents, and the study was approved by the internal review board of Sanggyepaik Hospital.

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Viral RNA was extracted from each sample by using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), and reverse transcription of 0.5 μg of each RNA sample was performed. All nasopharyngeal aspirates from the study population were tested for common respiratory viruses such as human respiratory syncytial virus (hRSV), influenza virus A and B, parainfluenza virus (PIV), and adenovirus by using multiplex reverse transcription–PCR (RT-PCR) (*6*,*7*). The rest of each specimen was then frozen at –80°C until tested. RT-PCR assays were performed for rhinovirus (RV), human metapneumovirus (hMPV), human coronavirus (hCoV)–NL63, hCoV-OC43, hCoV-229E, and hCoV HKU-1, as described (*8*–*13*). Positive and negative controls were included in each experiment.

DNA was extracted from the aspirates by using a QIAamp DNA Blood Mini Kit (QIAGEN GmbH). PCR assays were performed to detect human bocavirus (hBoV) by using primers for the nonstructural-1 and nucleoprotein-1 genes, as described (*14*). To detect WUPyV, PCR was performed by using primers AG0044 (5′-TGT TAC AAA TAG CTG CAG GTC AA-3′) and AG0045 (5′-GCT GCA TAA TGG GGA GTA CC-3′); confirmation was performed by using primers AG0048 (5′-TGT TTT TCA AGT ATG TTG CAT CC-3′) and AG0049 (5′-CAC CCA AAA GAC ACT TAA AAG AAA-3′), as described (*4*). KIPyV was detected by nested PCR assays that used primers POLVP1-39F, POLVP1-363R, POLVP1-118F, and POLVP1-324R, as described (*5*). The plasmids containing major capsid protein (VP)–1 region of KIPyV and VP-2 region of WUPyV as positive control were donated by Tobias Allander and David Wang. All PCR products for WUPyV and KIPyV were sequenced to confirm the specificity for each virus. PCR product was examined after electrophoresis on a 1% agarose gel. Amplicon was purified and sequenced in both directions. Nucleotide sequences were aligned by using BioEdit version 7.0 (www.mbio.ncsu.edu/bioedit/bioedit.html) and presented in a topology tree, prepared in MEGA 3.1 (*15*). Using SAS software version 8.02 (SAS Institute, Inc., Cary, NC, USA), we performed the Fisher exact test to compare the proportion of symptomatic WUPyV patients with those who were in the control groups.

Of the 486 children who were hospitalized with acute respiratory tract infections, median age was 9 months (range 1–69 months); of the 72 asymptomatic children, median age was 14 months (range 1–77 months). The age distribution of patients with respiratory tract infections was 220 (45.3%) <12 months, 196 (40.3%) 12–23 months, and 70 (14.4%) 24–69 months. The male:female ratios of the symptomatic children (1.9:1) and asymptomatic children (1.7:1) did not differ significantly. The clinical diagnoses for the 486 symptomatic children were bronchiolitis for 250 children, pneumonia for 201, and croup for 35. Most

children in both groups had no additional underlying medical conditions at the time of admission.

Viruses were detected in 407 (83.7%) of the 486 symptomatic children. The most frequently detected agents were hRSV in 101 (20.8%), RV in 91 (18.7%), hBoV in 51 (10.5%), PIV in 48 (9.8%), and hMPV in 34 (7.0%) (Table). WUPyV was found in 34 (7%) and KIPyV in 5 (1%) children in this group. A single virus infection with WUPyV was confirmed for 11 patients (2.2%) and KIPyV for 1 (0.2%). WUPyV was detected along with other viruses in 23 (67.6%) children.

In the asymptomatic group, KIPyV was not detected, but WUPyV was found in 3 (4.2%) children. Significant difference of prevalence between symptomatic and asymptomatic patients was not noted with WUPyV infection ($p = 0.6$) but was found with hBoV infection ($p = 0.001$). Most of the WUPyV infections were detected in May and June (Figure). The clinical symptoms of WUPyV infection in children were similar to those of other viral respiratory tract infections such as hRSV and hBoV. Gastrointestinal symptoms were found in 23.5% (8/34) of WUPyV-positive children. The 31 WUPyV strains detected in symptomatic children and 3 WUPyV strains in asymptomatic children, which were directly sequenced, clustered into 1 VP2 lineage (GenBank accession nos. EF639268–EF639288, EF655818–655825, and EU041602–041606). Our isolates showed 98%–100% nucleotide identity with the VP-2 region of the WUPyV reference strain (S1). Analysis of the KIPyV strains showed the same sequence with the VP-1 region of the KIPyV strain (GenBank accession nos. EF639289, EF655826–655827, and EU041609–041610).

Table. Detection of viruses among 486 children with acute lower respiratory tract infection, Seoul, South Korea*

Virus	No. positive (%)
Single virus infection	407 (83.7)
HRSV	101 (20.8)
RV	91 (18.7)
HBoV	51 (10.5)
PIV†	48 (9.9)
hMPV	34 (7.0)
WUPyV	34 (7.0)
AdV	22 (4.5)
HCoV‡	15 (3.0)
Influenza virus	6 (1.2)
KIPyV	5 (1.0)
Coinfection with polyomaviruses and other viruses	27 (5.5)
WUPyV + hRSV	6
WuPyV + RV	5
WUPyV + PIV	5
WUPyV + hMPV	2
KIPyV + PIV	2
KIPyV + hCoV-NL63	1
KIPyV + RV	1
WUPyV + RV + PIV	2
WUPyV + hBoV + PIV	1
WUPyV + hRSV + hBoV	1
WUPyV + hCoV-NL63 + PIV	1

*HRSV, human respiratory syncytial virus; RV, rhinovirus; hBoV, human bocavirus; PIV, parainfluenzavirus; hMPV, human metapneumovirus; WUPyV, WU polyomavirus; AdV, adenovirus; HCoV, human coronavirus; KIPyV, KI polyomavirus.
†PIV type 1 in 23 patients, PIV type 2 in 7, PIV type 3 in 10, and PIV type 4 in 8.
‡hCoV-NL63 in 3 patients, hCoV-OC43 in 1, and hCoV-229E in 1.

Conclusions

This prospective study shows that recently identified WUPyVs are prevalent in South Korean children with acute lower respiratory tract infections. Our detection of WUPyV in 34 (7.0%) of 486 children with acute lower respiratory tract infection suggests that the virus is prevalent in South Korea. Our finding of 27 (6.5%) WUPyV-positive samples among 416 patients <24 months of age compared with 7 (10%) positive samples from 70 patients >24 months of age suggests that WUPyV infection may occur more frequently in older children than in younger children; however, studies for latent infection of the virus are needed to confirm. Clinical diagnoses for patients with WUPyV-positive results only included bronchiolitis, tracheobronchitis, pneumonia, and croup. Coinfection of WUPyV with other respiratory viruses, especially hRSV, did not seem to influence the severity of disease, although we did not perform statistical analysis. Although our study tested for more viruses than previous studies and included a control group, whether detection of WUPyV in nasopharyngeal specimens means infection or just transmission in the respiratory tract remains unclear. We performed PCR assays for WUPyV in stool samples from 72 children who had acute gastroenteritis during the same study period, but it was not detected (data not shown).

Our study's limitations include small population size, short study period, lack of testing for bacterial pathogens,

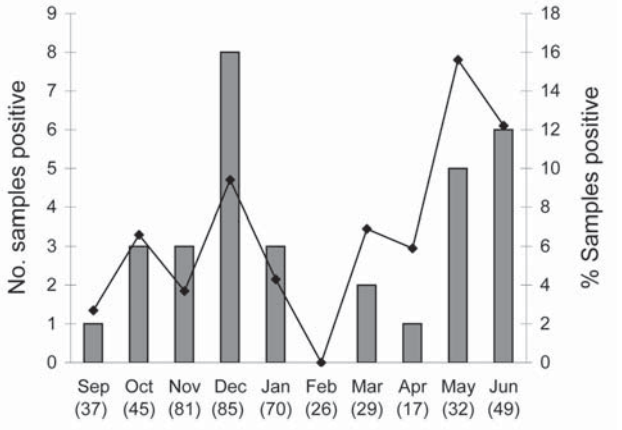

Figure. Seasonal distribution of WU polyomavirus (WUPyV) in children hospitalized with acute lower respiratory tract infection, September 2006–June 2007. Total no. WUPyV-positive samples = 34. Number in parentheses after each month is total number of samples tested.

and limitation to hospitalized patients. Our finding of KPyV in 5 (1.0%) of 486 children who had respiratory symptoms and were mostly coinfected with other respiratory viruses is similar to that of a previous study (*5*). In conclusion, WUPyV was frequently found in South Korean children with acute lower respiratory tract infections, but further studies are needed to distinguish the clinical role of this virus from that of other coinfecting respiratory viruses.

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Viral Load as Predictor of Crimean-Congo Hemorrhagic Fever Outcome

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We used quantitative real-time reverse transcription–PCR to measure viral load in serum from 24 patients in Kosovo who had acute Crimean-Congo hemorrhagic fever. Viral load correlated with clinical disease and antibodies and could be used as a predictor of disease outcome.

Crimean-Congo hemorrhagic fever (CCHF), caused by CCHF virus, is a potentially fatal infection in Africa, Asia, Eastern Europe, and the Middle East. CCHF virus is transmitted to humans by bites of *Ixodid* ticks and from person to person by contact with blood or blood-containing body fluids. Therefore, nosocomial and intrafamiliar cases are frequently reported in CCHF outbreaks (*1*). CCHF can be treated with ribavirin, but the decision about which CCHF patients should be given the drug may be difficult (*2*,*3*). Classification of patients according to criteria of disease severity is an important step in deciding whether to initiate antiviral therapy and is based on clinical data and biochemical test results (*2*,*4*). Because of severe side effects from ribavirin treatment, early laboratory confirmation would be desirable to establish stronger criteria for case classification of CCHF.

Real-time PCR has enabled measurement of viral loads, monitoring of antiviral treatment effects and emergence of antiviral resistant strains, and prediction of disease progression and outcome (*5*). For many viral diseases, including hemorrhagic fevers, viral load measurement has become an integral part of disease management (*6*–*9*). However, to our knowledge, the usefulness of viral load monitoring for CCHF has never been investigated. Given the importance of predicting CCHF disease severity and risk for death, our aim was to measure viral load in CCHF patients and to correlate it with other laboratory parameters and disease outcomes.

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The Study

Serum samples were obtained from CCHF patients from Kosovo in 2001 (www.who.int/csr/don/2001_06_29e/en), 2003, and 2005. Clinical and biochemical data were provided by the Clinic of Infectious Diseases, Pristina, Kosovo. Presence of an acute febrile syndrome characterized by malaise, nausea, fever, and bleeding from various sites was reported as well as possible modes of infection. Leukocyte and platelet counts, aspartate and alanine aminotransferase levels, activated partial thromboplastin times, and creatinine values were available for most patients. Patients were categorized into 3 groups according to disease severity: fatal, severe, or moderate cases. On the basis of classification by Swanepoel (*4*), severe cases were defined by the presence of hemorrhagic manifestations (epistaxis, hematemesis, and melena), lowered blood pressure (<100/60 mm Hg), and raised serum creatinine and transaminase levels.

Serologic testing for anti-CCHF virus immunoglobulin (Ig) M and IgG was done by ELISA. Molecular data were obtained by real-time reverse transcription–PCR (RT-PCR) with a limit of detection of 240 copies/mL of sample as recently described (*10*). Some assay modifications were necessary for accurate quantitation of viral load. Synthetic RNA was generated as a quantitative calibrator, and a competitive internal control was constructed as previously described to detect possible influences of PCR inhibitors (*11*). The original real-time RT-PCR protocol was complemented by the addition of 200 pmol/μL of internal control probe YFP2 (5′-ROX-ATCGTTCGTTGAGCGATTAG-CAG-BBQ-3′). This probe recognizes an alternative binding site introduced in the target gene by overlap-extension PCR (*11*). The standard curve for CCHF virus quantitation was based on synthetic calibrator RNA with concentrations from 24×10^5 to 24×10^1 copies/mL. Statistical analysis was performed with statistical software R version 2.2.1 (www.r-project.org) and the Statgraphics 5 package (Manugistics, Dresden, Germany).

A total of 24 patients had clinical, serologic, and molecular confirmation of CCHF (Table). All 24 patients had an acute febrile syndrome, 8 reported tick bites, and 2 had been exposed in a hospital. For the 9 patients who died, only 1 serum sample was available from each. For the 9 patients with severe disease and the 6 with moderate disease, 2–3 consecutive samples were available.

From the 24 patients, 43 serum samples were tested by real-time RT-PCR and ELISA (Table). Viral loads ranged from 10^2 to 10^{10} copies per milliliter of serum, depending on the day of illness, the severity of disease, and the results of serologic analyses (Table). Whether early laboratory findings could serve as prognostic markers for outcome was explored. Because prognostic information is most relevant in the first week of disease, only samples taken up to

day 7 of symptoms from any patient were included in the analysis. Average sampling days did not differ between patients who died and those who survived (5.0 and 3.9 days, respectively, p = 0.28, analysis of variance [ANOVA]). No patient from either group had detectable IgG in the first week. Although we suspected that early development of IgM might correlate with good outcome, such correlation was not found. Of those patients in whom IgM was detected up to day 7, 5 (62%) died. Of those without IgM in the first week, 3 (42%) died (insignificant difference in 1-way ANOVA, p = 0.6). Furthermore, the presence of IgM in the first week did not correlate with viral load, which suggests that virus levels in the first week can be regarded as an independent prognostic parameter. Namely, viral load seemed to be strongly related to the clinical classification ($p \leq 0.001$), with the average log value 9.25 (1.78×10^9) in the group of patients who died and 6.91 (8.06×10^6) in the group who survived (Figure).

To determine whether IgG could influence viremia, viral loads were correlated with log-transformed reciprocal antibody titers. Quantitative IgG levels showed a highly significant inverse correlation with viral loads ($p<0.0001$) (Figure). It was thus reasoned that IgG levels could influence the later course of disease and, in particular, could re-

Table. Detection of CCHF viral load by real-time RT-PCR in serum of patients with acute CCHF, Kosovo*

Patient no.	Disease severity	Day of illness	IgM titer	IgG titer	Viral load (copies/mL)
1	Fatal	2	Neg	Neg	6.5100×10^8
2	Fatal	3	Neg	Neg	2.5040×10^9
3	Fatal	4	Neg	Neg	2.7400×10^9
4	Fatal	9	Neg	Neg	3.3840×10^9
5	Fatal	4	1,600	Neg	1.0160×10^8
6	Fatal	6	3,200	Neg	1.3450×10^{10}
7	Fatal	7	400	Neg	1.8675×10^9
8	Fatal	7	800	Neg	3.4800×10^9
9	Fatal	7	800	Neg	1.2920×10^9
10	Severe	8	>6,400	Neg	1.6050×10^6
		18	>6,400	400	1.1500×10^5
11	Severe	9	Neg	Neg	2.3250×10^7
		24	>6,400	>6,400	Neg
12	Severe	4	800	Neg	3.3900×10^6
		16	6,400	3,200	Neg
13	Severe	2	Neg	Neg	1.0430×10^9
		9	>6,400	400	3.1200×10^3
		42	>6,400	3,200	Neg
14	Severe	8	Neg	Neg	8.1000×10^6
		14	6,400	Neg	2.0100×10^3
15	Severe	3	Neg	Neg	3.8100×10^7
		14	6,400	200	Neg
16	Severe	3	>1,600	Neg	2.5235×10^6
		6	>800	>800	2.2350×10^4
17	Severe	4	Neg	Neg	3.3600×10^7
		10	>6,400	100	3.8100×10^4
18	Severe	2	Neg	Neg	7.8500×10^7
		9	6,400	400	3.2000×10^3
		23	>6,400	>6,400	Neg
19	Moderate	12	>6,400	Neg	5.7525×10^4
		17	>6,400	800	Neg
20	Moderate	9	>6,400	1,600	1.9600×10^3
		11	>6,400	3,200	Neg
21	Moderate	12	>6,400	800	1.9191×10^5
		13	>6,400	3,200	1.0240×10^4
		32	>6,400	>6,400	Neg
22	Moderate	10	>6,400	Neg	4.6400×10^4
		19	>6,400	>6,400	1.2000×10^3
		26	>6,400	>6,400	Neg
23	Moderate	9	6,400	6,400	7.6800×10^3
		20	>6,400	>6,400	7.5000×10^2
24	Moderate	7	400	Neg	7.4400×10^5
		18	>6,400	6,400	Neg

*CCHF, Crimean-Congo hemorrhagic fever: RT-PCR, reverse transcription–PCR; Ig, immunoglobulin; neg, negative result.

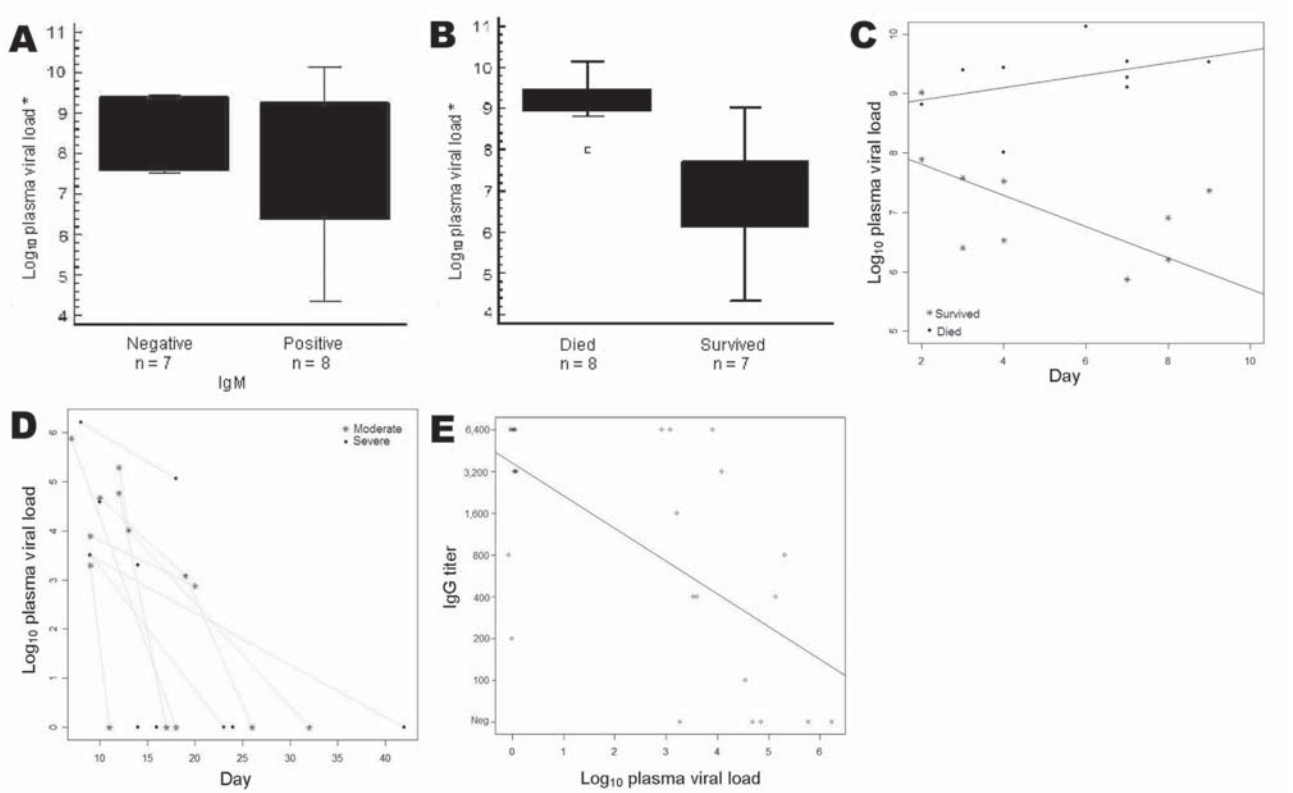

Figure. Correlation between clinical outcome, serologic data, and Crimean-Congo hemorrhagic fever (CCHF) viral load measurements. A) Viral load versus immunoglobulin (Ig) M result taken during the first week of illness. B) Viral load versus outcome. Average viral loads were 1.6×10^9 copies/mL in persons who died and 5×10^6 copies/mL in persons who survived (difference highly significant, $p<0.0001$). The dot is a datum point that has been identified as an outlier. C) Statistically significant difference ($p<0.001$) in CCHF viral load and day of illness between group who died and group who survived. D) No correlation in viral load and day of illness between severe and moderate CCHF cases. E) Inverse correlation of quantitative IgG levels with viral loads ($p<0.0001$) in samples taken after first week of illness. Black dot, >1 sample; *, first week samples.

flect a discrimination between severe and moderate cases. In samples from both categories, no relationship between IgG and clinical classification ($p = 0.65$) was determined after day 7. Also, no significant relationship was found between clinical classification and viral load ($p = 0.74$) in severe versus moderate cases. On average, viral load log value was 2.38 in severe cases and 2.69 in moderate cases (Figure).

Conclusions

This study describes the differential influences of CCHF viral load, IgM, IgG, and clinical outcome. CCHF viral load, but not IgM, could be used as a predictor of CCHF outcome. It was unexpected that IgM correlated with neither outcome nor viral load. On the contrary, quantitative IgG levels inversely correlated with viral loads, which suggests that IgG might neutralize virus in vivo. The fact that virus titers decreased in survivors independent of antibodies during the first week implies involvement of innate or cellular immune mechanisms in the elimination of CCHF virus.

CCHF viral load ranged from 10^2 to 10^{10} copies/mL in the serum samples. It was shown that viral load of $\geq 10^8$ copies/mL is a strong factor ($p \leq 0.001$) for differentiating CCHF patients who died from those who survived. However, viral load does not help differentiate between severe and moderate cases according to common case definitions (*4*). The same was true for IgM levels. Viral load is also useful for estimating need for infection control measures. Viral loads measured in our patients were high, $>10^9$ copies/mL, higher than viral loads in other arboviral diseases that are not easily transmitted in the hospital, e.g., dengue (*12*). This finding could help explain why CCHF virus causes nosocomial infections on a regular basis. Another use for this finding is systematic monitoring of patients receiving ribavirin therapy. In the absence of sufficiently large numbers of treated patients, however, we could not investigate this application.

Acknowledgments

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Characterization of Hemorrhagic Fever with Renal Syndrome Caused by Hantaviruses, Estonia

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Thirty cases of hemorrhagic fever with renal syndrome (HFRS) due to Puumala virus (PUUV), Saaremaa virus (SAAV), and Dobrava virus infection were confirmed in Estonia. Except for the levels of serum creatinine, no remarkable differences were found in the clinical course of HFRS caused by PUUV and SAAV.

Hemorrhagic fever with renal syndrome (HFRS) is an endemic zoonosis in Eurasia. In Europe, HFRS is caused by 3 hantaviruses: Puumala virus (PUUV), carried by the bank vole (*Myodes glareolus*); Dobrava virus (DOBV), carried by the yellow-necked mouse (*Apodemus flavicollis*); and Saaremaa virus (SAAV), carried by the striped field mouse (*A. agrarius*) (*1*).

In Estonia, PUUV and SAAV were found in wild rodents. High prevalence of human antibodies has been detected against SAAV (23%) on Saaremaa Island and against PUUV (18%) in central Estonia (*2*,*3*). In contrast to Slovenia, Croatia, and Bosnia-Herzegovina, where a milder HFRS caused by PUUV and a more severe form caused by DOBV have been reported (*4*,*5*), no severe forms of HFRS have been observed in Estonia thus far. Our first report of HFRS in Estonia included cases caused by PUUV and SAAV (*6*). (At that time, SAAV was mistakenly designated DOBV; therefore, DOBV and SAAV infections could not be distinguished.)

No fatal cases have been reported on Saaremaa Island, where 23% of the population has antibodies against SAAV; this finding strongly suggests that SAAV causes a milder form of HFRS. To validate this hypothesis, we analyzed clinical data and laboratory findings in Estonian HFRS patients infected by different hantaviruses.

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The Study

During 1999–2004, serum samples from 321 patients with suspected HFRS were analyzed by using ELISA. Of these, 30 were confirmed for hantavirus infection by the Department of Virology, National Institute for Health Development (Tallinn). Blood samples were collected from patients during the acute (at admission to a hospital) and convalescent phases (Table 1). The acute-phase serum samples were tested by immunoglobulin (Ig) M and IgG ELISA as described (*7*,*8*). Convalescent-phase serum samples were collected from 25 patients, and the causative hantavirus was typed by focus reduction neutralization test (FRNT) as described earlier (*7*). We found that 21 patients were infected by PUUV, 3 by SAAV, and 1 by DOBV (at least a 4-fold higher endpoint titer).

Complete clinical documentation was available for 25 patients, and their case records were reviewed. One patient with PUUV infection, a 1-year-old child, was excluded from our study.

The most common clinical symptoms were acute onset of disease, fever, and back pain (Table 2). Impaired renal function was also noted in almost all patients. Proteinuria and microscopic hematuria were reported for all PUUV- and SAAV-infected patients, but hematuria was not found in the DOBV-infected patient. Oliguria (<400 mL/24 h) was noted for 55% of the PUUV patients and all SAAV patients; subsequent polyuria (>2,500 mL/24 h) was noted for 45% of the PUUV-infected patients and in 67% of the SAAV patients. Mild oliguria (380 mL/24 h) and polyuria (5,000 mL/24 h) also developed in the patient with DOBV infection. One patient with PUUV infection and 1 with SAAV infection required hemodialysis because of symptoms of uremia. An increased level of serum creatinine was found in all patients with PUUV and SAAV infections. The mean level of serum creatinine was significantly higher in the SAAV patients ($p = 0.043$).

Other common abnormalities in laboratory findings were the elevated levels of C-reactive protein, serum urea, and transaminases. We observed normal mean levels of platelets; however, thrombocytopenia was found in 57.1% of the PUUV-infected patients and only in 33% of the SAAV-infected patients.

In summary, no remarkable differences in the clinical course of HFRS caused by PUUV and SAAV were found. The comparison between the 3 groups was difficult because of the small number of patients infected by SAAV and DOBV. The only patient with DOBV infection demonstrated a mild clinical course of disease.

Table 1. Hantavirus-specific antibodies in 30 HFRS patients in Estonia as determined by ELISA and FRNT*

Patient no.	ELISA			FRNT of convalescent-phase serum				
	Time after onset, d	PUUV IgM/IgG	SAAV IgM/IgG	Time after onset	PUUV	SAAV	DOBV	SEOV
681	13	**1.095/0.556**†	0.075/0	5 mo	**640**‡	40	<40	<40
715	12	**1.147/0.258**	0.052/0.045	21 d	**640**	<40	<40	<40
716	17	**1.072/0.383**	0.028/0	1 mo	**2,560**	<40	<40	<40
718	7	**1.079/0.142**	0/0.019	1 mo	**2,560**	<40	<40	<40
728	24	**0.602/0.727**	0.039/**0.145**	9 mo	**640**	<40	<40	<40
731	8	**1.43/0.309**	0/**0.102**	24 d	**2,560**	<40	<40	<40
761	9	**1.048/0.213**	0.034/**0.138**	ND	ND	ND	ND	ND
763	9	**1.309/0.631**	0.031/0.088	ND	ND	ND	ND	ND
766	15	**1.340/0.586**	0.036/0.043	7 mo	**640**	<40	<40	<40
767	16	**1.840/0.627**	0.040/0.034	12 mo	**640**	<40	<40	<40
770	13	**0.995/0.127**	**0.231**/0.031	5 mo	**160**	<40	<40	<40
772	6	**0.331**/0.044	**0.137**/0.021	11 mo	**160**	<40	<40	<40
774	4	**1.361/0.863**	**0.103**/0.088	11 mo	**160**	<40	40	<40
800	8	**1.151/0.152**	0/0.099	ND	ND	ND	ND	ND
812	9	**1.144/0.244**	0.026/0.046	9 mo	**640**	<40	<40	<40
813	7	**1.058**/0.089	0.082/0.06	2 mo	**640**	<40	<40	<40
840	8	**0.724**/0.038	0.083/0.055	9 mo	**640**	<40	<40	<40
841	8	**0.704/0.142**	0.073/0.013	20 d	**2,560**	160	160	<40
855	24	**0.791/0.152**	**0.632**/0.090	1 mo	**640**	40	40	<40
857	5	**1.133**/0.043	**1.053**/0.067	ND	ND	ND	ND	ND
861	23	**1.051/0.184**	**0.559**/0.035	1.5 mo	**640**	<40	<40	<40
895	5	**0.957/0.135**	ND/0.011	1 mo	**2,560**	<40	<40	<40
899	13	**0.797/0.120**	ND/0.005	1.5 mo	**160**	<40	<40	<40
927	11	**0.833/0.279**	ND/0.004	1.5 mo	**640**	<40	40	<40
933	5	**0.569/0.464**	ND/**0.116**	18 mo	**160**	<40	40	<40
691	6	**0.355**/0.047	**1.014**/0.081	4 mo	<40	**160**	40	<40
769	10	**0.271**/0	**0.218/0.254**	20 d	<40	**2,560**	640	<40
801	14	0/0.038	**0.394/1.548**	1.5 mo	<40	**640**	160	<40
849	4	0.011/0	**0.599/0.133**	ND	ND	ND	ND	ND
795	12	**0.144**/0	**0.442/362**	11 mo	<40	160	**640**	<40

*HFRS, hemorrhagic fever with renal syndrome; FRNT, focus reduction neutralization test; PUUV, Puumala virus; SAAV, Saaremaa virus; Ig, immunoglobulin; DOBV, Dobrava virus; SEOV, Seoul virus; ND, not done. Numbers in **boldface** indicated positive results in ELISA and at least 4-fold higher titer in FRNT. The cut-off value for ELISA was set at OD = 0.100.
†Mean optical density (OD) for duplicate samples.
‡Reciprocal endpoint titer at an 80% reduction.

Conclusions

Because at least 2 hantaviruses, PUUV and SAAV, circulate in Estonia, our main aim was to describe the clinical courses of HFRS caused by different hantaviruses. We found that HFRS cases caused by PUUV, SAAV, and DOBV all occurred in Estonia. Approximately 75% of the Estonian patients were infected with PUUV, a finding that agrees with the more frequent detection of hantavirus antigen in *M. glareolus* in mainland Estonia. DOBV antibodies have been detected in HFRS patients (the present study) and in blood donors (*9*), but the virus has thus far not been detected in rodents captured in Estonia.

Hallmarks of HFRS, such as blurred vision and hemorrhages, were not frequent among the patients in our study. Although acute transient myopia was observed in 25% of PUUV-infected patients, the differences between the groups were not statistically significant. In Fennoscandia, where only PUUV infections have been reported, visual dysfunction has been reported for 31%–36% of the patients (*10*,*11*). In Slovenia, 83% of PUUV-infected patients and 42% of DOBV-infected patients reported blurred vision (*4*). The reason for different prevalence rates of this symptom between countries is unclear, but the type of causative hantavirus or lack of a diagnosis of visual disturbance might play a role. Hemorrhagic manifestations were found in 3 patients (2 PUUV and 1 SAAV), but no statistically significant differences were found between the groups. The patient with a DOBV infection did not show

Table 2. Frequency of clinical symptoms and laboratory findings in 24 HFRS patients, Estonia*

Findings	No. patients (%)		
	PUUV, n = 20 (%)	SAAV, n = 3 (%)	DOBV, n = 1
Signs and symptoms			
Fever	20 (100)	3 (100)	1
Headache	8 (40)	3 (100)	0
Abdominal pain	11 (55)	3 (100)	1
Back pain	16 (80)	2 (67)	1
Nausea/vomiting	11 (55)	2 (67)	1
Hemorrhage	2 (10)	1 (33)	0
Visual impairment	5 (25)	0	0
Diarrhea	3 (15)	2 (67)	1
Oliguria (<400 mL/d)	11 (55)	3 (100)	1
Polyuria (>2,500 mL/d)	9 (45)	2 (67)	1
Hematuria (microscopic)	20 (100)	3 (100)	0
Hemodialysis	1 (5)	1 (33)	0
Laboratory findings	Mean value (% patients with abnormal findings), range		
Leukocyte count, 4–10 × 10^9/L	11 (58), 5.7–24.4	9 (33), 8.1–11.3	5
Thrombocyte count, 100–400 × 10^9/L	120 (57), 12.6–350	126 (33), 90–163	174
C-reactive protein <10 mg/L	84 (94), 9.7–229.8	69 (100), 52–95	168
Serum urea, 2.8–8.3 mmol/L	23 (94), 5.0–87.0	31 (100), 20.1–42.5	ND
Serum creatinine, 35–115 μmol/L	303 (100), 120–1,124	606 (100)†, 489–796	98
AST <26 U/L	74 (100), 28–205	56 (100), 39–77	122
ALT <35 U/L	62 (88), 20–233	71 (100), 41–123	ND
Proteinuria (g/L)	3 (95), 0.25–11.8	7 (100), 1.06–17.8	1

*HFRS, hemorrhagic fever with renal syndrome; PUUV, Puumala virus; SAAV, Saaremaa virus; DOBV, Dobrava virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
†Statistically significant result, p<0.05.

any signs of hemorrhages. Normal levels of mean platelet counts were found in all groups of our HFRS patients, although in 57% and 33% of PUUV- and SAAV-infected patients, respectively, a mild thrombocytopenia was found. Slovenian DOBV-infected patients had significantly lower platelet counts than PUUV-infected patients (*4*). Thrombocytopenia was also observed for 75% and 52% of patients in Finland and Sweden, respectively (*10*,*11*).

Gastrointestinal symptoms are common in hantavirus infections. We did not, however, find significant differences between the PUUV- and SAAV-infected patients, although diarrhea and abdominal pains were more common in SAAV- than PUUV-infected patients. In our study, transient impairment of renal function was noted in all patients, together with elevated levels of serum urea and creatinine, findings in line with those from previous studies in other countries. Our study demonstrated that HFRS symptoms were not generally distinguishable between the PUUV- and SAAV-infected patient groups. Only the level of serum creatinine was significantly higher in the SAAV-infected patients.

Two forms of HFRS have been reported from the Balkans, a mild form with mortality rates up to 0.1% caused by PUUV, and a severe form with mortality rates >10% caused by DOBV. On the other hand, in Estonia and other countries such as Germany, Denmark, Slovakia, Latvia, and Lithuania, where SAAV (or DOBV-Aa, as SAAV is occasionally designated) circulates, no fatal cases have been reported.

The symptoms caused by different hantaviruses range from subclinical or mild to very severe. Knowledge concerning all the parameters involved in the disease severity is still lacking. Besides the genotype of the causative hantavirus, human HLA-B8, -DR3, and -DQ2 alleles may be associated with more or less serious symptoms in HFRS (*12*). Our study included only hospitalized HFRS patients. More attention should be paid to outpatients referred to family doctors, infectious disease specialists, and nephrologists.

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Human Multidrug-Resistant *Salmonella* Newport Infections, Wisconsin, 2003–2005

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We conducted a retrospective study of *Salmonella* Newport infections among Wisconsin residents during 2003–2005. Multidrug resistance prevalence was substantially greater in Wisconsin than elsewhere in the United States. Persons with multidrug-resistant infections were more likely than persons with susceptible infections to report exposure to cattle, farms, and unpasteurized milk.

During the past decade, multidrug-resistant (MDR) *Salmonella enterica* serotype Newport strains in the United States have increased substantially (*1*). The prevalence of the most common MDR *S.* Newport phenotype, Newport-MDRAmpC, increased from 1% of human *S.* Newport isolates tested in 1998 to 21% of isolates tested in 2003 (*2*). Newport-MDRAmpC is resistant to at least chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, and cephalothin. This phenotype exhibits decreased susceptibility to ceftriaxone (*2*), a critically important antimicrobial agent for treating invasive salmonellosis in children (*3*).

Studies suggest that dairy cattle are a major US reservoir for MDR *S.* Newport (*4*–*6*). However, data documenting the prevalence of MDR *S.* Newport among infected human case-patients in dairy-intensive states are limited. To assess the prevalence of resistance among *S.* Newport isolates in Wisconsin, which in 2002 had the greatest density of milk cows in the United States (*7*), we evaluated antimicrobial susceptibility data from *S.* Newport infections among Wisconsin case-patients during 2003–2005. We also compared information on potential exposures for case-patients with Newport-MDRAmpC and susceptible infections.

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The Study

Surveillance data were electronically compiled for laboratory-confirmed *S.* Newport infections among Wisconsin residents with illness onsets from January 1, 2003, through December 31, 2005. Providers and local health departments reported hospitalization status; travel history; and exposure to raw milk, cattle, horses, reptiles, and dead animals. The study population included case-patients whose isolates were tested for antimicrobial drug susceptibility at the Wisconsin State Laboratory of Hygiene. Identification and susceptibility testing were conducted on isolates from stool, urine, and blood samples.

Serotype identification was performed according to the Kauffmann-White scheme (*8*). Slide and tube agglutination were used for identification of O (somatic) and H (flagellar) antigens, respectively. All isolates were tested for susceptibility to ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole, by using the Kirby-Bauer disk diffusion method. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (*9*). Antimicrobial agents were categorized into CLSI antimicrobial subclasses, and each isolate was assigned to ≥1 categories according to its antimicrobial resistance phenotype and the number of subclasses to which it was resistant (National Antimicrobial Resistance Monitoring System for Enteric Bacteria [NARMS], pers. comm.; Table 1). Pansusceptible isolates were defined as isolates that had no detected antimicrobial drug resistance. Because isolates were not tested for ceftiofur resistance, our definition of Newport-MDRAmpC did not include resistance to this drug.

The prevalence of each type of resistance among *S.* Newport isolates from Wisconsin case-patients was compared with that reported elsewhere in the United States, by using 2003 and preliminary 2004 NARMS data. Data were analyzed by using Epi Info 2002, version 3.3.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA); to assess associations between antimicrobial resistance and reported exposures, odds ratios and Mantel-Haenszel and Fisher exact 2-tailed p values were calculated where appropriate.

Serotyping and antimicrobial drug susceptibility testing were conducted on *S.* Newport isolates from 268 case-patients. Median age was 34 years (range <1–96 years); of 267 case-patients for whom sex was reported, 57% were female. Resistance patterns are provided in Table 1. Among the 5 (2%) quinolone-resistant isolates (2 resistant to nalidixic acid and ciprofloxacin, 2 resistant to nalidixic

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Table 1. Antimicrobial drug resistance patterns of human *Salmonella* Newport isolates among case-patients*

Resistant to	Frequency (%)	
	Wisconsin (n = 268), 2003–2005	Rest of United States (n = 402), 2003–2004
None detected	95 (35)	317 (79)
≥1 CLSI subclass†	173 (65)	85 (21)
≥2 CLSI subclasses	150 (56)	81 (20)
≥3 CLSI subclasses	150 (56)	77 (19)
≥4 CLSI subclasses	150 (56)	74 (18)
≥5 CLSI subclasses	146 (55)	71 (18)
At least ACSSuT‡	139 (52)	69 (17)
At least ACSuTm§	7 (3)	4 (1)
At least MDRAmpC¶	137 (51)	68 (17)
Quinolone and cephalosporin (third generation)#	5 (2)**	2 (0.5)

*Based on data from the National Antimicrobial Resistance Monitoring System for Enteric Bacteria.
†CLSI, Clinical and Laboratory Standards Institute. Subclasses included aminoglycosides (kanamycin, gentamicin, streptomycin), aminopenicillins (ampicillin), β-lactamase inhibitor combinations (amoxicillin-clavulanic acid), first-generation cephalosporins (cephalothin), third-generation cephalosporins (ceftriaxone), cephamycins (cefoxitin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), phenicols (chloramphenicol), quinolones (nalidixic acid, ciprofloxacin), sulfonamides (sulfisoxazole), and tetracyclines (tetracycline).
‡ACSSuT, ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole, tetracycline.
§ACSuTm, ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole.
¶At least drugs to which MDRAmpC is resistant: chloramphenicol, streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, ampicillin, cefoxitin, cephalothin, and ceftriaxone. Note: the Wisconsin State Laboratory of Hygiene does not routinely test *Salmonella* isolates for resistance to ceftiofur, a third-generation cephalosporin that is related to ceftriaxone.
#Resistant to ciprofloxacin and/or nalidixic acid, and ceftriaxone.
**1 isolate in this category was also MDRAmpC.

acid only, and 1 resistant to ciprofloxacin only), 4 were ceftriaxone resistant and 1 was MDRAmpC resistant. The frequencies of antimicrobial drug resistance among Wisconsin *S.* Newport isolates were substantially greater for all resistance subgroups than frequencies reported elsewhere in the United States during 2003 and 2004 (NARMS, pers. comm.; Table 1; Figure).

Of 194 case-patients for whom hospitalization status was reported, 46 (24%) had been hospitalized. Of case-patients with Newport-MDRAmpC and pansusceptible infections, similar proportions were hospitalized (26% and 24%, respectively) and had known hospitalization status (72% and 73%, respectively). Two case-patients died: an 84-year-old woman and a 37-year-old man for whom salmonellosis was not considered the probable cause of death. The 2 associated isolates were pansusceptible.

Persons infected with Newport-MDRAmpC were significantly more likely than persons infected with pansusceptible *S.* Newport to be male and to have had contact with cattle, to have drunk unpasteurized milk, and to live on or have visited a farm or petting zoo (Table 2). Reported exposure to reptiles was significantly associated with pansusceptible infection (Table 2). No association was found between hospitalization and resistance (odds ratio [OR] 1.09, p = 0.81).

Conclusions

We describe a substantially greater prevalence of MDRAmpC resistance among Wisconsin case-patients with *S.* Newport infections that occurred during 2003–2005, compared with data reported elsewhere in the United States (NARMS, personal communication, 2007). This finding is of particular concern because Newport-MDRAmpC exhibits decreased susceptibility to ceftriaxone, a third-generation cephalosporin that is the treatment of choice for invasive salmonellosis in children (*3*). Additionally, because the bla_{CMY-2} gene that confers ceftriaxone resistance in Newport-MDRAmpC is located on a plasmid that was readily transferred between *Escherichia coli* in laboratory assays (*10*), propagation of Newport-MDRAmpC could increase the spread of CMY-2 plasmids to other bacteria.

Patients with Newport-MDRAmpC infection were more likely than patients with pansusceptible infections to

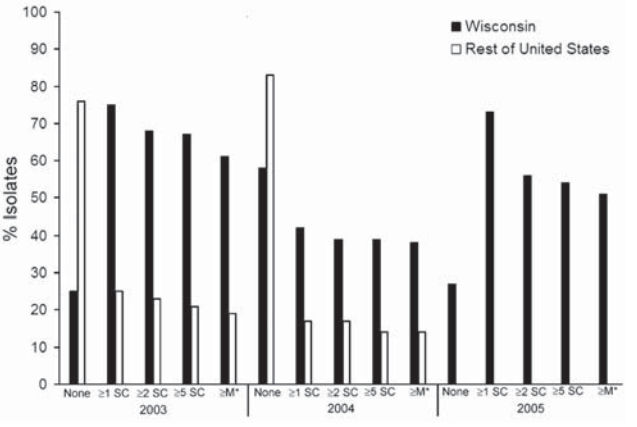

Figure. Antimicrobial drug resistance patterns of human *Salmonella* Newport isolates from Wisconsin (2003–2005) and elsewhere in the United States (2003–2004), based on data provided by the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS). 2005 NARMS data were not available at the time of publication of this report. Antimicrobial subclasses are as defined by the Clinical and Laboratory Standards Institute (*9*). SC, subclass; M*, MDRAmpC.

Table 2. Association between reported demographic and exposure variables and *Salmonella* Newport-MDRAmpC infections in Wisconsin case-patients, 2003–2005*

Variable†	Infection, n (%)		Odds ratio	p value
	MDRAmpC‡ (n = 137)	Pansusceptible (n = 95)		
Male	71(52)	30 (32)	2.33	0.002§
Contact with cattle	20 (15)	0	UD	0.0001§
Farm residence or farm or petting zoo visit¶	14 (10)	0	UD	0.001§
Consumption of raw milk	10 (7)	0	UD	0.006#
Contact with horses	2 (2)	0	UD	0.514#
Foreign travel	0 (0)	1 (1)	0	0.409#
Contact with dead animal	1 (7)	0	UD	1.000#
Contact with pet reptile	0 (0)	7 (7)	0	0.002#

**Salmonella* Newport–multidrug-resistant AmpC (MDRAmpC) is resistant to at least chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, and cephalothin and shows decreased susceptibility to ceftriaxone. Table includes case-patients with Newport-MDRAmpC and pansusceptible infections only. UD, undefined.
†A specific exposure period was not assessed except for travel. Other exposures reported for case-patients included eating raw ground beef (1 MDRAmpC), eating raw cookie dough (1 MDRAmpC and 1 pansusceptible), preparing a raw chicken pet diet (1 pansusceptible), contact with an ill family member (1 MDRAmpC and 1 pansusceptible), and attending a pig roast (6 MDRAmpC and 1 pansusceptible).
‡At least MDRAmpC resistant.
§Mantel-Haenszel χ^2.
¶Exposure to farms and petting zoos was not explicitly assessed by the case reporting form. In all, 14 case-patients reported this exposure; all associated isolates were Newport-MDRAmpC.
#Fisher exact test.

report contact with cattle, farms, and unpasteurized milk. These exposures are likely to be more common among patients with Newport-MDRAmpC infection than among the general Wisconsin population, which suggests that dairy cattle are an important reservoir for Newport-MDRAmpC. Increased prevalence of Newport-MDRAmpC in Wisconsin may be due to selective pressure from the use of antimicrobial drugs on dairy farms (*1*), particularly ceftiofur, an extended-generation cephalosporin closely related to ceftriaxone (which is commonly used in cattle) (*11*). Clonally and independently acquired CMY-2–associated ceftiofur resistance has been identified among *Salmonella* strains isolated from dairy farms (*12*).

Few published data are available on the prevalence of Newport-MDRAmpC in other dairy-intensive states. Minnesota, which in 2002 had the eighth-greatest density of milk cows in the United States (*7*), reported a significant increase in MDR *S.* Newport among human isolates during 1996–2003, including an increase in the percentage of isolates with decreased susceptibility to ceftriaxone (*13*). However, NARMS reported a similar increase in Newport-MDRAmpC prevalence nationally during 1998–2003 (*2*). Analyses of unpublished data provided by the Minnesota Department of Health indicated that 22% of 147 human isolates tested had antimicrobial drug resistance profiles consistent with the Newport-MDRAmpC phenotype during 2003–2005; this prevalence is much lower than that among Wisconsin case-patients who were ill during the same period. Although differences in enteric disease surveillance could partially explain this discrepancy, Newport-MDRAmpC's emergence in dairy cattle is likely to be associated with several factors.

Our findings underscore the need for intensive Newport-MDRAmpC surveillance in major dairy states. Efforts to promote the conservative and appropriate use of ceftiofur and other antimicrobial drugs in dairy cattle are indicated; they should be complemented by strategies to discourage transmission of MDR *Salmonella* among cattle, such as separating ill from parturient animals and disinfecting environmental niches (*14*). Furthermore, providers should be discouraged from prescribing antimicrobial drugs to patients with low-risk *Salmonella* infections (*15*), and public health messages should emphasize the importance of pasteurizing milk and cooking meat appropriately.

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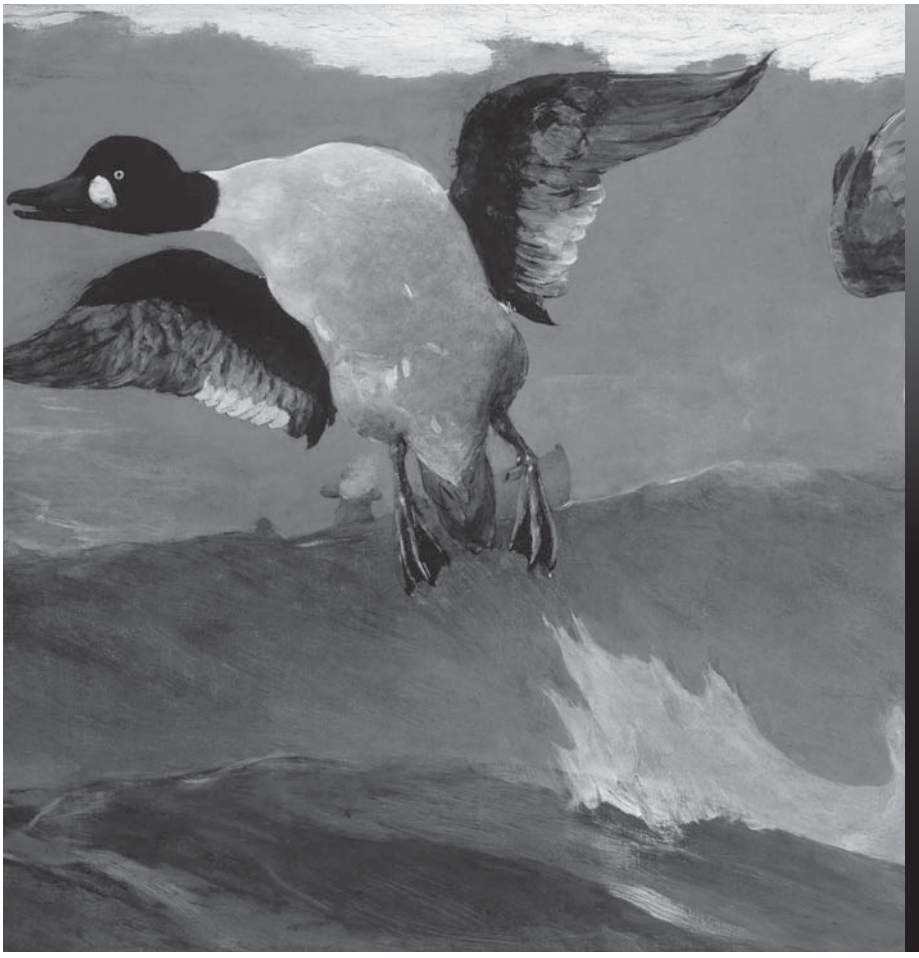

Medical Students and Pandemic Influenza

Benjamin Herman,* Rhonda J. Rosychuk,* Tracey Bailey,* Robert Lake,* Olive Yonge,* and Thomas J. Marrie*

To assess knowledge of pandemic influenza, we administered a questionnaire to all medical students at the University of Alberta; 354 (69%) of 510 students responded. Data from questionnaires such as this could help determine the role of medical students during a public health emergency.

Scientists believe we may be on the verge of the next great pandemic. Health Canada predicts that 4.5–10.6 million Canadians will become clinically ill and 11,000–58,000 will die in such an event (*1*). The current strain of influenza A (H5N1), which has been circulating in Southeast Asia among birds, with limited spread to humans, has fueled this concern (*2*,*3*). An influenza pandemic would cause a shortage of healthcare professionals due to illness and an increased demand for their services (*1*). Consequently, Canada and other countries have begun developing plans to deal with the anticipated health crisis (*1*), and medical students could play an important role.

The Study

As part of pandemic influenza planning at the University of Alberta, researchers developed a Web-based questionnaire (*4*) comprising 42 questions to assess demographic information, risk perception of pandemic influenza (likelihood of developing and dying from the illness), general knowledge about pandemic influenza, willingness to volunteer, and suggested consequences of not volunteering during a pandemic. Response options were generally either Yes/No or a 5-point Likert-type scale (e.g., ranging from very unlikely to very likely). The final version was administered through the Internet from September 12, 2006, until October 31, 2006. The 5-point scale responses were dichotomized. Respondents with missing values were excluded from data summaries and statistical tests.

All 510 medical students at the University of Alberta were invited by email to complete the questionnaire; 354 (69%) responded. Most (62%) respondents were women, and all but 1 were reported to be in good health. A variety of past volunteer activities were reported. Although medical students obtained health information from varying sources, they had the highest degree of confidence in the information provided by physicians.

Medical students' knowledge regarding the spread, prevention, and treatment of influenza is summarized in Table 1. Also included is the perceived likelihood of infection and outcome of such infection; 146 (41.2%) of students believed they were likely to be infected, but only 16 (4.5%) believed they would die from such an infection. Regarding volunteering during a future pandemic, most (247) believed that healthcare students have an obligation to do so (Table 2). A minority (30) agreed with penalties for heathcare students and academic staff who refused to

Table 1. Knowledge of medical students about influenza spread, prevention, treatment, likelihood of infection, and outcome of such infection*

Survey item	No. (%)
Influenza spread through	
Close contact with infected person	287 (81.1)
Blood transfusion	88 (24.9)
Sexual contact	75 (21.2)
Cough/sneeze from infected person	338 (95.5)
Touching door knobs	263 (74.3)
Contact w/infected wild birds, chickens	182 (51.4)
Influenza prevention	
Nothing can prevent pandemic influenza	5 (1.4)
Hand washing	331 (93.5)
Cover mouth when coughing/sneezing	289 (81.6)
Vaccination	257 (72.6)
Vitamins and herbal supplements	47 (13.3)
Antiviral drugs	121 (34.2)
Antibiotics	14 (4.0)
Quarantine	241 (68.1)
Staying home/avoiding public places	253 (71.5)
Moving to area with no influenza	56 (15.8)
Wearing protective equipment in public	211 (59.6)
Eating sauerkraut	8 (2.3)
Treatment	
Nothing can treat pandemic influenza	16 (4.5)
Antibiotics	28 (7.9)
Antibacterial drugs	208 (58.8)
Vaccination	43 (12.1)
Bed rest	275 (77.7)
Fluids	280 (79.1)
Complementary medicine	19 (5.4)
Chicken soup	66 (18.6)
Likelihood of infection if pandemic influenza is in Edmonton	
Very unlikely	9 (2.5)
Likely or very likely	146 (41.2)
Likelihood (likely or very likely) of the following outcomes if you have pandemic influenza	
Won't miss school or work	42 (11.9)
Will miss some school or work	251 (70.9)
Hospitalization but will recover	79 (22.3)
Death	16 (4.5)

*For spread, prevention, and treatment, right-hand column indicates positive response to item or statement. Multiple responses were possible within each category. For likelihood, right-hand column indicates responses for each category on a 5-point Likert-like scale ranging from "very unlikely" to "very likely."

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Table 2. Responses from medical students about volunteering for healthcare work during an influenza pandemic

Statement	No. (%) positive responses
Health sciences students should be encouraged to volunteer in the event of healthcare worker shortage	226 (63.8)
Retired healthcare workers should be encouraged to volunteer	185 (52.3)
Healthcare students have moral/ethical/professional obligation to volunteer	247 (69.8)
Government is justified in requiring people to work if there are insufficient volunteers	15 (43.8)
Healthcare students should be penalized for refusal to comply with government requirement	30 (8.5)
Healthcare academic staff should be penalized for refusal to comply with government requirement	76 (21.5)
Expulsion from school/termination from work	34 (9.6)
Jail time	11 (3.1)
Fine	128 (36.2)
Other	62 (17.5)
None of the above	154 (43.5)

provide services as required by the government. Healthcare and emergency workers were given the highest priority when assigning scarce resources; politicians were assigned the lowest priority.

Conclusions

This study shows that medical students have knowledge gaps with respect to various aspects of pandemic influenza. Most medical students knew that the main route of influenza transmission is by direct contact and respiratory droplets from coughing and sneezing. However, despite no supporting medical evidence, 88 (24.9%) medical students responded that blood transfusion is a major route of transmission (*5*); 75 (21.2 %) students also indicated influenza could be transmitted through sexual contact. Although direct sexual contact can transmit influenza, it is unlikely that this would be a major route of transmission.

Preventive measures are important not just during a pandemic, but at all times to decrease the risk of transmission of bacterial and viral illnesses. Most medical students (93.5% and 81.6%, respectively) correctly identified hand washing and cough etiquette, respectively, as effective preventive measures (it is important to note, however, that there is a difference between students knowing and complying). Social distancing can help reduce the peak incidence of an epidemic and spread it over several weeks instead of a few, thus avoiding a healthcare surge (*6*). Household quarantine is also effective at reducing attack rates in the community but only if the compliance rate is high (*7*). Knowledge regarding treatment of pandemic influenza is difficult to analyze because determining what measures would be effective is complex. Given the time lag between identification of the pandemic strain and the time needed to produce a vaccine, any known treatment strategy would not be effective unless it could be applied to the strain that is causing the pandemic.

Although it is encouraging that most students believe healthcare students have an obligation to volunteer during a pandemic, a dichotomy exists when it comes to setting a policy for actual deployment. One argument is that medical students should be encouraged to volunteer during a crisis because this will provide valuable medical training at a time when a great deal of the workforce—including physicians and nurses—will be ill and unable to work. During the 1918 influenza pandemic, some medical students in the United States graduated early from medical school and had expedited medical board examinations to increase the number of medical personnel available to provide services (*8*). Conversely, some may argue that placing the next generation of doctors on the front lines as volunteers will put them at increased risk for contracting illness. It is possible in this situation that the numbers of future physicians could decrease and adversely affect recovery efforts after a pandemic. During the severe acute respiratory syndrome crisis in Toronto, medical students were removed from the wards (D. Low, pers. comm.) (*9*). Although this was done with good intentions and safety in mind, students experienced a great deal of frustration regarding the suspension of their education and research activities (*9*).

Other recent events may offer insight into the desirability of using medical students as volunteers. Anecdotes from the terrorist attacks in New York City on September 11, 2001, relate how medical students helped in various capacities immediately following the tragedy (*10*). However, no official school or government mandate was issued forcing these persons to volunteer. In contrast, after the devastation of Hurricane Rita, college leaders at Texas A&M University College of Medicine made a decision to cancel classes and provided orientation sessions for students so that they could begin volunteering as soon as possible (*11*). Volunteer tasks in this situation were limited, and many voiced frustration that their relatively limited medical skills greatly restricted their ability to contribute. In a more positive vein, students also claimed that "the experience made them even more driven to become the best doctors they can" (*11*).

Our study was limited in that only 69% of the medical students at 1 medical school in Canada responded. However, the results do show that planning and debate should begin before a pandemic occurs and that expectations of the role of medical students must be explicit (*12*). A government must consider the virulence and characteristics of the

illness as well as the number of volunteers needed before deciding on an appropriate course of action. Ultimately, it remains to be seen what the climate of the next pandemic will be.

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Mr Herman is a third-year medical student at the University of Alberta. His research interests are evolving.

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Newfound Hantavirus in Chinese Mole Shrew, Vietnam

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Sequence analysis of the full-length medium segment and the partial small and large segments of a hantavirus, detected by reverse transcription–PCR in lung tissues of the Chinese mole shrew (*Anourosorex squamipes*) captured in Cao Bang Province, Vietnam, in December 2006, indicated that it is genetically distinct from rodentborne hantaviruses.

Insectivores (or soricomorphs) have been largely ignored as being important in the evolutionary dynamics of hantaviruses, despite the isolation of Thottapalayam virus (TPMV) from the Asian house shrew (*Suncus murinus*) (*1*,*2*) and the detection of hantavirus antigens in tissues of the Eurasian common shrew (*Sorex araneus*), alpine shrew (*S. alpinus*), Eurasian water shrew (*Neomys fodiens*), and common mole (*Talpa europea*) (*3*). Recently, genetically distinct hantavirus sequences have been found by reverse transcription–PCR in the Therese shrew (*Crocidura theresae*) in Guinea (*4*) and the northern short-tailed shrew (*Blarina brevicauda*) in the United States (*5*). In addition, a phylogenetically distinct hantavirus has been isolated from lung tissues of the Ussuri shrew (*C. lasiura*), captured along the Imjin River near the demilitarized zone in South Korea (J.-W. Song and R. Yanagihara, unpub. data).

The Study

To further investigate the existence and phylogeny of nonrodentborne hantaviruses, we analyzed lung and other visceral tissues, collected in RNAlater Stabilization Reagent (QIAGEN, Valencia, CA, USA), from 24 soricomorphs, including 9 white-toothed shrews (*Crocidura* spp.), 3 Chinese mole shrews (*Anourosorex squamipes*), and 12 long-nosed moles (*Euroscaptor longirostris*), captured in northern, central, and southern Vietnam during November and December 2006. RNA, extracted from 20–50 mg of each tissue by using the RNA-Bee isolation kit (TEL-TEST, Inc., Friendswood, TX, USA), was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and the primer 5′-TAG-TAGTAGACTCC-3′. Oligonucleotide primers for subsequent nested PCR were designed from consensus regions of TPMV and other hantaviruses (Table 1).

Gene-amplification reactions were performed in 50-μL reaction mixtures, containing 200 μmol deoxyribonucleoside triphosphate, 0.5 U of super-therm polymerase (PureTech Co., Ltd, Seoul, South Korea), 1 μg of cDNA, and 10 pmol of each primer. Initial denaturation, at 94°C for 5 min, was followed by touchdown cycling with denaturation at 94°C for 40 s, annealing from 50°C to 37°C for 40 s, elongation at 68°C for 1 min 20 s, then 25 cycles of denaturation at 94°C for 40 s, annealing at 40°C for 40 s, and elongation at 68°C for 1 min 20 s in a Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany). PCR products were purified by the Wizard PCR Preps DNA Purification System (Promega). DNA sequencing of at least 3 clones of each amplicon was performed in both directions with the dye primer cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (Model 377, Perkin Elmer Co., Waltham, MA, USA) (*6*).

Hantavirus sequences were not detected in tissues of the white-toothed shrews and long-nosed moles. By contrast, the full-length 3,637-nt (1,139-aa) medium (M) segment was amplified from lung tissues of 3 Chinese mole shrews, captured in Thanh Cong commune, Nguyen Binh District, Cao Bang Province, along the southern border of the People's Republic of China. Designated Cao Bang virus (CBNV), the newly identified hantavirus exhibited low nucleotide and amino acid sequence similarity to representative hantaviruses harbored by Murinae, Arvicolinae, Neotominae, and Sigmodontinae rodents, ranging from 62.6% (nt) and 61.2% (aa) for Hantaan virus (HTNV) 76–118; 62.3% (nt) and 61.7% (aa) for Dobrava virus (DOBV) Greece to 58.1% (nt) and 52.0% (aa) for Puumala virus Sotkamo; and 58.8% (nt) and 54.7% (aa) for Sin Nombre virus NMH10 (Table 2).

Pairwise alignment and comparison of a 1,185-nt coding region of the small (S) segment showed similar degrees of sequence identity between CBNV and rodentborne hantaviruses, ranging from 61.4% for HTNV 76–118 to 58.0% for TULV (Tula virus) M5302v. Much higher sequence similarity was found in a 412-nt coding region of the large (L) segment, ranging from 72.6% for HTNV 76–118 and 75.2% for DOBV Greece, to 67.2%–71.6% for hantaviruses harbored by Arvicolinae, Neotominae, and Sigmodontinae rodents. CBNV sequences were similarly divergent from Tanganya virus (TGNV) Tan826: for S segment, 63.5% (nt) and 65.0% (aa) similarity; for L segment, 71.3% (nt) and 77.0% (aa) similarity.

Phylogenetic trees based on sequences of the full-length M segment and partial S and L segments, generated

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Table 1. Oligonucleotide primers for amplification of Cao Bang virus

Segment	Primer	Sequence (5′→3′)	Polarity
Small	OSM55	TAG TAG TAG ACT CC	+
	OSM47	GGC CAG ACA GCA GAT TGG	+
	CBS1063F	ATK GCA TCH AAR ACA GTN GGN A	+
	CBS1016F	GGA GRA CWC AAT CAA TGG GT	+
	CBS1195F	GCN TGG GGN AAR GAG GCW GT	+
	CBS593R	GAC TGG GCA TTN GGC ATN GA	–
	CBS506R	ATH CTT GTC CCY TTR TTA TC	–
	S6	ACG TCI GGA TCC ATI TCA TC	–
	CBS-3′endR	TAG TAG TAK RCT CCY TRA A′	–
Medium	OSM55	TAG TAG TAG ACT CC	+
	G1–1	TAG TAG TAG ACT CCG CAA	+
	OSV697	GGA CCA GGT GCA DCT TGT GAA GC	+
	G2F1	TGG GCT GCA AGT GC	+
	CBM2762F	GGN AAY AHN GTC TCA GGN TAT	+
	CBM2804F	GAT TCH TTY CAA TCA TTY AA	+
	CBM2858F	GAR TGG GNA GAT CCW GAT	+
	CBM479R	AND TTG CAN GCA TGA ATA GG	–
	CBM505R	CCA ATS CAA NMA KAC AGC TT	–
	CBM1272R	TTH TGY TTW GAN ACA AGG CA	–
	CBM1322R	CHA CTC TYT GRC AMA CAA A	–
	T-M1442R	CCA TGN AAN CCT GGA ACA CA	–
	T-M1485R	CCA GCC AAA RCA RAA TGT	–
	CBM2256R	CAN GCM CCA TAR CAA TGA AA	–
	T-M2957R	GAA CCC CAD GCC CCN TCW AT	–
	G2T	TAG TAG TAK ACW CCG CA	–
Large	OSM55	TAG TAG TAG ACT CC	+
	PHL-173F	GAT WAA GCA TGA YTG GTC TGA	+
	PHL-2111F	CAG TCW ACA RTT GGT GCA AGT GG	+
	PHL-2935F	YTM ATG TAT GTT AGT GCA GAT GC	+
	TPMV-L195R	TTR TCA GAC CAD TCA TG	–
	TPMV-L345R	TRT AAT TRT CAG GTG T	–
	PHL-3445R	GRT TAA ACA TAC TCT TCC ACA TCT C	–
	PHL-3388R	AAA CCA TTC AGT TCC ATC ATC	–

by the maximum likelihood and neighbor-joining methods using the GTR+I+G model of evolution, showed similar topologies supported by bootstrap analysis, in which CBNV was relatively distinct from rodentborne and other shrewborne hantaviruses (Figure). A strong association with TGNV was observed on the basis of the S segment (1,185 bases), however. Further sequence information will clarify the relationship between CBNV and other soricidborne hantaviruses and whether these form a monophyletic group in parallel with the evolution of Soricinae and Crocidurinae shrews. If one judges by the distant evolutionary relationship between shrews and rodents, future sequences of other soricidborne hantaviruses will provide considerable insights into their evolutionary origins.

Conclusions

Designing suitable primers for the amplification of CBNV presented unanticipated challenges. Ironically, the recently acquired full genome of TPMV (J.-W. Song and R. Yanagihara, unpub. data) was not particularly helpful, since CBNV was genetically more divergent from TPMV than from well-characterized rodentborne hantaviruses (78% bootstrap support, S segment). Also, because the tissues were collected in RNAlater, virus isolation attempts could not be performed. As such, progress in obtaining the full-length sequence of CBNV has been slow.

A forest-dwelling soricine typically residing at elevations of 1,500–3,000 m, the Chinese mole shrew (family Soricidae, subfamily Soricinae) has a vast geographic range, extending from western and central People's Republic of China, northern Myanmar, northern Thailand, Assam, Bhutan, northern Vietnam, Taiwan, and possibly Lao People's Democratic Republic. That a hantavirus has been identified in the Chinese mole shrew was not completely unexpected, in view of the isolation of a HTNV-like virus from this species in Sichuan Province in 1986 (*7*). However, those authors may have prematurely concluded that their hantavirus isolate was closely related to HTNV, since no genetic analysis was performed.

Viewed within the context of newly identified, genetically distinct hantaviruses in the northern short-tailed shrew (*B. brevicauda*), Eliot's short-tailed shrew (*B. hylophaga*),

Table 2. Pairwise nucleotide and amino acid sequence analysis of the full-length M segments of Cao Bang virus and other hantaviruses*

Hantavirus	CBNV	HTNV	SEOV	SOOV	DOBV	PUUV	PHV	TULV	SNV	ANDV
CBNV TC-3	–	62.6	62.4	62.5	62.3	58.1	57.3	58.3	58.8	58.0
HTNV 76–118	61.2	–	71.3	80.6	70.7	58.0	57.9	58.8	57.4	57.6
SEOV 80–39	60.9	77.1	–	71.1	70.4	58.6	57.5	58.8	56.7	57.2
SOOV SC-1	60.8	91.2	76.6	–	70.4	57.8	58.1	59.4	58.1	57.4
DOBV Greece	61.7	77.3	77.2	60.8	–	58.3	57.1	58.0	58.3	57.6
PUUV Sotkamo	52.0	53.8	53.8	54.1	53.4	–	70.1	71.4	65.5	64.8
PHV PH-1	52.4	54.3	53.6	54.2	53.5	75.6	–	73.0	65.5	63.6
TULV M5302v	52.9	55.7	54.7	54.8	55.0	78.9	80.4	–	66.4	64.8
SNV NMH10	54.7	54.7	53.1	55.2	54.1	66.9	67.6	69.2	–	70.6
ANDV Chile	54.2	54.7	54.1	55.3	54.4	66.8	66.7	67.8	77.9	–

*Pairwise distances are presented as a triangular matrix, with nucleotide on the top half and amino acid similarities on the bottom half. GenBank accession nos.: Cao Bang virus (CBNV TC-3), EF543526; Hantaan virus (HTNV 76–118), NC_005219; Seoul virus (SEOV 80–39), NC_005237; Soochong virus (SOOV SC-1), AY675353; Dobrava virus (DOBV Greece), NC_005234; Puumala virus (PUUV Sotkamo), NC_005223; Prospect Hill virus (PHV PH-1), X55129; Tula virus (TULV Moravia 5302v), NC_005228; Sin Nombre virus (SNV NMH10), NC_005215; and Andes virus (ANDV Chile-9717869), NC_003467.

masked shrew (*Sorex cinereus*), and dusky shrew (*S. monticolus*) in the United States (S. Arai and R. Yanagihara, unpub. data)—as well as in the Eurasian common shrew in Switzerland, Hungary, and Finland (J.-W. Song, S. Arai, and R. Yanagihara, unpub. data), the Ussuri shrew in South Korea (J.-W. Song and R. Yanagihara, unpub. data), the Asian house shrew in India (*1*,*2*), and the Therese shrew in Guinea (*4*)—the detection of a newfound hantavirus in the Chinese mole shrew would predict that hantaviruses harbored by shrews are as geographically widespread as those harbored by rodents. Preliminary studies indicate CBNV-like sequences in the liver tissue of Chinese mole

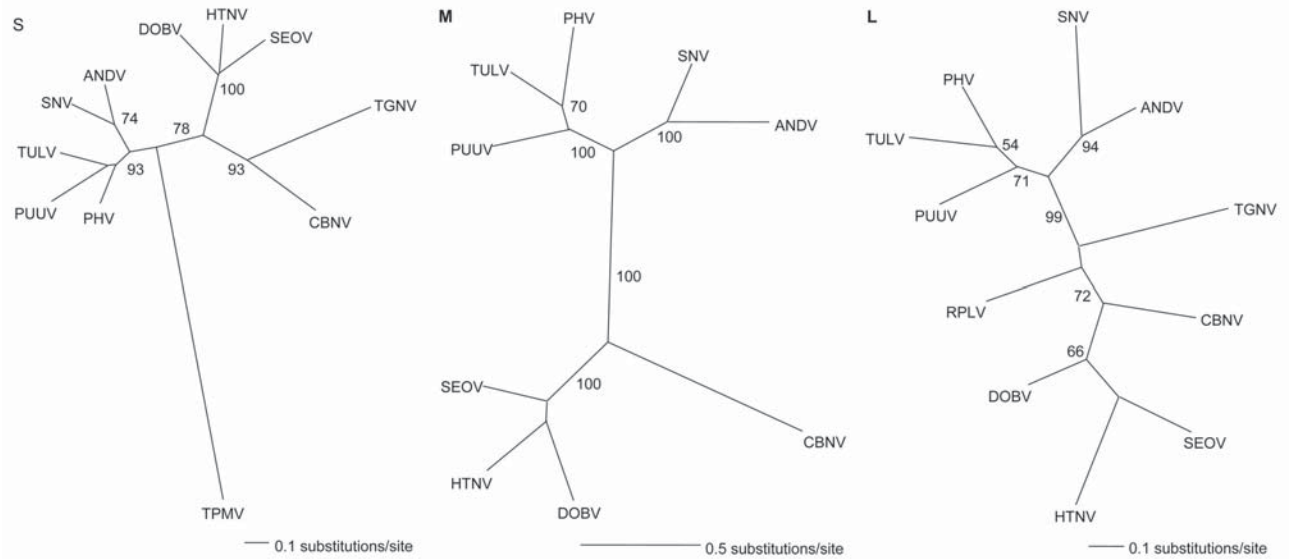

Figure. Phylogenetic trees based on the 1,185-nt partial small (S), 3,637-nt full-length medium (M), and 412-nt partial large (L) segments of Cao Bang virus (CBNV). The depicted S tree was generated by the neighbor-joining (NJ) method, by using the GTR+I+G model of evolution as estimated from the data. The M and L trees were generated by the maximum likelihood (ML) method, using the same model of evolution. The phylogenetic position of CBNV is shown in relationship to representative Murinae rodentborne hantaviruses, including Hantaan virus (HTNV 76–118, GenBank accession nos. NC_005218, NC_005219, NC_005222), Dobrava virus (DOBV Greece, GenBank accession nos. NC_005233, NC_005234, NC_005235), and Seoul virus (SEOV 80–39, GenBank accession nos. NC_005236, NC_005237, NC_005238); Arvicolinae rodentborne hantaviruses, including Tula virus (TULV M5302v, GenBank accession nos. NC_005227, NC_005228, NC_005226), Prospect Hill virus (PHV PH-1, GenBank accession nos. Z49098, X55129, EF646763) and Puumala virus (PUUV Sotkamo, GenBank accession nos. NC_005224, NC_005223, NC_005225); and Sigmodontinae and Neotominae rodentborne hantaviruses, including Andes virus (ANDV Chile 9717869, GenBank accession nos. NC_003466, NC_003467, NC_003468) and Sin Nombre virus (SNV NMH10, GenBank accession nos. NC_00521, NC_005215, NC_005217). Also included are Thottapalayam virus (TPMV VRC-66412, GenBank accession no. AY526097), Tanganya virus (TGNV Tan826, GenBank accession nos. EF050454, EF050455), and Camp Ripley virus (RPLV MSB89863, GenBank accession no. EF540771). NJ, ML, and maximum parsimony phylogenetic methods yielded similar topologies with only minor cosmetic differences. Host identification was confirmed by diagnostic mitochondrial DNA sequences (GenBank accession no. EF543528). The numbers at each node are bootstrap probabilities (expressed as percentages), as determined for 1,000 (NJ) and 100 (ML) iterations by PAUP* version 4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA, USA) (http://paup.csit.fsu.edu). GenBank accession nos. for CBNV: S (EF543524); M (EF543526); and L (EF543525).

shrews captured in Taiwan (S. Arai and R. Yanagihara, unpub. data). Also, investigations on the genetic diversity of CBNV and other newly identified members of the *Hantavirus* genus will provide additional insights into the phylogeography and co-evolution of hantaviruses and their soricid reservoir hosts. One or more of these newfound shrewborne viruses may yield valuable clues about the molecular determinants of hantavirus pathogenicity.

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Environmental Predictors of Human West Nile Virus Infections, Colorado

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To determine whether environmental surveillance of West Nile Virus–positive dead birds, mosquito pools, equines, and sentinel chickens helped predict human cases in metropolitan Denver, Colorado, during 2003, we analyzed human surveillance data and environmental data. Birds successfully predicted the highest proportion of human cases, followed by mosquito pools, and equines.

The United States has experienced numerous locale-specific West Nile virus (WNV) epidemics since the infection was first identified in New York in 1999 (*1*). Since then, the Centers for Disease Control and Prevention has recommended that jurisdictions consider improving surveillance based on probability of arbovirus activity and available resources (*2*). As a result, many states have developed or enhanced environmental surveillance systems to detect WNV activity within their jurisdictions.

Tri-County Health Department (TCHD), a local public health agency in metropolitan Denver, Colorado, serves >1 million people in Adams, Arapahoe, and Douglas Counties, and spans urban and rural regions. In response to the anticipated arrival of WNV, the state of Colorado added WNV serologic testing to its existing sentinel chicken surveillance (which included serologic testing for western equine encephalitis and St Louis encephalitis), and initiated surveillance of mosquito pools, dead birds, and equines. This retrospective study aims to utilize both epidemiologic and spatial tools to determine whether the enhanced environmental surveillance system was able to predict human infections in space and time during an epidemic year of WNV activity. In addition, this study assesses whether predictability differed by urban/rural location and month of onset of human disease.

The Study

Since 2002, healthcare providers and laboratories have been required to report patients with laboratory evidence of acute WNV infection in Colorado if testing identified WNV-specific immunoglobulin M antibodies in either cerebral spinal fluid or serum by ELISA, or blood donors with a positive nucleic acid test result. In addition to laboratory confirmation, patients had to exhibit clinical features consistent with an acute WNV infection, including encephalitis, meningitis, flaccid paralysis, or WNV fever; detailed case definitions have previously been described (*3*). Human surveillance data were downloaded from the Web-based statewide notifiable disease reporting system.

Environmental data included specimens of birds, mosquito pools, equines, and sentinel chickens that were tested for WNV. Bird oral swab specimens and mosquito pools were tested by reverse transcription–PCR or VecTest (Medical Analysis Systems, Inc., Camarillo, CA, USA) at Colorado Department of Public Health and Environment's (CDPHE) laboratory or regional local health department laboratories. Equine specimens were tested at the Department of Agriculture and Colorado State University Veterinary Diagnostic Laboratories on a fee-for-service basis by IgM antibody capture–ELISA (MAC-ELISA) at a 1:400 dilution to eliminate false-positive results due to vaccination (*4*). Sentinel chickens were set at 3 permanent sites and tested weekly by MAC-ELISA at the Weld County Health Department laboratory. All laboratory results were reported to CDPHE and forwarded to TCHD.

A total of 408 human WNV cases were reported in Adams, Arapahoe, and Douglas Counties during 2003. TCHD's environmental surveillance system identified 109 (50.0%) of 218 birds that tested positive for WNV, 62 (54.9%) of 113 equines that tested positive, 58 (21.7%) of 267 mosquito pools that tested positive, and 45 (12.7%) of 354 sentinel chickens that tested positive. Geographic locations were determined for all but 4 (99.0%) of the infected humans, for 96.0% of environmental species with positive results, and for 97.4% of environmental species with negative results. Human and environmental surveillance data were mapped in ArcView, version 3.3 (ESRI, Redlands, CA, USA) (Figure). The space–time relationships between human infections and environmental positive results were computed for all humans. For space, a distance threshold of 2 km was used, well within the dispersal distances identified for *Culex tarsalis* (*5*), the primary vector of WNV in Colorado. For time, the temporal threshold for environmental positive results was any time before, and up to, the time of the onset of human infection. This assumes that once an environmental positive result was documented at a particular location, WNV would remain at that location for the rest of the season. Using a script written in the Avenue programming language for ArcView 3.3, if a person was within the threshold for both space and time in relation to an environmental positive result, then we concluded that

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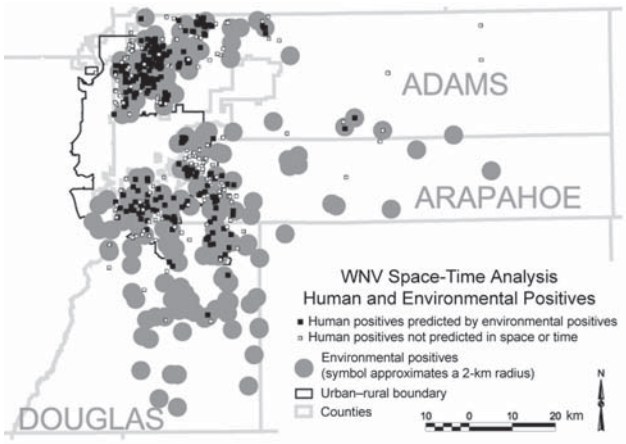

Figure. Human infections and positive environmental results, Adams, Arapahoe, and Douglas Counties, Colorado, 2003.

the occurrence of human infection was successfully predicted by the environmental surveillance system. Human cases that were identified as being successfully predicted and all environmental positive results are indicated in the Figure.

To determine whether environmental surveillance was more accurate at predicting human cases by urban than rural location and month of human infection onset, we calculated the proportions of human cases that were successfully predicted. Urban regions were identified as those census tracts with densities ≥1,600 persons per square mile (PPSM) based on Census 2000 data. In contrast to the Census Bureau's "urban area" classification of 1,000 PPSM, this density value was derived statistically by using Colorado census tract data and did not include census blocks of low population surrounding highly built-up areas. SAS software (SAS Institute Inc., Cary, NC, USA) was used to determine measures of association by Wald χ^2 tests with a significance level of 0.05.

Overall, the 4 types of environmental surveillance were able to predict 64.6% of all reported human cases of WNV infection (Table 1). When the 4 types of environmental specimens were analyzed separately, birds successfully predicted the highest proportion of human cases, followed by mosquito pools and equines. The accuracy rates for birds were higher in urban than in rural locations and were better in the latter half of the season. Although findings were not significant, human infections were more successfully predicted by both mosquito pools ($p = 0.0874$) and equines ($p = 0.0782$) in rural areas.

To validate our findings, we assessed the data in a different manner to determine how well positive environmental results predicted human infections and how well negative environmental results predicted the absence of human infections. Similar to the previous analysis, the spatial cutoff was 2 km, and the temporal threshold for environmental positive results was any time before, and up to, the time of human infection onset. Environmental negative results were expected to be followed by no human infections for at least 2 weeks. In this analysis, 50.8% of positive environmental results were followed by a human infection at some point after the environmental positive result was detected, and 86.0% of negative environmental results were followed by a lack of human cases for at least the next 2 weeks (Table 2).

Conclusions

This study evaluated whether resources dedicated to environmental surveillance for the detection of WNV activity can predict human cases. More specifically, we assessed the predictability of 4 different types of environmental surveillance to identify where and when these methods were most successful in predicting human infections.

Overall, an environmental indicator preceded almost two thirds of human infections, and half of positive environmental results were followed by a human infection. Although more tests were performed for sentinel chickens and mosquito pools, birds were better predictors of human infections. Because bird surveillance depends on the public identifying and bringing in birds for testing, bird surveil-

Table 1. Number and proportion of human infections successfully predicted by positive environmental specimens, Tri-County Health Department, Colorado, 2003

	Total no. human infections	No. (%) human Infections successfully predicted by				
		Any environmental specimen	Birds	Mosquito pools	Equines	Sentinel chickens
Region						
Urban (reference value)	292	202 (69.2)	176 (60.3)	51 (17.5)	35 (12.0)	3 (0.9)
Rural	112	59 (52.7)*	30 (26.8)*	28 (25.0)	21 (18.8)	1 (1.0)
Onset date†						
June/July	79	28 (35.4)*	17 (21.5)*	9 (11.4)*	8 (10.1)	0
August	273	196 (71.8)	157 (57.5)	54 (19.8)	41 (15.0)	4 (1.5)
September (reference value)	47	34 (72.3)	30 (63.8)	14 (29.8)	6 (12.8)	0
Total	404‡	261 (64.6)	206 (51.0)	79 (19.6)	56 (13.9)	4 (1.0)

*Significant at the p = 0.05 level.
†Five human case-patients had missing onset date.
‡Four human case-patients could not be geo-located.

Table 2. Relationship between positive or negative environmental specimens and human infections, Tri-County Health Department, Colorado, 2003

Specimens	No. positive results followed by human infection/ all positive results (%)	No. negative results followed by human infection/ all negative results (%)
All specimens	133/262 (50.8)	591/687 (86.0)
Birds	62/105 (59.0)	82/98 (83.7)
Mosquito pools	38/57 (66.7)	154/203 (75.9)
Equines	17/55 (30.9)	30/32 (93.8)
Sentinel chickens	16/45 (35.6)	325/354 (91.8)

lance was dramatically more accurate in urban areas with high human population densities. Sentinel chicken surveillance had extremely low predictive success, which supports the subsequent decision to discontinue this surveillance method in Colorado in 2004.

This study uses a combination of epidemiologic and geographic tools to analyze WNV data spatially, temporally, and categorically. These findings would be valid when sufficient environmental samples with accurate geo-location data are submitted for testing. As with any surveillance system, environmental surveillance is likely to be more predictive during epidemic levels of virus transmission. However, the goal of state and county WNV surveillance is not to document endemic virus circulation, but rather predict increased risk for human transmission and epidemic level activity in time to initiate public notification and preventive measures.

Several other studies have illustrated the utility of dead bird testing (*6*–*8*) and dead bird clustering (*9*–*11*) in predicting human infections. Another study looked at county-level data to monitor dead birds and WNV-positive birds, as well as WNV-positive mosquito pools to predict human risk (*12*). Our study incorporates dimensions of space and time to assess the overall success of environmental surveillance at a local level. Methods used in this analysis could potentially be applied to other vectorborne diseases.

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Primary versus Nonprimary Cytomegalovirus Infection during Pregnancy, Israel

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We examined prospectively the outcome of primary and nonprimary maternal cytomegalovirus (CMV) infection during pregnancy among 88 and 120 women, respectively. The risk for vertical transmission was 1.83× higher for primary infection than for nonprimary infection. Nonetheless, congenital CMV disease was diagnosed in both infection groups at similar rates.

Cytomegalovirus (CMV) infection is the most frequent congenital infection and a common cause of deafness and intellectual impairment, affecting 0.5%–2.5% of all live births (*1*–*3*). Intrauterine infection occurs in 40% of primary maternal infections, with delivery of 10% to 15% symptomatic newborns and late neurologic sequelae in 10% of those asymptomatic at birth (*1*).

Although preexisting maternal immunity reduces maternal-fetal transmission, the severity of congenital CMV disease is similar following primary or nonprimary infection (*4*–*7*). Yet, several reports found increased vertical transmission after nonprimary CMV infection (*4*–*9*). Therefore, our objective was to examine the outcome of primary and nonprimary maternal CMV infections during pregnancy.

The Study

Institutional Ethics Committee approval was obtained. Women with positive CMV immunoglobulin (Ig) M (n = 208), referred for risk for CMV infection between January 1998 and December 2001, were enrolled in this prospective cohort observational study. Clinical and pregnancy-related information was obtained. Serum CMV IgG and IgM were measured by enzyme immunoassay and CMV-IgM immunofluorescence assay (*10*). IgG avidities ≤25% indicated recent infection (*10*).

Ultrasonographic examinations were performed between the 15th and 21st weeks of pregnancy. The reference method for prenatal diagnosis of CMV, requiring combined viral isolation and positive CMV PCR from amniotic fluid after gestational week 21 or 7 weeks after maternal symptoms (*3*,*11*), was applied for all amniocenteses. Amniotic fluid samples were inoculated onto MRC5 monolayers for CMV isolation (*10*), and DNA was amplified by PCR (*10*,*12*). Parents made decisions regarding amniocentesis and the fate of pregnancy after medical, and sometimes rabbinical, consultations. Elective terminations of pregnancy (ETOP) required external committee approval. Available aborted fetuses were examined for CMV-induced histopathologic changes. Immediately after birth, neonatal urine and anti–CMV IgM were examined. Subsequently, the newborns underwent cerebral ultrasound and auditory and ophthalmologic assessment.

Primary infection was defined as the occurrence of anti–CMV IgG seroconversion during pregnancy (*1*). Women who were seropositive for anti–CMV IgM and anti–CMV IgG when first evaluated during pregnancy and with IgG avidity >35% were considered to have nonprimary infection (*12*). The latter were divided into those with preconception evidence of anti–CMV IgG and negative anti–CMV IgM (group 1) and those without prior tests for CMV (group 2). Vertical transmission was declared if the amniotic fluid contained CMV virus or DNA, if pathologic features of CMV disease existed in the aborted fetus, or if neonatal IgM or urine cultures were positive for CMV.

Analysis of variance and the Kruskal-Wallis or Mann-Whitney tests were used. Frequencies were compared by χ^2 or Fisher exact tests. Relative risk was calculated with Epi Info 2000 software (available from www.cdc.gov/epiinfo).

Of the 208 enrolled women, 88 (42.3%) had primary CMV infection; 120 (57.7%) had nonprimary CMV infection, 36 (17.3%) from group 1 and 84 (40.4%) from group 2. The mothers' ages were similar in both groups. The median gestational age upon referral was 15 weeks (9.5–19.0 weeks), and the median number of pregnancies was 3 (range 1–10). CMV serologic testing was part of the routine gynecologic examination in 127 (61.0%) of the women: 35 (39.8%) after primary infection and 92 (76.6%) in the nonprimary infection group ($p<0.001$). Clinical signs of CMV infection triggered 52 (25%) of the tests, while patient anxiety induced the rest. Clinical CMV symptoms were more common with primary than with nonprimary infections (53 [60.2%], and 44 [36.6%], respectively, $p = 0.002$).

Pregnancies with primary infection had significantly fewer live births than those with nonprimary infection (Table 1). Primary infections in the first 20 gestational weeks resulted in 46.5% live births, 46.5% ETOP, and 7% miscarriages, while pregnancies with such infections after week 23 were 100% full term ($p = 0.004$).

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Table 1. Outcome of pregnancies by type of CMV infection*

Outcome of pregnancy	Primary infection (%)	Nonprimary infection (%)	Total	p value
Live birth	51 (58.0)	97 (80.8)	148	<0.001
ETOP*	30 (34.0)	21 (17.5)	51	0.006
Miscarriage†	7 (8.0)	2 (1.7)	9	0.038
Total	88	120	208	

*CMV, cytomegalovirus; ETOP, elective termination of pregnancy. Thirty ETOP were performed during the first trimester, 21 between the 21st and 23rd weeks of pregnancy.
†Mean gestational age of miscarried fetuses was 7 wk.

The following analysis included 169 women (excluding 39 with miscarriages or ETOP before week 21). Of them, 100 had amniocentesis, with most in the nonprimary infection group 2, 62.7% (52/83), and the rest similarly distributed between nonprimary group 1, 42.9% (15/35), and primary infection, 40.7% (33/81). Approximately half of the amniocenteses provided evidence of fetal CMV infection, both in primary infection (16/33) and nonprimary infection group 1 (7/15), but only in 17.3% (9/52) of group 2 ($p<0.001$). Vertical transmission was determined from the amniotic fluid by culture (n = 12) or PCR (n = 30), abortus pathology (n = 6), or positive neonatal IgM (n = 2) or urine cultures (n = 13). Vertical transmission rates were 35.8% (24/76), 30.0% (9/30), and 15.3% (11/72) in the primary and nonprimary infection groups 1 and 2, respectively ($p = 0.017$). The relative risk for vertical transmission in primary infection was 1.83 (95% confidence interval 1.1–3.03, $p = 0.019$) versus nonprimary infection.

All liveborn babies had similar natal characteristics regardless of the maternal infection group. Four newborns and 7 aborted fetuses (6.5%, 11/169) had congenital CMV disease (Table 2), 6.0% (4/67) after primary CMV infection and 6.9% (7/102) after nonprimary infection, with 13.3% (4/30) in group 1 and 4.2% (3/72) in group 2 ($p = 0.26$). Three of the 4 mentally retarded neonates were born after nonprimary infection. CMV infection was not detected in newborns of mothers with negative prenatal diagnostic tests (n = 68).

Table 2. Characteristics of aborted fetuses and neonates with congenital CMV disease*

Case no.	Maternal infection	Maternal symptoms	Week of infection	US	AF	Outcome, wk	Natal/abortion status	Follow-up, 2 y
1	Primary	Flu	<20	IUGR microcephaly	+	Live born, 38	Congenital disease	Sensorineural hearing loss, PMR
2	Primary	None	<18	Hyperechoic bowel	ND	Abortion, 19	Postmortem: disseminated CMV	
3	Primary	Fever	7	Hyperechoic bowel	+	Abortion, 23	Postmortem: disseminated CMV	
4	Primary	Fever	12	None	+	Abortion, 24	Postmortem: disseminated CMV	
5	Nonprimary (group 1)	None	Unclear	IUGR	ND	Live born, 38	Brain calcifications	PMR
6	Nonprimary (group 1)	Flu	22	Tricuspid regurgitation	ND	Abortion, 33	Postmortem: disseminated CMV	
7	Nonprimary (group 1)	Fever	20	IUGR pericardial fluid, brain calcifications	+	Abortion, 30	Postmortem: disseminated CMV	
8	Nonprimary (group 1)	Flu	<20	None	+	Live born, 40	Congenital disease	PMR
9	Nonprimary (group 2)	Fever	12	ND	+	Abortion, 24	Postmortem: disseminated CMV	
10	Nonprimary (group 2)	Flu	4	ND	ND	Live born, 38	Congenital disease	Sensorineural hearing loss, PMR
11	Nonprimary (group 2)	None	14	Liver calcifications	+	Abortion, 24	Postmortem: disseminated CMV	

*CMV, cytomegalovirus; US, ultrasonography; AF, amniotic fluid; IUGR, intrauterine growth retardation; +, positive according to PCR, culture, or both; ND, not done; PMR, psychomotor retardation.

Conclusions

To our knowledge, this is the first cohort in which the natural history of nonprimary CMV infection was evaluated prospectively in the mother from pregnancy to its conclusion, in contrast to published studies that determined it from established neonatal infection and retrospective assessment of the mothers' serologic test results (*2*,*4*–*8*,*13*). Most Israeli physicians obtain CMV serologic test results in pregnancy in response to women's demands and as a precaution, but the results, mainly of nonprimary infection, are confusing. Our findings suggest that nonprimary infection is also dangerous. While vertical transmission after primary CMV infection was similar to the reported 30% to 40% (*1*,*3*,*11*), after nonprimary infection it was 19.6%, much higher than the published 2.2% (*1*–*3*,*13*). Furthermore, of the 11 cases of congenital CMV disease, 7 were associated with nonprimary maternal infection.

The high rate of vertical transmission in the nonprimary infection group attests to the numerous amniocenteses performed in Israel to exclude fetal infection. The procedure is considered safe because neonatal loss rates in those undergoing it and not undergoing it are identical (*14*), and the risks of undergoing the procedure do not outweigh the risks for congenital CMV infection. In the nonprimary group, 67 (55.8%) had amniocentesis, but in the primary infection group, only 33 (37.5%) underwent the procedure, which reflects the increased early ETOP rate with primary infection.

Traditionally, vertical transmission after nonprimary infection was established by isolating CMV from neonatal saliva or urine, or the presence of neonatal IgM (*4*–*8*). We, however, determined transmission also from CMV in the amniotic fluid, and the observed difference between amniotic CMV and neonatal CMV disease suggests fetal virus elimination between amniocentesis and birth, probably by preexisting maternal CMV-specific antibodies. The high transmission rate could have resulted also from reinfection by CMV strains different from the primary strain (*6*), further enhanced by increased virulence of present-day strains.

The high proportion (>40%) of religiously observant women accounts for the reluctance of some to terminate pregnancy without ultrasonographic and amniocentesis evidence of fetal infection and also for the increased early ETOP (permitted by Jewish law only before the second trimester). We are aware that data were obtained for only 169/199 (84.9%) of fetuses or newborns and that only 31/51 (60.8%) of the aborted fetuses were available for examination, but omissions were random. Possible referral selection bias and the low number of affected women discourage definitive conclusions. Nevertheless, both primary and nonprimary CMV infection during pregnancy are clearly important causes of congenital disease.

Dr Rahav is the director of the Infectious Diseases Unit, Sheba Medical Center, Tel-Hashomer, Israel. Her research interests include mycobacterial infections, bacterial strain typing, HIV-1 reverse transcriptase, and congenital CMV infection.

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Rickettsia felis in Chile

To the Editor: Rickettsiosis due to *Rickettsia felis* is an emerging disease that has been reported worldwide (*1*). Fever, headache, myalgia, and macular rash have been attributed to *R. felis* infection in humans (*1*). In South America, *R. felis* infection in fleas (mostly *Ctenocephalides* spp.) has been reported only in Brazil, Peru, and Uruguay (*2*–*3*). Although a growing number of articles have reported that *R. felis* is transmitted by fleas, the acquisition mechanism of *R. felis* by vertebrates or uninfected fleas in nature remains unknown (*4*).

Cats experimentally exposed to *R. felis*–infected fleas have been shown to become seropositive (*5*). However, neither serologic nor molecular evidence of *R. felis* infection has been reported in cats under natural conditions, despite the fact that most *C. felis* fleas are infected by *R. felis* (*6,7*).

In November 2006, we investigated the presence of rickettsial DNA in 30 *C. felis* fleas randomly collected from 22 domestic cats privately owned and housed indoors in a single household in Santiago, Chile. To detect rickettsial DNA in each individual flea, PCRs were performed that targeted a 398-nt fragment of the rickettsial *gltA* gene and an 856-nt fragment of the rickettsial *ompB* gene (*7,8*).

A total of 21 individual fleas (70%) yielded expected PCR products for both *gltA* and *ompB* genes. PCR *gltA* products from the 21 fleas and *ompB* products from 5 fleas were subjected to DNA sequencing as described (*7*). The *gltA* partial sequences obtained from 21 fleas were identical, as were the *ompB* partial sequences from 5 fleas. These sequences were 100% identical to corresponding sequences in the *R. felis* genome (GenBank accession no. CP000053).

Blood serum samples were collected from the 22 cats and tested by indirect immunofluorescence assay (IFA) with crude antigens derived from 6 *Rickettsia* isolates from Brazil: *R. bellii*, *R. amblyommii*, *R. rhipicephali*, *R. rickettsii*, *R. parkeri*, and *R. felis* (*7,9*). Serum was considered to contain antibodies against rickettsiae if it displayed a reaction at 1:64 dilution. End-point titers against each *Rickettsia* species were determined by testing serial 2-fold serum dilutions. Reactive serum specimens were tested in 2 or 3 replications by 2 readers before the end-point titer was determined. Serum showing a *Rickettsia* species titer at least 4-fold higher than those observed for the other *Ricketttsia* species was considered homologous to the first *Rickettsia* species or to a very closely related genotype (*7,9*). In each slide, a nonreactive cat serum specimen (negative control) and a known reactive cat serum specimen (positive control) were tested at the 1:64 dilution (*7*).

IFA detected antibodies reactive with *R. felis* (titer ≥64) in 16 (72.7%) of 22 cats. Among those, 5 (22.7%) also reacted with *R. rhipicephali*, 4 (18.2%) with *R. bellii*, 3 (13.6%) with *R. parkeri*, 2 (9.1%) with *R. rickettsii*, and 1 (4.5%) with *R. amblyommii*. No serum reacted with any other *Rickettsia* species without reacting with *R. felis* (Table). Four cat serum specimens (cats 1, 3, 8, and 11) showed titers to *R. felis* at least 4-fold higher than those to any of the other 5 antigens. The antibody titers in these 4 animals were considered to have been stimulated by *R. felis* infection. For the remaining 12 seropositive cats, we could not discern whether *R. felis* had been the infection agent because the results displayed a single titer of 64 for *R. felis* or showed similar titers for other *Rickettsia* species.

We report 70% *R. felis*–infected fleas in this study on the basis of the concordant results of 2 PCR amplifications (*gltA* and *ompB*) and DNA

Table. End-point titers of indirect immunofluorescence assay (IFA) for 6 *Rickettsia* species in cats from a household in Santiago, Chile*

Cat no.	IFA titers for *Rickettsia* antigens						PAIHR
	R. felis	*R. rhipicephali*	*R. bellii*	*R. parkeri*	*R. rickettsii*	*R. amblyommii*	
1	128	–	–	–	–	–	*R. felis*
2	256	256	128	–	–	–	
3	512	128	64	–	–	–	*R. felis*
5	64	–	–	–	–	–	
6	64	64	–	–	–	–	
7	64	–	–	–	–	–	
8	128	–	–	–	–	–	*R. felis*
9	64	–	–	–	–	–	
10	64	–	–	–	–	–	
11	128	–	–	–	–	–	*R. felis*
12	64	–	–	–	–	–	
14	128	–	64	128	–	128	
15	64	–	–	–	–	–	
19	64	–	–	–	–	–	
21	128	64	128	128	128	–	
22	64	64	–	64	64	–	

*PAIHR, possible antigen involved in a homologous reaction (serum showing for a *Rickettsia* species titer at least 4-fold higher than that observed for any other *Ricketttsia* species was considered homologous to the first *Rickettsia* species); –, nonreactive at titer ≥64.

sequencing. This infection rate is within the range (13.5%–90%) that has been reported for *R. felis* infecting *Ctenocephalides* fleas in Brazil and Uruguay (*2*,*3*,*7*). Sixteen (72.7%) cats contained *R. felis*–reactive antibodies; 4 of them showed titers to *R. felis* at least 4-fold higher than those to the other 5 rickettsial strains, findings that enabled us to technically conclude that these cats were exposed to *R. felis* or a closely related organism (*1*,*7*,*9*). Our finding of 70% *R. felis* infection in fleas infesting the cats indicates that cats acquired the infection through infected fleas. However, the mechanism of *R. felis* transmission by fleas is yet to be demonstrated under experimental conditions.

To our knowledge, the presence of *R. felis*, or a spotted fever group *Rickettsia* species, has not been reported in Chile. Recent investigations have provided clinical and serologic evidence of canine (*10*) and human (K. Abarca and J. Lopez, unpub. data) infection by spotted fever rickettsia in Chile, confirmed by IFA that used *R. conorii* commercial antigen. Since substantial serologic cross-reaction occurs between *R. conorii* and *R. felis* antigens (*1*), *R. felis* could be causing infection in dogs or humans in Chile.

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Possible Typhoon-related Melioidosis Epidemic, Taiwan, 2005

To the Editor: Melioidosis is a severe infection caused by *Burkholderia pseudomallei*. This organism is present in tropical and subtropical regions where melioidosis is endemic. Before 1995, melioidosis was rare in Taiwan. In 2001, when the annual number of cases of melioidosis in Taiwan was determined to be 1–3 per year from 1996 to 2000, the idea was first proposed that the disease was endemic (*1*).

From July 21 through August 24, 2005, an unusually large number (54) of melioidosis cases occurred in Taiwan. This number exceeded the average case number of 9.4 per year from 2001 to 2004. Since this outbreak appeared to be a common-source epidemic, all persons were suspected of becoming infected from this source at the same time.

To determine this common source, we investigated the role of Typhoon Haitang, which hit Taiwan on July 18 and 19, 2005, and resulted in heavy rainfall. Because the date of this typhoon overlapped the incubation period (1–21 days in most cases) (*2*) and rain is a factor in outbreaks of melioidosis (*3*), Typhoon Haitaing may have been the cause.

All 57 clinical strains of *B. pseudomallei* isolated during this outbreak were typed by pulsed-field gel electrophoresis (PFGE) DNA macrorestriction analysis (*4*). A higher incidence rate (8.86% per million) and clonal diversity (9 PFGE types) of *B. pseudomallei* were observed in the subtropical zone (south of 23.5°N) of Taiwan than in the temperate zone (north of 23.5°N) (0.18% per million and 2 PFGE types) (Table). Because clonal diversity in outbreaks of melioidosis is characteristic of extreme weather (*5*), these data support possible involvement of the typhoon in this outbreak.

Table. PFGE patterns of clinical isolates of *Burkholderia pseudomallei* obtained before and after Typhoon Haitang, Taiwan, 2005*

PFGE types	No. clinical isolates					
	Before (Jan–Jun)			After (Jul–Sep)		
	Subtropical zone, no.	Temperate zone, no.	Total, no. (%)	Subtropical zone, no.	Temperate zone, no.	Total, no. (%)
S1	0	0	0	31	0	31 (57.4)
S1a	0	0	0	1	0	1 (1.9)
S2	0	0	0	0	1	1 (1.9)
S3	0	0	0	3	1	4 (7.4)
S3a	2	0	2 (66.6)	10	0	10 (18.5)
S3b	0	0	0	2	0	2 (3.7)
S3c	0	0	0	2	0	2 (3.7)
S4	0	0	0	1	0	1 (1.9)
S5	1	0	1 (33.3)	0	0	0
S6	0	0	0	1	0	1 (1.9)
S7	0	0	0	0	1	1 (1.9)
Total	3	0	3 (100)	51	3	54 (100)
Incidence rate†	0.52	0	0.13‡	8.86§	0.18§	2.38‡

*Typhoon Haitang hit Taiwan on July 18, 2005. Logistic regression analyses evaluating the associations were conducted with SAS software version 6.12 (SAS Institute, Cary, NC, USA). PFGE, pulsed-field gel electrophoresis.
†Per million population. At the end of June 2005, the population of subtropical counties was 5,753,647, and the population of temperate zone counties was 16,936,127. In 2005, the population at risk for melioidosis in Taiwan was 22,689,774. Data obtained from the Department of Taiwan Internal Affairs.
‡Odds ratio (OR) 17.99, 95% confidence interval [CI] 5.63–57.54, p = 0.0001.
§OR 0.019, 95% CI 0.006–0.060, p = 0.0001.

Because *B. pseudomallei* can grow at a temperature as low as 4°C (*6*) and the possible spread of melioidosis into temperate zones has been reported (*7*), the epidemic distribution of *B. pseudomallei* in the temperate zone of Taiwan is still not clear. Determining the role of Typhoon Haitang in exposing microbes distributed in the soil, as described by Thomas et al. (*8*), may provide evidence of differences in the distribution of *B. pseudomallei* in the soil of subtropical and temperate zones of Taiwan.

Most clones of *B. pseudomallei* in this study were isolated in the subtropical zone of Taiwan, but 2 clones (S2 and S7) that each caused 1 case of melioidosis were found in the temperate zone. The 2 patients infected with the S2 and S7 clones lived ≈200 km north of the boundary between the subtropical and temperate zones and had not crossed this boundary for ≥3 years. Although the incubation period for *B. pseudomallei* may be as long as 62 years (*9*), and the presence of this organism in the temperate zone before Typhoon Haitang cannot be excluded, we believe that these 2 patients are newly infected cases in the temperate zone.

The 2 predominant clones in this outbreak, S1 and S3a, caused 30 and 10 cases of melioidosis, respectively. Since the appearance of predominant clones, a case-cluster of melioidosis been regarded as an indicator of contamination of an environmental source (*5*). This clustering suggests contamination of soil in the subtropical zone of Taiwan with the S1 and S3a clones.

Patients in this outbreak had severe symptoms of melioidosis, including fever (38/54), cough (16/54), pneumonia (12/54), septic shock (9/54), shortness of breath (4/54), and chest pain (2/54). Eleven of the 54 patients died. Because few patients had skin injuries and most (32/54) had a short incubation period of 1–9 days, inhalation may have been the route of transmission. Increased inhalation of *B. pseudomallei* has been reported in cases of melioidosis during heavy monsoonal rain and wind (*3*).

In conclusion, Typhoon Haitang likely had a role in an outbreak of melioidosis in the subtropical zone of Taiwan that showed high incidence rates and clonal diversity of isolates of *B. pseudomallei*. Our findings showed differences in distribution of *B. pseudomallei* in the soil of subtropical and temperate zones of Taiwan. *B. pseudomallei* clones found only in the temperate zone warrant further study to help prevent their spread. Some clones predominant in the subtropical zone may be suitable for vaccine development.

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Instructions for Authors

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.

Human Bocavirus in Infants, New Zealand

To the Editor: In 2005, a parvovirus, subsequently named human bocavirus (HBoV), was discovered in respiratory samples taken from infants and children hospitalized at Karolinksa University Hospital, Sweden, with lower respiratory tract infection (*1*). HBoV has since been identified in infants and children with respiratory illness in >17 countries, at frequencies ranging from 1.5% to >18.0%.

In the past decade New Zealand has experienced increasing bronchiolitis hospitalization rates, currently >70 admissions per 1,000 infants. To determine the contribution of HBoV to New Zealand's bronchiolitis disease prevalence, we tested samples collected from infants hospitalized with community-acquired bronchiolitis (*2*) during 3 consecutive winter epidemics (June to October, 2003; July to October, 2004; and June to October, 2005) in Wellington, NZ, for HBoV by PCR. The Central Regional Ethics Committee approved the study. Written, informed consent was obtained from the parent or guardian.

Demographic, clinical, and laboratory data were collected during hospitalization. Ethnicity of those who ascribe to >1 group was determined by using a national census method that prioritizes ethnicity as follows: Māori>Pacific>Other>New Zealand European. Oxygen requirement was determined to be the best measure of bronchiolitis severity (*2*). Infants who needed assisted ventilation or continuous positive airway pressure were classified severe; those who required oxygen supplementation moderate; and infants who were hospitalized but did not require supplemental oxygen mild.

Nucleic acid was extracted from thawed nasopharyngeal aspirates (stored at 80°C) by using a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Auckland, NZ). The HBoV nonstructural protein (NP-1) gene was amplified by using primers 188F (5′-GAGCTCTGTAAGTACTATTAC-3′) and 542R (5′-CTCTGTGTTGACTGAATACAG-3′) (*1*) with Expand High Fidelity DNA Polymerase (Roche Diagnostics, Basel, Switzerland) for 35 cycles. Products (354 bp) were purified and sequenced from primers 188F and 542R on an ABI3730 Genetic Analyzer by using a BigDye Terminator version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequences were submitted to GenBank under accession nos. EF686006–13.

Alignments of NP-1 gene sequences from nucleotides (nt) 2410–2602, and NP-1 predicted amino acid sequences from amino acids (aa) 1–97 were constructed by using ClustalW version 1.83 (available from www.ebi.ac.uk/tools/clustalw/index.html) and compared with HBoV prototype sequences from GenBank (DQ00495-6). Nasopharyngeal aspirates were also screened for respiratory syncytial virus (RSV) by reverse transcription–PCR (RT-PCR) and nested PCR (*3*) and for human metapneumovirus (*4*), influenza A (H1, H3), and influenza B by RT-PCR (*5*).

Eight (3.5%) of 230 samples, collected from infants hospitalized with bronchiolitis during the 2003–2005 winter epidemic seasons, were positive for HBoV. In 5 HBoV-positive infants no other pathogens were identified, but RSV was detected in 3 (Table). The 8 HBoV-positive infants had a median age of 9.5 months, and the male:female ratio was 1:1. The median length of hospital stay was 5.5 (range 1–16) days.

As expected, because HBoV NP-1 is highly conserved, sequence variation among New Zealand isolates and the prototype Stockholm ST-1 and ST-2 (*1*) NP-1 sequences was limited. Alignments of the partial NP-1 sequence (nt 2410–2602) of New Zea-

Table. Summary of 8 infants with human bocavirus infection hospitalized with bronchiolitis, New Zealand, 2003–2005*

Infant no.	Date admitted	Sex/ age, mo	Ethnicity	Attended daycare?	Length of hospital stay, d	Illness severity	Apnea	Underlying conditions/ comorbitities	RSV subtype	Highest temp., °C	Enteritic symptoms
1	Jul 2003	M/9	Pacific	No	16	Mod	–	–	A	40.1	Diarrhea
2	Aug 2003	F/4	Pacific	No	6	Sev	–	–	B	38.4	Diarrhea
3	Sep 2003	F/11	NZ European	No	1	Mod	–	–	–	38.1	–
4	Sep 2003	F/10	Pacific	No	4	Sev	–	33 weeks' gestation	–	38.3	Diarrhea
5	Aug 2004	M/8	Pacific	No	2	Mod	–	*Haemophilus influenzae* conjunctivitis	–	37.7	–
6	Jul 2005	M/10	Chinese	No	10	Mod	–	34 weeks' gestation, repaired esophageal atresia and tracheomalacia	–	37.7	–
7	Aug 2005	F/9	Pacific	No	9	Sev	+	30 weeks' gestation	A	39.2	–
8	Sep 2005	M/13	NZ European	Yes	5	Mod	–	Hydronephrosis, *Pseudomonas aeruginosa* urinary tract infection	–	37.4	–

*Temp., temperature; Mod, moderate; Sev, severe; –, absent; NZ, New Zealand; +, present.

land isolates with those of ST-1 and ST-2 were identical, except for a G→A change at nt 176 in 2 New Zealand isolates (from infants 5 and 8 years of age), which resulted in a predicted amino acid exchange of S→N at aa 59. In addition, an A→T change at nt 274 in all 8 NZ isolates resulted in a predicted amino acid substitution of T→S at aa 92, a change that has been reported previously in Japanese isolates (*6*).

This study reaffirms previous reports of finding HBoV in a subset of infants with bronchiolitis (*7*). It is also, to our knowledge, the first study of its kind in New Zealand infants, confirming wide distribution of HBoV. In the northern hemisphere, HBoV circulates primarily during the winter months, although it continues circulating until early summer, later than most other seasonal respiratory viruses (*8*). Therefore, this study may underestimate the percentage of New Zealand infants with bronchiolitis whose HBoV test results were positive because sample collection ceased in October (southern hemisphere spring) at the end of the bronchiolitis epidemic. The small number of HBoV-positive infants prevents conclusions concerning ethnicity, coinfection, and bronchiolitis severity.

Although detection of viral nucleic acid by PCR in infants with bronchiolitis does not prove that the virus is the cause of the disease, it raises a hypothesis worthy of investigation. Further studies are required to determine the role of HBoV as a human pathogen. Although coinfection is common, HBoV detection appears to be infrequent in asymptomatic controls (*9*). In our study RSV was detected in 3 (37.5%) HBoV-positive samples. We may have underestimated additional coinfection because we did not test for several respiratory agents, including parainfluenza viruses, rhinoviruses, or the newly discovered coronaviruses.

Finally, HBoV has recently been detected in fecal samples (*10*). Because 3 HBoV-positive infants had diarrhea in addition to bronchiolitis, knowing prevalence of HBoV in fecal specimens from asymptomatic New Zealand children and in those with acute gastroenteritis would be of interest.

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Lyme Disease in Urban Areas, Chicago

To the Editor: Lyme disease is a multisystem illness caused by infection with the tickborne spirochete *Borrelia burgdorferi*. Most infections in the United States occur in the Northeast and upper Midwest, and the midwestern focus now includes Illinois (*1*,*2*). Previously, the greatest risk of contracting Lyme disease in the Midwest was confined to the northernmost states (Wisconsin and Minnesota) and did not encroach into heavily populated areas around the city of Chicago. However, we showed recently that *B. burgdorferi*–infected *Ixodes scapularis* ticks were recovered from sites in Cook and DuPage counties (*3*), but the percentages of infected ticks were low (≤5%). Since that time, however, reports of Lyme disease in Cook County have been reviewed and individual *I. scapularis* tick submissions from Lake County, north of Chicago, have been received. We therefore surveyed new areas north of Chicago (closest was <1 mile from the city limits; farthest was ≈25 miles from the city limits) and examined additional ticks for infection with *B. burgdorferi*.

From December 2006 to May 2007, we collected 172 adult *I. scapularis* ticks from sites to the north and northwest of Chicago (Figure). Adult ticks were collected because nymphal ticks are more difficult to obtain, and the infection rate in adult ticks is similar (*1*). The tick midguts were removed aseptically, inoculated into tubes containing 1 mL of modified Barbour-Stoenner-Kelly medium (*4*), incubated at 35ºC, and examined for spirochetes for up to 3 weeks. Spirochetes were recovered from 21 (32%) of 65 ticks and 40 (37%) of 107 ticks collected from sites in Cook and Lake counties, respectively. In addition, PCR using primers specific for outer surface protein A (*5*) confirmed that the spirochetes were *B. burgdorferi*.

The findings demonstrate that the midwestern endemic focus of

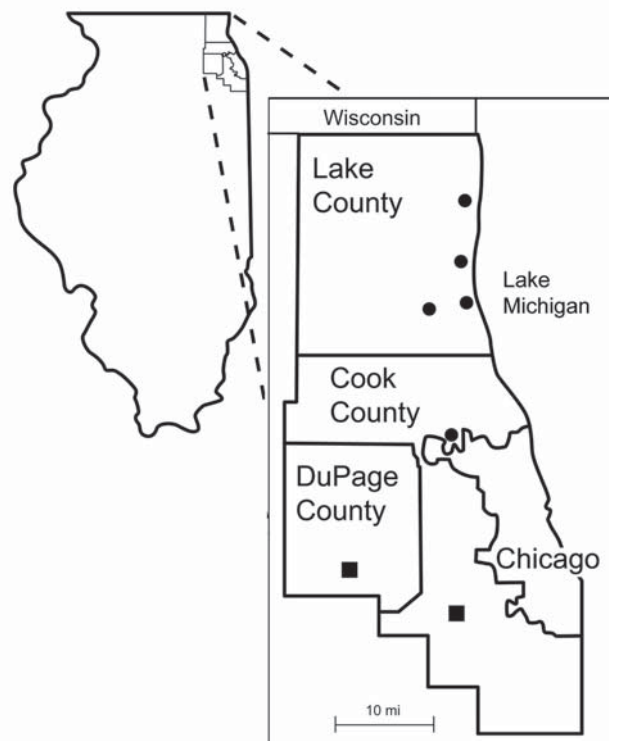

Figure. Sites surrounding Chicago from which *Borrelia burgdorferi*–infected *Ixodes scapularis* ticks were recovered in 2005–2006 (■) and 2006–2007 (●).

B. burgdorferi–infected *I. scapularis* now includes northern Cook and Lake counties. More importantly, the high percentage of *B. burgdorferi*–infected ticks in this region confirms a newly recognized significant risk of Lyme disease in suburban areas adjacent to Chicago (population ≈7 million). Recently, the Infectious Diseases Society of America recommended that clinicians consider prescribing a single prophylactic dose of doxycycline (200 mg) when patients have received tick bites in areas where the percentage of *B. burgdorferi*–infected *I. scapularis* exceeds 20% (*6*,*7*). The high percentage of infected adult ticks identified in this survey highlights the need for physicians in the Chicago area to become familiar with this recommendation, especially considering the high likelihood that nymphal *I. scapularis* ticks are similarly infected (*1*). Moreover, confirmation of the increasing risk of contracting Lyme disease near metropolitan Chicago should provide impetus for more comprehensive studies to completely define the risk of this potentially serious illness.

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Oral Versus IV Treatment for Catheter-related Bloodstream Infections

To the Editor: I read with interest the article by Halton and Graves on the economics of catheter-related blood stream infections (*1*). The most important determinants of infection in a temporary central venous catheter (CVC) are location and duration (*2*). Also important are aseptic CVC insertion and maintenance. Reducing the economic effects of CVCs is important, but I believe clinicians should use oral antimicrobial agents more often in place of intravenous (IV) antimicrobial therapy.

The economic and clinical benefits of using oral versus intravenous antimicrobial therapy are considerable. Oral therapy has important advantages over intravenous therapy administered via CVCs. Clinical advantages of oral antimicrobial therapy include the elimination of phlebitis CVC line infections. At equivalent doses, acquisition costs of oral agents are less than intravenous counterparts. Healthcare institutions charge IV administration fees per antimicrobial intravenous dose. Administrative cost for intravenous antimicrobial agents is US $10/dose. Intravenous antimicrobial administration costs are eliminated when drugs are administered orally. In hospitalized patients, oral antimicrobial therapy results in a decreased hospital length of stay with its attendant economic implications.

Oral therapy for serious systemic infections should be with high bioavailability drugs, i.e., ≥90%, which results in essentially the same serum/tissue levels as if when administered by IV. Because all parenteral antimicrobial agents do not have an oral formulation, clinicians should select an equivalent oral agent with the same spectrum as its parenteral counterpart

to treat most serious systemic infections (*3*). Currently, oral antimicrobial agents are available to treat infections formerly only treatable with intravenous drugs, e.g., vancomycin-resistant enterococci, methicillin-resistant *Staphylococcal aureus*, and *Pseudomonas aeruginosa* (*4*,*5*).

Whenever possible, clinicians should opt for oral therapy instead of IV therapy. Oral antimicrobial therapy is not an initial option in critically ill patients requiring intravenous therapy and in those who are unable to absorb oral drugs. Fortunately, most patients are candidates for oral therapy or intravenous to oral switch therapy.

Substantial savings can be realized by using oral antimicrobial therapy initially or as soon as possible after initial IV therapy. The take-home message is, with the exception of critically ill patients and those unable to absorb oral drugs, clinicians should consider oral therapy before resorting too quickly to IV antimicrobial agents via CVC. Nosocomial (CVC) infections are important from a clinical and economic perspective. Clinicians should consider oral antimicrobial agents more frequently instead of having CVC lines placed for IV drug administration. Currently, oral agents are available to treat nearly all pathogens, even those formerly only treatable with intravenous antimicrobial drugs.

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In Response: In his letter Cunha suggests that oral antimicrobial drug therapy is safer and less expensive than intravenous therapy via central venous catheters (CVCs) (*1*). CVCs are often used in critically ill patients to deliver antimicrobial drug therapy, but they expose patients to a risk of catheter-related bloodstream infection (CRBSI). Our current knowledge about the cost-effectiveness of allocating resources toward interventions that prevent CRBSI in patients requiring a CVC has already been reviewed (*2*). If antimicrobial drug therapy can be delivered orally for some patient groups, instead of through a CVC, then the costs and benefits of this alternate strategy should be evaluated.

Like any decision that involves the reallocation of resources toward a different clinical practice, this decision should not be based on instinct but subjected to a rigorous economic appraisal using a cost-effectiveness framework. The decision requires consideration of all relevant alternative modes of delivery, as identified with the help of clinical experts. Depending on the clinical context, options may include delivery via CVCs or peripheral lines, use of intravenous to oral switch therapy, or oral administration with a variety of dosing schedules.

To identify the most efficient mode of antimicrobial drug delivery, all relevant costs and benefits of each option should be specified and each mode of delivery compared in terms of a common outcome (e.g., the incremental cost per quality-adjusted life year). Financial costs or cost-savings are important, but not the sole consideration for a decision maker (*3*). Having identified the "best" option given our current understanding of the problem, we must then incorporate the residual uncertainty surrounding this choice into the evaluation, explore the level of confidence in the decision, and identify what future research is needed (*4*).

The Centre for Healthcare Related Infection Surveillance and Prevention (CHRISP), Queensland Health, provided funding to the Queensland University of Technology for the development and publication of this research.

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AIDS Vaccine Development: Challenges and Opportunities

Wayne C. Koff, Patricia Kahn, and Ian D. Gust, editors

Caister Academic Press, Wymondham, UK, 2007
ISBN: 9781904455110
Pages: 151; Price: US $99.00

A quarter of a century after AIDS became known as a frightening new disease, substantial progress has been attained on treatments that convert this once certain death sentence into a manageable chronic disease. While some prevention successes have been attained (e.g., screening of the blood supply in industrialized countries), a safe and effective vaccine—the "holy grail" of public health prevention—remains elusive.

In the late 1990s, the first HIV vaccine taken to phase III trial, the VaxGen gp120 vaccine (VaxGen, Brisbane, CA, USA), became the basis for substantial debate and controversy between empiricists (generally public health persons who believed that the urgency of the pandemic required taking some risks, including a potentially low-efficacy vaccine as a first step) and reductionists (generally basic scientists and researchers who felt that the gp120 vaccine was unlikely to work given our state of knowledge and who wanted to wait for a better candidate vaccine). With trial results now available, we know that this vaccine was not efficacious. We also know that a phase III trial, although challenging to organize and conduct among persons at high risk, is doable. What else we do and do not know scientifically is summarized nicely in the 19 chapters of this excellently edited, concise (150 pages), softbound book.

The book is organized into 5 parts: Global Overview; What Does a Vaccine Need to Do?; Preclinical Development: Design Challenges; Clinical Trials; and From Testing to Deployment. Each chapter, written by experts in each field, is impressive in its balance of compactness (3–4 double-sided pages, including references), technical content, and user-friendliness (abstract and conclusion for each chapter make quick review easy).

The authors and editors are to be commended for bringing each of the key topics relevant to HIV vaccines to the reader in a highly accessible form. Key topics include HIV pathogenesis; the twists and turns of what specific knowledge of simian immunodeficiency virus and nonhuman primates is or is not applicable to HIV and humans; and the highly technical nature of modern immunology, virology, and structural biology. The editors were careful to include chapters on important nonscientific aspects of HIV vaccine development, such as clinical site development, regulatory issues, scale-up, and manufacturing.

This book provides an excellent introductory overview for the beginning HIV vaccine researcher or any person who needs a more technical primer on the various aspects of the HIV vaccine challenge. The number of HIV vaccine researchers is now increasing, given the support of several organizations (e.g., Bill and Melinda Gates Foundation) and collaborations (e.g., Global HIV Vaccine Enterprise, the Partnership for AIDS Vaccine Evaluation, and the Center for HIV-AIDS Vaccine Immunology). These organizations, collaborations, and researchers are attempting to better organize the human and technical resources needed to challenge this formidable foe on the scale of the Manhattan Project or the March of Dimes search for a polio vaccine. Let us hope that they will eventually succeed.

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Bird Flu: A Virus of Our Own Hatching

Michael Gerber

Lantern Books, Brooklyn, New York, USA, 2006
ISBN: 159056081
Pages: 465; Price: US $30.00

Bird Flu: A Virus of Our Own Hatching, by Michael Gerber, MD, is written for a nonprofessional audience. A professional audience would quickly put it aside for more factually correct sources of information.

Dr. Gerber is the director of Public Health and Animal Agriculture at the Humane Society of the United States. Much of the book is devoted to criticism of the commercial farming of

birds and other animals; agricultural practices are blamed for the threat of pandemic influenza. He neglects the fact that most of the cases of human infection with influenza A (H5N1) have come from family farms in Asia, rather than the large commercial ventures.

The science relating to the current subtype H6N1 is changing so rapidly that any book is out-of-date by the time it is published. The book contains 90 pages of references, mostly from the popular press. Few current peer-reviewed sources are cited.

The need for authoritative information on avian influenza (H5N1) for the lay public is great, but unfortunately, this book does not meet that need. It focuses heavily on doomsday scenarios and offers little in terms of practical advice to the public. For those interested in the book, it can be found online at www.birdflubook.com.

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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.

A Country Story

Kenneth Fields

"When I was a little girl back in East Texas,"
My mother's mother, Beulah, used to tell,
"There was an outbreak of the German measles,
Mama was pregnant, so I went away
To a neighbor lady's, three or four miles from home
When the first signs showed. I was just eight, and sick,
And lonesome for Mama. One day she came for me.
My little sister had broken out, and Mama
Figuring she would die, and the baby, too,
Wanted us all together for those last weeks.
She wanted me home with her. As it turned out
My sister had been reading by the fire
And broke out from the heat, and it was me
That carried the measles home. After Mama died
I used to think of seeing her out the window
Talking to the neighbor lady on that day,
Crying and wiping her eyes with her apron hem."

Pieter Bruegel the Elder (c. 1525–1569). From The Seasons (1565), Return of the Herd.
Oil on panel (160 cm × 120 cm). Kunsthistorisches Museum, Vienna, Austria

The Panoramic Landscape of Human Suffering

Polyxeni Potter*

The Old Masters were never wrong about suffering, wrote W.H. Auden. They understood how it takes place, "While someone else is eating or opening a window or just walking dully along" (*1*). Auden was referring to the work of Pieter Bruegel the Elder, which dwelled on suffering, along with labor and merrymaking, the lot of simple folk. He painted them with such dedication it earned him the title "Peasant Bruegel."

He so delighted in the behavior of peasants, he disguised himself as one, and went out into the countryside to mingle with them during their feasts and weddings, "... brought gifts like the other guests, claiming relationship or kinship with the bride or groom." He observed "how they ate, drank, danced, capered, or made love, all of which he was well able to reproduce cleverly and pleasantly," wrote chronicler Karel van Mander, "... men and women of the Campine and elsewhere—naturally, as they really were" (*2*). So well did he represent them and through them all of humanity, that in the words of his friend the famed cartographer Abraham Ortelius, "he painted many things that cannot be painted" (*3*).

He was held in high esteem by scholars of his day, among them poet and engraver Dierick Volckherzoon Coornhert, who once was so impressed by Bruegel's work, he wrote, "I examined it with pleasure and admiration from top to bottom for the artistry of its drawing and the care of the engraving…methinks I heard moaning, groaning and screaming and the splashing of tears in this portrayal of sorrow" (*3*).

What we know about the artist comes from Karel van Mander's Painter's Book, published in 1604, some 35 years after Bruegel's death. He was likely born in the late 1520s in Breda (modern Netherlands); lived and worked in Antwerp and Brussels; and apprenticed with sculptor, architect, painter, designer of tapestry and stained glass Pieter Coecke van Aelst, whose daughter he later married. The apprenticeship had little influence on his style but did introduce him to humanist circles and the work of Maria Verhulst Bessemers, his mother-in-law, a skilled miniaturist and illuminator who experimented with tempera on linen (*4*).

After 1559, he dropped the "h" from his name, though his sons, Jan and Pieter the Younger, retained the original Brueghel spelling. Too young at the time of his death to learn from their father, the sons studied with their grandmother and became important artists in their own right, part of a brilliant legacy of four generations in the 16th and 17th centuries.

Like many northern artists, Bruegel traveled to Italy. He visited Naples and Messina and lived in Rome, where he worked with Giulio Clovio, the "prince of miniaturists"

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according to Giorgio Vasari. Inspired by Italian landscape painting, he "did many views from nature so it was said of him when he traveled through the Alps that he had swallowed all the mountains and rocks and spat them out again, after his return, onto his canvases and panels, so closely was he able to follow nature there and in his other works" (*3*). Drawings of the Alpine landscape published as engravings when he returned to Antwerp brought him early fame. They were completed during his long association as draftsman for leading print publisher Hieronymus Cock.

Along with drawing and designing for Cock's engravings, Bruegel continued to paint. He favored multifigure compositions in which groups were seen from above. Some of his paintings recalled the fantastic landscapes of the ever popular Hieronymus Bosch (c. 1450–1516). So successful was the resemblance that humanist Domenicus Lampsonius complimented Bruegel by calling him "a second Bosch." But the Master's interest in the burlesque was brief.

Bruegel came to landscape painting from the tradition of Joachim Patinir and the Netherlandish painters, inventors of the genre, and from the Venetians, whose work so impressed him. But his genius went far beyond these. His compositions, carefully structured and realistic, were spare, ahead of their time in their focus on shape and movement. Intrigued by the workings of nature, he turned away from idealized landscapes. Familiar with the common people, he translated moralizing and proverbial tales into vernacular earthy scenes infused with humor and whimsy. "There was always more than he painted" (*3*).

Landscape painting has been linked to the rise of Antwerp. The city on the Schelde was a prosperous commercial and publishing center. Demand for luxury goods created a flourishing art market, for as Karel van Mander put it, "art gladly resides with wealth" (*5*). Antwerp's guild, in which Bruegel was accepted as Master, boasted 300 artists, at a time when the city supported 169 bakers, 78 butchers, and 75 fishmongers. Landscapes were painted for the open market, and prints were big business.

Unlike many of his contemporaries who struggled to compete, Bruegel was patronized by connoisseurs and earned fame and prestige during his lifetime. Wealthy merchant Niclaes Jongelinck owned 16 of his works. On commission, Bruegel painted for Jongelinck's home a series representing the seasons. Five of likely six panels survive, among them, Return of the Herd, on this month's cover. Though created in the medieval tradition of calendar scenes, each panel focused not on the labors of the season alone but on the transformations of nature and its interrelationships with humans.

The Seasons represents the mature work of a man called by his contemporaries "the most perfect artist of the century" and contains many innovations used to express weather conditions, light effects, and human behavior (*3*). Symbolic color was used to invoke seasonal atmosphere. Precise execution gave way to faster, sketchier, more spontaneous technique, allowing greater naturalness and expression in the figures. Paint was thinner to let underpaint show. Peter Paul Rubens later studied this technique.

Return of the Herd has a circular rhythm linking the foreground with the background, the ritual return of the herd with mountains and gathering clouds. The high-horizon vista of trees, running water, and hills dominates. Yet, tempered by the presence of unvarnished humanity, the cosmographic vision turns parochial. Winter is just around the corner. Humans and animals head for cover.

"Nature herself feared being outdone by Bruegel" (*3*). But his fertile metaphorical terrain, with its rich tonal variations, rhythmical movement, and insuppressible aura of death and regeneration, without parallel in the 16th century, may have found its match in our times. The landscape of emerging infections in our November issue, broad, diverse, and fueled by human activity, rivals Bruegel's in geographic expanse, topographical detail, and the threat of unrelenting human hazard: antimicrobial drug resistance, violent conflict, HIV/AIDS, vectorborne infections, epizootic (H5N1) and pandemic flu, ecologic disasters, community- and hospital-acquired infections. Human suffering, as Auden put it, so well understood by the Old Masters, continues unabated, "... in a corner, / some untidy spot / where the dogs go on with their doggy life and the torturer's horse / scratches its innocent behind on a tree" (*1*).

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Impact of Globalization and Trade in Animals on Infectious Disease Ecology

Effects of Local Anthropogenic Changes on *Anopheles hyrcanus* and *Culex modestu*, Camargue, France

Need for Improved Methods to Collect and Present Spatial Epidemiologic Data for Vectorborne Diseases

Emergence of Methicillin-Resistant *Staphylococcus aureus* of Animal Origin in Humans

Swine Workers and Swine Influenza Virus Infection

Epidemiology and Viral Molecular Characterization of Reemerging Rabies, South Africa

Pig Herds Free from Human Pathogenic *Yersinia enterocolitica*

Fishborne Zoonotic Intestinal Trematodes, Vietnam

Hospitalizations and Deaths Caused by Methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005

Invasive Group A Streptococcal Infection in Older Adults, United States, 1998–2003

Possible Community Transmission of Swine Influenza (H3N2) Infection in a Child, Canada

Marburg Virus Reservoir Hosts

Human Brucellosis in Germany, 1962–2005

African Swine Fever Virus DNA in Soft Ticks, Senegal

Rhodococcus equi Infection After Alemtuzumab Therapy for T-cell Prolymphocytic Leukemia

Complete list of articles in the December issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 3–7, 2007
American Public Health Association Annual Meeting: Politics, Policy and Public Health
Washington, DC, USA
http://www.apha.org/meetings

November 21–24, 2007
Genetics and Mechanisms of Susceptibility to Infectious Diseases
Institut Pasteur
Paris, France
http://www.pasteur.fr/infosci/conf/sb/host_genetics

January 23–25, 2008
International Symposium on Avian Influenza: Integration from Knowledge to Control
Bangkok, Thailand
http://www.biotec.or.th/AIconf2008

February 3–6, 2008
15th Conference on Retroviruses and Opportunistic Infections
Hynes Convention Center
Boston, MA, USA
http://www.retroconference.org

March 16–19, 2008
International Conference on Emerging Infectious Diseases
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

April 8–11, 2008
Genomes 2008 - Functional Genomics of Microorganisms
Institut Pasteur
Paris, France
http://www.pasteur.fr/infosci/conf/sb/genomes_2008

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING
INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://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 (http://www.cdc.gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. 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 of first author—both authors if only 2. 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 of first author—both authors if only 2. 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 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 of first author—both authors if only 2. 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.