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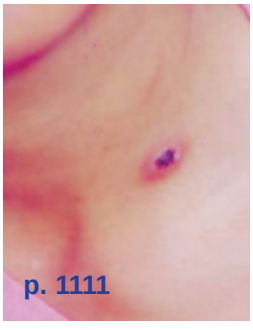
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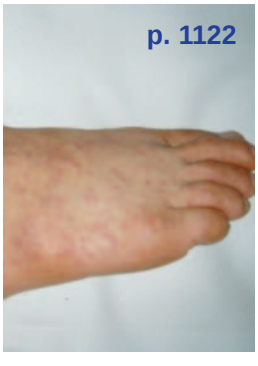
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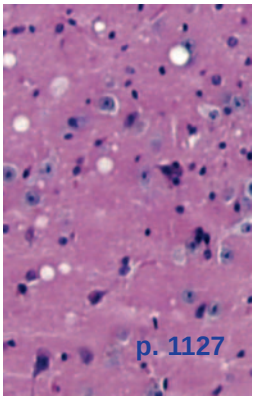

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News & Notes

Emerging Infectious Determinants of Chronic Diseases

Siobhán M. O'Connor,* Christopher E. Taylor,† and James M. Hughes‡

Evidence now confirms that noncommunicable chronic diseases can stem from infectious agents. Furthermore, at least 13 of 39 recently described infectious agents induce chronic syndromes. Identifying the relationships can affect health across populations, creating opportunities to reduce the impact of chronic disease by preventing or treating infection. As the concept is progressively accepted, advances in laboratory technology and epidemiology facilitate the detection of noncultivable, novel, and even recognized microbial origins. A spectrum of diverse pathogens and chronic syndromes emerges, with a range of pathways from exposure to chronic illness or disability. Complex systems of changing human behavioral traits superimposed on human, microbial, and environmental factors often determine risk for exposure and chronic outcome. Yet the strength of causal evidence varies widely, and detecting a microbe does not prove causality. Nevertheless, infectious agents likely determine more cancers, immune-mediated syndromes, neurodevelopmental disorders, and other chronic conditions than currently appreciated.

Infectious agents have emerged as notable determinants, not just complications, of chronic diseases. Not infrequently, infection may simply represent the first misstep along a continuum from health to long-term illness and disability. Preventing or treating infection or the immune response to infection offers a chance to disrupt the continuum, avoiding or minimizing a chronic outcome. To capitalize on these opportunities, clinicians, public health practitioners, and policymakers must recognize that many chronic diseases may indeed have infectious origins.

A diverse spectrum of agents, pathways, outcomes, and co-factors characterize the already well-established causal associations. Together, this group affects all populations around the globe—regardless of country, region, race/ethnicity, socioeconomic status, or culture. Expectations are that additional etiologic relationships will emerge over the coming decades, influenced by ever-evolving populations, ecology, and economies as well as by advances in science and technology (*1*,*2*). The true potential to avoid or minimize chronic disease by preventing or treating infections may yet be substantially underestimated.

Controlling infectious diseases remains paramount to the health and well-being of persons and populations worldwide. The breakdown of public health and prevention measures leads to the resurgence of old and new microbial threats. Nevertheless, implementing and maintaining infection control measures is shifting disease patterns, so that today chronic diseases represent the major health burden of established economies (>90 million people in the United States) and are a rapidly growing burden in developing economies (http://www.cdc.gov/nccdphp/overview.htm) (*3*). This fact implies that preventing or mitigating chronic diseases of infectious etiology could have considerable positive impact on global and domestic health. Add to this the potential benefits of minimizing infections that influence the morbidity of preexisting chronic conditions. The result is a tremendous opportunity to reduce long-term illness and disability worldwide by maximizing infection prevention and control.

In this perspective, we focus on (non-HIV) infectious determinants of chronic diseases, in which ≥1 infectious agent(s) causes, precipitates, or drives the chronic disease or its long-term sequelae. Expanding on previously published discussions (*4*–*7*), we outline the causal connections and reasons for their emergence, describing the breadth of the field and the diverse pathways from microbial exposure to chronic disease. Lastly, we present a complex systems framework for the multifactorial interactions that often lead to long-term sequelae, citing current and emerging opportunities for research to prevent chronic diseases of infectious etiology and discussing the potential impact of these benefits.

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Infectious Disease–Chronic Disease Connections

For centuries, physicians and scientists hypothesized that infection might explain some chronic syndromes. Proof, however, lagged behind speculation. A paucity of tools to detect many agents and the challenges of linking past infection—sometimes decades in the past—with present chronic illness perpetuated the idea that most infectious diseases are acute illnesses, and that chronic diseases have noninfectious causes. By the latter third of the 20th century, however, exceptions to this dogma began to emerge. For example, hepatitis B virus (HBV) infection came to explain a large proportion of chronic liver disease (CLD) and hepatocellular carcinoma (HCC) in areas of endemic infection (*8*) (http://www.cdc.gov/ncidod/diseases/hepatitis). However, it was the discovery that *Helicobacter pylori* can induce gastric inflammation that truly transformed conventional thinking about the noncommunicable nature of many chronic conditions (*9*); in recognition of this groundbreaking achievement, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine 2005. Researchers have subsequently demonstrated that eradication of *H. pylori* can cure most cases of peptic ulcer disease, a chronic condition long attributed to noninfectious factors such as stress, diet, smoking, and family history (*7*,*9*,*10*). Today, scientists and physicians widely recognize the plausibility of infectious agent origins for chronic diseases.

The causal relationships fall into 3 basic categories. First, an infectious agent produces chronic illness or long-term disability through progressive tissue pathology or organ decompensation (e.g., HBV-associated CLD and HCC), attributable to direct effects of past or persistent infection (e.g., transformation of host cells, tissue invasion); or immune response to the persistent infectious agent; or ongoing immune response after the infectious agent(s) is cleared. Second, the initial stages of infection cause permanent, lifelong deficits or disability (e.g., poliovirus-induced permanent paralysis). Third, infection indirectly predisposes a person to chronic sequelae (e.g., maternal infection during pregnancy leads to preterm delivery that, with or without infection of the infant, increases the child's risk for chronic neurologic and pulmonary deficits). Together, these diverse relationships create a cascade of opportunities to reduce the impact of chronic disease by interrupting infection before the outcome is irreversible.

Stimulated by changing scientific perceptions, the advent of polymerase chain reaction (PCR) and other molecular techniques, and advances in immunology and culture methods, a succession of discoveries from 1975 to 1995 greatly expanded the number of recognized infectious determinants of chronic diseases (Figure 1). We now know that HBV and hepatitis C virus (HCV) infections account for most CLD and HCC cases worldwide (*8*). In fact, HCC was the first recognized vaccine-preventable cancer (through HBV immunization). Blood donor screening, along with programs to prevent HBV and HCV transmission, now further reduces the risk for CLD and HCC (http://www.cdc.gov/ncidod/diseases/hepatitis) (*11*–*13*).

Today, immunization against human papillomavirus (HPV) promises to make cervical cancer—the second leading cause of cancer mortality in women worldwide—the next vaccine-preventable malignancy (*3*). Until now, cervical cancer prevention has hinged on early detection and ablation of precancerous and malignant lesions through lifelong Papanicolaou cervical smear screening of all women. While successful where economically feasible, this strategy does not address the infectious etiology of cervical cancer; studies associate HPV with 90% to 99.7% of malignant lesions (high-risk viral subtypes HPV-16 and HPV-18 with 65% to 70% of lesions), and HPV-induced oncoproteins are implicated in the pathway from infection to malignancy (*14*,*15*).

Microbes also cause nonmalignant chronic diseases. For example, *Borrelia burgdorferi* infections can result in chronic Lyme arthritis. In the absence of that discovery, an infectious portion of chronic inflammatory arthritis might still be categorized as a noninfectious autoimmune syndrome; *B. burgdorferi* and *B. garinii* infections also induce the chronic central nervous system manifestations of neuroborreliosis (*16*,*17*). These examples illustrate only a few of the numerous causal associations identified over the past 50 years; yet even they forecast the possibility that many other chronic conditions await the identification of infectious determinants.

Although the pace of discoveries has slowed over the past decade, at least 13 of the ≈39 most recently described infectious agents induce at least 1 distinct chronic syndrome (*1*,*13*,*16*,*18*–*20*). Most recently, a poliomyelitislike paralysis following West Nile virus infection expanded the list (*20*). With ample precedent, researchers, clinicians, and veterinarians can anticipate that infectious determinants of chronic diseases will continue to emerge.

Reasons for Emergence

Evolving ecology and changing human behavior, such as migration, recreation, work, and culture, influence human exposures to the infectious determinants of chronic as well as acute illnesses (*1*,*2*). Microbial virulence factors, wildlife behavioral traits, zoonotic infections, and the environment all converge to determine both the infectious capacity of potential pathogens and the likelihood of human exposure. Superimposed on human genetics and biology, the milieu shapes individual and population risk profiles for the causal infections agents and their chronic sequelae (*7*,*14*,*21*).

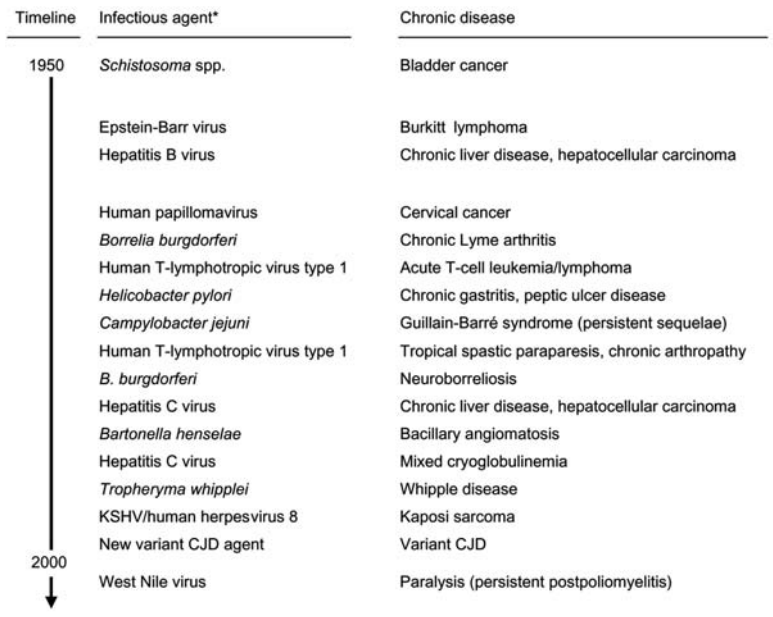

Figure 1. Emergence timeline for infectious determinants of chronic diseases. For references to support this figure, see online version (available from http://www.cdc.gov/ncidod/EID/vol12no07/06-0037-G1.htm)

Over recent years, the powerful tools of molecular biology, particularly PCR, plus advances in immunologic and other techniques, have exposed new causal links by detecting difficult-to-culture and novel agents in chronic disease settings. Microbes can now be irrefutably linked to pathology without meeting Koch's postulates, Hill's epidemiologic criteria, or even the revised criteria of Hill and Evans (*22*). For example, applying recombinant immunoscreening for the first time, investigators cloned the previously undescribed agent of most transfusion-associated (non-A, non-B) hepatitis and the cause of a major portion of chronic hepatitis, HCV (*23*). Innovative sequence-based analysis (broad-range PCR) and phylogenetic relationships finally identified *Tropheryma whipplei* as the elusive microbial source of Whipple disease (*19*,*22*). Improved culture techniques subsequently facilitated propagation of the bacterium. Now evidence confirms neurologic and ocular manifestations of this chronic gastrointestinal syndrome. Representational difference analysis identified the viral cause of Kaposi sarcoma (KS) in HIV-positive gay men (*24*). Later, researchers also linked the KS-associated herpesvirus to endemic or classic KS in the absence of HIV infection.

Today, technical advances boost the armory of detection tools available to uncover new infectious determinants of chronic diseases, including the following: broad-range amplification of bacterial ribosomal targets, gene expression arrays (microarrays) that detect microbes or characterize host response to specific agents, degenerate probe screens for families or groups of viruses, mass spectrometry, electron microscopy, enhanced antigen and antibody detection techniques, and growth-promoting factors that improve microbe cultivation (*1*). The highly successful sensitivity of these tools, however, can be a double-edged sword. Detecting an infectious agent, its nucleic acid, or other biomarkers of infection in the setting of chronic disease does not prove it caused disease. Neither does the presence of antibodies to pathogens, for immunoglobulin G signifies previous infection but not necessarily causation (*22*). This fact is particularly true for ubiquitous infections. For example, chronic Lyme disease, reactive arthritis, CLD or HCC, peptic ulcer disease, cervical cancer, and Chagas cardiomyopathy develop only in some of the many people infected with *B. burgdorferi*, *Chlamydia trachomatis* or *Salmonella* species, HBV or HCV, *H. pylori*, HPV, and *Trypanosoma cruzi*, respectively. In contrast, the inability to detect an agent in the setting of chronic disease does not rule out infectious etiology. Existing tools and methods may not be sensitive enough to link known agents with chronic disease, or they may be unable to detect as yet uncharacterized novel or emerging microbes. Diagnostic assays might not access intracellular, sequestered, or nonreplicating agents. Testing may occur too long after the exposure, particularly when years of pathology precede diagnosis of the chronic condition, or persistent immune response to an already cleared infectious agent accounts for chronic disease. Studies that focus on the wrong group of people or the wrong tissue cannot support or refute causality. In all these circumstances, a true infectious determinant might remain unidentified.

Breadth of the Field

A broad spectrum of infectious agents and their chronic outcomes compose this evolving field. Every

organ system or tissue has been a target. Bacteria, fungi, parasites, viruses, and the recently discovered prions are all implicated, and as yet unidentified etiologic agents will likely be described over the coming years (Figure 2).

Already established causal associations prove that certain infectious agents evoke only 1 type of chronic pathology (e.g., poliovirus-induced persistent flaccid paralysis). Yet single agents can also produce multiple distinct syndromes in different organ systems. HBV-associated CLD, HCC, and polyarteritis nodosa, as well as HCV-associated CLD, HCC, mixed cryoglobulinemia, and arthropathy demonstrate this phenomenon (http://www.cdc.gov/ncidod/diseases/hepatitis) (*13*,*23*,*25*,*26*). So do 3 very different outcomes of human T-lymphotropic virus type 1 (HTLV-1) infection: acute T-cell leukemia/lymphoma, tropical spastic paraparesis/HTLV-1-associated myelopathy, and chronic arthropathy (*27*,*28*). On the other hand, disparate infections sometimes lead to 1 common chronic clinical syndrome, likely through converging pathogenic mechanisms (e.g., chronic HBV and HCV-related CLD or HCC; reactive arthritis following *Salmonella*, *Shigella*, *Klebsiella*, or *Chlamydia trachomatis* infections) (*21*,*23*, *25*,*26*,*29*).

A person's age at the time of infection—from intrauterine or perinatal, through childhood and adolescence, to adulthood and the elder years—may further influence the risk for chronic outcome. For example, perinatal HBV infection dramatically increases the risk of developing adult or pediatric CLD with or without HCC (*11–13*,*30*) (http://www.cdc.gov/ncidod/diseases/hepatitis). Recurrent infections or perhaps serial infections with certain agents might also determine a person's risk for chronic outcome.

Currently, the strength of causal evidence ranges from confirmed to speculative. Reproducible epidemiologic and laboratory data unambiguously establish that certain infectious agents directly lead to 1 or more distinct chronic outcomes, globally or in unique populations. Animal models often illustrate the plausibility of human pathogenesis. Sometimes clinical trials and surveillance further demonstrate that preventing or treating the culprit infection(s) avoids or eliminates the long-term sequelae. Consider HBV-associated CLD. Sound scientific evidence now confirms that immunization and behavioral interventions prevent CLD and HCC by preventing infection and transmission (http://www.cdc.gov/ncidod/diseases/hepatitis) (*11–13*). Similarly, appropriate antimicrobial drug therapy can eliminate group A *Streptococcus* infections before rheumatic valvular disease develops and cure *H. pylori*–associated chronic gastritis and peptic ulcer disease (*7*,*9*,*10*). Unfortunately, the translation of infectious disease knowledge into programs that minimize pathology and the human suffering produced by chronic disease often lags, even when all evidence supports causality.

At the opposite end of the evidence spectrum, only preliminary or inconclusive findings, conflicting or inconsistent data, case series or small studies, anecdotal reports, or unreproduced single-source data support certain hypotheses. A lack of sensitive or specific detection assays, analyses that target the wrong tissue, or investigations that seek infectious agents too long after the initial infection might explain such observations. Suboptimal study designs also hamper the ability to reproduce or compare research results and to correctly infer causality. For example, if investigations examine only persons at low risk, only those at high risk, too few exposed or at-risk persons, or too many people not even at risk for the chronic outcome, then positive or negative findings can produce faulty conclusions. Studies lacking appropriate controls also convey uninterpretable results. On the other hand, evidence for or against an infectious etiology of chronic disease can change over time, influenced by new and sometimes contradictory findings, improved detection tools, and data interpretation. Onchocerciasis is an intriguing example of this fluidity. Infection with the filarial parasite *Onchocerca volvulus* is the long-established cause of river blindness. Recent evidence, however, suggests that the *Onchocerca* endosymbiont bacterium, *Wolbachia wuchereria*, may stimulate the pathogenic inflammation responsible for this

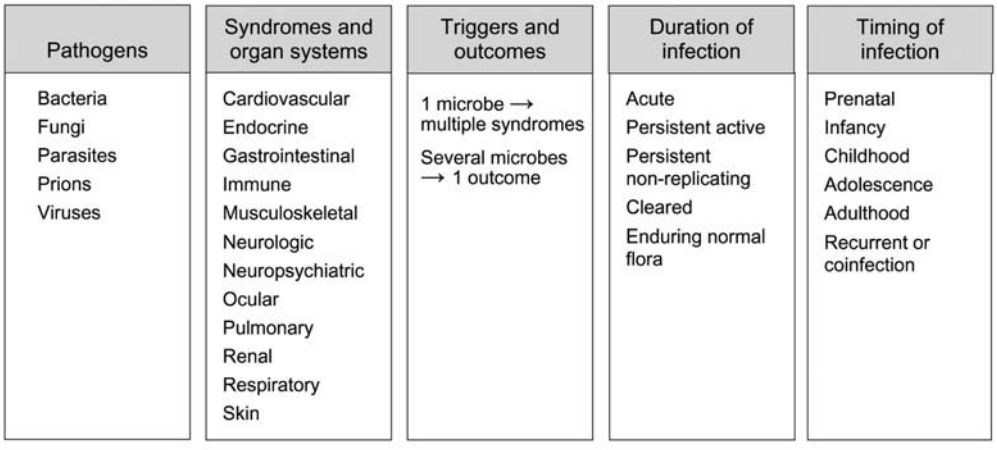

Figure 2. Infectious determinants of chronic diseases.

tragic, preventable lifelong disability (*31*). If so, could *Wolbachia* also influence in whom *W. bancrofti*–associated lymphatic filariasis develops, potentially opening new therapeutic avenues to prevent this major cause of global disability (*32*)?

Despite the challenges, researchers continue to pursue elusive but plausible infectious agent origins of chronic syndromes such as systemic lupus erythematosus, rheumatoid arthritis and other inflammatory arthritides, Crohn disease, type 1 diabetes, multiple sclerosis, neuropsychiatric and developmental disorders, leukemias and lymphomas, and other malignancies (*33*–*44*). In concert, previously unrecognized long-term effects of known infectious agents continue to emerge.

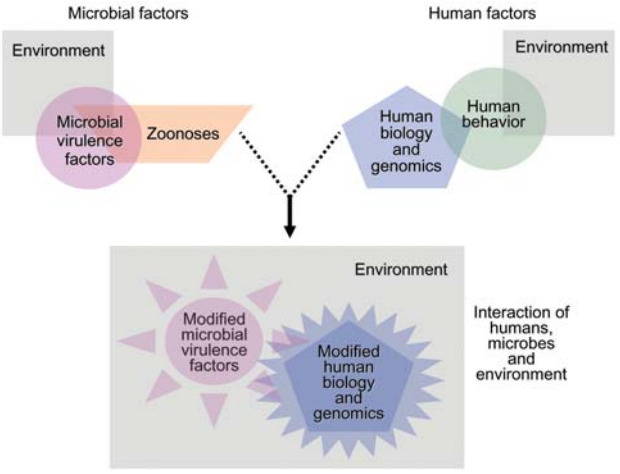

Figure 3. Complex systems framework, showing interaction of multiple factors leading to chronic sequelae of infections.

Range of Pathways

Directly or indirectly, infectious agents produce long-term outcomes through pathways that include acute infection, persistent active infection, persistent nonreplicating (latent) infection, immune response to an infectious agent that may not commonly be pathogenic, and malignant transformation. Direct tissue damage or genomic integration explain certain chronic sequelae, but an inflammatory immune response—one of the body's primary means to protect against infection—defines multiple established infectious causes of chronic diseases, including some cancers (*1*,*5*,*7*,*14*,*15*,*17*,*21*,*23*,*28*,*29*) (http://www.cdc.gov/ncidod/diseases/hepatitis). Inflammation also drives many chronic conditions that are still classified as (noninfectious) autoimmune or immune-mediated (e.g., systemic lupus erythematosus, rheumatoid arthritis, Crohn disease) (*33*–*35*,*38*). Both innate and adaptive immunity play critical roles in the pathogenesis of these inflammatory syndromes (*34*,*35*). Therefore, inflammation is a clear potential link between infectious agents and chronic diseases. Aberrant cellular and humoral responses to infections could launch the continuum from infection to long-term sequelae, consistent with the proposed damage-response framework (*6*).

Biofilms, or microbial communities that behave like biofilms, also represent potential, unrecognized stages in the pathways from infectious agent exposure to chronic disease. In both situations, cultures and even PCR results can be negative. For example, tympanic fluid cultures from animal models of chronic *Haemophilus influenzae* otitis media, associated with biofilms, are frequently negative (*45*); uropathogenic *Escherichia coli* can invade bladder epithelial cells to establish intracellular communities that behave like biofilms, evade immune surveillance, and produce sterile urine cultures (*46*). Similarly, imbalances within communities of normal gut flora or between commensals and pathogens residing in the gut are proposed to produce or exacerbate chronic syndromes such as Crohn disease (*35*–*37*). These observations suggest that novel and already characterized infectious agents are likely to determine a substantially greater—and potentially preventable—portion of chronic disease than yet realized. If so, upstream (earlier) primary and secondary prevention of infection will become opportunities to avoid irreversible or severe chronic disease across large populations.

Frequently, the opportunity to identify new infectious determinants of chronic diseases may lie in the study of complex systems. Chronic diseases are often multifactorial, with established noninfectious risk factors. Yet infection actually defines more than a few of these conditions (e.g., cervical cancer, reactive arthritis). In such settings, complex systems, interactions between human, microbe, and the environment, tempered by time, determine microbial exposure, human infection, and the development of chronic sequelae (Figure 3). Simulating the balance, flux, and networks of multicomponent systems biology, many factors can converge to produce chronic disease, among them genetic susceptibility to infection or to adverse chronic outcome, duration of infection, co-infections, microbial factors, host microbial communities, age, micronutrient status, sex hormones, behavior-dependent exposures such as smoking and diet, chemical exposures, zoonoses, and the strength of an exposed person's immune response to an infectious agent(s) (*1*,*14*,*15*,*17*,*21*,*24*,*25*,*47*–*49*) (http://www.cdc.gov/ncidod/diseases/hepatitis). Human migration or travel, human-human interactions, evolving economies, political change, education, new medical interventions, changes in climate and ecology, and other factors further influence these complex systems.

Also diverging from the usual perceptions of causality, some hypotheses propose that infections may actually protect against certain chronic conditions; some microbial

exposures may be critical to normal human immune development. Perhaps reduced or delayed exposure(s) to an infectious agent(s), or alterations in the balance of normal flora, increase a person's susceptibility to inflammatory conditions like asthma and Crohn disease (*37*,*50*).

Current and Emerging Discovery and Prevention Opportunities

Chronic diseases do often stem from infections. Numerous causal associations are established, and progress in the field is certain to detect and confirm additional links. These developments should lead to new treatment regimens and public health programs that substantially reduce and even prevent chronic diseases worldwide, intervening before or during the early stages of disease to avoid or minimize the chronic sequelae of infections. If a mere 5% of chronic disease is attributable to infectious agents, in the United States alone 4.5 million of the 90 million people living with chronic disease might benefit from strategies designed to prevent or appropriately treat selected infections. Worldwide, the impact could be far greater. Avoiding exposure, reducing transmission, vaccinating to avert infection, and treating infection early could realize this prevention potential, dramatically reducing the global impact of chronic disease measured by disability-adjusted life years or other measures (*51*). The strategies must, however, build on sound scientific evidence.

Continued pathogen discovery and improved detection of infectious agents with sensitive, specific, reproducible assays are crucial to these efforts. In many settings, the systems biology approach will advance the timely recognition, characterization, and mitigation of infectious determinants of chronic diseases (*49*). Combining proteomics, genomics, microarrays, nanotechnology, and mass spectrometry with traditional detection tools such as histopathology may better confirm or refute hypotheses of causation, but only when applied to appropriate specimens from well-designed epidemiologic studies in the appropriate populations (*1*). Advances in information technology will be key to these efforts. The nature of chronic disease further demands longitudinal and prospective assessments since the symptoms of chronic disease may not appear until years after exposure to an infectious agent.

At present, cancers, autoimmune or immune-mediated diseases, and neurodevelopmental disorders are leading candidates for infectious agent origins. Yet other chronic conditions must also remain under consideration. Together, infectious determinants of chronic diseases offer a spectrum of research and prevention possibilities—opportunities that could substantially affect global health by reducing chronic disease worldwide. Not all chronic conditions will have infectious agent roots. Nevertheless, the broad prevention potential presented by these causal relationships has emerged as an important, cross-cutting clinical and public health issue, a result of the increased risk posed by newly recognized agents and changing population exposures as well as an increased appreciation for the causal links.

Dr Siobhán O'Connor has been assistant to the director of the National Center for Infectious Diseases, Centers for Disease Control and Prevention, for Infectious Causes of Chronic Diseases, and is currently coordinator, Linking Infectious Agents and Chronic Diseases, Coordinating Center for Infectious Diseases. Her research interests focus on identifying and preventing recognized and potential infectious determinants of chronic diseases, particularly cancers and immune-mediated syndromes.

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Global Public Health Surveillance under New International Health Regulations

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The new International Health Regulations adopted by the World Health Assembly in May 2005 (IHR 2005) represents a major development in the use of international law for public health purposes. One of the most important aspects of IHR 2005 is the establishment of a global surveillance system for public health emergencies of international concern. This article assesses the surveillance system in IHR 2005 by applying well-established frameworks for evaluating public health surveillance. The assessment shows that IHR 2005 constitutes a major advance in global surveillance from what has prevailed in the past. Effectively implementing the IHR 2005 surveillance objectives requires surmounting technical, resource, governance, legal, and political obstacles. Although IHR 2005 contains some provisions that directly address these obstacles, active support by the World Health Organization and its member states is required to strengthen national and global surveillance capabilities.

On May 23, 2005, the World Health Assembly adopted the new International Health Regulations (IHR 2005) (*1*) as an international treaty. This step concluded the decade-long effort led by the World Health Organization (WHO) to revise the old regulations (IHR 1969) to make them more effective against global disease threats. Originally adopted in 1951 (*2*) and last substantially changed in 1969 (*3*), IHR 1969 had lost its effectiveness and relevance by the mid-1990s, if not earlier (*4*).

The resurgence of infectious diseases noted in the first half of the 1990s showed IHR 1969's limitations. For example, after smallpox was eradicated in the late 1970s, IHR 1969 only applied to the traditionally "quarantinable" diseases of cholera, plague, and yellow fever. In addition, IHR 1969 restricted surveillance to information provided only by governments, lacked mechanisms for swiftly assessing and investigating public health risks, contained no strategies for developing surveillance capacities and infrastructure, and failed to generate compliance by WHO member states. WHO began revising IHR 1969 in 1995 (*5*), and IHR 2005's adoption completed the modernization of this important body of international law on public health.

IHR 2005 departs radically from IHR 1969 and represents a historic development in international law on public health (*6*). IHR 2005 expands the scope of the regulations' application, strengthens WHO's authority in surveillance and response, contains more demanding surveillance and response obligations, and applies human rights principles to public health interventions. The most dramatic of these changes involves a new surveillance system that far surpasses what the IHR 1969 contained. After reviewing key surveillance concepts and frameworks, this article describes IHR 2005's surveillance regime and assesses its likely performance. It concludes by discussing obstacles that could prevent IHR 2005 from becoming an effective global public health surveillance system and addressing how these obstacles might be overcome.

Key Surveillance Concepts and Evaluation Framework

Public health surveillance has been defined as "the ongoing systematic collection, analysis, and interpretation of outcome-specific data for use in the planning, implementation, and evaluation of public health practice" (*7*). A surveillance system requires structures and processes to support these ongoing functions (*7*).

The Centers for Disease Control and Prevention (CDC) developed guidelines that identify the essential elements and attributes for an effective public health surveillance

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system (*8*). According to these guidelines, evaluating surveillance systems involves 2 main steps: 1) describing the purpose, operation, and elements of the system and 2) assessing its performance according to key attributes. This article uses this 2-step approach to evaluate the global public health surveillance system prescribed by IHR 2005.

Surveillance System Specified in IHR 2005

In the CDC framework, describing a surveillance system includes 4 main elements: 1) health-related events under surveillance and their public health importance, 2) purpose and objectives of the system, 3) components and processes of the system, and 4) resources needed to operate it (*8*).

Health-related Events under Surveillance

IHR 2005 identifies health-related events that each country that agrees to be bound by the regulations (a "state party") must report to WHO. In terms of health-related events that occur in its territory, a state party must notify WHO of "all events which may constitute a public health emergency of international concern" (article 6.1). These events include any unexpected or unusual public health event regardless of its origin or source (article 7). IHR 2005 also requires state parties, as far as is practicable, to inform WHO of public health risks identified outside their territories that may cause international disease spread, as manifested by exported or imported human cases, vectors that may carry infection or contamination, or contaminated goods (article 9.2).

IHR 2005 provides guidance to assist state parties' compliance with these obligations in 4 ways. First, IHR 2005 defines a "public health emergency of international concern" (PHEIC) as "an extraordinary event which is determined [by the WHO Director-General]… (i) to constitute a public health risk to other States through the international spread of disease and (ii) to potentially require a coordinated international response" (article 1.1). Unlike IHR 1969's limited scope of application to just 3 communicable diseases (*3*), IHR 2005 defines disease as an illness or medical condition that does or could threaten human health regardless of its source or origin (article 1.1). This scope therefore encompasses communicable and noncommunicable disease events, whether naturally occurring, accidentally caused, or intentionally created.

Second, IHR 2005 contains a "decision instrument" (annex 2) that helps state parties identify whether a health-related event may constitute a PHEIC and therefore requires formal notification to WHO (Figure 1). The decision instrument focuses on risk assessment criteria of public health importance, including the seriousness of the public health impact and the likelihood of international spread.

Figure 1. International Health Regulations (IHR) 2005 decision instrument (simplified from annex 2 of IHR).

Third, IHR 2005 includes a list of diseases for which a single case may constitute a PHEIC and must be reported to WHO immediately. This list consists of smallpox, poliomyelitis, human influenza caused by new subtypes, and severe acute respiratory syndrome (SARS). A second list of diseases exists (Figure 1) for which a single case requires the decision instrument to be used to assess the event, but notification is determined by the assessment and is not automatic. Finally, IHR 2005 also encourages state parties to consult with WHO over events that do not meet the criteria for formal notification but may still be of public health relevance (article 8).

IHR 2005's expansion of the range of public health events under surveillance and the use of risk assessment criteria in deciding what is reportable is possibly the single most important surveillance advance in IHR 2005. This change greatly enhances effective surveillance of emerging infectious diseases, which are "infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range" (*9*). IHR 2005's surveillance strategy, especially the decision instrument, has been specifically designed to make IHR 2005 directly applicable to emerging infectious disease events,

which are usually unexpected and often threaten to spread internationally.

In addition to events that may constitute a PHEIC, IHR 2005 also requires state parties to report the health measures (e.g., border screening, quarantine) that they implement in response to such events (article 6). State parties are also specifically required to inform WHO within 48 hours of implementing additional health measures that interfere with international trade and travel, unless the WHO Director-General has recommended such measures (article 43).

Purpose and Objectives of Surveillance under IHR 2005

IHR 2005's purpose is to prevent, protect against, control, and facilitate public health responses to the international spread of disease (article 2), and IHR 2005 makes surveillance central to guiding effective public health action against cross-border disease threats. The regulations define surveillance as "the systematic ongoing collection, collation and analysis of data for public health purposes and the timely dissemination of public health information for assessment and public health response as necessary" (article 1.1). Surveillance is central to IHR 2005's public health objectives, which explains why IHR 2005 requires all state parties to develop, strengthen, and maintain core surveillance capacities (article 5.1). This obligation goes beyond anything concerning surveillance in IHR 1969, which did not address surveillance infrastructure and capabilities beyond a general requirement for a state party to notify WHO of any outbreak of a disease subject to the regulations.

Components and Processes of IHR 2005 Surveillance

IHR 2005 describes key aspects of the surveillance process from the local to the global level. As part of IHR 2005's core surveillance and response capacity requirements, each state party has to develop and maintain capabilities to detect, assess, and report disease events at the local, intermediate, and national levels (article 5.1, annex 1). Officials at the national level must be able to report through the national IHR focal point to WHO when required under IHR 2005 (articles 4.2 and 6). The regulations also mandate that WHO establish IHR contact points that are always accessible to state parties (article 4.3). Connecting these levels produces the surveillance architecture illustrated in Figure 2.

Requiring that a national IHR focal point be established is another surveillance initiative in IHR 2005. The focal point is designed to facilitate rapid sharing of surveillance information because it is responsible for communicating with the WHO IHR contact points and disseminating information within the state party (article 4.2). By linking national IHR focal points through WHO, IHR 2005 establishes a global network that improves the real-time flow of surveillance information from the local to the global level and also between state parties (article 4.4).

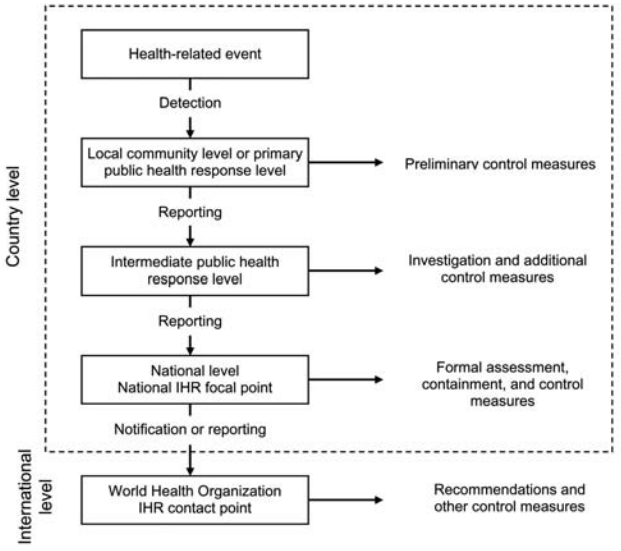

Figure 2. Public health surveillance structures and processes specified in International Health Regulations (IHR) 2005.

Resources Needed to Operate IHR 2005's Surveillance System

Building and maintaining the surveillance system envisioned in IHR 2005 will require substantial financial and technical resources. State parties will be primarily responsible for providing resources needed to develop their core surveillance capacities. Each state party has to assess its ability to meet the core surveillance requirements by June 2009. In addition, each state party has to develop and implement a plan for ensuring compliance with core surveillance obligations (articles 5.1 and 5.2, annex 1).

WHO is obliged to assist state parties in meeting their surveillance system obligations (article 5.3), but this provision does not allocate any WHO funds for this purpose. State parties are required to collaborate with each other in providing technical cooperation and logistical support for surveillance capabilities and in mobilizing financial resources to facilitate implementation of IHR 2005 (article 44.1).

Evaluating the IHR 2005 Surveillance System's Attributes and Potential Performance

Key attributes of effective surveillance systems identified by CDC are usefulness, sensitivity, timeliness, stability, simplicity, flexibility, acceptability, data quality, positive predictive value, and representativeness. Of these

attributes, usefulness, sensitivity, timeliness, and stability will be most critical to the success of the IHR 2005 surveillance system. Simplicity, acceptability, and flexibility will affect the establishment and sustainability of the surveillance system. Data quality, positive predictive value, and representativeness are central to accurately characterizing health-related events under surveillance. Table 1 summarizes these attributes, provides commentary on their relevance to effective surveillance under IHR 2005, and assesses the likely performance of the IHR 2005 surveillance system for each attribute. The following paragraphs concentrate on assessing IHR 2005 with respect to the key attributes of usefulness, sensitivity, timeliness, and stability.

Usefulness of the Surveillance System

The central premise of IHR 2005 is that rapidly detecting PHEIC will support improved disease prevention and control both within and between state parties. Ample evidence shows that delayed recognition and response to emerging diseases may result in adverse consequences in terms of illness and death, spread to other countries, and disruption of trade and travel (*10*). The usefulness of surveillance under IHR 2005 represents the sum of all the critical system attributes and can only be assessed after the system is in operation, so this attribute is not discussed here. However, for the future sustainability and development of IHR 2005, we must evaluate its overall usefulness and document its contribution to prevention and control of adverse health events. IHR includes mechanisms to review and, if necessary, amend its provisions and in particular requires periodic evaluation of the functioning of the decision instrument (article 54).

Sensitivity of the Surveillance System

The IHR 2005 surveillance provisions imply 100% sensitivity as a standard, namely the reporting of all events that meet notification requirements. The use of risk assessment criteria (Figure 1) also allows for higher sensitivity for PHEIC than would be possible with a list of predetermined disease threats (as in IHR 1969). To test the potential sensitivity of the decision instrument proposed in drafts of the revised IHR in 2004, investigators in the United Kingdom applied the then-proposed decision instrument to all events (N = 30) that were important enough to have been published in the national surveillance bulletin for England and Wales during 2003 (*11*). According to this method, 12 of the 30 events would have been reportable under the decision instrument. These events included all those that were considered potential PHEIC. Investigators concluded that the decision instrument was highly sensitive for selecting outbreaks and incidents that require reporting under the proposed IHR revision.

The sensitivity of the IHR 2005 surveillance system will probably be affected by 2 factors. First, in all likeli-

Table 1. International Health Regulations (IHR 2005) assessed according to attributes of public health surveillance systems (adapted from [8])

Attribute	Attribute details	Relevance to IHR 2005
Usefulness	Contribution to prevention and control of adverse health-related events	Design and scope imply improved usefulness compared with IHR 1969, but attribute must be evaluated after IHR 2005 has operated for a period
Sensitivity	Proportion of true events detected by system and ability to detect outbreaks	Specifies notification of all potential public health emergencies of international concern (PHEIC) and provides multiple pathways to increase sensitivity
Timeliness	Speed between steps particularly from event onset to response	Specifies assessment within 48 h and reporting within 24 h by state parties and prescribes immediate reporting of events at local and intermediate levels within state parties
Stability	Reliability and availability of surveillance system	All state parties must notify all potential PHEIC from June 2007 and establish capacity to detect, assess, and report events by 2012, with potential extensions to 2016
Simplicity	Simplicity of structure and ease of operation	Architecture of surveillance system is streamlined and transparent, especially at international level
Flexibility	Ability to adapt to changing information needs and operating conditions	Use of risk assessment criteria means that surveillance applies to new as well as established disease threats
Acceptability	Willingness of persons and organizations to participate	Establishment of surveillance in international law represents commitment by state parties to participate
Data quality	Completeness and validity of recorded data	Specifies information to be reported and includes provisions for validation and assessment of all reports to separate rumors from real events
Positive predictive value	Proportion of reported events that are true events	Oriented toward high sensitivity with correspondingly low specificity and positive predictive value, so WHO will not declare most notified events to be PHEIC
Representativeness	Ability to describe events over time and their distribution by place and person	Likely to be increased after validation and assessment, as for data quality

hood, inadequate capacities at the local and intermediate levels within state parties will limit the system's sensitivity more than capacities at the national level. Second, state parties may not always be willing to comply with their reporting obligations in the face of possible adverse political and economic consequences that may result from alerting the world to a disease event in their territories. Fear of such adverse consequences undermined reporting obligations in IHR 1969.

IHR 2005 incorporates strategies to address these potential limitations. First, as noted above, IHR 2005 requires state parties to build and maintain core local, intermediate, and national surveillance capabilities (article 5.1, annex 1). Fulfillment of this obligation will improve surveillance capacity vertically, from local to national levels, which should support higher sensitivity.

Second, IHR 2005 permits WHO to improve sensitivity by collecting and using information from multiple sources. IHR 1969 only allowed WHO to use information provided by state parties (*3*), and failure of state parties to abide by their reporting obligations adversely affected WHO surveillance activities (*5*). Under IHR 2005, WHO can collect, analyze, and use information gathered from governments, other intergovernmental organizations, and nongovernmental organizations and actors (article 9.1). By permitting WHO to cast its surveillance network beyond information it receives from governments, IHR 2005 creates opportunities for WHO to improve the sensitivity of the surveillance system and avoid being blocked by governmental failure to comply with reporting requirements.

Timeliness of the Surveillance System

Public health practitioners understand how timely notification of public health risks is necessary for effective intervention strategies (*12*,*13*), lessons reiterated in the SARS pandemic (*14*). Timely surveillance is also stressed in connection with strategies to deal with pandemic influenza (*15*,*16*). Timeliness may be the most important attribute that IHR 2005 will have to demonstrate to be effective.

IHR 2005 contains several provisions that relate to timeliness. National-level assessments with the decision instrument must be completed within 48 hours (annex 1, part A, 6[a]). State parties must then notify WHO within 24 hours of assessing any event that may constitute a PHEIC or that is unexpected or unusual (articles 6.1 and 7). The same 24-hour requirement applies to reporting public health risk outside a state party's territory that may constitute a PHEIC (article 9). State parties must also respond within 24 hours to all requests that WHO makes for verification of health-related events in their territories (article 10.2).

Timeliness of reporting is likely to be affected more by actions taken at local and intermediate levels than national-level provision of information to WHO. In this regard, IHR 2005 includes the core surveillance capacity that local and intermediate public health entities must be able to carry out their reporting responsibilities immediately (annex 1).

WHO's ability to draw on a wide array of sources of information, including the Internet and nongovernmental organizations and actors, may enhance the timeliness of the IHR 2005 surveillance system (*13*,*17*). In countries that have less well-developed local, intermediate, and national surveillance systems, nongovernmental sources of information can often provide information faster than governments. Accessing this type of information early and often helps WHO contact countries sooner, which increases the chances of more effective interventions.

Stability of the Surveillance System

The obligations each state party has to build and maintain core capacities in surveillance at the local, intermediary, and national levels, combined with the responsibilities for surveillance WHO has globally, should construct a global surveillance system that will be stable and reliable over time. Recognizing that core capacities at the national level and below will not develop overnight, IHR 2005 gives state parties until June 2012 to develop these capacities (article 5.1). State parties can obtain a 2-year extension on this deadline by submitting a justified need and an implementation plan and can request an additional 2-year extension, which the WHO Director-General has the discretion to approve or deny (article 5.2).

The 5-year grace period, and the possibility of 2-year extensions, was a necessary compromise and reflects the difficulties many developing states will have in improving their surveillance systems. The stability and reliability of the IHR 2005 surveillance system are designed to increase steadily as the grace period and any extensions come to an end.

Potential Obstacles to Achieving IHR 2005 Surveillance System Objectives

Continued lamentations about the weaknesses of public health surveillance nationally and globally (*18*) illustrate that achieving useful, sensitive, timely, and stable surveillance through IHR 2005 will be a challenge for states and the international community. Several potential obstacles, including technical, resource, governance, legal, and political concerns, will complicate and frustrate efforts to improve national and global surveillance capabilities. Table 2 summarizes these potential barriers and possible responses.

Technical Issues

Emerging infectious diseases often create technical challenges for surveillance, even for the most technologi-

Table 2. Barriers to International Health Regulations (IHR) 2005 surveillance effectiveness, and potential responses

Barrier	Description	Potential responses
Technical	Difficulty detecting previously unrecognized pathogens, especially those with asymptomatic transmission	Specialized surveillance approaches such as syndromic surveillance; improved diagnostic technologies; training and support for epidemiology, laboratory, and other staff
Resource	Limited resources for public health surveillance, particularly in developing countries	Systematic global strategy for assessment and development of surveillance and response capacities, particularly in developing countries
Governance	Lack of awareness about limitations of existing surveillance and lack of governance capabilities to develop and manage sophisticated systems	Training and support for public health professionals and managers; periodic surveillance system evaluations; performance monitoring focusing on attributes such as sensitivity and timeliness
Legal	Potential for countries to make reservations to some obligations in IHR 2005 and concerns it may not be consistent with domestic law in some countries	Formulation of reservations to ensure minimal effects on public health surveillance; development of "model" public health legislation that can be adapted for use in many countries
Political	Concern about potential negative effects on trade and tourism from reporting disease events	Strategies to limit excessive responses; fostering a collaborative, measured response to public health emergencies of international concern; awareness of self-defeating effects of withholding information

cally advanced and well-resourced countries. The sensitivity of surveillance systems for new pathogens has historically been limited, particularly if such pathogens presented themselves in unusual or unexpected ways. Recent modeling has shown that the ability to control the spread of a new pathogen is influenced by the proportion of transmission that occurs before the onset of overt symptoms or through asymptomatic infection (*19*). This property explains why diseases such as influenza and HIV may be more difficult to control than smallpox or SARS.

Consequently, surveillance needs to be sufficiently sensitive to detect infectious agents that have not yet resulted in large numbers of diagnosed cases. One approach to this challenge is syndromic surveillance (*20*), but such surveillance has not been effective in detecting emerging infectious diseases early (*21*). In fact, WHO abandoned syndromic surveillance as a strategy for the revised IHR after pilot studies demonstrated that it was not effective (*22*). Improved diagnostic technologies may also help public health authorities identify new pathogenic threats (*23*). Strategies for enhancing reporting processes have been well described (*24*).

Resource Issues

The demands of IHR 2005 surveillance obligations will confront many countries, particularly developing countries, with resource challenges. IHR 2005 does not include financing mechanisms, which leaves each state party to bear the financial costs of improving its own local, intermediate, and national level surveillance capabilities. The obligation on state parties and WHO to collaborate in mobilizing financial resources (article 44) is a weak obligation at best. The lack of economic resources will, if not more vigorously addressed as recommended by the UN Secretary-General (*25*), retard progress on all aspects of the upgraded surveillance system. WHO, in conjunction with the United Nations and the World Bank, could consider developing a global strategy to support the development and maintenance of core surveillance capacities.

Governance Issues

Governance obstacles include managerial and administrative weaknesses in countries from the local to the national level. Few countries have conducted a systematic review of their surveillance systems, and thus most lack detailed knowledge of gaps and limitations in their surveillance infrastructures and how to address these problems (*26*). Only a few states have assessed their ability to detect and respond to emerging disease threats, such as those posed by bioterrorism agents (*27*). The IHR 2005 requirement that each state party assess the condition of its public health surveillance within 2 years of the regulations' entry into force should help countries improve their national governance for surveillance purposes. Again, many states will need external assistance with such work.

Legal Issues

State parties may face legal complications in implementing IHR 2005 within their national legal and constitutional systems. For example, the United States has indicated that requirements of US federalism may affect its compliance with IHR 2005 (*28*). The US position suggests that other countries may also wish to formulate reservations to IHR 2005 to account for the demands of their national constitutional structures and systems of law (*29*). Whether such reservations will undermine the IHR 2005 surveillance system cannot be assessed, but this concern has to be monitored closely as countries determine whether reservations are required under their national constitutional systems. IHR 2005 also specifies that domestic legislation and administrative arrangements be adjusted fully with IHR 2005 by June 2007, or by June 2008 after a

suitable declaration to the WHO Director-General (article 59.3). Helping state parties update their public health law may be technical assistance that industrialized countries can provide.

Political Issues

Questions remain about the level of political commitment countries will demonstrate in implementing IHR 2005. IHR 1969 suffered because state parties frequently failed to report notifiable diseases and routinely applied excessive trade and travel restrictions (*4*). The relevance of such trade and travel concerns was most recently illustrated during the SARS pandemic through China's initial fears that disclosing the pandemic would harm its economy and foreign trade (*30*,*31*). WHO's access to nongovernmental sources of surveillance information reduces the incentives that state parties once had to hide disease events, as was demonstrated during the SARS pandemic (*32*). In addition, IHR 2005 includes provisions that require WHO to recommend, and state parties to use, control measures that are no more restrictive than necessary to achieve the desired level of health protection (articles 17, 43). Uncertainty lingers, however, as to whether these obligations will fare better in terms of state party compliance than similar ones in IHR 1969.

Conclusion

Establishing effective global public health surveillance is at the heart of IHR 2005. Evaluating the surveillance system specified by IHR 2005 is necessary to understand the potential for this new set of international legal rules to contribute to global health governance. IHR 2005 prescribes essential elements of a surveillance system and seeks to achieve the critical attributes of usefulness, sensitivity, timeliness, and stability. These features resonate with other aspects of IHR 2005 that make it a seminal development for global health governance. In May 2006, the World Health Assembly adopted a resolution urging WHO member states to comply immediately, on a voluntary basis, with IHR 2005 in light of the threat posed by avian influenza (*33*).

The task of turning the IHR 2005 vision of an effective global public health surveillance system into reality is daunting. Of the obstacles complicating this challenge, lack of financial resources to upgrade surveillance systems, especially in developing countries, will be the most difficult to overcome. In IHR 2005, public health has been given a governance regime unlike anything in the history of international law on public health. Turning the blueprint detailed in IHR 2005 into functional architecture that benefits all is one of the great public health challenges of the first decades of the 21st century.

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Neisseria meningitidis Sequence Type and Risk for Death, Iceland

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Invasive meningococcal infections are hyperendemic in Iceland, a relatively isolated country in the mid-Atlantic. We performed a nationwide study on all viable meningococcal strains (N = 362) from 1977 to 2004. We analyzed the association of patient's age and sex, meningococcal serogroups, and sequence types (STs) with outcomes. Overall, 59 different STs were identified, 19 of which were unique to Iceland. The most common STs were 32 (24.6%), 11 (19.9%), and 10 (10.2%). The unique ST-3492 ranked fourth (7.7%). The most common serogroups were B (56.4%), C (39.8%), and A (2.2%). Age ($p<0.001$) and infection with a unique ST ($p = 0.011$) were independently associated with increased death rates, whereas isolation of meningococci from cerebrospinal fluid only was associated with lower death rates ($p = 0.046$). This study shows evolutionary trends of meningococcal isolates in a relatively isolated community and highlights an association between unique STs and poor outcome.

Invasive infections caused by *Neisseria meningitidis* (meningococci) cause high rates of illness and death worldwide (*1*–*3*). Meningococci have frequently caused epidemics in Iceland, a relatively isolated community in the mid-Atlantic (*4*,*5*). To more fully understand the phylogeny of meningococcal strains, various typing methods have been used, including serogroup and serotype classifications. Epidemiologic studies have used more discriminating methods, such as multilocus enzyme electrophoresis, based on electrophoretic variation of several chromosomally encoded cytoplasmic "housekeeping" enzymes (*6*). More recently, sequence-based molecular methods have been used to type meningococci. Multilocus sequence typing (MLST) uses neutrally selected housekeeping genes (*7*), which are sequenced with automated equipment (*8*). This method gives all the information obtained by multilocus enzyme electrophoresis and improves on it in several ways (*7*). MLST is not dependent on the researcher's interpretation, and no reference standards are necessary. The data are portable; they are easily stored and transmitted and can therefore be easily compared.

We have generated a population-based registry of invasive meningococcal infections in Iceland since 1975. Iceland is well suited for studies of meningococcal infections, since the population is well defined, patient follow-up information is relatively accessible, and meningococcal isolates dating back to 1977 are stored centrally. We used MLST to study the evolutionary dynamics of invasive meningococcal infections in Iceland during a 28-year period, 1977–2004. The purpose of this long-term, nationwide study was 2-fold: 1) compare Icelandic strains with those circulating globally and 2) study the association between patient demographics, sequence types (STs), serogroups, and outcomes.

Materials and Methods

Setting

Iceland is a 103,000-km^2 island in the mid-Atlantic, with a population of 220,918 at the beginning of the study period and 293,577 at the end of 2004. Every citizen has access to government-based health care. Currently, 2 university hospitals and 14 community hospitals exist in the country. Since 1975, blood cultures for the whole country have been processed at only 3 sites. This study was approved by the National Bioethics Committee of Iceland and the Data Protection Authority of Iceland.

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Case Definitions and Collection of Data

A prospective registry of all invasive cases of meningococcal disease since 1975 has been generated. This registry includes all patients with a diagnosis of infection, confirmed by culture of blood, cerebrospinal fluid (CSF), or joint fluid. It also includes patients with clinical illness compatible with meningococcal disease and a positive culture from a throat specimen or a positive Gram-stain smear, latex agglutination test, or polymerase chain reaction (PCR) of CSF, blood, or joint fluid. The registry also includes information regarding patient age, sex, and residence and location of hospital where treatment was administered. We calculated the death ratio for patients with meningococcal disease during hospitalization or within 4 weeks of diagnosis by hospital records and the national population registry of Iceland (http://www.statice.is/). Imported cases were excluded.

Microbiology

All invasive meningococcal isolates are sent for serogrouping and susceptibility testing at the Department of Clinical Microbiology at Landspitali University Hospital, the national reference laboratory for the country. The oldest invasive isolates in the collection date from 1977. In total, 362 isolates from January 1, 1977, to December 31, 2004, were viable and thus available for further study. Serogrouping was performed by using standard antisera (Difco Laboratories, Detroit, MI, USA). When an unusual relationship was observed between serogroups and STs, serogrouping was performed at least twice. MICs for penicillin, sulfadiazine, and rifampin were measured by using the Etest (AB Biodisk, Solna, Sweden) according to Clinical Laboratory Standards Institute criteria (*9*).

MLST

MLST was performed by determining the nucleotide sequences of 7 housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, *pgm*) as previously described (*8*). Alleles and sequences were assigned by using the MLST database (http://neisseria.org/nm/typing/mlst/). Sequence typing data were analyzed as described previously (*10*). Data were submitted to the MLST database from January 30, 2004, to August 10, 2004, and STs that had not been previously described were assigned a new number. Strains with STs that were found exclusively in Iceland were classified as "unique" in the context of statistical analysis. The allelic profiles were used to study the relatedness of the STs by using the unweighted pair-group method with arithmetic mean (UPGMA). Phylogenetic trees were constructed with the Sequence Type Analysis and Recombinational Tests (START) suite of programs (http://pubmlst.org/software/).

BURST (Based Upon Related Sequence Types, http://pubmlst.org/software/) was also used to examine the relationships within clonal complexes, while the relationships between different clonal complexes were ignored. BURST required allelic profile data only, and these also contained their ST numbers. For MLST data based on 7 loci, a cutoff point of 5 identical loci allows inclusion of strains that belong to a single clonal complex, while excluding those that do not.

Statistical Analysis

The collective term "ST group" was used to differentiate unique STs, defined as strains found exclusively in Iceland, from other STs, which have been described elsewhere. We used the Pearson χ^2 test and the Fisher exact test as appropriate to assess the bivariate relationship between categorical variables, in particular how death rate was related to the other variables, including ST group. Patient age, based on ST group of the isolate and patient status, was compared by using the Mann-Whitney test. To further assess factors associated with death, we performed multivariable logistic regression analysis with death as the dependent variable. Controlling for age, we tested each of the following variables in separate models: sex of the patients, serogroup (B, C, and others), ST group (unique STs vs. other STs), residence (capitol area vs. rural), hospital location (capitol area vs. rural), and finally, we examined the site of the positive bacterial culture in 2 different ways, in 4 categories (blood, CSF only, both blood and CSF, and other sites), and in 2 categories (CSF only vs. all other sites). Variables that remained significant in the model after controlling for age were evaluated in further models to assess independent associations with death. Level of significance was set at $p<0.05$. All tests were 2-tailed. Statistical analysis was performed by using SPSS version 10.5 (SPSS Inc., Chicago, IL, USA).

Results

Epidemiology of Invasive Meningococcal Disease

The number of registered cases in Iceland varied greatly from 1977 to 2004, ranging from 55 cases/year during the epidemic of 1977 to 7–8 cases/year in 1988 and 2003. The average incidence of invasive meningococcal disease during this 28-year period was 7.1 cases/100,000 population/year, but if the epidemic year of 1977 is excluded, it drops to 6.4 cases/100,000 population/year. A detailed description of the study cohort and serogroups of the organisms is given in Table 1. Meningococci were most commonly isolated from CSF only (39.7%). Serogroups varied substantially within the study period (Figure 1).

Sequence Typing of Meningococcal Isolates

MLST was performed on all 362 viable strains, which were responsible for 72.7% of all documented cases of

Table 1. Description of the patient cohort

Parameter	No. (%)
Patients	362
Male	185 (51.1)
Female	177 (48.9)
Children*	244 (67.4)
Adults	118 (32.6)
Strain isolated from cerebrospinal fluid only	144 (39.8)
Strain isolated from blood only	105 (29.0)
Strain isolated from cerebrospinal fluid and blood	78 (21.5)
Strain isolated from joint fluid	10 (2.8)
Strain grown from throat culture†	25 (6.9)
Serogroup A	8 (2.2)
Serogroup B	204 (56.4)
Serogroup C	144 (39.8)
Serogroup Y	3 (0.8)
Serogroup W135	3 (0.8)

*<16 years of age at the time of diagnosis.
†Positive throat culture in the setting of invasive meningococcal disease, diagnosed clinically.

invasive meningococcal disease in the country during the study period. Overall, 59 STs were observed. A summary of the MLST results is given in Table 2, and the association between STs and serogroups is shown in Table 3. Missing isolates were predominantly from the first 2 years of the study. During the epidemic of 1977, pathogens were genetically homogenous, however, as all strains were ST-10 (Table 2).

Strains of 8 different STs caused 75% of all infections. ST-32 was most common, causing 24.6% of all cases. It was predominantly of serogroup B and endemic during almost the entire period. ST-32 also caused a small epidemic in the country in 1993 and 1994. The second most common ST was ST-11 (19.9% of all cases), which was predominantly of serogroup C. It was first seen in Iceland in 1989 and was the main culprit in invasive meningococcal disease from 1999 to 2002. ST-10 caused 10.2% of all infections and dominated during the first 3 years of the study, but it has not been seen since 1983. In 1983, a new type emerged, ST-3492 from the ST-41/44 complex; it was the fourth most common ST in Iceland overall and caused 7.7% of all infections. ST-3492 was predominantly serogroup C. This ST was the most common cause of invasive disease in 1989 and 1990 but disappeared after 1996.

During the study, 19 STs that were unique to Iceland were described, and these accounted for 14.6% of all invasive infections. Most of these emerging STs (14 of 19) caused only single infections (3.9% of all episodes). The remaining 5 STs caused 10.8% of all invasive disease in the country.

In general, good concordance was seen between STs and serogroups. Nevertheless, isolates exhibiting both serogroup B and C capsules were observed among the 4 most common STs (Table 3).

Dendrogram and Clonal Complexes

The phylogenetic tree of isolates in this study is shown in Figure 2. All STs that were encountered in ≥3 clinical cases and all new STs are shown. ST-2148 was remarkably similar to ST-32, differing only at 1 locus. This clone emerged in 1999 and, like ST-32, was found in both serogroups B and C. ST-11 was most closely related to ST-8 and ST-10, which had 4 and 3 genes, respectively, in common with ST-11. However the ST-8 and ST-10 clones had different serogroups. The relationships within clonal complexes and their association with death is shown in Table 4. We identified 9 complexes and 17 singletons; most isolates (26.5%) fell within group 2, in which ST-32 was the ancestral strain.

Routine vaccination was initiated among children and young adults (<18 years of age) in late 2002 with a conjugated meningococcal vaccine against serogroup C (NeisVac-C, Baxter, Orth/Donau, Austria); >90% of all Icelanders <18 years of age were vaccinated. Three years later, no evidence has been seen for capsule switching.

Antimicrobial Drug Susceptibility

All meningococcal isolates were susceptible to penicillin (MIC 0.012–0.125 μg/mL). All strains were also susceptible to rifampin (MIC 0.008–0.19 μg/mL) (*9*). In contrast, 148 isolates (40.9%) were resistant to sulfadiazine (MIC >8 μg/mL). Most commonly, these meningococci were ST-32, ST-1, and ST-11.

Patient Outcomes

During the 28-year study period, 31 (8.6%) of 362 patients died after the infection. Higher case-fatality ratios were associated with higher age (p = 0.001), but no significant difference was seen between men and women (p = 0.953), residents in the capitol area and rural areas (p = 0.259), or patients who received treatment in hospitals in the capitol area versus in rural hospitals (p = 0.239). When

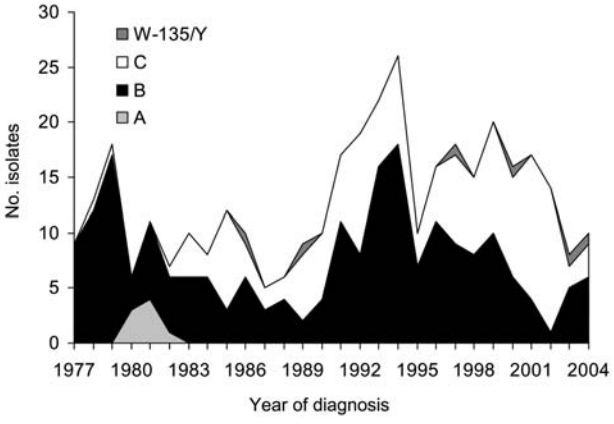

Figure 1. Annual number of invasive meningococcal isolates by serogroup, Iceland, 1977–2004.

Table 2. Epidemiology of invasive meningococcal disease in Iceland*

Year (no. cases)	No. isolates	Sequence type (ST)																			
		1	8	10	11	32	40	41	44	60	162	206	275	1314	1323	*2148*	2266	*3435*	*3464*	*3492*	Other STs
1977 (55)	9			9																	
1978 (21)	13			10																	334, 2986, *3333*
1979 (25)	18			13		1												2			13, 1011
1980 (16)	6	3		1																	*3501*, *4013*
1981 (18)	11	4		2		2															1423, 2320, *3471*
1982 (10)	7	1		1		2								1	1			1			
1983 (19)	10			1		2							1	2	2					1	43
1984 (13)	8					6									1				1		
1985 (13)	12									2		1			3				1	3	1328 (×2)
1986 (11)	10					1				2		1			2					3	23
1987 (9)	5											1	2	1					1		
1988 (7)	6					1				2				1						2	
1989 (14)	9				1									1						5	785, *3502*
1990 (17)	10					2						1		1						4	2843, *3508*
1991 (18)	17				1	5					1	2		1						3	1015, 1154, *3509*, *5119*
1992 (21)	19				4	4						3	1	1						3	34, *3710* (×2)
1993 (23)	22				5	13		2	1											1	
1994 (30)	26		1		5	16								1						1	286, *3756*
1995 (14)	10				2	5			1											1	46
1996 (17)	16		1		3	8								1						1	43,467
1997 (20)	18		6		2	5			1	1											22, 944, 1671
1998 (16)	15		4		3	7											1				
1999 (20)	20		1		10	4		1								1					352, *3757*, *3758*
2000 (18)	16		3		6	2					1					1					23,34,*3759*
2001 (19)	17				12	1			1							1					33, 1943
2002 (16)	14				13												1				
2003 (8)	8				2						1						3				1163, *3334*
2004 (10)	10				3	2	3														1281, *4178*
Total	362	8	16	37	72	89	3	3	4	7	3	9	4	11	9	3	5	3	3	28	45

*The total number of cases is shown with the number of ST isolates available for multilocus sequence typing. Unique STs, those found exclusively in Iceland, are shown in *italics*. The most prevalent STs for each year are shown in shaded cells.

results were analyzed by source of culture, patients with a positive culture from CSF only had significantly lower death ratios than other patients in the cohort (4.2% vs 11.5%, p = 0.02). These patients were younger than the remainder of the cohort, and as a result, this difference was of borderline significance when age was corrected for (p = 0.059).

The association between the most common STs, serogroups, and patient outcomes is summarized in Table 3. Unique STs were more frequently found in isolates with serogroup C capsule (19 of 204 with B, 33 of 144 with C, and 1 of 13 with other serogroups; p = 0.001). Death was not associated with particular serogroups, however (14 of 204 with B, 14 of 144 with C, and 3 of 14 with other serogroups, p = 0.138). The case-fatality ratio among patients infected by meningococci with previously described STs was 7.1% (22/309) compared to 17.0% (9/53) of patients infected by unique STs (p = 0.03). No significant difference was found between the age of patients with unique and previously described STs (p = 0.686) and source of isolates among patients with unique and previously described STs (p = 0.511).

We then performed multivariable logistic regression analysis to study the association of these parameters with outcome, age and sex of the patient, residence, location of hospital, source of positive culture, serogroup of the isolates, and ST group (unique STs in comparison to other STs). In the final model (Table 5) 3 parameters were independently associated with outcomes. Higher age was highly significantly associated with death, followed by infection with a unique ST. Isolation of meningococci from CSF only was associated with lower case-fatality ratios. Age and ST group remained significant when epidemic cases from 1977 were excluded from the analysis (data not shown).

Discussion

To our knowledge, this population-based, longitudinal study is the first of its kind to examine the molecular epidemiology of all viable invasive meningococcal strains by using MLST. The 28-year observation period started in 1977, during an epidemic of meningococcal disease in Iceland. The well defined population of Iceland, with excellent follow-up information on patients and its relative

Table 3. Association between meningococcal sequence types (STs), serogroups, and death, Iceland, 1977–2004*

ST	Serogroup A		Serogroup B		Serogroup C		Total	
	No. isolates	No. deaths	No. isolates	No. deaths	No. isolates	No. deaths	No. isolates	No. deaths (%)
32			86	7	3		89	7 (8)
11			5		67	6	72	6 (8)
10			36	1	1	1	37	2 (5)
3492			2		26	4	28	4 (14)
8					16		16	
1314			11				11	
206			1	1	8		9	1 (11)
1323					9		9	
1	8						8	
60			7				7	
2266			5				5	
44			4				4	
275			4	1			4	1 (25)
40			3				3	
41			3	1			3	1 (33)
162			3				3	
2148			2	1	1		3	1 (33)
3435			3	2			3	2 (67)
3464					3		3	
23							2†	1 (50)
34			1		1		2	
43			1		1		2	
1328					2	1	2	1 (50)
3710			2				2	

*All STs that were encountered more than once are shown. Blank cells indicate zero values. Unique STs are shown in *italics*; 19 unique STs were found. Of 362 cases, 53 were caused by strains with unique STs and 309 by other STs.
†Both isolates were serogroup Y.

isolation make it an ideal setting for studies of this nature.

In the current study, the 362 isolates had 59 different STs, and of those, 19 were exclusively found in Iceland. These unique STs accounted for 14.4% of all infections during the 28-year period, and ST-3492 was by far the most common.

Although both long-term studies and population-based studies are lacking, other investigators have used MLST to study selected meningococcal strains from individual countries (*8*,*11*–*13*). For example, Murphy et al. analyzed 56 Irish meningococcal strains by this method, collected during a 4-year period. Of the invasive isolates, 26 different STs were identified, including 5 new ones (*12*). Takahasi et al. found 65 different STs among 182 isolates, 42 of which were unique to Japan, in a survey of Japanese strains (*13*). The distribution of some STs therefore seems to be fairly restricted geographically, which is also manifested by the fact that 41.1% of the Icelandic isolates characterized in this study are exclusively associated with Scandinavia.

ST-32 was the most common type found in Iceland, causing almost one fourth of all infections. It has been reported to cause numerous outbreaks worldwide and has a tendency to cause hyperendemic disease, particularly septicemia with a high death rate (*12*). However, in our study, the case-fatality ratio in patients infected by ST-32 did not differ from that in patients infected by other STs. The second most common type, ST-11, caused most cases of serogroup C disease during the second half of the study. This type has also been reported in several countries, with a propensity to spread rapidly once introduced into the population (*8*,*12*,*14*). The third most common type, ST-10, was mostly serogroup B. It was the primary cause of the meningococcal outbreak in 1977, but it disappeared after 1983. The most closely related type, ST-8, was first detected in the country more than a decade later, but this ST uniformly belonged to serogroup C. ST-3492 was the fourth most common type; it had not been described previously. It was almost uniformly serogroup C, with a tendency to cause worse outcomes.

By comparing the 7 housekeeping genes used in MLST, a close relationship between ST-11, ST-8, and ST-10 was observed. However, ST-11 and ST-8 are primarily serogroup C, whereas ST-10 is primarily serogroup B. These results could indicate genetic transfer and possible capsular switch. Analysis of genetic relatedness also shows a close relationship between ST-206 and ST-3492, which were most commonly serogroup C. However, both ST-206 and ST-3492 are part of the ST41/44 complex, which is predominantly associated with serogroup B meningococci, thus highlighting genetic transfer between closely related STs.

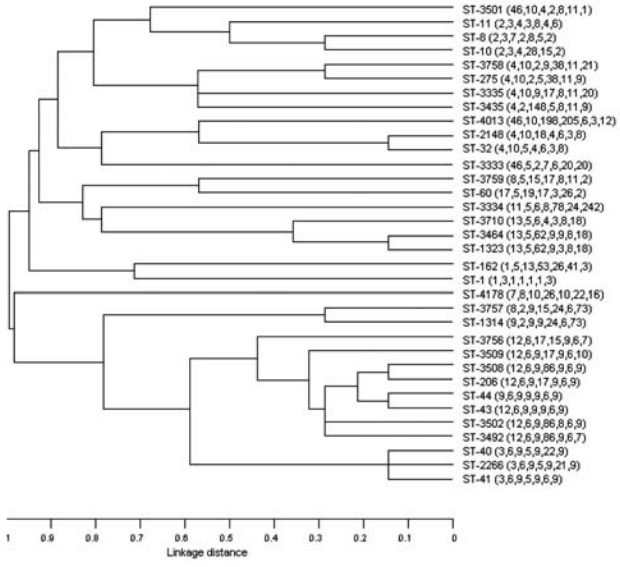

Figure 2. Phylogenetic relationships of invasive meningococcal isolates in Iceland, 1977–2004. Unweighted pair-group method with arithmetic mean (UPGMA) was used to construct the tree with the Sequence Type Analysis and Recombinational Tests (START) suite of programs. Sequence types (STs) and allelic profiles are given on the right. All STs that were encountered in ≥3 clinical cases and all new STs are shown.

All of our strains were susceptible to penicillin. This contrasts with the situation in southern Europe, in particular, where resistance is increasingly reported (*15*–*17*). Likewise, none of the isolates in our study exhibited resistance to rifampin, which still seems to be rare (*18*).

To our knowledge, this is the first study to look at associations between STs and patient outcomes. By multivariable analysis, age and infection by a unique ST were independently associated with higher death rates. Lower death rates were observed among patients with a positive culture from CSF only than among other patients. Age has previously been shown to be associated with worse outcome in patients with meningitis (*19*), but infection with a novel or unique ST has not. At least 2 potential explanations could explain this difference. First, these strains likely represent evolutionary changes within the meningococcal population; therefore, a lower level of immunity against the unique STs within the population could translate into greater disease severity. In the case of pneumococcal infections, for example, the spread of clonal types can be influenced by herd immunity (*20*). Second, the difference in outcomes may indicate greater virulence of unique STs. Although data on this topic are lacking for meningococci, Sandgren et al. have shown that pneumococci with identical serotypes but different clonal types can have different invasive potentials (*21*,*22*). We therefore propose that meningococcal expression of virulence traits, other than the capsule type, may be linked to certain STs. Indeed, recent data suggest that serogroup C capsule expression may contribute to the invasive character of ST-11 meningococci (*23*). A more detailed analysis of virulence properties of specific meningococcal STs, including capsule expression, and their association with clinical characteristics is warranted. Judging from clinical experience, increased awareness during meningococcal epidemics may

Table 4. Association between *Neisseria meningitidis* CC, ST, and patient deaths, Iceland, 1977–2004*†

CC	ST	Deaths (freq)	SLV	DLV	SAT
1	10	2 (37)	0	1	0
	8	0 (16)	0	1	0
2	**32**	**7 (89)**	**3**	**1**	**0**
	34	0 (2)	1	2	1
	1015	0 (1)	2	2	0
	2148	1 (3)	1	2	1
	33	0 (1)	3	1	0
3	*3756*	0 (1)	0	1	14
	3492	4 (28)	1	6	8
	43	0 (2)	3	5	7
	44	0 (4)	1	5	9
	46	0 (1)	1	2	12
	206	**1 (9)**	**4**	**4**	**7**
	3509	0 (1)	1	4	10
	41	1 (3)	3	4	8
	2266	0 (5)	2	1	12
	2320	0 (1)	0	1	14
	1423	0 (1)	0	1	14
	3502	1 (1)	1	4	10
	3508	0 (1)	4	4	7
	1328	0 (2)	0	1	14
	2843	0 (1)	1	4	10
	40	0 (3)	2	1	12
4	1314	0 (11)	0	1	0
	3757	0 (1)	0	1	0
5	467	0 (1)	0	1	0
	13	0 (1)	0	1	0
6	**275**	**1 (4)**	**3**	**1**	**1**
	1163	0 (1)	2	1	2
	1671	0 (1)	1	2	2
	3758	0 (1)	0	1	4
	352	0 (1)	2	2	1
	3435	2 (3)	0	1	4
7	1154	0 (1)	1	0	0
	60	**0 (7)**	**1**	**0**	**0**
8	*3710*	0 (2)	0	2	1
	1323	0 (9)	1	2	0
	3464	0 (3)	1	0	2
	334	0 (1)	0	2	1
9	*3501*	0 (1)	1	0	0
	2986	0 (1)	1	0	0

*CC, clonal complex; ST, sequence type; freq, frequency of isolates with the ST; SLV, single locus variant; DLV, double locus variant; SAT, satellite.
†We identified 9 clonal complexes and 17 singletons (11, 286, *5119*, 162, 1, *3759*, 23, 1943, 22, 944, *3471*, *3333*, 1011, 785, *3334*, *4178*, and *4013*), defined as strains that do not fit in any group in the collection. Some groups have an ST that is the ancestral strain for that group (shown in **boldface**). Unique STs are shown in *italics*.

Table 5. Multivariate analysis of death rate in patients with invasive meningococcal disease, Iceland, 1977–2004*

Variable	OR (95% CI)	p value
Age	1.031 (1.016–1.048)	0.0001
Unique ST†	3.225 (1.311–7.934)	0.011
Positive CSF culture‡	0.381 (0.147–0.984)	0.046

*OR, odds ratio; CI, confidence interval; ST, sequence type; CSF, cerebrospinal fluid.
†Infection with an isolate with an ST unique to the country, in comparison to other, previously described STs.
‡Positive CSF culture only, in comparison to all other sources of positive culture, including blood only and blood and CSF.

speed diagnosis and improve prognosis, which could bias our results since an epidemic of meningococcal infections was ongoing in 1977, when this study began. The epidemic was primarily caused by ST-10, an "old" ST, which accordingly could be associated with lower death ratio. However, the 2 risk factors for poor outcome remained significant even when epidemic cases were excluded, which argues against this hypothesis. When the outcomes were analyzed by source of the isolate, having a positive culture from CSF only was associated with lower risk for death. Although patients with CSF isolates were younger, this parameter remained significant when we corrected for age and ST category of the isolate. We do not have detailed information regarding patients' clinical signs and symptoms. Nevertheless, this part of the cohort most likely represents patients with meningitis, who generally have lower death ratios than do those with sepsis.

One limitation of the study is that submission date of MLST data ultimately determined whether we classified STs as old or new, which may bias the results. However, most data were submitted within a relatively short period, which should minimize this risk. As a result, more than a year from the original submission of the data (December 2005), we checked whether subsequent isolates with these novel STs had been identified, and none were found. Since routine vaccination was implemented in Iceland, meningococcal C disease has only been seen among unvaccinated adults. The rise in serogroup B is of concern, but a longer observation period is required before a conclusion can be reached regarding the issue of serogroup replacement.

In summary, this long-term, nationwide study looked at evolutionary trends of invasive meningococcal isolates in a well-defined setting, where invasive meningococcal disease has been hyperendemic. Although the most common STs have been described previously, we describe a high number of emerging STs. In particular, one ST, unique to Iceland, ranked fourth in prevalence. This study highlights the interplay between epidemiologic and evolutionary processes, which ultimately may produce unique meningococcal strains that lead to worse outcomes. More studies on virulence properties and host immunity are warranted to advance preventive strategies against meningococcal disease.

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Tickborne Arbovirus Surveillance in Market Livestock, Nairobi, Kenya

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To identify tickborne viruses circulating in Kenya and the surrounding region, we conducted surveillance at abattoirs in Nairobi, Kenya. Species of ticks collected included *Rhipicephalus pulchellus* (56%), *Amblyomma gemma* (14%), *R. appendiculatus* (8%), *A. variegatum* (6%), and others. A total of 56 virus isolates were obtained, 26 from *A. gemma*, 17 from *R. pulchellus*, 6 from *A. variegatum*, and 7 from other species. Virus isolates included Dugbe virus (DUGV), an unknown virus related to DUGV, Thogoto, Bhanja, Kadam, Dhori, Barur, and foot-and-mouth disease (FMDV) viruses. This is the first report of Dhori virus isolation in East Africa and the first known isolation of FMDV associated with tick collection. Our results demonstrate the potential for tickborne dissemination of endemic and emergent viruses and the relevance of *A. gemma* in the maintenance of tickborne viruses in this region.

Viruses transmitted by blood-feeding arthropods (arboviruses) are responsible for some of the most serious emerging infectious disease problems facing the world today. Arthropodborne viruses constitute the largest biologic group of vertebrate viruses. Their considerable number and diversity suggest that arthropod vector transmission offers distinct survival benefits for the virus. Approximately 50% of arbovirus isolations from field-collected arthropods are from mosquitoes, and 25% are from ticks; however, this difference may represent a sampling bias, since many more mosquitoes are collected and tested for virus than ticks. To investigate the abundance of tickborne arboviruses in Kenya and the surrounding region, we collected and tested ticks infesting livestock driven to market at 2 major abattoirs in Nairobi, Kenya. These abattoirs receive the bulk of animals slaughtered for Nairobi and its environs, which is the largest livestock market in the country. Approximately 30% of animals slaughtered in these abattoirs come from within Kenya; the rest are from neighboring countries, including Ethiopia, Sudan, Somalia, and Tanzania.

Among pastoral communities in this region, livestock are frequently maintained in enclosures close to human habitation, and small ruminants sometimes sleep inside houses overnight for security reasons. Such practices increase the potential for zoonotic virus transmission between animals and humans. Poor husbandry and grazing practices put great pressure on land resources, which results in the need to continuously move large numbers of animals, especially cattle, in search of pasture. In some parts of East Africa, these pastoral communities exist near wildlife parks, and wildlife and livestock sometimes mix, which allows transfer of ticks and possibly viruses between these animal groups. Additionally, livestock marketing practices allow movement of animals across borders in the region, which allows ticks and tickborne viruses to move between countries.

Previous surveillance reports based on virus isolations or serologic studies in cattle from Kenya, the Central African Republic, and South Africa have identified tickborne arboviruses from the *Bunyaviridae*, *Flaviviridae*, *Rhabdoviridae*, *Reoviridae*, and *Orthomyxoviridae* (*1–4*). The genus *Nairovirus*, family *Bunyaviridae*, includes 37 named viruses that are principally tickborne (*5–7*). The most serious human pathogen among the tickborne viruses in the African region is Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the *Nairovirus* genus that can cause fatal hemorrhagic disease (*8,9*). Outbreaks of

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Crimean-Congo hemorrhagic fever have occurred in People's Republic of China, South Africa, Pakistan, and Russia (*10–12*). The first reported human case of this disease in Kenya occurred recently at a farm that was heavily infested by ticks (*13*). Nairobi sheep disease virus (NSDV), also in the genus *Nairovirus*, causes fever, hemorrhagic gastroenteritis, and abortion in sheep and goats in East Africa (*14*). Epizootics of NSDV have been reported in parts of Africa where susceptible herds of sheep have been moved to NSDV-endemic areas, resulting in decimation of whole herds (*14*). Dugbe virus (DUGV), another member of the *Nairovirus* genus, has been repeatedly confirmed in tickborne virus surveys in Africa and causes febrile illness and thrombocytopenia in humans (*2*). Bhanja virus (BHAV), an unassigned member of the *Bunyaviridae* family, has also been isolated in this region (*1,2*). Other tickborne viruses present in Africa include Thogoto virus (THOV) (genus *Thogotovirus*, family *Orthomyxoviridae*), isolated in Kenya; Barur virus (family *Rhabdoviridae*), isolated in Somalia; and Kadam virus (KADV) (genus *Flavivirus*, family *Flaviviridae*) and Chenuda virus (genus *Orbivirus*, family *Reoviridae*), confirmed serologically in cattle in South Africa (*1,2,4*).

Our study isolated and identified 6 previously known tickborne arboviruses, including DUGV, BHAV, THOV, Dhori virus (DHOV), KADV, and Barur virus. In addition, 2 viruses related to DUGV were isolated. An unexpected result of this study was the isolation of foot-and-mouth disease virus (FMDV) from tick pools.

Materials and Methods

Tick Collection and Processing

Ticks were collected from the hides of flayed animals between September and November 1999 at the Njiru and Dagoretti abattoirs, on the outskirts of Nairobi, Kenya. Attached ticks were pulled off manually and placed in sterile plastic vials, which were loosely capped and transported to the laboratory. The origin of individual sampled animals could not be determined. All animals to be slaughtered for the day were put in 1 enclosure, irrespective of origin.

In the laboratory, ticks were washed twice with sterile water to remove excess particulate contamination from animal hides, rinsed once with 70% ethanol, and then rinsed twice with minimum essential medium (MEM), with antimicrobial agents (100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B). Ticks were identified by sex and species by using appropriate identification keys (*15,16*), transferred to sterile vials, and stored at –80°C until homogenized for virus isolation. Voucher specimens were prepared in ethanol, and identifications were reviewed at the International Centre of Insect Physiology and Ecology, Nairobi. Ticks were later thawed at room temperature, identifications were confirmed, and ticks were pooled into groups of 2 to 50, depending on the size of the ticks and according to species, collection dates, and sites. Each pool was homogenized by using 90-mesh alundum in a prechilled, sterile mortar and pestle with 1.6–2 mL ice-cold bovine albumin (BA)-1 medium (1× medium 199 with Earle's salts, 1% BA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B). The homogenates were clarified by low-speed centrifugation at 1,500 rpm for 15 minutes at 4°C, and supernatants were aliquoted and stored at –80°C. In the case of *Hyalomma* species, the primary vectors of CCHFV, each pool was screened by reverse transcription–polymerase chain reaction (RT-PCR) for CCHFV before tissue culture injection was conducted (Table 1).

Virus Isolation

For virus isolation in cell culture, Vero cells were grown in 25-cm^2 cell culture flasks to 80% confluency in MEM with 10% fetal bovine serum (FBS), 2% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B. Cells were rinsed with sterile saline, and 0.2 mL clarified tick homogenate was added followed by injection at 37°C for 45 minutes to allow virus adsorption. After incubation, cells were rinsed with saline and maintenance medium (MEM with Earle's salts, with 5% FBS, 2% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μL/mL amphotericin B) was added. Cells were incubated at 37°C and observed daily for signs of cytopathic effects (CPE). The pooled infection rate program (PooledInfRat, Centers for Disease Control and Prevention, Fort Collins, CO, USA; http://wwwcdcgov/ncidod/dvbid/westnile/softwarehtm) was used to compare virus infection rates in the tick species collected and processed in this study.

Virus Identification

Agents causing CPE in tissue culture were initially identified to virus group by using the indirect fluorescent antibody assay (IFA) on spot slides of infected Vero cells with polyvalent mouse hyperimmune ascitic fluids obtained from the National Institutes of Health Reference Reagents Program. Fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin G was the secondary antibody.

RT-PCR was also used to identify most of the virus isolates obtained from tissue culture. RNA was extracted from cell culture supernatants with the QIAamp Viral RNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended protocol. RT-PCR was performed with the Titan One Tube RT-PCR kit (Roche, Indianapolis, IN, USA) with primers mainly targeting the

Table 1. Reverse transcription–polymerase chain reaction primers used for arbovirus isolation, Nairobi, Kenya

Virus target	Target gene	Primer designation	Primer sequences*
Crimean-Congo hemorrhagic fever	N	CCHF F2	TGGACACCTTCACAAACTC
		CCHF R3	GACAAATTCCCTGCACCA
Dugbe	N	DG S1	TCTCAAAGACAAACGTGCCGCAG
		DG S5	TGCAACAACTGGATGTGTGA
Nairobi sheep disease	N	NSD 12f	GAATGGTCGAACGTGGAC
		NSD 16r	TGCTGTCAGGACACCAGG
Thogoto	N	THO NF	CCTGCAGGGGCGGAAGTTATG
		THO NR	AAAATCCTCGCAGTTGGCTATCA
Flaviviruses	NS5	FLAVI fu2	GCTGATGACACCGCCGGCTGGGACAC
		FLAVI cfd3	AGCATGTCTTCCGTGGTCATCCA
Bunyamwera and California serogroups	N	BCS82C	ATGACTGAGTTGGAGTTTCATGATGTCGC
		BCS332V	TGTTCCTGTTGCCAGGAAAAT
Rift Valley fever	G2	RVF-M-727f	GGAACCCCTGCATGAAAGAGA
		RVF-M-1565r	CGATCCTGTGACGCAAACTC
Dhori	N	DHO NF2	TGGTACCCTTTTCTTGCTTCACTCC
		DHO NR2	TGCTCTTCCTCGGCTCAAACACCA
Babanki	C/E3	BAB 1007f	TGGCCATGGAAGGTAAGGTAAT
		BAB 1569r	TATGGCGTTGAGCAGGGTATC
Hazara	N	HAZ 803f	CTGGTTGAGCTAGAGGGGAAAGACG
		HAZ 1304r	GGGCGGCATCATCGGGACTG
Coxsackie B4	Pol	COX 6749f	ACTTTGTGAGAGGGGGTATGC
		COX 7151r	ACGTGGTATTGGGTGTTTTT
Koutango	NS5	KOU 176f	TCAGGGAGGTGGGAGGTAAAC
		KOU 734r	TCATGCCATCCAACAGAAGGT
Saboya	NS5	SAB 226f	GCAGGCTGGGACACAAAGAT
		SAB 815r	CTACAAGGGGCAATGATGGTTC
Ndelle	λ3	NDE 655f	GGGGTTTTCTGGCTAATGTCAC
		NDE 1238r	GGGCCTGTCCAGTCTTTTTG
Middleburg	E2	MID 1939f	TACATGCCCCGAAGGTGACT
		MID 2458r	CGGGATGGTGTTCGGTAAAG

*Primer sequences 5′–3′.

known African tickborne viruses (Table 1). References are available from the authors for previously published primers. All other primers were designed for this study to amplify a specific fragment from the virus listed and have not been tested for cross-reactivity with other related or unrelated viruses. RT-PCR was also performed on RNA extracted from uninfected Vero cells as a negative control. Amplified DNA fragments were visualized by electrophoresis on 0.8%–1.0% agarose gels. DNA fragments were extracted from gels with the QIAquick Gel Extraction Kit (Qiagen), and DNA was eluted in 20 μL 10 mmol/L Tris-HCl, pH 8.5, and stored at –20°C. RT-PCR fragments were sequenced with the CEQ DCTS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA, USA) with listed primers and analyzed with a CEQ 8000 automated sequencer (Beckman Coulter, Inc.). Both strands of DNA were sequenced. Nucleic acid sequences were compared with the GenBank database by using the BLAST program (http:www.ncbi.nlm.nih.gov/BLAST).

Additional methods used to identify selected isolates included complement fixation (CF) and hemagglutination-inhibition tests (*17*) and panviral microarray-based genotyping (*18*). Alignment of nucleic acid and deduced protein sequences was conducted by using the MegAlign program (Lasergene 6.1, DNASTAR, Inc., Madison, WI, USA); phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.0 (http://www.megasoftware.net).

Results

Tick Collection

A total of 15,851 ticks were collected and processed in 1,071 pools for virus isolation. Species of ticks collected and their proportions in the collection are shown in Table 2. The predominant species collected was *Rhipicephalus pulchellus* (56%), followed by *Amblyomma gemma*, *R. appendiculatus*, and *A. variegatum*. Other species were sampled in smaller numbers. However, the calculated virus pooled infection rate was higher for *A. gemma* than for *R. pulchellus* (Table 2). The number of *Hyalomma* specimens collected was comparatively small (3%). These species are the primary vectors of CCHFV; this agent was not among the viruses isolated.

Table 2. Tick species collected and their virus yield, Kenya

Species	No. collected	No. virus isolates	Pooled infection rate
Amblyomma variegatum	994	6	6.16
A. gemma	2,160	26	11.05
A. lepidum	963	4	4.24
A. coharens	4	0	0
Boophilus decoloratus	985	1	1.01
Hyalomma truncatum	270	0	0
H. albiparmatum	112	0	0
H. rufipes	96	0	0
H. dromedarii	1	0	0
Rhipicephalus appendiculatus	1,228	2	1.63
R. evertsi	146	0	0
R. pulchellus	8,892	17	2.48
Total	15,851	56	

Virus Isolation and Identification

A total of 56 virus isolates were obtained from 51 tick pools; 52 of the 56 viruses were identified (Table 3). Five pools contained 2 different viruses. All of the isolated viruses caused CPE in Vero cells. The observed onset of CPE was 4–10 days postinfection. In the initial identification by IFA, 6 isolates reacted positively with the Thogoto group–specific antiserum (polyvalent 4), 33 isolates reacted with the Congo group–specific antiserum, 1 isolate reacted with the flavivirus group–specific antiserum (group B), and 1 isolate reacted with antiserum that included specificity to DHOV (polyvalent 10).

Forty-five virus isolates were identified by using RT-PCR and nucleic acid sequencing with primers specific to known tickborne viruses or by CF assay or microarray-based genotyping. The identified isolates included 26 DUGV, 6 THOV, 6 Barur virus, 3 FMDV, 2 BHAV, 1 DHOV, and 1 KADV. DUGV was isolated most frequently (46%). Most DUGV isolates were recovered from *A. gemma* (62%), whereas the most commonly sampled tick, *R. pulchellus*, yielded only 2 DUGV isolates (8%) (Table 3).

Two of the virus isolates that were IFA-positive with Congo group antiserum were RT-PCR negative when primers specific for DUGV, CCHFV, Hazara virus, or BHAV, all of which were represented in the antiserum, were used. However, RT-PCR using NSDV nucleocapsid–specific primers and RNA extracted from these isolates produced 3 major bands, including one ≈880 bp in size; the expected band size for the NSDV-specific fragment was 887 bp. The 880-bp fragment was sequenced, and an alignment of 513 nt (nt) of this sequence with nucleocapsid sequences from DUGV, CCHFV, and NSDV showed 71%, 58%, and 60% homology, respectively, which suggests that these isolates were most closely related to DUGV. Alignment of sequences from the 2 isolates showed them to be 95% homologous. Specific primers were designed for this DUGV-like virus from sequence of the fragment described above. RT-PCR conducted with these primers produced bands of correct size and sequence with RNA from the DUGV-like virus isolates, while RT-PCR results using these primers with RNA from DUGV and BHAV were negative. RT-PCR and sequencing of all of the virus isolates using the primers designed from the DUGV-like virus sequence showed 4 additional isolates of this DUGV-like virus; 2 were from pools that also contained DUGV. Sequence homology between all 6 DUGV-like isolates was 95%–100%, based on a 508-nt alignment of the S segment of the virus RNA. Of the 6 isolates of this

Table 3. Virus isolates obtained from ticks collected in Nairobi, Kenya

Species	DUGV	DUGV-like	BHAV	THOV	DHOV	KADV	BARV-like	FMDV	Unknown	Total
Amblyomma variegatum	6	0	0	0	0	0	0	0	0	6
A. gemma	16	5	0	4	0	0	0	0	1	26
A. lepidum	2	1	0	1	0	0	0	0	0	4
A. coharens	0	0	0	0	0	0	0	0	0	0
Boophilus decoloratus	0	0	1	0	0	0	0	0	0	1
Hyalomma truncatum	0	0	0	0	0	0	0	0	0	0
H. albiparmatum	0	0	0	0	0	0	0	0	0	0
H. rufipes	0	0	0	0	0	0	0	0	0	0
H. dromedarii	0	0	0	0	0	0	0	0	0	0
Rhipicephalus appendiculatus	0	0	1	0	0	1	0	0	0	2
R. evertsi	0	0	0	0	0	0	0	0	0	0
R. pulchellus	2	1	0	1	1	0	6	3	3	17
Total	26	7	2	6	1	1	6	3	4	56

*DUGV, Dugbe virus; BHAV, Bhanja virus: THOV, Thogoto virus; DHOV, Dhori virus; KADV, Kadam virus; BARV, Barur virus; FMDV, foot-and-mouth disease virus

virus, 5 (83%) were from *A. gemma* pools, and 1 (17%) was from a pool of *R. pulchellus.* One additional isolate, obtained from a pool of *A. lepidum*, was identified as being DUGV-like by CF test. RT-PCR conducted on RNA extracted from this isolate with the primers designed for the DUGV-like virus described above produced a weak band. Sequence of this product was ≈80% homologous to the other isolates of DUGV-like virus and 70%, 63%, 57%, and 55% homologous to DUGV, NSDV, CCHFV, and Hazara virus, respectively, which suggests that it may be a different DUGV-like isolate.

Six isolates of THOV were obtained: 4 from pools of *A. gemma*, l from *A. lepidum*, and 1 from *R. pulchellus*. Since the THOV genome is segmented, a portion of each of the 6 genome segments from each isolate was sequenced and compared with available sequence from other THOV isolates to determine if reassortment of virus genome segments had occurred. No evidence was found for reassortment of virus segments. Phylogenetic analysis showed that the THOV isolates were most closely related to other African isolates from Uganda (1996), Kenya (1960), and Nigeria (1969) (data not shown).

The single isolate of DHOV, another member of the tickborne orthomyxovirus group, was obtained from a pool of *R. pulchellus*. A single isolate of KADV, the only African member of the tickborne flavivirus group, was recovered from a pool of *R. appendiculatus*. Six isolates were found by CF test to be indistinguishable from Barur virus, a rhabdovirus. Further characterization of these isolates was not conducted.

Three isolates of FMDV were identified by using panviral microarray-based technology. RNA isolated from viral culture of 1 isolate was reverse transcribed, randomly amplified, and hybridized to panviral DNA microarrays as described (*19*). Analysis of the hybridization patterns showed extensive hybridization to oligonucleotides derived from FMDV. Based on this result, PCR primers were designed from conserved regions of FMDV to confirm the identity of the virus. A PCR product of ≈600 bp was generated; it possessed 97% nucleotide identity to FMDV serotype SAT3. Two other isolates were identical by CF test.

Four isolates remained unidentified. Three of these were recovered from *R. pulchellus* and 1 from *A. gemma*. All of these isolates failed to react with the hyperimmune ascites grouping fluids used for the IFA identification procedure and produced negative results in RT-PCR tests when primers specific to known African tickborne viruses were used.

Discussion

In this study, *A. gemma* ticks were incriminated for the first time as key vectors or reservoirs of tickborne viruses in the East African region; 46% of our virus isolates were obtained from this species. Distribution limits of ticks are variable and are influenced by several factors, including climate, vegetation, host density, host susceptibility, and host grazing habits. During previous studies conducted at the Lake Victoria basin in Kenya (*1*), *A. gemma* was not collected, most likely because this species is limited to more arid zones. *A. gemma* is found only in the dry zones of bushwillows (*Combretum*) and shrub steppe and is much more restricted than *A. lepidum* to very dry areas. DUGV and DUGV-like viruses were the most frequently isolated viruses in this study (33/56, 59%), and 64% of these isolates were from pools of *A. gemma*. Four of the 6 THOV isolates were obtained from pools of this species as well. Our results suggest that viruses are being actively transmitted in the drier parts of East Africa where *A. gemma* is more common. The pastoral regions, which supply many of the animals slaughtered at abattoirs in Nairobi, are predominantly dry and therefore likely to harbor this tick species in abundance.

The proportion of *R. appendiculatus* collected in this study was small when one considers the distribution of this tick in Kenya and its importance as a pest, a finding that suggests that most sampled cattle came from climatic zones where this species is not abundant. In Tanzania and Kenya, *R. appendiculatus* is most abundant in areas receiving >1,000 mm mean annual rainfall. It is absent from xerophytic and dry thicket zones with overgrazed pastures and little grass cover (*15*). NSDV is mainly transmitted by *R. appendiculatus*, and the virus is found only in areas where this species is abundant (*14*); therefore, the relatively low numbers of this species collected may explain why NSDV was not isolated in this study. Pools of *R. appendiculatus*, however, did yield single isolates of BHAV and KADV. BHAV has been isolated previously in Kenya and Nigeria (*1*,*20*). The medical implications of this virus for humans and animals in this region have not been determined, although the virus has been associated with human infection and illness in eastern Europe and West Africa (*21*–*23*). KADV is the only known African tickborne flavivirus. The virus was first isolated from *R. pravus* ticks taken from a cow in Uganda (*24*,*25*) and later in Kenya from *A. variegatum* and *R. pulchellus* (*14*). Although KADV pathogenicity is not evident in humans, antibodies against KADV were detected in human sera during a serosurvey in Uganda (*26*).

DUGV is commonly isolated in surveillance studies conducted in Africa (*1*,*2*,*27*,*28*), and it appears to be endemic in most of the drier parts of the continent. The implications of DUGV for human health have not been evaluated in Kenya, although reports from other regions in Africa suggest that human infection and illness caused by DUGV infection occur (*2*,*22*,*27*). Johnson et al. (*1*), in an

earlier study conducted around Lake Victoria, recovered more DUGV isolates than any other virus and observed that more tick pools from dry scrub land (away from the lake) were infected with DUGV than pools from the swamp edge. These researchers also observed that 12 of the 39 DUGV isolates recovered varied in their behavior in cell culture and in suckling mice, which suggests that some of the DUGV strains isolated were different. In our study, in addition to the 26 isolates of DUGV, we identified 2 DUGV-like viruses (6 isolates of one, 1 isolate of another), which were found to differ significantly in S segment nucleotide sequence from previously published DUGV sequences. Further investigation of these isolates is necessary to determine their relatedness to DUGV.

THOV was first isolated in Kenya from *Rhipicephalus* species and *Boophilus decoloratus* in the 1930s (*29*) and has been isolated repeatedly from various tick species in Kenya, West Africa, Europe, and Asia (*30*). Two THOV infections have been reported in humans, with 1 fatality (*22*). In our survey, THOV was isolated from pools of *A. gemma* (4), *A. lepidum* (1), and *R. pulchellus* (1). DHOV, also a member of the *Thogotovirus* genus in the *Orthomyxovirdae* family, has been previously isolated in Europe, Asia, and the Middle East (*31–34*). Human DHOV infection has been evidenced by serologic survey results and human illness (*23*,*34*,*35*). We report here the first isolation of DHOV in East Africa. This finding suggests a southward spread of the virus that is supported by the presence of competent tick vectors in the region and demonstrates the potential for other tickborne viruses circulating in Europe and Asia to spread to the African continent. Such spread would have adverse consequences for large, immunologically naive populations whose pastoral practices provide for closer human-animal contact.

An unexpected finding in this study was the isolation of FMDV from 3 pools of *R. pulchellus*. FMDV is endemic in many parts of Africa; however, it has not previously been identified in association with tick surveillance or transmission studies (*36*). This finding does not constitute evidence that FMDV replicates in or can be transmitted by ticks; in fact, previous reports indicate that the virus is not transmissible by *Rhipicephalus* ticks or blood-feeding flies (*37*,*38*). However, the virus has been demonstrated to persist in ticks for up to several days after feeding on an infected animal (*37*). The ticks in our study were not held for any length of time to allow for blood in the ticks to be digested before processing for virus isolation. Therefore, FMDV may have been present in undigested blood in ≥1 ticks in each pool. FMDV is present in the blood of an infected animal, skin lesions, and skin areas that do not contain lesions (*39*). The virus persists in skin up to 4 days beyond the period of viremia and for extended periods in preserved hides (*40*). Therefore, mouth parts of ticks feeding on FMDV-infected cattle might have become contaminated with the virus, which was then not sufficiently exposed to the external rinsing procedures to which the ticks were subjected before processing. Further investigation is necessary to clarify the mechanism of these FMDV isolations and the implications of these findings.

Our study illustrates the potential for tickborne dissemination of endemic and emergent viruses, some of which are human pathogens, among livestock as well as the potential for transmission of these pathogens to humans. Regular surveillance is warranted to monitor the presence and spread of these and other viruses facilitated through livestock rearing, marketing, and movement in Africa.

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Rodent-associated *Bartonella* Febrile Illness, Southwestern United States

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Serum specimens from 114 patients hospitalized with a febrile illness were tested with an indirect immunofluorescence assay (IFA) using *Bartonella* antigens prepared from 6 species of sigmodontine rodents and 3 known human *Bartonella* pathogens: *B. henselae*, *B. quintana*, and *B. elizabethae.* Acute- and convalescent-phase serum samples from 5 of these patients showed seroconversion with an IFA titer ≥512 to rodent-associated *Bartonella* antigens. The highest titer was against antigen derived from the white-throated woodrat (*Neotoma albigula*), although this rodent is not necessarily implicated as the source of infection. Three of the 5 who seroconverted showed no cross-reaction to the 3 *Bartonella* human pathogens. Common clinical characteristics were fever, chills, myalgias, leukopenia, thrombocytopenia, and transaminasemia. Although antibodies to *Bartonella* are cross-reactive, high-titer seroconversions to rodent-associated *Bartonella* antigens in adults with common clinical characteristics should stimulate the search for additional *Bartonella* human pathogens.

The discovery of hantavirus pulmonary syndrome and its high death rate in the southwestern United States resulted in greater vigilance in evaluating patients with acute febrile illness, particularly those with thrombocytopenia (*1*). Clinicians soon became aware of substantial numbers of hospitalized patients with a severe flulike prodrome and thrombocytopenia. In spite of conventional culture and serologic analysis for known pathogens and diseases, including hantaviruses, plague, tularemia, relapsing fever, spotted fever, murine typhus, and Q fever, no diagnosis could be made. To assist physicians in identifying treatable pathogens, we submitted serum to reference laboratories for diagnostic seroassays directed at known pathogens and organisms not previously associated with human disease. A concept of the role of rodent-associated bartonellae as a cause of unexplained febrile illness in the western United States has been recently developed (M. Kosoy, pers. comm.). We considered the possibility that some cases in our study were caused by *Bartonella* species.

Among at least 20 known species and subspecies of *Bartonella*, 5 have been identified as causes of human disease in North America (*2*,*3*). *B. henselae* causes cat-scratch disease with regional lymphadenitis and occasionally hepatosplenic disease in the immunocompetent host, and bacillary angiomatosis, cerebritis, or peliosis hepatis in the immunocompromised host (*4–6*). Louseborne *B. quintana* causes trench fever, aseptic meningitis, bacteremia, endocarditis, or bacillary angiomatosis (*4*,*7–9*). Recently isolated cases of infection with *B. elizabethae* (*10*), *B. vinsonii* subsp. *arupensis* (*11*), and *B. washoensis* (*12*) suggest that the spectrum of *Bartonella* infections may continue to expand.

Many mammals, including numerous species of rodents, are commensally infected with *Bartonella* species in North America (*12–15*). We sought serologic evidence for human bartonellae infection in serious febrile illnesses in the Four Corners region, using diverse *Bartonella* antigens in an indirect immunofluorescence assay (IFA) (*13*). We report 7 years' cumulative experience in diagnostic referrals, including 5 cases showing seroconversion, and

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4 cases with a single high titer, to *Bartonella* antigens derived from strains isolated from rodents, particularly the white-throated woodrat (*Neotoma albigula*) captured in New Mexico.

Materials and Methods

Patients

From July 1993 to June 2001, 114 patients 15–78 years of age were referred by their physicians for assistance in diagnosing a febrile illness with a duration <12 days at the time of admission. One hundred patients were hospitalized in New Mexico, 10 in Arizona, and 4 in Colorado. All patients were hospitalized on the basis of the attending physician's decision concerning severity of illness, the possibility of hantavirus infection in the prodrome phase, and the need for diagnostic studies, supportive care, and presumptive antimicrobial-drug therapy. At the time specimens were collected, results of conventional microbiologic assays and diagnostic serologic analysis were negative or unavailable.

Patients were divided into 4 clinical groups according to conventional diagnostic results (Table 1). Seventy-six patients (group A) had an acute undifferentiated febrile illness without pulmonary, cardiac, or renal manifestations. Twelve patients (group B) had bacterial lobar pneumonia (11 patients) or acute respiratory distress syndrome (1 patient) diagnosed by typical signs and symptoms, hypoxemia, pulmonary infiltrates, and prompt clinical response to β-lactam antimicrobial drugs (*16*,*17*). Twelve patients (group C) had hantavirus cardiopulmonary syndrome diagnosed by strip immunoblot serology (*18*) and reverse transcription–polymerase chain reaction (RT-PCR) of serum (*19*). Fourteen patients (group D) had an acute febrile syndrome without pulmonary manifestations and with a diagnosis established by conventional blood culture, serology, or PCR; this group included 3 patients with *Escherichia coli* sepsis, 2 with *E. coli* pyelonephritis, 3 with Rocky Mountain spotted fever, 1 with acute *Staphylococcus aureus* aortic valve endocarditis, 1 with bubonic plague, 1 with acute Q fever, 1 with parvovirus infection, 1 with acute rheumatic fever, and 1 with acute lupus erythematosis. All patients (except those in group D) had at least 2 negative blood cultures, negative spinal fluid cultures and cytometrics when appropriate, negative hantavirus serologic results (except group C), and negative serologic results for plague, tularemia, Q fever, spotted fever, and *Ehrlichia* species ordered at the discretion of the attending physician. Except for hypertension (5 patients) and chronic alcoholism (12 patients), no patient had underlying disease such as diabetes, malignancy, or HIV infection. The charts were reviewed retrospectively by the investigators. The study was approved by the institutional review boards of the University of New Mexico and the Navajo Nation.

Serologic Analysis

Citrated and clotted blood was collected within 24 hours of admission from 90 patients (acute-phase sample), 7–42 days after admission from 10 patients, and at admission and during convalescence from 14 patients (all in group A). Plasma was immediately frozen at –80°C. An IFA was performed as previously described (*13*). All antigens were prepared at the Bacterial Zoonoses Branch, Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado.

Vero E6 monolayers were infected separately with 1 of 9 strains of *Bartonella*: 3 strains (*B. quintana, B. henselae*, and *B. elizabethae*) were isolated from humans and 6 strains were isolated from the meadow vole (*Microtus pennsylvanicus*), white-throated woodrat (*N. albigula*), deer mouse (*Peromyscus maniculatus*), cotton rat (*Sigmodon hispidus*), Ord kangaroo rat (*Dipodomys ordi*), and rock squirrel (*Spermophilus variegatus*). Plasma was diluted 1:32 in phosphate-buffered saline, placed in antigen-containing wells, incubated at 37°C for 30 minutes, washed, and incubated at 37°C for 30 minutes with rabbit antihuman immunoglobulin (Ig) conjugated with fluorescein isothiocyanate. Positive samples were then tested in serial 2-fold dilutions on monolayers infected with 1 of 9 *Bartonella* strains. Mouse hyperimmune sera were produced by injection of BALB/c mice with the same *Bartonella* strains that were used for the antigen preparations. These sera were used as IFA-positive controls (titers >1,000 in each assay). Results were tabulated without knowledge of the patient's clinical status.

Results

Serum samples from 114 patients with acute febrile illness, including 14 with both acute- and convalescent-phase serum samples, were tested at a dilution of 1:32 by

Table 1. Rodent-associated *Bartonella* serologic results in 114 adults with acute febrile illness, southwestern United States

Clinical diagnosis	Total	No. thrombocytopenic	No. leukopenic	Titer to *Neotoma albigula*–associated *Bartonella* antigen				
				≤64	128	256	512	≥1,024
Undifferentiated fever	76	55	43	52	11	2	5	6
Bacterial pneumonia	12	4	4	12	0	0	0	0
Hantavirus pulmonary syndrome	12	12	0	11	1	0	0	0
Other febrile illnesses	14	9	4	11	0	1	0	2

IFA with a panel of 9 *Bartonella* antigens. All positive samples were retested at a dilution of 1:32 and at doubling dilutions to 1:4,096. In 12 of 13 cases with titers ≤512 to any rodent-associated antigen, the titer to the *N. albigula*–associated *Bartonella* antigens (NA-AB antigens) were the highest measured. Therefore, only the titers to NA-AB antigens are shown in Table 1. IFA titers to NA-AB ≥128 were observed more often in undifferentiated febrile illness (group A, 24 of 76) than in the 3 groups with specific diagnoses (groups B–D, 4 of 38) ($\chi^2 = 4.98$, p = 0.026, using Yates' correction). Among 24 patients in group A with titers ≥128, a total of 11 had convalescent-phase titers ≥512. Clinical information was sufficient to analyze for 9 of these 11 patients: 5 patients with both acute- and convalescent-phase titers (Table 2) and 4 patients with only a convalescent-phase titer (Table 3). Nine patients in group A with both acute- and convalescent-phase serum samples showed no increase in titer or a titer >64.

Of 24 patients with pneumonic disease (groups B and C), only 1 had a titer of 128 to NA-AB antigens. Of 14 patients with other diagnosed febrile illnesses (group D) not listed in Tables 2 and 3, three had high titers to NA-AB antigens (Table 1). A 35-year-old man with aortic valve endocarditis and cultures of blood and valve positive for *S. aureus* had an NA-AB titer of 1,024 on admission and the following day. A 30-year-old man with fever, myalgias, headache, thrombocytopenia, and leukopenia with admission serum positive by PCR for *Borrelia hermsii* (tick-borne relapsing fever) had an acute-phase (day 1) titer of 256 and a convalescent-phase (day 24) titer of 1,024 to NA-AB antigens. A 23-year-old woman with fever and acute hepatic injury had positive convalescent-phase (day 28) IgM phase I (512) and IgG phase II (1,024) titers for *Coxiella burnetii* antigens and an NA-AB antigen titer of 256 in a convalescent-phase serum sample.

Five of the 14 patients with acute- and convalescent-phase serum samples in group A showed a ≥4-fold increase in titer to NA-AB antigens and convalescent-phase titers >512 on days 14, 7, 7, 12, and 42, respectively, after admission (Table 2). Each of the 5 who seroconverted had a clinical syndrome characterized by fever (temperature >39°C), chills, pronounced myalgias in the back and thighs, nausea, and headache. Two who seroconverted had a sore throat and 2 had diarrhea, but none had other upper or lower respiratory symptoms, abnormal chest radiograph results, lymphadenopathy, hepatosplenomegaly, bleeding, rash, altered consciousness, or abnormal neurologic findings. Thrombocytopenia and leukopenia were common (Tables 2 and 3), but no patients had evidence of coagulopathy, or cardiac, pulmonary, renal, or neurologic disease.

Four other patients in group A had a single titer >512 to NA-AB antigens on days 21, 7, 20, and 23, respectively, after admission (Table 3). This group had elevated levels of serum transaminase, bilirubin, and alkaline phosphatase, which is indicative of active hepatitis. These 4 patients were treated with doxycycline, and all recovered without sequelae. Of the 9 patients listed in Tables 2 and 3, one had a diagnosis of chronic alcoholism (patient 6, Table 3). All 9 were negative for hepatitis A, B, and C; Q fever; Rocky Mountain spotted fever; murine typhus; leptospirosis; granulocytic or monocytic ehrlichiosis; plague; and tularemia; they also had negative titers for HIV, hantavirus, and antinuclear antibody. Patients 6, 8, and 9 were tested for antibody to hepatitis E at the Hepatitis Branch of CDC in Atlanta, Georgia, and were negative (M. Favorov, pers. comm.). Patients 1, 4, and 6 had 6-, 3-, and 3-fold lower titers, respectively, to the known *Bartonella* pathogen antigens compared with the titer to NA-AB antigens (Tables 2 and 3).

Table 2. Clinical and laboratory data of 5 adults with undifferentiated fever and seroconversion to *Neotoma albigula*–derived *Bartonella* antigens*

									Doubling dilution end titer (acute/convalescent phases)§				
Patient no., age (y), sex	DOI	T (°C)†	Leukocytes $\times 10^3/\mu L$‡	PLT $\times 10^3/\mu L$‡	HCT (%)†	AST (U/L)†	BIL (mg/dL)†	LDH (U/L)†	*Bartonella* from *Neotoma*	*B. vinsonii* from *Microtus pennsylvanicus*	*B. quintana*	*B. henselae*	*B. elizabethae*
1, 55, F	5	39.7	2.7	147	44	183	1.5	167	256/4,096	64/64	<32/64	<32/<32	64/64
2, 30, M	5	39.3	3.2	110	50	85	1.5	206	256/1,024	128/1,024	64/512	64/256	64/512
3, 34, F	6	39.7	3.5	95	44	324	1.7	190	<32/1,024	<32/256	<32/64	64/256	<32/64
4, 29, M	2	39.2	17.9	226	48	ND	ND	ND	<32/512	32/64	<32/<32	<32/<32	32/32
5, 23, F	2	38.8	5.0	125	40	ND	ND	130	32/512	<32/64	<32/<32	<32/128	<32/64

*DOI, day of symptomatic illness at hospitalization; T, temperature; PLT, platelet count; HCT, hematocrit ; AST, aspartate aminotransferase; BIL, bilirubin; LDH, lactate dehydrogenase; ND, not determined.
†Highest value during 2–6 d of hospitalization.
‡Minimum value.
§Convalescent-phase titers 2–6 wk after hospital admission.

Table 3. Clinical and laboratory data of 4 adults with undifferentiated fever and a single convalescent-phase titer to *Neotoma albigula*–derived *Bartonella* antigens*

Patient no., age (y), sex	DOI	T (°C)†	Leukocyte × 10^3/μL‡	PLT × 10^3/μL‡	HCT (%)†	AST (U/L)†	BIL (mg/dL)†	LDH (U/L)†	Doubling dilution end titer (convalescent phase)§				
									Bartonella from *Neotoma*	*B. vinsonii* from *Microtus pennsylvanicus*	*B. quintana*	*B. henselae*	*B. elizabethae*
6, 42, M	2	39.1	23.3	11	47	4,580	4.3	16,000	2,048	64	<32	<64	128
7, 17, M	3	38.8	3.3	108	43	60	1.4	229	1,024	256	256	64	512
8, 23, F	3	39.0	3.9	245	44	834	5.2	NA	512	256	256	64	64
9, 32, M	7	39.0	2.9	35	44	1,049	3.8	1,248	512	NA	NA	NA	NA

*DOI, day of symptomatic illness at hospitalization; T, temperature; PLT, platelet count; HCT, hematocrit; AST, aspartate aminotransferase; BIL, bilirubin; LDH, lactate dehydrogenase; NA, not available.
†Highest value during 2–6 d of hospitalization.
‡Minimum value.
§Convalescent-phase titers 2–6 wk after hospital admission.

Discussion

This study provides preliminary serologic evidence for a *Bartonella* or *Bartonella* cross-reactive species that is causing acute febrile illness in immunocompetent adults in the rural southwestern United States. Five patients who seroconverted to rodent-associated antigen had fever, myalgias, headache, and chills with varying degrees of leukopenia, mild hepatitis, and thrombocytopenia. Four other patients with a single elevated titer 2–5 weeks into their illness had more severe hepatic injury. In the absence of culture- or PCR-positive evidence of *Bartonella* infection in any of these patients, the interpretation of these serologic observations is related to the cross-reactivity between *Bartonella* species as well as non-*Bartonella* species, interpretation of the quantitative IFA titer, variations among pathogens to stimulate antibody responses, timing of serum specimen collection, and the route of exposure.

Although antigens derived from *Bartonella* isolated from *N. albigula* were used, this process does not imply that the human infection was caused by a *Bartonella* strain that naturally infects *N. albigula*. Serologic cross-reactions among *Bartonella* species are common (*20*), and the IFA is unable to distinguish between infection with *B. quintana* or *B. henselae* (*21*). The cross-reactivity between rodent-associated and known *Bartonella* pathogen–associated antigens was expected and found to some degree in nearly all cases. We did not find clear evidence for infection with *Bartonella* species known to cause disease in humans, including *B. henselae, B. quintana, B. vinsonii*, and *B. elizabethae*, in the sense that titers to rodent-associated, particularly NA-AB, antigens were always higher than those for known human *Bartonella* species. The lack of cross-reactivity in 3 patients is consistent with a rodent-associated *Bartonella* infection, although infection with a *Bartonella* associated with a nonrodent animal cannot be ruled out (*22*).

Identification of *Bartonella* infections in humans in the southwestern United States is important because cat-scratch disease is not common in this region, and cat fleas, presumed vectors for *B. henselae*, do not naturally exist in such arid environments (*23*). Cross-reactivity between *Bartonella* antigens and antigens of *C. burnetii* and *Chlamydia* species has been demonstrated (*24*,*25*). Except for the woman in group D who had clear evidence of acute Q fever hepatitis, significant *Bartonella* titers ≥128 were not associated with detectable antibody to phase I or II *Coxiella* antigens in the complement fixation test in all 8 patients tested. None of the patients had a condition associated with nonspecific immune stimulation such as HIV infection, injection drug use, or collagen vascular disease that could account for false-positive results.

The IFA was developed at CDC (*21*) and has been assessed most extensively in the diagnosis of *B. henselae* and *B. quintana* infection in the United States (*20*). At the National Referral Center of CDC, a titer of 64 is considered positive (*20*). When a strict case definition is used for cat-scratch disease, this titer has a sensitivity of ≈80% and a specificity of 93% to 96% (*20*,*21*,*26*). Other investigators have found greater specificity when titers of 128 (*27*), 256 (*25*), or 512 (*28*) were used to diagnose cat-scratch disease. An IFA titer of 512 to *B. henselae* in adults with no exposure to cats or illness compatible with cat-scratch disease was uncommon (<1%) in 1 study in Germany (*27*). We used a conservative threshold IFA titer of 512 to present clinical data on 9 patients based in part on this experience with cat-scratch disease, recognizing that immunogenicity to immunodominant antigens may vary among species of the same genus. The usefulness of a single titer of 1:512 to NA-AB antigens (Table 3) is unknown because IFA titers to *B. henselae* persist during the first year after infection (*20*).

The clinical syndrome associated with seroconversion to NA-AB antigens was characterized by either a brief undifferentiated febrile illness or fever accompanied by hepatic injury. Clinical evidence for inflammation in the lung, heart, kidney, and nervous system was not apparent. Infection with *B. henselae*, particularly in immunocompromised hosts, has been documented to involve the liver

(*2*). Moreover, thrombocytopenia and leukopenia, which were common in our small sample of febrile patients, have also been associated with *B. quintana* infection (*29*) in immunocompetent adults and with *B. henselae* infection in immunocompromised adults (*2*). No patient had intraerythrocytic bacilli visible on Giemsa-stained blood smear (*30*) (F. Koster, unpub. data). A clear definition of the syndrome awaits definitive identification based on culture of the pathogenic species from patients. Thus, a concerted effort to identify acute infections with rodent-associated *Bartonella* should be undertaken with specific serologic assays as well as intensive PCR-based diagnostics and culture techniques specific to the fastidious *Bartonella* genus.

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Migratory Passerine Birds as Reservoirs of Lyme Borreliosis in Europe

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To define the role of birds as reservoirs and disseminators of *Borrelia* spirochetes, we characterized tick infestation and reservoir competence of migratory passerine birds in Sweden. A total of 1,120 immature *Ixodes ricinus* ticks were removed from 13,260 birds and assayed by quantitative polymerase chain reaction (PCR) for *Borrelia*, followed by DNA sequencing for species and genotype identification. Distributions of ticks on birds were aggregated, presumably because of varying encounters with ticks along migratory routes. Lyme borreliosis spirochetes were detected in 160 (1.4%) ticks. *Borrelia garinii* was the most common species in PCR-positive samples and included genotypes associated with human infections. Infestation prevalence with infected ticks was 5 times greater among ground-foraging birds than other bird species, but the 2 groups were equally competent in transmitting *Borrelia*. Migratory passerine birds host epidemiologically important vector ticks and *Borrelia* species and vary in effectiveness as reservoirs on the basis of their feeding behavior.

Recent outbreaks of West Nile virus infection or avian influenza indicate that birds participate in the ecology of zoonotic infections, an important cause of illness and death in humans and animals (*1*). The emergence of these threats underscores the need for understanding the maintenance of bird-associated infections in nature, which is prerequisite for disease prevention.

Migratory birds are known to carry several microbial agents of human disease, including viruses, chlamydiae, and enterobacteria (*2*,*3*). Evidence of the last 2 decades indicates that birds in North America and Eurasia host vectorborne pathogens, such as *Anaplasma* species and Lyme borreliosis (LB) spirochetes (*4*–*6*). LB is the most common vectorborne zoonosis in temperate regions of the Northern Hemisphere and is transmitted to humans by *Ixodes* ticks (*7*). *Borrelia* spirochetes infect naive *Ixodes* larvae when they feed on a reservoir host and are transmitted back to the reservoir population by infected nymphs. Rodent species, such as the white-footed mouse (*Peromyscus leucopus*) in the northeastern United States and *Apodemus* and *Clethrionomys* species in continental Europe, are common hosts of both immature ticks and LB spirochetes (*8*,*9*). However, recent field vaccination and biodiversity studies suggest that alternative hosts play a greater role than expected in the natural cycle of LB (*10*,*11*).

In comparison with studies of mammals as LB reservoirs, few studies have been conducted on the role of birds as hosts of *Borrelia*. The natural cycle of LB spirochetes, in particular *Borrelia garinii*, involves seabirds in northern Europe and game birds in the United Kingdom, which are the most studied models (*12*,*13*). However, the relationship between migratory passerine birds and *Borrelia* is less understood. Although experimental studies on avian infection have been conducted (*14*–*17*), less is known about reservoir competence of natural bird populations, especially those that could transmit ticks that frequently bite humans (*5*,*18*–*20*).

Information that would allow comparison of the reservoir importance of bird and other vertebrate populations is not available or is controversial. Although 1 modeling study found that the frequency of LB cases was positively correlated with species diversity of ground-dwelling birds (*21*), other studies have found the contribution of birds in hosting and infecting ticks to be low (*22*,*23*). Another

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uncertainty is epidemiologic implications of LB group spirochetes associated with birds. For example, birds in Europe are reservoirs of *B. valaisiana*, which has not been associated with disease (*19*).

In the present study, we characterized tick infestation and *Borrelia* transmission from migratory passerine birds captured in southern Sweden to further define their importance as reservoirs and disseminators of these spirochetes. We found that these birds are hosts of epidemiologically important vector ticks and *Borrelia* species. However, exposure of birds to ticks, which depends on feeding habits, determines their effectiveness as *Borrelia* reservoirs.

Materials and Methods

Bird Capture and Tick Collection

Birds were captured at Ottenby Bird Observatory (www.sofnet.org/ofstn/Engelska) at the southern point of Öland Island in the Baltic Sea (56°12′N, 16°24′E) southeast of the Swedish mainland (Figure 1). Japanese mist nets and Helgoland traps were used for capture as previously described (*5*), and with the approval of the Swedish Museum of Natural History, Stockholm. Birds were trapped from March 17 to May 30, and from July 7 to November 13 of 2001, periods that are representative of spring and fall migrations, respectively. Trapped birds were banded and examined daily for ticks during these periods, except on April 2, September 17, 18, 22, and 24, and November 14, 16, and 17. Recaptured birds were not studied. Ticks attached to a bird's head were removed and, after species and stage identification, stored individually at −70°C.

DNA Extraction and Quantitative Real-Time PCR

Tick DNA was extracted by using the Puregene DNA isolation protocol (Gentra Systems, Minneapolis, MN, USA) and stored at −20°C. DNA extracts were assayed for LB and relapsing fever (RF) group *Borrelia* by using a quantitative real-time polymerase chain reaction (qPCR) assay with probes and primers specific for the 16S rRNA gene (*11*). Serially diluted *B. burgdorferi* B31 and *B. hermsii* HS1 DNA were used as standards (*11*).

Identifying and Genotyping *Borrelia* Species

Borrelia species were identified by direct sequencing of the amplicons generated from the *rrs* (16S)-*rrl* (23S) intergenic spacer (IGS) or 16S gene PCRs (*24*,*25*). When necessary, nested modification of these assays was used to increase success of amplification. In addition, we obtained *rrs-rrl* IGS sequences of *B. garinii* isolated from skin biopsy specimens of erythema migrans lesions from 11 LB patients from southern Sweden (*26*). Positions with at least 2 different character states in ≥2 sequences each were considered polymorphic and included in the typing matrix.

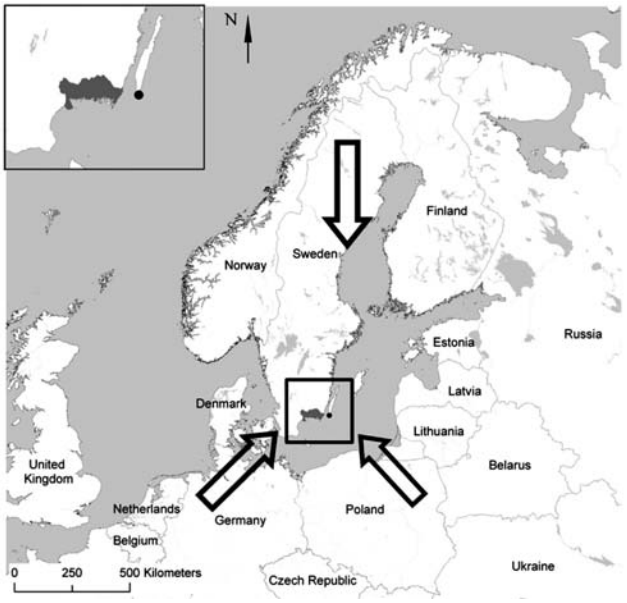

Figure 1. Scandinavian Peninsula in northern Europe. Location of Ottenby Bird Observatory (solid circle) on the southern tip of Öland Island in the Baltic Sea and nearby Blekinge County (shaded area) in mainland southern Sweden are shown in the inset. Directions of bird migration northward from outside northern Europe in the spring and back from Scandinavia and western Russia in the fall are shown by large arrows.

Sequences of new *B. garinii* IGS variants were deposited in GenBank database under accession nos. DQ307372–DQ307377.

Statistical Analyses

We used simple regression analysis, nonparametric Mann-Whitney U test, and standard parametric statistics giving the mean ± 95% confidence intervals (CIs) for continuous variables. We also used Fisher exact test, χ^2 goodness of fit, and odds ratio (OR) procedures for proportions. Statistical analyses were conducted with StatView version 5.0.1 (SAS Institute Inc., Cary, NC, USA) and StatXact version 6 (Cytel Software, Cambridge, MA, USA).

Results

Tick Infestation of Birds

According to the Ornithological Council's list of avian orders (available at www.nmnh.si.edu/BIRDNET/ORDERS/), 13,123 birds captured in this study were passerines (Passeriformes) (Table 1). In addition, there were 83 great spotted woodpeckers (Piciformes) and 54 sparrowhawks (Falconiformes). All studied birds were migratory. The 38 bird species studied comprised 6 ecologic guilds (*27*), each defined by a bird's foraging behavior. Three guilds comprised 19 species of ground-foraging

birds and included 4,614 invertebrate feeders, 906 granivores, and 125 insectivores. In addition, 500 wrens and 30 marsh warblers, which are herbaceous plant–foraging insectivores that predominantly feed on the ground, were included in this group. The remaining 3 guilds and 17 species, referred to as other birds, comprised 223 raptors, 6,612 arboreal insectivores, and 250 other reed-foraging insectivores.

Table 1. Infestation of migratory birds by *Ixodes ricinus* ticks and tick infection with Lyme borreliosis group spirochetes, Ottenby Bird Observatory study, Sweden, 2001

Bird species*	No. birds	No. ticks	No. (%) birds infested	Mean no. ticks/ infested bird	No. (%) birds with infected ticks	No. larvae	No. (%) positive larvae	No. nymphs	No. (%) positive nymphs
Ground foraging									
Erithacus rubecula	3,939	446	185 (5)	2.4	20 (11)	296	6 (2)	150	15 (10)
Luscina luscinia	32	9	4 (13)	2.3	2 (50)	5	0	4	2 (50)
Luscina svecica	85	8	5 (6)	1.6	1 (20)	0	0	8	1 (13)
Turdus philomelus	261	141	24 (9)	5.9	10 (42)	88	14 (16)	53	17 (32)
Turdus iliacus	51	22	9 (18)	2.4	2 (22)	5	0	17	4 (24)
Turdus merula	193	170	44 (23)	3.9	15 (34)	36	11 (31)	134	28 (21)
Turdus pilaris	23	6	3 (13)	2	1 (33)	3	1 (33)	3	1 (33)
Sturnus vulgaris	30	18	9 (30)	2	2 (22)	7	3 (43)	11	4 (36)
Prunella modularis	64	9	4 (6)	2.3	1 (25)	2	0	7	1 (14)
Anthus trivialis	61	29	11 (18)	2.6	6 (55)	17	8 (47)	12	6 (50)
Aluada arvensis	1	6	1 (100)	6	1 (100)	6	1 (17)	0	0
Fringilla coelebs	122	9	2 (2)	4.5	1 (50)	8	8 (100)	1	0
Carduelis flammea	441	1	1 (0.2)	1	0	1	0	0	0
Carduelis spinus	79	1	1 (1)	1	0	0	0	1	0
Pyrrhula pyrrhula	55	8	5 (9)	1.6	2 (40)	1	0	7	2 (29)
Carduelis chloris	73	5	5 (7)	1	0	1	0	4	0
Carduelis cannabina	26	1	1 (4)	1	0	0	0	1	0
Carpodacus erythrinus	55	1	1 (2)	1	0	0	0	1	0
Emberiza schoeniclus	54	1	1 (2)	1	0	1	0	0	0
Troglodytes troglodytes	500	33	17 (3)	1.9	0	25	0	8	0
Acrocephalus palustris	30	1	1 (3)	1	0	0	0	1	0
Other									
Accipiter nisus	54	2	1 (2)	2	0	0	0	2	0
Lanius collurio	169	7	2 (1)	3.5	0	4	0	3	0
Dendrocopus major	83	8	1 (1)	8	1 (100)	2	0	6	4 (67)
Hippolais icterina	87	15	2 (2)	7.5	0	15	0	0	0
Sylvia atricapilla	170	8	7 (4)	1.1	0	4	0	4	0
Sylvia borin	194	1	1 (0.5)	1	0	0	0	1	0
Sylvia curruca	621	11	8 (1)	1.4	2 (25)	4	0	7	2 (29)
Sylvia nisoria	13	4	3 (23)	1.3	0	1	0	3	0
Phylloscopus sibilatrix	65	1	1 (2)	1	0	1	0	0	0
Phylloscopus trochilus	2,116	21	19 (1)	1.1	1 (5)	9	0	12	1 (8)
Regulus regulus	2,212	1	1 (0.1)	1	0	0	0	1	0
Parus major	132	35	19 (14)	1.8	9 (47)	22	6 (27)	13	5 (39)
Parus caeruleus	541	9	6 (1)	1.5	0	1	0	8	0
Certhia familiaris	37	1	1 (3)	1	0	0	0	1	0
Phoenicurus phoenicurus	341	23	12 (4)	1.9	2 (17)	12	0	11	2 (18)
Sylvia communis	220	47	18 (8)	2.6	3 (17)	29	3 (10)	18	4 (22)
Acrocephalus scirpaceus	30	1	1 (3)	1	0	0	0	1	0
Total	13,260	1,120	437 (3)	2.6	82 (19)	606	61 (10)	514	99 (19)

*Ground-foraging species include invertebrate feeders (*Erithacus rubecula* through *Sturnus vulgaris*), insectivores (*Prunella modularis* and *Anthus trivialis*), granivores (*Aluada arvensis* through *Emberiza schoeniclus*), and herbaceous plant–foraging insectivores (*Troglodytes troglodytes* and *Acrocephalus palustris*). Other species include raptors (*Accipiter nisus* and *Lanius collurio*), arboreal insectivores (*Dendrocopus major* through *Phoenicurus phoenicurus*), and reed-foraging insectivores (*Sylvia communis* and *Acrocephalus scirpaceus*). The common names of the 38 bird species listed (from top to bottom) are European robin, thrush nightingale, bluethroat, song thrush, redwing thrush, blackbird, fieldfare, starling, dunnock, tree pipit, skylark, chaffinch, redpoll, siskin, bull finch, green finch, linnet, scarlet rosefinch, reed bunting, wren, marsh warbler, sparrow hawk, red-backed shrike, great spotted woodpecker, icterine warbler, blackcap, garden warbler, lesser whitethroat, barred warbler, wood warbler, willow warbler, goldcrest, great tit, blue tit, tree creeper, redstart, whitethroat, and reed warbler.

We measured bird infestation with ticks and then compared the occurrence of the ticks on the birds with different foraging habits. Overall, 1,127 ticks were removed from 437 (3.3%) of 13,260 birds (Table 1). Of these ticks, 606 (54%) were larvae, 514 (46%) were nymphs, and 7 (0.6%) were adults of *Ixodes ricinus*, confirming that subadult ticks predominate on birds (*5*). (Because of their low number, the adult ticks, as well as 4 *I. lividus* nymphs removed from 1 bird, were excluded from further analyses.). *I. ricinus* larvae and nymphs were found on 226 (52%) and 310 (71%) of 437 infested birds, respectively; 99 (23%) of these birds were infested with both stages. The proportion of birds infested with larvae was higher in fall than in spring: 188 (2.1%) of 9,145 birds versus 38 (0.9%) of 4,115 birds (OR 2.3, 95% CI 1.6–3.2). In contrast, the proportion of birds infested with nymphs was similar between the 2 collection periods: 212 (2.3%) birds in fall and 98 (2.4%) in spring (OR 1.0, CI 0.8–1.2). The counts of captured birds with no ticks or ≥1 subadult tick followed a negative binomial distribution and are shown in Figure 2. The counts of these ticks on infested birds more specifically corresponded to a Zipf distribution (Kolmogorov-Smirnov statistic 0.05, p = 0.3; inset in Figure 2). Aggregation of infestation risk was further indicated by the finding that once a bird is infested with 1 subadult tick, the likelihood of infestation with ≥2 such ticks was higher than expected from a Poisson distribution (p<0.0001).

Among infested birds, no correlation was found (R = 0.01) between the number of larvae and nymphs on a given bird, which is an indication that most larvae and nymphs were not host-seeking at the same time and place. Further support for this conclusion was an observed count of 99 birds co-infested with nymphs and larvae that was 38% lower than expected, if larval and nymphal infestations were fully covariant (z = 4.96, p<0.001). Co-infestation was lower than expected among both spring and fall migrants, especially in the latter group (z = 4.44, p<0.001). With regard to risk for infestation among different types of birds, prevalence was greater among ground foragers than other birds by group (335 [5.4%] of 6,175 vs. 102 [1.4%] of 7,085, OR 3.9; 95% CI 3.1–4.9). Infestation also differed by individual species (p<0.02, by Mann-Whitney U test) (Table 1).

We then retrospectively analyzed data on infestations of 15,839 birds captured in Scandinavia in 1991 that matched the species composition of this study (Table 2) (*5*). Similar to findings in the present collection, infestations with subadult stages were ≈3-fold more common among ground foragers (297 [3.5%] of 8,388) than in other birds by group (100 [1.3%] of 7,451, OR 2.7, 95% CI 2.1–3.4) and by individual species (p<0.02, by Mann-Whitney U test).

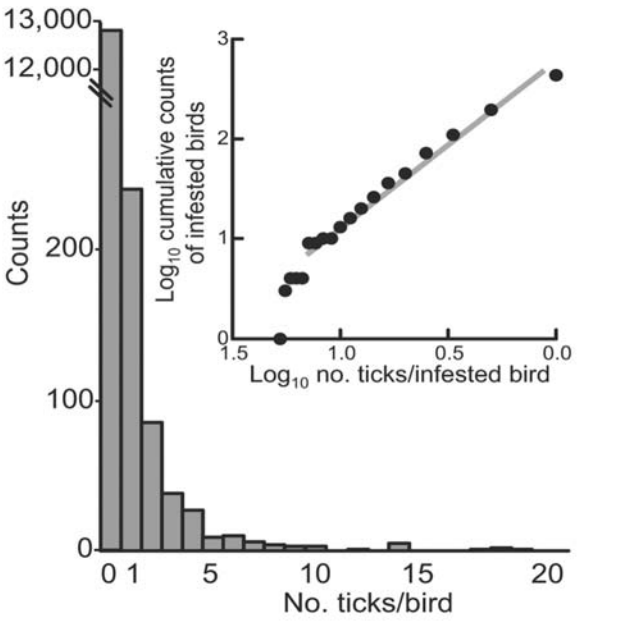

Figure 2. Frequency distribution of subadult tick infestations of migratory birds captured at Ottenby Bird Observatory, Sweden, 2001. Aggregation of risk of infestation is shown in the inset. Three birds with 26, 33, or 40 ticks, most of which were larvae, are excluded from the figure.

Borrelia Infection of Ticks

To characterize the role of birds in transmitting spirochetes to ticks, we determined the prevalence of *Borrelia* infection in larvae and nymphs by using multiplex qPCR, which also differentiates between LB and RF group spirochetes. LB spirochetes were found in 160 (1.4%) of 1,120 subadult *I. ricinus*, and were more common among nymphs than larvae (19.3% vs. 10.1%, OR 2.1, 95% CI 1.5–3.1) (Table 1), presumably a result of accumulation of infection in the former stage during consecutive feedings. Three samples (0.3%), 1 larva and 2 nymphs, were positive by qPCR for RF organisms.

LB spirochetes are rarely transmitted transovarially or during co-feeding (*28*,*29*), and their detection in feeding larvae is presumptive evidence of acquisition from the larva's host. To demonstrate that larvae in this study were infected by the birds, we analyzed 226 larvae-infested birds by comparing the proportion of the birds with infected larvae among the birds with a single larva (singly infested) and the proportion of the birds with ≥1 infected larva among the birds infested with ≥2 larvae (multiply infested). If the spirochetes were acquired transovarially, these indicators would not be expected to differ between the 2 groups. Conversely, a higher prevalence of infection in ticks from multiply infested birds in comparison to ticks from singly infested birds would be evidence of transmission from birds. Consistent with the latter hypothetical

Table 2. *Ixodes ricinus* ticks on migratory birds at bird observatories in Sweden and Denmark, 1991

Bird species*	No. birds examined	No. ticks collected	No. (%) birds infested	Mean no. ticks per infested bird
Ground foraging				
Erithacus rubecula	3,345	145	72 (2.2)	2.0
Luscina luscinia	204	90	28 (13.7)	3.2
Luscina svecica	301	18	11 (3.7)	1.6
Turdus philomelus	610	50	22 (3.6)	2.3
Turdus iliacus	457	100	38 (8.3)	2.6
Turdus merula	264	121	44 (16.7)	2.8
Turdus pilaris	109	5	4 (3.7)	1.3
Sturnus vulgaris	18	9	5 (27.7)	1.8
Prunella modularis	68	7	4 (5.8)	1.8
Anthus trivialis	237	105	47 (19.8)	2.2
Fringilla coelebs	169	3	3 (1.7)	1.0
Carduelis flammea	1,300	5	2 (0.2)	2.5
Pyrrhula pyrrhula	196	4	2 (1.0)	2.0
Carduelis chloris	93	1	1 (1.1)	1.0
Emberiza schoeniclus	187	2	2 (1.1)	1.0
Troglodytes troglodytes	674	12	6 (0.9)	2.0
Acrocephalus palustris	156	8	6 (3.8)	1.3
Other				
Accipiter nisus	93	2	2 (2.2)	1.0
Sylvia atricapilla	501	11	5 (1.0)	2.2
Sylvia borin	285	1	1 (0.3)	1.0
Sylvia curruca	450	4	4 (0.9)	1.0
Regulus regulus	2,566	5	5 (0.2)	1.0
Parus major	454	2	2 (0.4)	1.0
Phoenicurus phoenicurus	688	70	44 (6.4)	1.6
Sylvia communis	650	38	22 (3.4)	1.7
Acrocephalus scirpaceus	1,764	15	15 (0.9)	1.0
Total	15,839	833	397 (2.5)	2.1

*Ground-foraging species include invertebrate feeders (*Erithacus rubecula* through *Sturnus vulgaris*), insectivores (*Prunella modularis* and *Anthus trivialis*), granivores (*Fringilla coelebs* through *Emberiza schoeniclus*), and herbaceous plant–foraging insectivores (*Troglodytes troglodytes* and *Acrocephalus palustris*). Other species include raptors (*Accipiter nisus*), arboreal insectivores (*Sylvia atricapilla* through *Phoenicurus phoenicurus*), and reed-foraging insectivores (*Sylvia communis* and *Acrocephalus scirpaceus*). The common names of the 26 species listed (from top to bottom) are European robin, thrush nightingale, bluethroat, song thrush, redwing thrush, blackbird, fieldfare, starling, dunnock, tree pipit, chaffinch, redpoll, bull finch, green finch, reed bunting, wren, marsh warbler, sparrow hawk, blackcap, garden warbler, lesser whitethroat, goldcrest, great tit, redstart, whitethroat, and reed warbler.

outcome, the proportions in singly infested and multiply infested birds were 7 (5.5%) of 128 and 21 (21.4%) of 98, respectively (OR 4.7, 95% CI 1.9–11.6).

In another approach with multiply infested birds, we compared the count of infected larvae expected at 5.5% prevalence of infection (as found for the larvae of singly infested birds) with that observed in the larvae after the first positive larva has been identified. The observed and expected count of positive larvae was 33 and 6, respectively ($p = 0.004$), which is additional evidence of transmission of spirochetes from birds to larvae.

Excluding the 1 skylark in the study, infestation by infected ticks was higher (3.0%) in 20 ground-foraging species than in 17 other species (0.6%) ($p<0.05$, by t-test) (Table 1). These rates correlated with the overall infestation rate of birds ($R^2 = 0.77$; $p<0.001$), which is another indication that ticks were being infected by birds (Figure 3). We also compared the frequency of infection among birds in the 2 groups by measuring the ratio of birds with infected larvae to the number of larvae-infested birds. Infection with LB spirochetes was more common in ground-foraging birds than in other bird species: 17 (34.0%) of 50 birds of 12 species versus 5 (7.8%) of 64 birds of 13 species, respectively ($p<0.001$, OR 6.1, 95% CI 2.1–18.0). We excluded from this analysis 106 European robins (*Erithacus rubecula*), which predominated among ground foragers but were infested by larvae with an unusually low infection prevalence of 2%.

Borrelia Species Composition

Eighty-eight (55%) of 160 samples that were positive by qPCR with LB probe produced amplicons in *rrs-rrl* IGS or 16S PCR. The latter PCR was performed on 12 samples that in qPCR with LB group-specific probe showed a distinct amplification pattern presumably attributable to *B. valaisiana* DNA and were negative in the IGS PCR. Sequence analysis of the amplicons showed *B. garinii* in 75 (85%) samples, *B. valaisiana* in 6 (7%) samples,

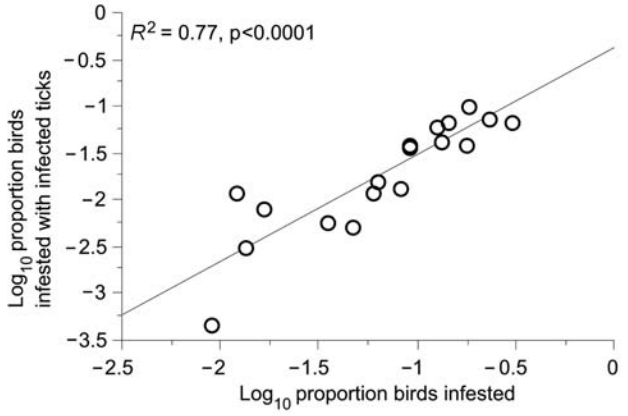

Figure 3. Relationship between tick infestation of birds and infestation with ticks infected with Lyme borreliosis group spirochetes.

B. afzelli in 4 (5%) samples, and *B. burgdorferi* in 3 (3%) samples (Table 3). The 3 samples positive by qPCR with the probe for RF spirochetes were identified by *rrs-rrl* IGS sequencing as *B. miyamotoi* group spirochetes (*30*).

To determine epidemiologic importance of *B. garinii* variants that are disseminated or maintained by migratory birds, we typed and compared the *rrs-rrl* IGS region of 47 of 75 *B. garinii* samples from bird ticks and 11 erythema migrans isolates of this species from LB patients from nearby Blekinge County in mainland Sweden (Figure 1). *B. garinii* PCR samples from ticks produced 11 variants; 6 of these variants, represented by 31 (66%) samples, were also found in LB patients. Larvae were infected with 3 *B. garinii* variants also found in biopsy specimens, which indicates that migratory birds serve as hosts for *B. garinii* strains that are pathogenic to humans.

Reservoir Competence of Migratory Birds

With the exception of pheasants in the United Kingdom (*13*), the reservoir competence of other bird groups or species, including migratory birds, is not fully understood (*31*). The efficiency of transmission of spirochetes, as measured by their prevalence in ticks, is 1 correlate of vertebrate host competence in maintaining the natural cycle of LB (*17*). To assess such competence of migratory birds, we measured and compared the spirochete count and infection prevalence in larvae and nymphs collected from these birds. Inasmuch as birds migrate in regularly alternating periods of 1 day resting and 6 days flying (*32*), we presumed that the ticks collected from the birds represent a random collection with respect to the degree of their engorgement. The frequency of spirochete counts in the larvae followed a normal distribution (Figure 4). In contrast, it was bimodal for the nymphs, which suggests that 2 populations of this stage are present: 1 with low spirochete counts and 1 with higher spirochete counts.

To further distinguish between infections of larvae and nymphs, we compared the 2 stages with respect to the correlation between the spirochete load and infection prevalence among ticks collected from the same bird. These 2 variables showed a correlation for 56 larvae from 25 birds (R^2 = 0.39, p<0.01) but not for 95 nymphs collected from 63 birds (R^2<0.01, p>0.5) (Figure 5).

We next evaluated ground-foraging birds and birds of other species for efficiency of spirochete transmission to larvae by comparing the infection prevalence of larvae from individual birds. Twenty-three birds of 8 ground-foraging species and 5 birds of 2 other species were available for this analysis. The mean infection prevalence of individual collections of larvae from ground foragers and other birds was 61% (95% CI 46%–77%) and 77% (50%–100%), respectively, (p>0.4). To validate this result, which suggests that migratory passerines transmit LB spirochetes to ticks with similar efficiency, we compared LB spirochete counts in the larvae from the 2 bird groups. The cell counts were available for 52 larvae from 25 ground foragers and 9 larvae from 5 birds of other species. Weighted means of spirochetes per infected larvae from ground-foraging birds and other bird species were 135 (95% CI 21–862) and 23 (95% CI 2–318), respectively (p = 0.4). This was additional evidence that the 2 bird groups were equally competent in transmitting infection to larvae.

Discussion

This was the first large-scale study to show that migratory passerine birds participate in the enzootic maintenance of *Borrelia* spirochetes, including species and genotypes associated with LB in humans. By combining 2 approaches, quantification of infection in vector ticks and molecular typing, we demonstrate that these birds constitute an epidemiologically important alternative reservoir of LB, as well as a means for wide distribution of the pathogen.

This study's approach of characterizing *Borrelia* infection of ticks engorged on birds is analogous to xenodiag-

Table 3. *Borrelia* species in *Ixodes ricinus* ticks from migratory birds

	Larvae	Nymphs	Total
No. ticks tested*	606	514	1,120
No. (%) positive			
LB group†	61 (10.1)	99 (19.3)	160 (14.3)
B. garinii	27	48	75
B. burgdorferi	1	2	3
B. afzelli	0	4	4
B. valaisiana	1	5	6
RF group†	1 (0.2)	2 (0.4)	3 (0.3)
B. miyamotoi	1	2	3

*Quantitative polymerase chain reaction for Lyme borreliosis (LB) and relapsing fever (RF) including *B. miyamotoi* group spirochetes (*11*).
†*Borrelia* species was determined for 88 of 160 LB-positive samples and all 3 RF-positive samples by sequencing a partial rrs-rrl intergenic spacer region region (*25*) or, for *B. valaisiana*, a partial 16S rRNA gene (*24*).

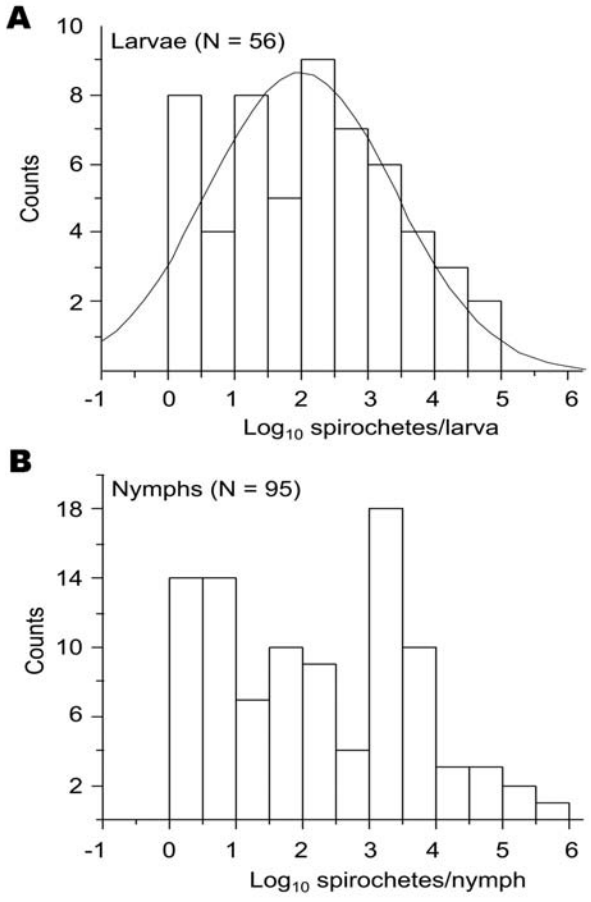

Figure 4. Frequency distribution of Lyme borreliosis group spirochete load in larvae (A) and nymphs (B). Normal comparison for the distribution of spirochete counts in larvae is shown. Values <1 cell/tick found in 5 larvae and 4 nymphs are excluded from the analysis.

nosis, which is commonly used in assessing reservoir competence in the laboratory (*17*). A correlation between rate of tick infestation and infestation with infected ticks is evidence of a bird source of infection. Consistent with this source, the proportion of birds infested with multiply infected larvae and the observed counts of infected larvae on individual birds exceeded the baseline values assumed to represent a hypothetical transovarial transmission. Furthermore, infection prevalence correlated with the number of spirochetes in larvae, which suggests a new variable for quantifying reservoir competence for *Borrelia* transmission. Finally, *Borrelia* species composition in larvae, namely, predominance of *B. garinii* and absence of *B. afzelii*, indicates the bird source of infection (*33*). However, inferring reservoir competence from measuring infection of naturally infesting ticks has drawbacks. Collection of only birds that had ticks on them at the time of capture could lead to an underestimation of the prevalence of infection among the studied bird population. Also, in this study we could not follow-up and quantify the infection of the nymphs that emerge from infected larvae, a transition that determines the ability of the nymphs to infect other hosts during subsequent feeding (*17*).

A negative binomial distribution of natural loads of subadult *I. ricinus* on migratory birds is a common characteristic of ectoparasitism (*34*,*35*), including infestation with ticks (*36*). Similar to other hosts, infestation of migratory birds is nonrandom, presumably due to different tick densities at stopover sites along the migration routes. These routes likely run in a south–north direction and within boundaries of central and northeastern Europe. Two indications of this are infestation of birds almost exclusively with *I. ricinus* ticks, which prevail in these regions, and the absence of *I. persulcatus*, a common bird parasite in eastern Europe and Asia (*37*).

Different activation times of larvae and nymphs along this geoclimatic axis also determine the dissociation between infestations with the 2 stages, as indicated by lack of correlation between their numbers on a given bird, as well as relatively infrequent co-infestations. This dissociation is further supported by the evidence of distinct

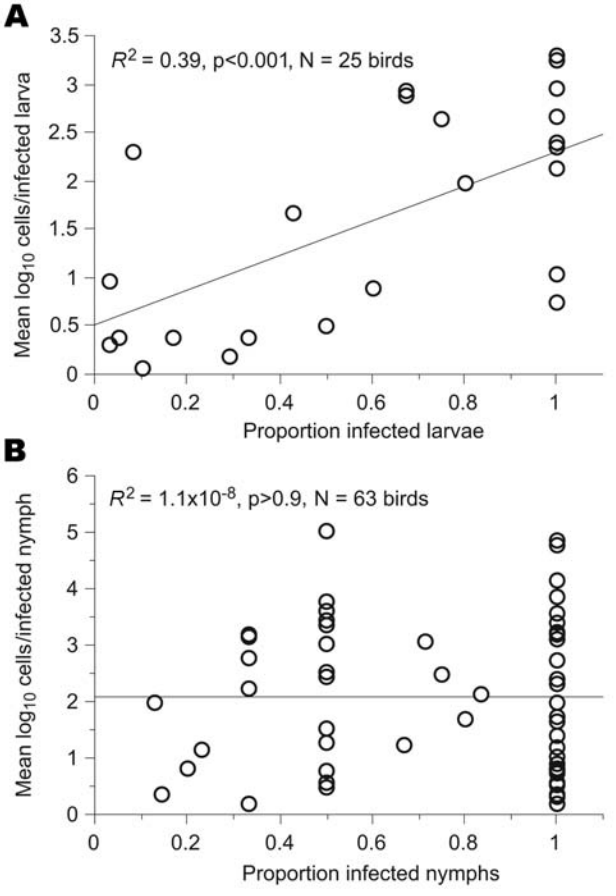

Figure 5. Relationship between Lyme borreliosis spirochete load and proportion of infected larvae (A) and nymphs (B). Values <1 cell/tick were excluded from the analysis.

histories of infection with LB spirochetes of larvae and nymphs: 1) greater prevalence of infection in nymphs than in larvae; 2) correlation between prevalence of infection and spirochete counts in larvae, but not nymphs; and 3) bimodal distribution of spirochete counts in nymphs, but not larvae, presumably due to residual infection in the nymphs acquired during feeding at larval stage. Thus, the 2 subadult tick stages represent different aspects of migratory birds' involvement in the maintenance of *Borrelia*. Whereas both stages contribute to the assessment of geographic dissemination and carrying capacity of infected vector ticks by birds, larvae provide a direct measure of birds' competence in transmitting the spirochetes.

In comparison with other hosts, birds appear to be infested with fewer ticks (*19*,*22*,*38*). For example, the 2.1–2.6 ticks per infested bird density found in this study is ≈20–30 times less than that found on rodents in south-central Sweden (*39*). Conversely, migratory bird population estimates suggest that their actual contribution in hosting, infecting, and disseminating ticks may be at least as important as that of other hosts. For example, ≈150 million migratory passerine birds come to their breeding grounds in Sweden in the spring (*40*), and at least 2 times that number migrate in the fall. Assuming that our findings are representative of these bird populations and at observed infestation and infection rates, ≈15 million infested birds would disseminate 40 million ticks, of which 5.6 million would be infected with LB group spirochetes. Five million of these ticks would carry *B. garinii*, and at least one third would be infected by birds. The 16% extrapolated prevalence of *B. garinii* found in nymphs feeding on migratory passerines in this study corresponds to ≈50% of that found in pheasants in the United Kingdom, where these birds are the major reservoir of this spirochete (*13*). Thus, migratory passerines contribute to influx of *B. garinii* into the natural circulation, where this species is known to adapt to local enzootic transmission cycle involving mammals.

Measuring the occurrence of ticks in 2 uniquely large migratory bird collections in Scandinavia at a 10-year interval provided consistent evidence of greater risk for exposure to ticks among ground-foraging birds. As a result of this increased risk, the infestation rate with infected ticks and the proportion of presumably infected birds were greater in ground feeders than in other birds. However, the transmission of spirochetes from bird to tick, defined as the amount and prevalence of infection in ticks, was similar between the 2 migratory bird groups. Thus, a bird's feeding behavior, rather than other biologic differences, is a critical determinant of its reservoir potential. Notwithstanding exceptions and as a group, those birds that spend time on the ground contribute most effectively to the maintenance of both the vector ticks and the spirochetes.

The agent of LB in North America, *B. burgdorferi*, is associated with different vertebrate reservoirs, including birds (*4*,*31*). The American robin, an abundant and commonly tick-infested passerine, is as effective as mice in reservoir competence for this bacterium (*17*). Understanding the contribution of this and other alternative reservoirs in enzootic maintenance of *B. burgdorferi* is prerequisite for advancing prevention strategies for LB (*11*).

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Mr Comstedt is a graduate student in the Department of Molecular Biology at Umeå University. His research interests include use of natural and experimental infection models in birds and mammalian hosts to understand the role of diverse reservoirs in dissemination and maintenance of *Borrelia* spirochetes, including Lyme borreliosis agents.

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Reinfestation Sources for Chagas Disease Vector, *Triatoma infestans*, Argentina

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Reinfestation by *Triatoma infestans* after insecticide spraying has caused elimination efforts in the dry Chaco region to fail repeatedly. The sources and spatial extent that need to be considered to understand the reinfestation pattern and to plan a comprehensive control program were studied in 2 adjacent rural communities in northwestern Argentina from 1993 to 1997. The effects of external, residual, and primary sources on the reinfestation pattern were evaluated by using geographic information systems, satellite imagery, spatial statistics, and 5-year retrospective data for 1,881 sites. The reinfestation process depended on primary internal sources and on surrounding infested communities. In the dry Chaco, successfully reducing the risk for reinfestation in a community depends on treating all communities and isolated sites within 1,500 m of the target community. In addition, during the surveillance phase, spraying all sites within 500 m of new foci will delay reinfestation.

Triatoma infestans, the main domestic vector of Chagas disease in Latin America, can disperse actively by flying or walking and passively through accidental carriage on humans and their belongings (*1*,*2*). Based mostly on the residual application of pyrethroid insecticides (*3*), an ongoing regional *T. infestans* elimination program has achieved only limited results in the dry Chaco region because of repeated reinfestation. Sources for reinfestation may be residual foci where triatomine bugs survived exposure to insecticides, preexisting foci overlooked by vector control staff, and adjacent infested communities left untreated (*4*–*7*). In northwestern Argentina and Bolivia, peridomestic foci of *T. infestans* detected just 1–3 months after applying pyrethroids were most probably residual foci (*5*,*8*–*10*). In the apparent absence of sylvatic foci of *T. infestans* in northern Argentina (*11*), the appearance of adult *T. infestans* can be explained by active dispersal from foci located in its flight distance (*12*–*14*). This flight distance is well within the range of clustering detected around external (up to 1,500 m) and internal sources (up to 400 m) observed in an earlier study (*7*).

Using geographic information systems, satellite imagery, spatial statistics, and retrospective data collected over 5 years, we identified *T. infestans* sources after community-wide insecticide spraying in an isolated rural community, Amamá, in northwestern Argentina (*7*). One year after spraying, an initial peridomestic focus was detected, and subsequent infestations clustered around it. This clustering suggested that residual spraying with insecticides in the colonized site and all sites in a radius of 450 m is necessary to prevent subsequent propagation of *T. infestans*. However, because the communities under surveillance are surrounded by other infested communities, preventing reinfestation is more complex. As part of a larger project on the ecoepidemiology and control of Chagas disease, we applied spatial tools (*7*,*15*) to analyze spatiotemporal *T. infestans* reinfestation patterns by following a blanket insecticide spraying in 2 adjacent rural communities surrounded by other communities with different histories of infestation. We evaluated the role of various types of *T. infestans* sources on the reinfestation pattern, with the long-term goal of building a metapopulation model of reinfestation.

Materials and Methods

Study Area

Field studies were conducted in the adjacent rural villages of Trinidad and Mercedes (27°12′33″S,

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63° 02′10″W), Santiago del Estero Province, Argentina. These communities were surrounded by other communities with diverse histories of infestation and insecticide spraying. Villa Matilde, San Luis, and San Pablo were close to Mercedes, and Pampa Pozo and a logging operation were close to Trinidad (Figure 1). All communities are located in a semiarid plain where a hardwood forest has been undergoing intensive exploitation. The area and history of infestation by *T. infestans* have been described previously (*5*,*6*,*10*). Communities consisted of 5 to 50 compounds. Most compounds include a domicile made of adobe walls and thatched roofs and a peridomestic area consisting of a patio and 3–8 structures (store rooms, kitchen, corrals, etc.) (*16*) and vary greatly in size. All domiciles were identified with a numbered plaque and mapped in 1992; new and abandoned structures were continuously recorded.

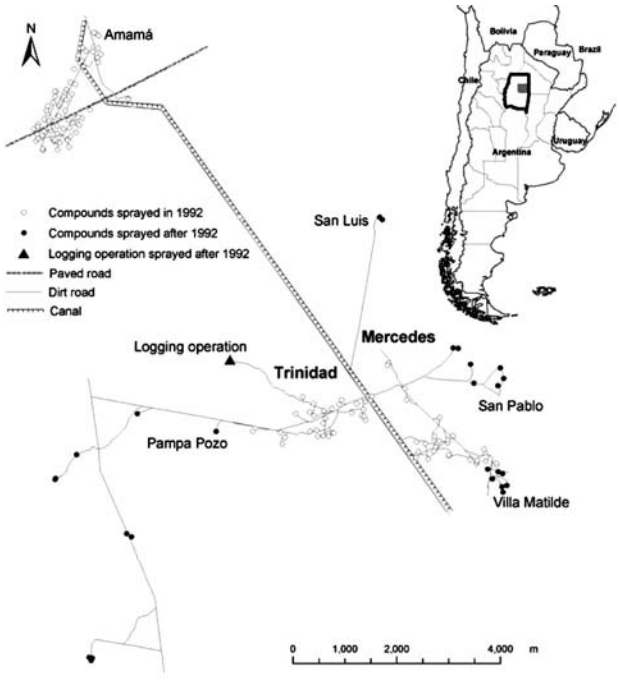

Figure 1. Study area: Trinidad, Mercedes, and neighboring communities, northwestern Argentina. Inset shows location of Moreno Department in Santiago del Estero Province.

Mapping and Geospatial Processing

An Ikonos satellite image (Space Imaging, Atlanta, GA, USA) sharpened to 1-m spatial resolution was georeferenced by global positioning system (GPS) (Trimble GeoExplorer II, Trimble Navigation Ltd., Sunnyvale, CA, USA) readings from landmarks in the field. The image and sketch maps from each compound were used to digitize structures that were not located originally with the GPS. The exact location of all structures (sites) was overlaid on the image by using sketch maps from each compound. The entomologic database from Trinidad, Mercedes, and neighboring communities was associated with geographic coordinates (in Universal Transverse Mercator, Zone 20S, WGS1984 datum) of each identified structure by using ArcGIS version 8.1 (Environmental Systems Research Institute, Redlands, CA, USA).

Field Surveys

In the baseline survey conducted in March 1992, *T. infestans* infested 88% of domiciles and 50% of peridomestic structures and colonized 79% and 38% of them, respectively (*10*). In October 1992, all compounds in Trinidad and Mercedes were sprayed with the pyrethroid deltamethrin (25 mg active ingredient/m^2) (K-Othrina, Agrevo, San Isidro, Argentina) by the Servicio Nacional de Chagas (NCS). The effectiveness of spraying was then assessed for each site by 2 technicians who spent 10 minutes per compound; all residual foci detected were immediately sprayed in December 1992 (*5*). The surveillance phase included community participation and selective insecticide spraying by NCS in sites with ≥1 *T. infestans* from 1993 to 1995 and by residents of compounds from 1996 to 2002 (*10*). The adjacent communities of Villa Matilde and Pampa Pozo were sprayed by NCS between October 1993 and May 1994; San Pablo was sprayed by residents in late 1994. The study objectives were explained to residents, and all participants signed an informed consent form.

Each domestic and peridomestic site in Trinidad and Mercedes was searched annually for triatomine insects from October 1993 to November 1997 (*17*). Two skilled insect collectors from NCS searched bedrooms, while another person searched peridomestic structures, for 30 minutes (1 person-hour and 0.5 person-hours, respectively) by using timed manual collections with 0.2% tetramethrin (Icona, Buenos Aires, Argentina) as an irritant agent (the flushing-out method). In peridomestic sites, additional searches for bugs were conducted in May 1995, 1996, and 1997 (0.5 person-hours per peridomestic compound). In May 1993, householders' collections in each compound were initiated by providing a labeled, self-sealing, plastic bag to each household. In addition, from May 1993 to November 1997, domestic sensor boxes (Biosensor, Biocientífica de Avanzada, Buenos Aires, Argentina) placed in bedrooms were inspected semiannually for evidence of infestation. In November 1995, intensive searches for insects by knock-down collections were done in a few domiciles (*17*). All bugs were identified to species and stage (*18*).

Statistical Analysis

We restricted the analysis of the reinfestation process to

1993 through 1997, when the system was less perturbed by selective insecticide spraying (the effects of which will be presented elsewhere) than thereafter. Infestation and total numbers of *T. infestans* in domestic sites were estimated on the basis of insects collected by flushing out, in sensor boxes, and by householders, and in peridomestic sites by flushing out. Prevalence and abundance of infestations were calculated for all types of peridomestic structures with ≥1 infested site detected from 1993 to 1997. In this study, cluster refers to an unusual aggregation of sites with high abundance of insects that are grouped together in time and space. Global (weighted K-function) and local (Gi[d]) spatial statistics were used to detect clustering of insects within the study area and to identify epicenters of infestation. The weighted K-function was used to analyze the spatial distribution patterns of abundance of *T. infestans* among all sites in the study area (*19*). A local spatial statistic, such as Gi[d] (*20*), can be used as a focal statistic when the weight of the point being evaluated is not included in the calculation (*7*,*21*,*22*). Gi[d] was used as a focal spatial statistic to measure spatial clustering of *T. infestans* abundance around known and suspected sources of *T. infestans* reinfestation and to calculate the range of distances over which such reinfestation occurred (*7*). Then, clustering occurs as long as Gi[d] values remain significant with increased distance, and peak clustering occurs when Gi[d] is maximized (*20*). When considering >1 site as a potential source, we corrected for multiple comparisons (*23*). Spatial analyses were performed by Point Pattern Analysis software (San Diego State University, San Diego, CA, USA) (*24*).

All sites that were positive after spraying were considered reinfested, including those that were newly infested, those where insects were discovered after intervention that may have survived treatment, and those with insects that had migrated into the trial site after intervention. Reinfestation sources of *T. infestans* were classified as follows: a) within communities, sources were residual if *T. infestans* colonies were detected in December 1992 immediately after the spraying and new otherwise; b) sources were primary if *T. infestans* colonies could not be attributed to other sources and secondary if they could be associated with an earlier primary source; c) internal sources occurred within the community, while external sources were outside the specific community (though they may have been internal to another community).

A compound was invaded when a single adult insect (or very few insects) was found in ≥1 structure in a given survey. A structure was infested when ≥1 insect was found in it, and a compound was infested when ≥1 structure in it was infested. A site was colonized when ≥1 nymph was found in it, and a compound was colonized when ≥1 structure in it was colonized.

Results

The overall prevalence of infestation in Trinidad-Mercedes was <3% through May 1995 and increased to 5%–8% thereafter (Figure 2). Colonization also increased from ≈1% through May 1995 to ≈3% through May 1997 and to >5% in November 1997. The geometric mean number of *T. infestans* per positive site fluctuated from 1 to 4, peaking in May 1996 and November 1997. Of 403 *T. infestans* captured, 248 (62%) were collected from peridomestic sites (Table). Goat corrals had more infested sites and larger *T. infestans* populations than other peridomestic structures.

The spatio-temporal reinfestation process varied between Trinidad and Mercedes (Figure 3). In Trinidad, ≈1.5 year after spraying (February 1994), the residents of 1 compound caught 25 bugs in a chicken coop. By November 1994, one domicile and 3 peridomestic sites (including a small granary) around this chicken coop were infested, and by May 1996, another colony was detected in a goat corral at the same compound. In western Trinidad, in November 1995, one colony was detected east of Pampa Pozo and south of the logging operation. Five years after spraying (1997), the number of infested sites and insects peaked; infestation clustered up to 600 m around a goat corral that hosted the largest colony detected after the 1992 spraying.

In Mercedes, in May 1995, only 2 adults (1 from each of 2 domiciles) and 1 nymph in a storeroom were captured. In November 1995, this storeroom was colonized, and adult insects were captured in the corresponding domicile. The infested site nearest to this storeroom was in the small community of San Pablo. Three years after spraying, insect populations were dispersed all over Mercedes, and by May 1996, the abundance of *T. infestans* per site was higher than ever.

Residual Foci of *T. infestans*

In Trinidad, 2 residual foci were detected in December 1992, but significant (Gi[d] >2.94, p = 0.05) clustering was detected only in May 1995 around 1 of them and only up to 50 m. Since the effects of this focus overlapped with the effects of the logging operation that was active from 1994 to 1996, this residual focus does not appear to be an independent source of *T. infestans*.

In Mercedes, 2 residual foci were detected in December 1992. Only around 1 of them, a storeroom, did we detect significant (Gi[d] >2.94, p = 0.05) clustering up to 100 m in May 1996, increasing to 250 m in November 1996 (Figure 3). This residual focus was not a likely source of *T. infestans* in 1996, given the time since this source was sprayed in 1992. A primary source detected in 1995 (with which the clustering effect of the residual focus overlapped) provided a more likely source for reinfestation in 1996.

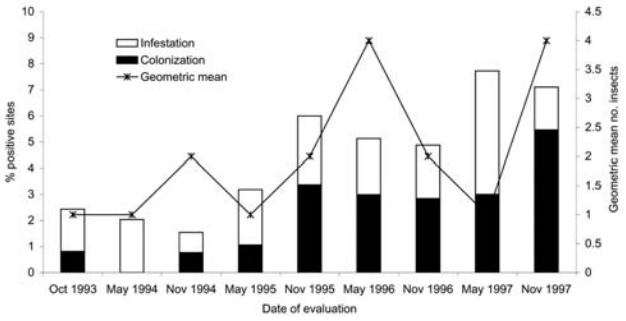

Figure 2. Percentage of infested and colonized sites and number of *Triatoma infestans* per positive site from 1993 to 1997 in Trinidad-Mercedes, Argentina.

Primary and Secondary Sources

In Trinidad, a chicken coop was a primary source of reinfestation in May 1994, with substantial focal clustering of insects up to 300 m around it in November 1994 and up to 200 m in May 1997 (Figure 3). Clustering at 300 m was also observed in May 1997 around a granary found to be colonized in November 1994, only 13 m from this primary source. In May 1997, several infested sites were detected in the influence area of the primary and secondary sources; the largest colony was associated with these sources, a goat corral in the center of Trinidad.

In Mercedes, a storeroom was considered a primary source of *T. infestans* in May 1995, when only 1 fifth-instar nymph was collected from it, and in November 1995 when it was found to be colonized and immediately sprayed. Significant focal clustering was registered up to 450 m around this storeroom in May 1996 and up to 500 m in November 1996 (Gi[d] >2.88, p<0.05) (Figure 3). This site was the nearest neighbor (900 m) to a compound in San Pablo infested with *T. infestans* in May 1997 and was believed to have been infested earlier. In February 1994, San Pablo was sprayed with residual insecticides by residents. Since San Pablo was not treated by professional spraying teams and because 1 of its compounds contained a dense colony, we considered it a potential external source of *T. infestans* for Mercedes until 1997.

External Sources

In Trinidad, 3 external sources of *T. infestans* were tested as potential sources for reinfestation. A small logging operation (Figures 1 and 3) in the northwestern extreme of Trinidad, 1,400 m from the nearest compound in Trinidad and overlooked during the 1992 spraying campaign, was found to be infested 2 years after spraying (1994) and remained infested until November 1996, when it was sprayed. Significant (Gi[d] >2.94, p<0.05) clustering around this site was registered at 1,450–1,700 m in May 1995 (Figure 3). The 5 compounds of the Pampa Pozo community were sprayed 1 year after blanket spraying of Trinidad and Mercedes. Three of the 5 compounds were infested before spraying, and the closest to Trinidad (650 m) was found to be colonized before being sprayed in late 1993. This compound was tested as a potential source of insects, and significant clustering (Gi[d] >2.94, p<0.05) was registered around it from 700 to 1,500 m in May 1995. Thus, the adult invasion registered from November 1994 to 1996 and several infestations in western Trinidad appear to have occurred while a stable focus in the logging operation and the more temporary focus at Pampa Pozo were present.

A compound in San Pablo that was infested in May 1997 and suspected of having been infested earlier was analyzed as an external source of *T. infestans* to Mercedes. A significant (Gi[d] >2.94) clustering at 950–1,450 m in November 1995, and at 950–1,200 m in 1996, was registered around this site (Figure 3). The nearest infested compound of Villa Matilde in October 1993, close to the southeastern extreme of Mercedes, was infested with only adults in November 1995 and May 1996 and was not a likely source of reinfestation.

We also considered infested sites at each of the 2 communities as potential sources for reinfestation in the neighboring community. None of the infested sites in Trinidad was found to have contributed to the reinfestation of

Table. Prevalence of infestation and number of *Triatoma infestans* insects by type of structure in Trinidad-Mercedes, Argentina, October 1993 to November 1997

Structure	No. sites inspected	No. positive sites (%)	Geometric mean no. bugs per infested site	No. *T. infestans*
Domicile	387	44 (11)	1.9	155
Goat corral	296	21 (7)	1.7	142
Kitchen or storeroom	248	15 (6)	2.4	78
Pig corral	239	3 (1)	1.6	15
Chicken coop	43	1 (2)	1	1
Tree (with or without chickens)	308	3 (1)	1	3
Other*	360	1 (0.3)	9	9
Total	1,881	88 (5)	2.2	403

*Included sheds with only a roof, bathrooms, cow and horse corrals, chicken roosts, wood piles, and small granaries. Of these, only 1 small granary was infested.

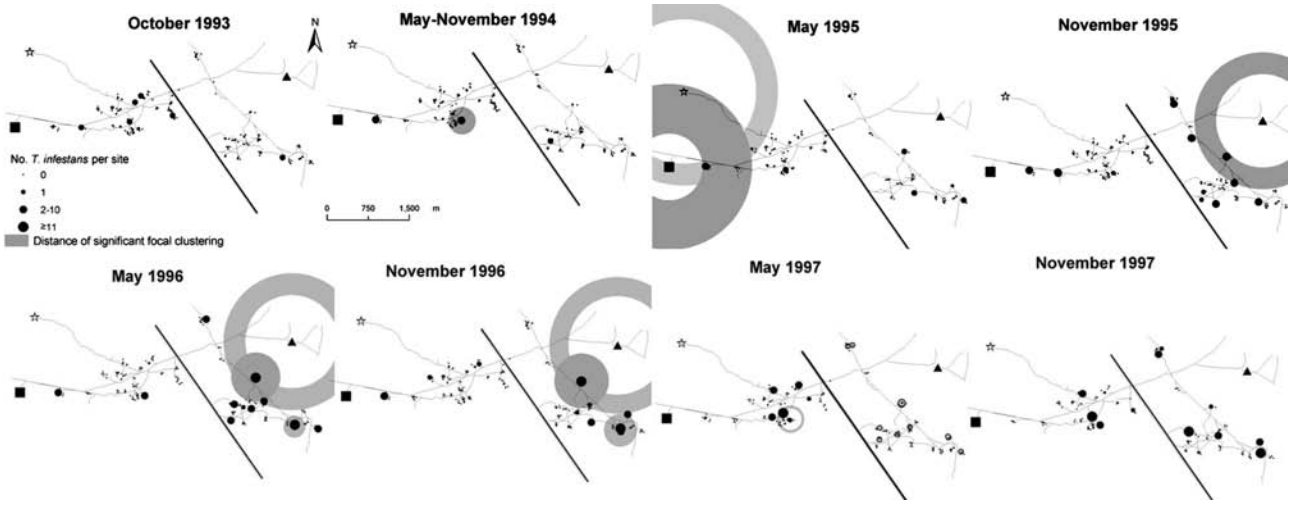

Figure 3. Abundance of *Triatoma infestans* in domestic and peridomestic sites: focal clustering distances of insect abundance around residual foci, primary and secondary sources, and external sources from 1993 to 1997 in Trinidad-Mercedes, Argentina. ☆, logging operation; ■, peridomestic structures in Pampa Pozo; ▲, peridomestic structures in San Pablo.

Mercedes, nor did the Mercedes sites appear to have contributed to the reinfestation of Trinidad.

In addition to active dispersal, 2 large triatomine colonies, which contained most instars, were detected in domiciles in Mercedes. According to householders' reports, we attribute these colonies to passive transport of *T. infestans* in bags and furniture brought from another logging operation ≈2,600 m away. A search of these belongings turned up numerous nymphs and adult bugs.

Discussion

The reinfestation dynamics of *T. infestans* in rural areas are heterogeneous in space and time and are a function of processes operating both within and between communities. Because control actions were not applied simultaneously throughout the target area of Trinidad and Mercedes, neighboring communities were external sources of reinfestation, while several peridomestic sites within the 2 communities became internal sources of reinfestation. In Mercedes, reinfestation was driven by a residual focus in 1992 and by a suspected external source in 1993, from which a primary internal source may have originated in 1995. In Trinidad, an internal source (a chicken coop in 1994) and 2 external sources (in 1993) were detected.

Residual foci were detected both in Trinidad and Mercedes, even when the insecticide spraying was performed by professional staff. Domestic residual foci were rare because pyrethroid insecticides have long-lasting residual effects indoors (*25*,*26*) but wane rapidly in peridomestic structures (*27*). Peridomestic residual foci are typically wooden structures with much of their surface exposed to extreme weather conditions and are difficult to spray adequately, as was also noted in the residual foci detected in Amamá in 1992 (*5*,*7*). The residual foci in Trinidad-Mercedes were not primary sources and acted only at relatively short distances (≤250 m) within a 30- to 48-month lag. Conversely, in the isolated Amamá, all reinfestation was driven by a residual focus (a pig corral) that became a primary source (*7*).

The effects of primary sources on reinfestation in Trinidad and Mercedes were similar, acting within a 6- to 30-month time lag and within a spatial range of 500 m. Primary sources that developed after blanket spraying produced more sites with high numbers of insects than did residual foci. The spatial range of infestation was notably similar to that registered previously in Amamá (*7*). Thus, primary sources appeared to act similarly in space and time on different types of landscape and arrangements of compounds and on areas with different histories of *T. infestans* infestation. Primary sources also appeared to have originated from external sources or residual foci, at least in our study area. In Mercedes, the primary source probably came from an external source, and in Trinidad it might have been a residual focus that was not detected by flushing-out searches after spraying in 1992 and 1993.

The large insect abundance found in February 1994 in a chicken coop, considered a primary source of reinfestation in Trinidad, indicated that the colony was founded ≥2 years previously (*28*). This source was probably originally a residual focus that then became a primary source, as with a pig corral in Amamá (*7*). The closer an external source is to the target community, the higher the risk that primary sources will appear in the community. The suspected external source in San Pablo, 600 m from Mercedes, apparently produced a primary focus, while the farthest source, 8 km away in Amamá, was not associated with reinfesta-

tion in Trinidad-Mercedes. Other external sources with persistent infestations (the logging operation and Pampa Pozo), located between 0.9 and 1.5 km away, did not produce any primary sources in Trinidad, but frequent findings of adult insects and colonized sites in western Trinidad can be attributed to them. The logging operation was more distant but lasted longer as a source (until it was sprayed in late 1996) and affected a wider area than the Pampa Pozo source that was sprayed earlier (in 1993). Thus, external sources had asynchronous dynamics with respect to internal sources, and their effects varied according to the distance from the target community and the history of infestation.

The primary sources for each community did not serve as external sources for each other, although they were only ≈500 m apart. In part, this lack of effect may be explained by the proportion of suitable habitat surrounding each source and the degree of spatial heterogeneity. Studies of mosquito vectors showed less dispersal of *Aedes aegypti* in areas where compounds were clustered than in areas where they were farther apart, and mosquito vectors tended to be spatially clustered at the household level in rural habitats with abundant human hosts and oviposition sites (*29*,*30*). In our study, internal sources were surrounded by more suitable sites for *T. infestans* than external sources, and the shorter distances between source and target increased the probability that insects would establish a new colony. Furthermore, the canal with running water between Trinidad and Mercedes may have been a barrier to *T. infestans* flight dispersal in each direction. A similar situation was found in Amamá where the northern infestation source was considered independent of the southern source, and the 2 were separated by a canal (*7*).

In addition to active dispersal, passive transport of *T. infestans* in workers' belongings provided an additional means of introducing bugs into communities. The weak local rural economy and unstable occupations of migrant workers enhanced this phenomenon. Contiguity and communication between more distant communities need to be considered for vector control programs in light of passive transport of *T. infestans*.

Our results suggest that control vector programs should cover potential external sources around the target community, at least up to 1,500 m, to reduce adult insect invasion; define the minimum control unit of *T. infestans* to increase cost-effectiveness of chemical control actions; and plan surveillance on the basis of residual spraying of recolonized sites and all sites within 450–500 m to prevent the subsequent propagation of *T. infestans*. Future work will aim to improve our understanding of the *T. infestans* reinfestation process under different regional conditions.

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Rickettsia sibirica Isolation from a Patient and Detection in Ticks, Portugal

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We report the first isolation of *Rickettsia sibirica* (strain mongolotimonae) from the blood of a patient and detection by polymerase chain reaction (PCR) of the rickettsia in a *Rhipicephalus pusillus* tick collected from a dead mongoose (*Herpestes ichneumon*) in the Alentejo region, Portugal. We describe also the first PCR detection of a new *Rickettsia* strain that is related to *R. sibirica*.

Rickettsia sibirica (strain mongolotimonae), initially named strain HA-91, was originally isolated from a *Hyalomma asiaticum* tick collected in the Alashian region of Inner Mongolia in 1991 (*1*). Since then, this emerging strain has been detected in other *Hyalomma* species, such as *H. truncatum* and *H. excavatum*, and in different areas of the world (*2*,*3*). In 1996, the first human case of infection caused by this rickettsia was described in France (*4*). This new strain was isolated from the blood and the skin of a patient admitted in March to the Hospital La Timone in Marseille. The patient had a mild illness with an eschar, rash, and fever. The unusual aspect of the case was its occurrence in March, when Mediterranean spotted fever (MSF) is rarely reported. Subsequently, other human cases were described in France, and diagnosis was confirmed by rickettsial isolation or polymerase chain reaction (PCR) detection of the agent in eschar and serum specimens. Cases outside of France have been reported in South Africa and Greece (*3*–*5*).

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In Portugal, the only previously recognized rickettsioses were caused by strains of *R. conorii* complex and *R. typhi* (*6*,*7*). However, *R. slovaca*, *R. aeschlimannii*, and *R. helvetica* have been isolated and detected by PCR in Portuguese ticks (*8*). We report the first isolation of *R. sibirica* (mongolotimonae strain) in Portugal from the blood of a patient with an initial clinical diagnosis of MSF and the detection of this rickettsia by PCR in a tick from the same region.

Case Report

A 73-year-old woman was admitted to Espirito do Santo Hospital in Évora, Alentejo region, on August 18, 2004. No history of travel, tick exposure, or direct contact with domestic animals was reported.

Before admission, the patient sought treatment from her family physician with redness and swelling of the third right toe. She was treated with 5 mg amlodipine. Three days later, her clinical symptoms had progressed. She exhibited fever, myalgia, prostration, and anorexia and was admitted to the hospital. On physical examination, the patient had a nonpuritic, generalized, erythematous, maculopapular rash involving the entire body, including the palms and soles. She was alert and oriented. Her mucous membranes appeared normal, and she had no jaundice or cyanosis. Physical examination found no difficulty in breathing, and her vital signs included temperature 39.6°C, respiratory rate 24 breaths/min, heart rate 81 beats/min, and blood pressure 156/72 mm Hg. Her heart and lungs were normal on examination. Her abdomen had normal peristaltic sounds, and she had no pain on superficial or deep palpation. The patient had a small, deep purple lesion on the anterior aspect of her right third toe. A presumptive

diagnosis of MSF was made, and treatment was initiated with penicillin G and 110 mg doxycycline, twice a day for 7 days; 48 hours later the patient was afebrile, and the rash had disappeared.

Laboratory evaluation showed a leukocyte count 7.8 × 10^3/μL with 86.4% neutrophils, hematocrit 42%, platelet count 177,000/μL, serum creatinine 1.0 mg/dL, alanine aminotransferase 93 IU/L, aspartate aminotransferase 116 IU/L, total bilirubin 0.8 mg/dL, creatine phosphokinase 267 IU/dL, lactate dehydrogenase 1,057 IU/L, and C-reactive protein 18.23 mg/dL. The chest radiograph did not show consolidation or other abnormality. Although the patient's condition gradually improved, her hepatic enzymes remained elevated.

Materials and Methods

Human Study

Isolation of Rickettsiae

A blood sample (5 mL) was collected from the patient in a sterile heparinized vacutainer (6 days after the onset of illness). The blood was left to sediment for 1 h, and the plasma, buffy coat, and erythrocytes were separated and stored in 1.8-mL tubes (Nunc) at −80°C. The buffy coat was added to a single shell vial seeded with Vero cells (African green monkey fibroblast cells) and centrifuged at 700 × g for 1 h in Eagle's minimal essential medium (MEM) at 22°C by using the centrifugation-enhanced shell-vial technique (*9*). After centrifugation, the supernatant was discarded, and 1 mL MEM was added. The shell vial was incubated at 32°C, and on day 6, the cell monolayer from the shell vial was scraped with glass beads and was transferred to a confluent monolayer of Vero cells in a 25-cm^2 culture flask, but no Gimenez staining or immunofluorescence assay (IFA) was conducted. For a period of 6 days, the monolayer was scraped daily, and a slide was prepared for Gimenez staining as previously described (*10*). At day 5, when microscopy showed rickettsial growth by Gimenez staining, a new slide was prepared to identify the bacterial growth by IFA, by using polyclonal human sera (pool of positive sera from patients containing immunoglobulin G (IgG) antibodies against *R. conorii*) as previously described (*11*). The cells were scraped with glass beads, 3 aliquots were stored in 1.8-mL tubes (Nunc) at −80°C, and the fourth was used to propagate the rickettsial isolate into a fresh confluent monolayer of Vero cells in a 25-cm^2 culture flask. After 8 days, the cells of the flask were scraped, and the cell suspension was harvested, centrifuged at 5,000 rpm for 30 min, and resuspended in phosphate-buffered saline (PBS) for DNA extraction.

Serologic testing of the patient's acute-phase serum (i.e., collected 6 days after the onset of illness) was performed by indirect IFA with antigens *R. conorii* Malish strain and *R. typhi* prepared at the Instituto Nacional de Saúde Dr Ricardo Jorge as previously reported (*11*). IgM titers ≥64 and IgG titers ≥128 for *R. conorii* and *R. typhi* were considered diagnostic of spotted fever or typhus rickettsiosis, respectively. After the isolate was characterized, the patient's serum was tested again by using the new *R. sibirica* (mongalotimonae strain) isolate as antigen.

DNA Extraction, PCR, and Sequencing

DNA was extracted from 200 μL of PBS cell suspension by using the DNeasy tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. PCR assays targeting the rickettsial genes for citrate synthase (*gltA*) and outer membrane protein A (*ompA*) were performed with specific primers. For citrate synthase gene (*gltA*), novel primers were designed, RpCS.415 (forward, 5′ GCTATTATGCTTGCGGCTGT 3′) and RpCS. 1220 (reverse, 5′ TGCATTTCTTTCCATTGTGC 3′), which amplify a 806-bp fragment. For the *ompA* gene, the primers Rr190.70p and Rr 190.602n, which amplify a 532-bp fragment, were used as previously described by Regnery et al. (*12*). Samples that yielded PCR products were confirmed by using a PCR assay incorporating the 120-M59′ and 120–807′ primer pair, which amplifies a 833-bp fragment of the *ompB* gene of *Rickettsia*, as previously described by Roux and Raoult (*13*). PCR was performed in a 50-μL reaction mixture containing 25 μL of the High Fidelity PCR Master Kit buffer (Roche Diagnostics, GmbH, Mannheim, Germany), 2 μL of each primer at 0.2 μmol/L, and 5 μL genomic DNA. Amplification was performed in a DNA thermocycler (T-3 thermoblock T, Biometra, Goettingen, Germany) under the following conditions: 2 min of initial denaturation at 94°C, then 35 cycles of 94°C for 30 s, 58°C (*gltA*) or 52°C (*ompA*, *omp*B) for 30 s, and 72°C for 90 s. Amplification was completed by holding the reaction mixture at 72°C for 7 min to allow complete extension of PCR products. For each reaction, a negative control (water) was included, and no positive control was used to avoid contamination. Five microliters of the PCR products were resolved by electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and examined by UV transillumination. PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen) as described by the manufacturer. The purified PCR products were sequenced in an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA) by using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the protocols supplied by man-

ufacturers. All sequences were determined by the consensus of the forward and reverse sequence analysis. The sequences of the *gltA*, *ompA*, and *ompB* amplicons were aligned with the corresponding sequences of other *Rickettsia* species available in GenBank/EMBL database, by using BLASTN software (*14*).

Phylogenetic Analysis

Phylogenetic relationships were inferred by using PAUP version 4b10 (*15*). For *ompA* gene analysis, a phylodendrogram was constructed by the neighbor-joining method, and distance matrixes were calculated by using the Kimura 2-parameter model to correct for multiple substitutions (*16*,*17*). Bootstrap values for the trees were obtained from 1,000 randomly generated trees.

Tick Study

Collection of Ticks

A total of 175 ticks were collected in different locations in the Alentejo region, for example, Beja, Ourique, Mourão (Alqueva Dam), and Mértola (Natural Park of Guadiana), during 2004. The species were identified on the basis of morphometric characteristics by 1 author (M. Santos-Silva), and ticks were kept in individual sterile tubes without any additive at –80°C until further processed.

DNA Extraction, PCR, and Sequencing

Ticks were washed for 5 min in iodinated alcohol and then in sterile distilled water for 5 min before being dried on sterile filter paper. DNA was extracted from ticks by using alkaline hydrolysis, as described previously (*18*). DNA from each tick was used as template in PCR assays targeting the rickettsial gene for citrate synthase (*glt*A) by using the same primer set (RpCS.415 and RpCS.1220) that was used for characterization of the human isolate. Samples that yielded PCR products were subsequently confirmed by another PCR by using primers Rr190.70p and Rr 190.602n for *ompA.* In 2 of the PCR-positive ticks, the presence of rickettsia was confirmed by using the primers 120-M59′and 120–807 for *ompB* to generate additional sequence data (*14*). PCR amplification, sequencing, and data analysis were performed as the protocol described above for the characterization of the rickettsial isolate obtained from the patient.

Nucleotide Sequence Accession Numbers

The GenBank nucleotide sequence accession numbers for partial sequences of *gltA*, *ompA,* and *ompB* genes generated in this study as follows. For PoHu10991, they are DQ423368, DQ423365, and DQ423364, respectively; for PoTiRb169, they are DQ423369, DQ423366, and DQ423363, respectively; and for PoTiRp53, they are DQ423370, DQ423367, and DQ423362, respectively.

Results

An isolate was obtained from the blood of the patient. The rickettsia was first detected in culture by Gimenez staining and IFA, and then the established isolate was characterized by PCR assays and sequencing. By BLAST analysis, the *gltA* sequence of the human isolate (PoHu10991) was 99.8% (653/654 bp), similar to that of *R. sibirica* (U59731). The *ompA* sequence was 99.8% (480/481 bp), similar to that of *Rickettsia* sp. HA-91 strain (U43796), and the *ompB* sequence was 100% (776/776), similar to that of *R. sibricia* (mongolotimonae strain) (AF123715). These data show that our isolate is definitively *R. sibirica* mongolotimonae strain.

The patient's acute-phase serum contained neither IgM nor IgG antibodies that reacted with *R. conorii* or *R. typhi* antigen by IFA. A second serum sample was not available.

Of the 175 ticks collected in nonsystematic schedule from March through August in different locations in the Alentejo region (Figure 1), 5 were *Rhipicephalus bursa*, 12 *R. turanicus*, 20 *R. pusillus*, 68 *R. sanguineus*, 59 *Hyalomma lusitanicum*, and 11 *Dermacentor marginatus*. The ticks were collected from different animals including Egyptian mongoose (*Herpestes ichneumon*), sheep (*Ovis aries*), cow (*Bos tauros*), dog (*Canis familiaris*), and vegetation (Tables 1 and 2). Rickettsial DNA was detected in 12 (6.9%) of the 175 ticks examined. Nine *Rhipicephalus* spp. and 3 *D. marginatus* contained rickettsiae detected by PCR (Table 2). All 59 *H. lusitanicum* were negative for rickettsial DNA. DNA from 1 male tick, identified as *R. pusillus*, collected in March from a dead Egyptian mongoose (*Herpestes ichneumon*) in the Alqueva Dam region (Figure 1), contained a rickettsia exhibiting nucleotide sequence of *gltA* 99.8% (654/655 bp) similar to *Rickettsia* sp. HA-91 (U59731). For *ompA* the sequence was 100% (484/484 bp) similar to *Rickettsia* sp. HA-91 (U43796), and the *ompB* sequence was 100% (660/660 bp) similar to

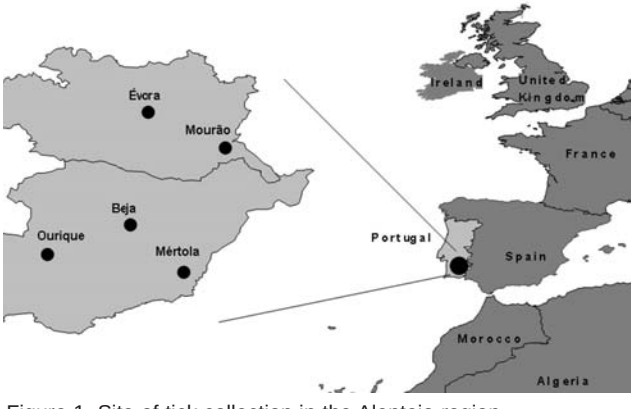

Figure 1. Site of tick collection in the Alentejo region.

Table 1. Number of *Rhipicephalus* spp. collected in the Alentejo region and identified rickettsiae

Month/site	Origin	Tick species (no., sex)*	Identified rickettsia
March			
Beja	Egyptian mongoose	*Rhipicephalus turanicus* (2M)	1 *Rickettsia* bar29
Mourão	Vegetation	*R. turanicus* (1M)	1 *Rickettsia* bar29
	Egyptian mongoose	*R. pusillus* (13F;7M)	1 *R. sibirica*
May			
Mértola	Dog	*R. sanguineus* (8F; 22M)	2 *Rickettsia* bar29
	Vegetation	*R. sanguineus* (2F; 4M)	–
		R. turanicus (2F)	–
	Cow	*R. sanguineus* (3F)	–
		R. bursa (2M)	–
	Sheep	*R. sanguineus* (1F)	–
		R. bursa (1F; 2M)	1 *Rickettsia* sp.
		R. turanicus (2F; 4M)	1 *Rickettsia* bar29
Ourique	Dog	*R. turanicus* (1M)	1 *Rickettsia* bar29
Beja	Dog	*R. sanguineus* (6 M)	1 *Rickettsia* bar29
		R. sanguineus (1M)	–
June			–
Ourique	Dog	*R. sanguineus* (10F; 7M)	–
August			
Mértola	Dog	*R. sanguineus* (2F, 2M)	–

*F, female; M, male.

R. sibirica mongolotimonae strain (AF123715). This Portuguese strain was designated PoTiRp53.

A second rickettsia species designated PoTiRb169 was identified in 1 *R. bursa* tick. The *gltA* sequence was 99.2% (655/660) similar to that of *R. sibirica* (U59734). The *ompA* was 97.5% (504/517) similar to that of *Rickettsia africae* (U83436), and the *ompB* was 98.6% (789/800) similar to that of *R. africae* (AF123706).

R. massiliae (bar 29 strain) was detected in 4 *R. turanicus* and 3 *R. sanguineus* ticks. *Rickettsia* sp. strain RpA4 was detected in 3 *D. marginatus* ticks. These data will be presented in a separate report.

Phylogenetic analysis based on the *ompA*-encoding gene showed that the human isolate PoHU10991 is most closely related to *R. sibirica* monglotimonae strain (GenBank accession no. U83439) as well as to the strain PoTiRp53, which was detected in *R. pusillus*. This cluster is supported by a high bootstrap value (>85%) (Figure 2). *Rickettsia* sp. PoTiRb169 strain is related to the *R. sibirica* cluster; however, the bootstrap value is low (52%), which means that this genotype was not accurately identified.

Discussion

To our knowledge, this is the first reported isolation of *R. sibirica* (strain mongolotimonae) from a patient's blood in Portugal. The patient, whose condition was originally diagnosed as MSF, sought treatment with 1 lesion on her toe that resembled a tick bite; fever; and maculopapular rash; these signs occurred in the month with the highest incidence of MSF. Therefore, no one suspected, on epidemiologic and clinical grounds, that she had a rickettsiosis that was different from MSF. The blood specimens were sent to our laboratory for routine serodiagnosis and blood culture. Our laboratory had long experience in isolation of rickettsiae from the blood of patients (>80 strains isolated) and performed the usual procedure for blood samples (*19*). The blood isolation was a marked achievement in identifying *R. sibirica* (strain mongolotimonae) because even if the patient had antibodies but no rickettsial isolation, we would have problems differentiating the illness from other rickettsial infections, since the serum cross-reacted with *R. conorii* antigen and in our laboratory, IFA for this rickettsia was not available. Determining

Table 2. Number of *Hyalomma lusitanicum and Dermacentor marginatus* collected in the Alentejo region and identified rickettsiae

Month/site	Origin	Tick species (no., sex)*	Identified rickettsia
March			
Mourão	Vegetation	*H. lusitanicum* (10F; 6M)	–
April			
Mértola	Vegetation	*D. marginatus* (2F; 3M)	–
May			
Mértola	Vegetation	*H. lusitanicum* (23F; 15M)	–
	Cow	*H. lusitanicum* (1F; 3M)	–
	Sheep	*H. lusitanicum* (2M)	–
	Vegetation	*D. marginatus* (6F)	*Rickettsia* sp. RpA4

*F, female; M, male.

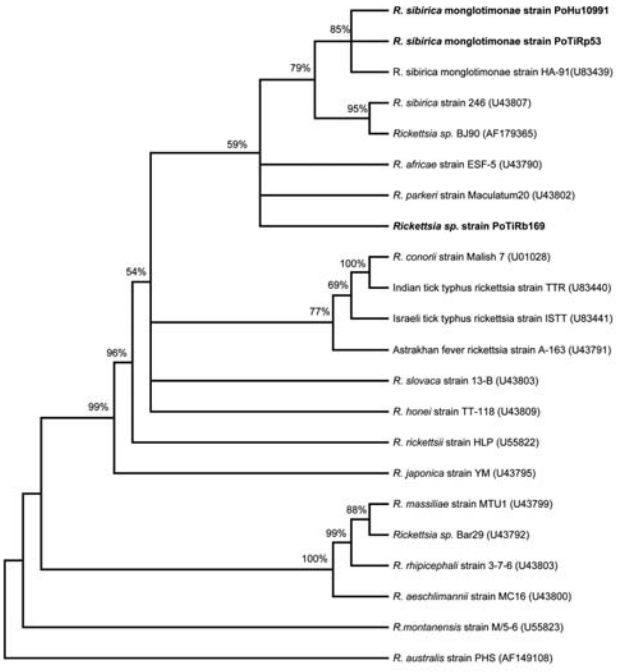

Figure 2. Unrooted consensus tree inferred from 1,000 replicated trees based on partial *ompA* gene sequence. Evolutionary distances were estimated by the Kimura 2-parameter model, and phylogenetic relationships were assessed by neighbor-joining method. Bootstrap values are indicated at the nodes. Branches with bootstrap values <50% are collapsed. Portuguese *Rickettsia* strains are indicated by **boldface** type. GenBank accession numbers are indicated for each rickettsia.

how many days were necessary for the patient's seroconversion would be useful, but a second serum sample was not available.

This patient exhibited clinical signs and symptoms similar to MSF, and she did not manifest lymphangitis or enlarged lymph nodes, a clinical feature that has been proposed as typical of *R. sibirica* (strain mongolotimonae) infection because it occurred in 44% of French patients with this infection (*20*). Characterizing and differentiating rickettsioses only on the basis of clinical manifestations is difficult since the same agent can exhibit different signs, depending on the host. In fact, in Israeli spotted fever, a study of Portuguese patients found no significant differences in the presence of eschars among patients infected with different strains of *R. conorii.* In contrast, in most cases reported from Israel, the eschar is rare or absent (*19*). Furthermore, infections caused by different *Rickettsia* spp. can cause the same sign, and not only the typical signs; for example, lymphangitis has been reported in African tick bite fever, *R. heilongjiangensis* infections, and as a reaction to argasid tick bites (*21–23*). Among 12 patients with *R. sibirica* mongolotimonae strain infection, 3 (25%), from Algeria, South Africa, and Portugal, were bitten by a tick on the foot, and in the last 2 patients, the eschar was found between the toes. Series of MSF cases have reported eschars on the leg but not in the foot (*6*,*19*).

Most of the cases caused by *R. sibirica* (strain mongolotimonae) reported in France have occurred in the spring, including only 1 case in early July, whereas the patients from South Africa and Greece were ill in winter. In contrast, our case occurred in August during the peak of the MSF season. Probably the occurrence of these cases in different months could be related to the differences in seasonal activity and population dynamics of different vectors. In countries such as Mongolia, Greece, Niger, and South Africa, *R. sibirica* is likely transmitted by *Hyalomma* ticks, but we report for the first time that a new tick host, *Rhipicephalus pusillus*, might be also implicated in the transmission of this rickettsia in Portugal. This finding alerts us to the possibility that the number of tick genera and species infected with *R. sibirica* (strain mongolotimonae) may be larger than the originally described *Hyalomma* spp. This fact is not surprising since *R. sibirica*, the agent of North Asian tick typhus, has been found in numerous different genera and species, including *Hyalomma* spp., *Dermacentor* spp., and *Haemaphysalis concinna* (*20*).

R. pusillus is present in all districts in the south of Portugal throughout the year, with a higher density from March to October (*24*). Also during this period, *R. sanguineus*, the vector implicated in transmission of the strains of *R. conorii*, exhibits higher density and activity. Although *R. sibirica* has been detected in *R. pusillus* in March, this species is also highly prevalent in August, when the human case was described. The higher density of other *Rhipicephalus* spp. such as *R. turanicus*, occurs in April and May, and for *R. bursa*, from May to August. In general, in Portugal, *Rhipicephalus* spp. are more prevalent in spring and summer. *Hyalomma* spp. are found in all seasons but are more prevalent from the end of summer through autumn and winter. *D. marginatus* prefers the cooler months (*24*). That *H. lusitanicum* ticks were not determined to be infected does not mean that they might not also be vectors of rickettsiae. This species has previously been found to harbor rickettsialike organisms, and *H. marginatum* has been found to be infected with *R. aeschlimannii* (*8*). All these ticks have been detected on humans in Portugal (*25*).

The role of *Rhipicephalus* spp. in the transmission of different rickettsiae in Portugal is corroborated by the finding of a new rickettsial strain, named PoTiRb169, detected in *R. bursa*. Although this strain differs from *R. sibirica* (strain mongolotimonae), it is closely related to this group.

The *ompA* phylogenetic analysis confirmed that rickettsial strain PoHu10991, isolated from a Portuguese

patient, and PoTiRp53 strain, isolated from *R. pusillus*, are similar to *R. sibirica* mongolotimonae strain HA-91. An identical cluster is obtained when a phylogentic tree is inferred from *gltA* gene sequences (data not shown). The *ompA* phylogeny has a low bootstrap value for the branching of *Rickettsia* sp. PoTiRb169. To establish the correct identification of this rickettsial strain according to genetic guidelines published by Fournier et al., other gene sequences (*rrs* and *sca4* genes) must be obtained, and more phylogenetic analysis must be performed (*26*).

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Dr de Sousa is an assistant researcher at National Health Institute Dr. Ricardo Jorge, Center for Vectors and Infectious Diseases Research. Her research has been focused on rickettsial agents with human health importance.

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Orientia tsutsugamushi in Eschars from Scrub Typhus Patients

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To verify the value of eschars for the diagnosis of scrub typhus and to characterize genotypes of *Orientia tsutsugamushi* in patients, we examined eschars and blood specimens of 7 patients from Shandong Province, People's Republic of China, for *O. tsutsugamushi* by polymerase chain reaction targeting the Sta56 gene. All 7 eschars and acute-phase blood samples were positive, while no specific DNA amplicons were obtained from the 7 convalescent-phase blood samples collected after antimicrobial drug therapy. The findings indicate that patients' eschars can be used for detection and genetic characterization of *O. tsutsugamushi* during the convalescent phase.

Scrub typhus, a widely endemic disease in Asian Pacific regions, is caused by *Orientia tsutsugamushi*, a gram-negative obligate intracellular bacterium in the family *Rickettsiaceae*. When the rickettsia is transmitted to a human through the bite of an infected mite, it begins to multiply at the bite site, and a characteristic skin lesion known as an eschar is formed. The pathogen then spreads systemically by the hematogenous and lymphogenous routes. Various clinical manifestations develop, including fever, rash, and lymphadenopathy (*1*).

Before 1986, scrub typhus was only found in southern China (south of the Yangtze River) primarily in the summer. *O. tsutsugamushi*, which causes "summer-type scrub typhus," is highly virulent and usually transmitted by the *Leptotrombidium deliense* mite. In 1986, scrub typhus was first reported in Mengyin County, Shandong Province, north of the Yangtze River. This newly recognized "autumn-winter type scrub typhus" is caused by a less virulent strain of *O. tsutsugamushi* and transmitted by the *L. scutellare* mite (*1*,*2*). Since then, cases of autumn-winter scrub typhus have been increasingly reported in many northern areas of China; eschars developed in 82%–91% of those infected (*1*,*2*).

Traditionally, the diagnosis of scrub typhus mainly relied on serologic tests. The disease could be retrospectively diagnosed in cases of seroconversion or a ≥4-fold rise in antibody titers between acute-phase and convalescent-phase serum specimens. The requirement of double serum specimens has limited its usage for diagnosis. Recently a polymerase chain reaction (PCR) assay was developed for detecting *O. tsutsugamushi* Sta56 gene in blood samples or isolates from patients (*3*–*8*). However, the test often gave a false-negative result because hemoglobin and other components in blood may inhibit PCR amplification (*3*,*4*,*9*). The commonly seen eschars in scrub typhus patients were suggested as alternative specimens for diagnosis (*9*). The objectives of this study were to verify the value of eschars for the diagnosis of scrub typhus by PCR assay and to characterize the genotype of *O. tsutsugamushi* during the convalescent phase.

Materials and Methods

Sample Collection

Seven scrub typhus patients reported at Feixian County (116°11′–118°18′E, 35°01′–35°33′N), Shandong Province, China, in September, October, and November of 2003–2004 were included in the study. The identification (ID) codes, age, sex, the locations of eschars, and other clinical characteristics on admission were documented (Table). The typical eschars of 2 patients (03PE1 and 04PE5) are shown in the Figure. After informed consent was obtained, 5 mL acute-phase blood was collected from each patient before treatment. Chloramphenicol was then

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Table. Clinical characteristics on admission and serologic results of 7 patients with scrub typhus*

Patient ID	Age, y (sex)	Fever, °C	Rash	Lymphadenitis	Location of eschars	Acute phase: Blood samples collected, d after onset	Acute phase: IgG titers of sera	Convalescent phase: Eschars and blood samples collected,† d after onset	Convalescent phase: IgG titers of sera
03PE1	35 (F)	38	+	+	Neck	3	N	9	320
03PE2	42 (M)	40	+	+	Umbilicus	7	80	8	320
03PE3	48 (M)	40	+	+	Right groin	5	N	10	320
03PE4	28 (F)	38	+	+	Left papilla	8	160	11	640
04PE5	53 (M)	38	+	+	Waist	2	N	6	320
04PE6	36 (M)	40	+	+	Behind right ear	10	80	15	1,280
04PE7	31 (F)	39	+	+	Left axilla	7	N	9	640

***F, female; M, male;** Ig, immunoglobulin; N, negative.
†Chloramphenicol was given to a patient immediately after the acute-phase blood was collected.

administered orally at a dosage of 1.5–2.5 g 4×/day for 4–5 days. Fever resolved for all 7 patients within 2 days of treatment. Eschar specimens and 5-mL convalescent-phase blood sample from each patient were collected at the time that the eschar spontaneously desquamated (6–15 days after treatment). Serum specimens were separated by centrifugation at 2,500× *g* for 10 min. All specimens from eschars, serum, and residual blood clots were kept at –70°C until use.

Detection of IgG Antibodies against *O. tsutsugamushi*

An indirect immunofluorescent antibody assay (IFA) was performed as described previously (*10*), by using mixed Gilliam, Karp, and Kato strains of *O. tsutsugamushi* as diagnostic antigen. Scrub typhus was diagnosed in the case of seroconversion or a ≥4-fold rise in IgG antibody titers between acute-phase and convalescent-phase sera.

DNA Extraction

Complete eschars (30–60 mg in weight) or 0.3 mL blood clot was homogenized with TE (10 mmol/L Tris Cl [pH 8.0] and 1 mmol/L EDTA) buffer and centrifuged at 3,000× *g* for 5 min; the supernatant was discarded. For the blood clot, the precipitate was resuspended and washed with TE buffer 3 times to eliminate the residual inhibitors in blood. Then 400 μL lysis buffer (10 mmol/L Tris [pH 8.0], 0.1 mol/L EDTA, 0.5% sodium dodecyl sulfate), 10 μL proteinase K (20 mg/mL; Promega Corp., Madison, WI, USA), and 2 μL lysozyme (4 mg/mL; DingGuo Biotech. Co. Ltd, Beijing, People's Republic of China) were added, and incubated at 50°C for 6 h. DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated in ethanol. Finally, the DNA was washed with 75% ethanol and dissolved in 20 μL distilled water.

PCR Amplification

PCR amplification of the Sta56 gene was performed by using species-specific primers, Pr1 (5′-tac att agc tgc agg tat gac-3′) and Pr2 (5′-AAT TCT TCA ACC AAG CGA TCC-3′) (*3*,*4*,*10*). The amplifications were performed in a volume of 50 μL with a Perkin-Elmer model 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The amplification program consisted of 1 cycle for 5 min at 94°C, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min. This process was followed by a final extension at 72°C for 5 min. The amplification products then underwent electrophoresis in a 2% agarose gel containing ethidium bromide and visualized under UV light.

The acute- and convalescent-phase blood samples were processed and run through the PCR instrument at the same time as the eschar specimens. DNA from reference strains of Gilliam, Karp, and Kato was used as positive controls, and distilled water was used as a negative control in each amplification. To avoid contamination, DNA extraction, reagent setup, PCR, and electrophoresis were performed in separate rooms.

Sequence Analysis

The purified PCR amplicons of all the positive samples were sequenced by Shanghai Invitrogen Biotechnology Co. Ltd (Shanghai, People's Republic of China). The sequences were compared with all available reported *O. tsutsugamushi* Sta56 gene sequences in GenBank by using BLAST (Basic Local Alignment Search Tool) program (available from www.ncbi.nlm.nih.gov/BLAST). The GenBank accession numbers of sequences obtained from the 7 patients in this study are DQ188085, DQ188086, DQ188087, DQ188088, DQ188089, DQ188090, and DQ188091, respectively.

Results

Seroconversion or a >4-fold rise in titers of IgG antibody to *O. tsutsugamushi* was observed in all 7 patients (Table), thus confirming the diagnosis of scrub typhus. Seven eschars and 7 acute-phase blood samples from the patients were positive by PCR targeting the Sta56 gene,

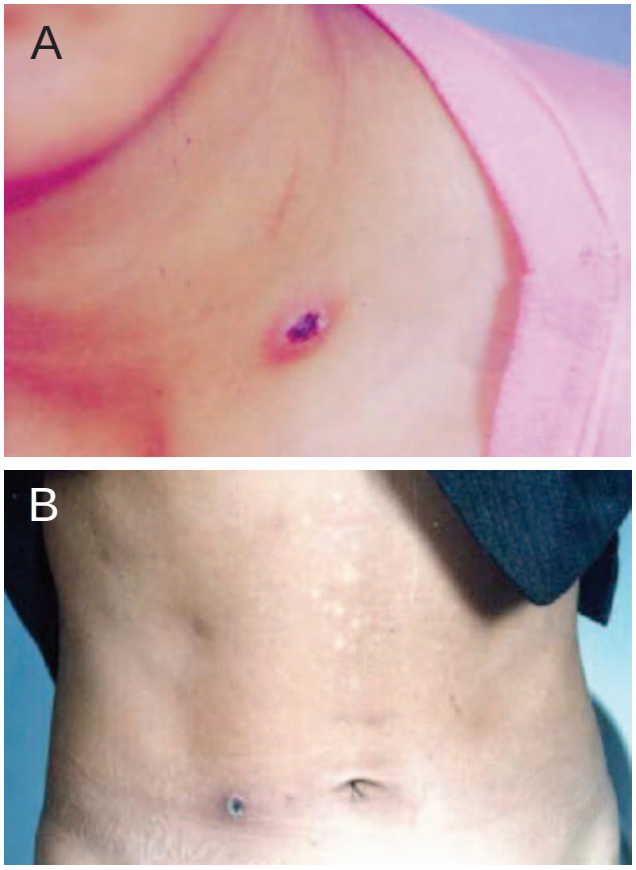

Figure. The locations of typical eschars in 2 representative patients with scrub typhus. A) An eschar on the neck of a patient (03PE1). B) An eschar on the waist of a patient (04PE5).

while 7 convalescent-phase blood samples collected after antimicrobial drug treatment were all PCR-negative.

The 317-bp partial sequence of the *O. tsutsugamushi* Sta56 gene amplified from each eschar was identical to that of its corresponding acute-phase blood sample. The sequences from the 7 patients differed from each other by 1 or 2 bp; two sequences were identical. The nucleotide sequences were 95.6%–97.8% homologous with the corresponding parts of Kawasaki strain Sta56 gene deposited in GenBank (accession no. M63383), while the sequence homologies with other strains such as Karp, Kato, Kuroki, Shimokoshi, and Je-cheon were all <75.87%.

Discussion

Previous studies used spleen tissues of infected mammals or acute-phase blood from patients to detect *O. tsutsugamushi* by PCR (*3*,*4*). However, PCR amplification of *O. tsutsugamushi* DNA from blood often lacks sensitivity because some hemoglobin, iron porphyrin, and other factors may inhibit the PCR, although obtaining and processing the blood that avoids the inhibitors is possible (*3*,*4*,*9*). Ono et al. previously found *O. tsutsugamushi* DNA (identified as Kawasaki type) in only 1 patient's eschar before antimicrobial drug treatment but not in the acute-phase blood sample (*9*). In the present study, 7 scrub typhus patients were examined, and *O. tsutsugamushi* DNA was successfully detected in their spontaneously desquamated eschars and acute-phase blood samples. These findings further proved that eschars could be used as an alternative, easily acquired, and sensitive sample for the diagnosis of *O. tsutsugamushi* infection, particularly when persons are reluctant to provide a blood sample because of cultural or other reasons.

We described a new simple confirmatory diagnostic assay in which eschars are used as an alternative to serologic tests such as IFA, which usually requires double blood samples from acute and convalescent phases. In addition, from the successful and efficient detection of the *O. tsutsugamushi* DNA in naturally desquamated eschars, we can infer the presence of the agent in eschars before beginning antimicrobial drug therapy. If eschars had been sampled during the acute phase by punch biopsy, this method could be used for the early diagnosis of scrub typhus.

A previous study carried out in Thailand detected *O. tsutsugamushi* DNA in convalescent-phase blood of patients after a single dose of doxycycline (*10*). However, in the present study, *O. tsutsugamushi* DNA was not persistent in the convalescent-phase blood of patients after 4–5 days of chloramphenicol treatment. Whether a lack of PCR sensitivity or difference in the treatment regimens explains the apparent lack of *O. tsutsugamushi* DNA in the convalescent-phase blood samples is not known. A possible reason is that the patients in the present study were infected with the less virulent strain of *O. tsutsugamushi* (*11*), which may only persist in blood for a short period after antimicrobial drug treatment. Our previous study indicated that to isolate *O. tsutsugamushi* from patients with autumn-winter scrub typhus, cyclophosphamide (0.25 mg/g of body weight) had to be injected into the experimental mice after injection of patients' blood to suppress immunity (*11*).

Sequence analysis of partial Sta56 gene clarified that the genotypes of *O. tsutsugamushi* in the scrub typhus patients from Shandong Province, China, were more closely related to Kawasaki type, which is less virulent than other genotypes and only caused a mild syndrome (*1*). The finding has applications for physicians to treat patients and prescribe medicine.

Dr Liu is a professor of epidemiology at the Beijing Institute of Microbiology and Epidemiology. His research interests focus on vectorborne infectious diseases and highly pathogenic avian influenza.

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Epidemiologic and Molecular Analysis of Human Tularemia, United States, 1964–2004

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Tularemia in the United States is caused by 2 subspecies of *Francisella tularensis*, subspecies *tularensis* (type A) and subspecies *holarctica* (type B). We compared clinical and demographic features of human tularemia cases from 1964 to 2004 from 39 states in which an isolate was recovered and subtyped. Our data indicate that type A and type B infections differ with respect to affected populations, anatomic site of isolation, and geographic distribution. Molecular subtyping with pulsed-field gel electrophoresis further defined 2 subpopulations of type A (type A-east and type A-west) that differ with respect to geographic distribution, disease outcome, and transmission. Our data suggest that type A-west infections are less severe than either type B or type A-east infections. Through a combined epidemiologic and molecular approach to human cases of tularemia, we provide new insights into the disease for future investigation.

Tularemia is a zoonotic disease caused by the gram-negative bacterium *Francisella tularensis* (*1*). Transmission occurs through arthropod bites (especially ticks and deerflies), ingestion of contaminated food or water, inhalation of contaminated aerosols, and handling of infected animal tissues. Human illness usually takes 1 of several clinical forms. The most common is ulceroglandular tularemia; more serious forms include pneumonic, typhoidal, and meningitic tularemia.

Nearly all human cases of tularemia in the United States are caused by *F. tularensis* subspecies *tularensis* (type A) or *F. tularensis* subspecies *holarctica*, (type B) (*2*). Type A and B isolates can be differentiated on the basis of glycerol fermentation, virulence in animal models, and by polymerase chain reaction (*3–5*). Recently, molecular assays have been developed to further discriminate within subspecies by using pulsed-field gel electrophoresis (PFGE), multiple-locus variable–number tandem repeat analysis (MLVA), whole-genome microarrays, and single nucleotide variations (*6–9*). In nature, the 2 subspecies are thought to be maintained in distinct but incompletely defined cycles (*10–12*).

Although both subspecies of *F. tularensis* cause human illness (*10*), the clinical and epidemiologic features of type A and type B infections have not been systematically compared for a substantial number of cases. Furthermore, the implications of other subgroupings, as defined by molecular techniques, are largely unknown. We identified to subspecies level all human *F. tularensis* isolates that were submitted to the Centers for Disease Control and Prevention (CDC) for a 40-year period and further subtyped a portion by PFGE. Our findings demonstrate distinct subpopulations of *F. tularensis* that differ in their clinical manifestations, geographic location, and likely modes of transmission.

Methods

We analyzed all available *F. tularensis* isolates from humans (n = 316) recovered by or submitted to CDC by state and local health departments from 1964 through 2004. All work with *F. tularensis* cultures was performed in a biosafety level 3 (BSL-3) laboratory using BSL-3 safety precautions. Isolates were confirmed as *F. tularensis* by characteristic growth on agar and direct fluorescence antibody staining. Type A and type B isolates were differentiated by biochemical subtyping (glycerol fermentation)

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with the 96-well automated MicroLog MicroStation System with GN2 Microplates (Biolog Inc, Hayward, CA, USA) (*13*).

Demographic and clinical data on source patients were extracted from submission forms that accompanied the isolates. Extracted information included patient age and sex, date of disease onset, form of clinical disease, anatomic source of isolate, underlying illness, outcome, county where infected, and likely mode of transmission. Isolates received after 1990 (n = 155) were matched with patients reported through the National Electronic Telecommunication Surveillance System to extract county of exposure. Information was verified by contacting the reporting state health department.

PFGE subtyping was performed on a subset of isolates with a modified version of the PulseNet 1-day standardized protocol for subtyping foodborne pathogens (*14*,*15*). A total of 41 type A and 22 type B isolates were used. DNA-embedded agarose plugs were prepared and lysed under BSL-3 conditions. PFGE plugs were cut (2.0 mm) and digested with 40 U *Pme*I enzyme (New England Biolabs, Beverly, MA, USA) for 6 hours at 37°C under BSL-2 conditions. *Salmonella enterica* serotype Braenderup (H9812) was used as a reference standard, and DNA plugs were digested with 50 U *Xba*I enzyme (Roche Diagnostics, Indianapolis, IN, USA) for 3 hours at 37°C. Seakem agarose gels (1%) were prepared with 0.5× Tris-borate-EDTA buffer (Sigma, Saint Louis, MO, USA), and digested DNA plugs were loaded on the comb. Electrophoresis was performed with a CHEF Mapper (Bio-Rad, Hercules, CA, USA) with switch times of 1.79 to 10.71 s at 6V/cm for 17.5 h at 14°C. Gels were stained with ethidium bromide (1 mg/mL) and gel images captured by using a Gel Doc 1000 imager (Bio-Rad).

Analysis of PFGE gels was performed with BioNumerics software version 3.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Gels were normalized by using the *Salmonella* reference strain. A dendrogram was constructed with Dice similarity coefficients and unweighted pair group method with averages (UPGMA). Demographic and clinical data were analyzed with EpiInfo 2002 (CDC, Atlanta, GA, USA). To analyze categorical data and nonparametric tests for continuous data, χ^2 was used.

Results

A total of 316 *F. tularensis* isolates from 39 states were available for analysis; 208 (66%) were type A, and 108 (34%) were type B (Table 1). Among the 10 states submitting at least 10 isolates, the distribution of the 2 subspecies was nonrandom ($\chi^2 = 34$, $p < 0.0001$). Overall, the 2 subspecies segregated into several geographically distinct clusters (Figure 1). Most isolates on the eastern seaboard, in and around Arkansas and Oklahoma, and in the broad area from the Colorado Rockies west to the Sierra Nevada Mountains were type A. In contrast, most isolates from the northern Pacific Coast and along tributaries of the Mississippi River were type B.

To further understand this geographic clustering, we developed a PFGE subtyping method for *F. tularensis* using *Pme*I. PFGE differentiated both between type A and B strains and among type A strains (Figure 2). Electrophoresis conditions were optimized to resolve restriction fragments between 25 kb and 125 kb for both *F. tularensis*

Table 1. Number of type A and type B *Francisella tularensis* isolates, United States, 1964–2004*

State	Type A	Type B	Total
Alaska	2	0	2
Arizona	4	0	4
Arkansas	16	0	16
California	3	7	10
Colorado	19	8	27
Delaware	3	0	3
Georgia	5	1	6
Idaho	1	2	3
Illinois	2	6	8
Indiana	1	6	7
Iowa	2	1	3
Kansas	9	0	9
Kentucky	3	20	23
Louisiana	7	0	7
Maine	4	0	4
Maryland	5	0	5
Michigan	1	2	3
Minnesota	0	1	1
Mississippi	2	1	3
Missouri	12	8	20
Montana	0	1	1
Nebraska	6	1	7
Nevada	9	0	9
New Jersey	1	0	1
New Mexico	6	0	6
New York	5	3	8
North Carolina	4	0	4
North Dakota	3	5	8
Ohio	1	1	2
Oklahoma	14	1	15
Oregon	8	14	22
Pennsylvania	1	0	1
South Dakota	9	4	13
Tennessee	4	0	4
Texas	4	1	5
Utah	8	0	8
Virginia	9	1	10
Washington	0	8	8
Wyoming	13	3	16
Unknown	2	2	4
Total	208	108	316

*Submitted to the Centers for Disease Control and Prevention.

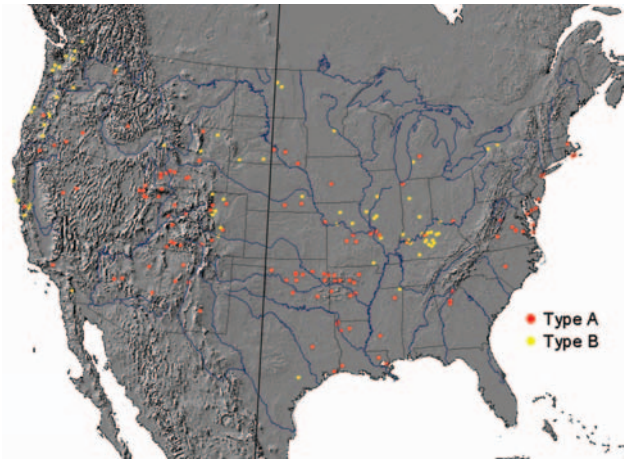

Figure 1. Geographic distribution for type A (red circles) and type B (yellow circles) *Francisella tularensis* isolates from humans, contiguous United States, 1964–2004. Each circle represents 1 isolate. Isolates were plotted randomly within the county of exposure. The 100th meridian is indicated by the black line transecting the United States. County of exposure was known for 198 (63%) isolates.

and the *Salmonella* reference strain. Reproducibility testing verified that no differences in PFGE patterns were observed between experiments.

Comparison of PFGE patterns for a subset of isolates (41 type A, 22 type B) from distinct locations (Figure 3) showed that all 22 type B isolates yielded the same PFGE pattern, which is consistent with previous data showing that type B strains exhibit little genetic diversity (*7*). In contrast, the type A isolates yielded PFGE patterns that fell into 2 main clusters (Figure 3; 32 type A-east, 9 type A-west). Isolates from 1 cluster came from states completely west of the 100th meridian (type A-west), while the remainder came from states transecting or east of the 100th meridian (type A-east, Figure 1). Based on the PFGE clustering, subsequent epidemiologic analysis was performed separately for type A isolates from the eastern (n = 133; type A-east) and western (n = 71; type A-west) contiguous United States.

Information on age and sex was known for 290 (92%) and 312 (99%) source patients, respectively. Males accounted for 75% (157/208) of type A and 72% (75/104) of type B infections. Patients with type A infections were significantly younger than patients with type B infections (median age 38 years vs. 50 years, $p<0.01$), and patients with type A-west infections were significantly younger than those with type A-east infections (median age 33 years vs. 44 years, $p<0.02$). An immunocompromising condition (e.g., malignancy, organ transplant, and HIV infection) was reported for 11 (10%) of 108 type B–infected patients, 6 (5%) of 133 type A-east–infected patients, and none (0%) of 68 type A-west–infected patients ($p<0.01$). For the 235 (74%) source patients for whom outcome was known, the overall case-fatality rate was 9% (20/235), with similar rates for infections caused by type A (9%, [15/161]) and type B (7% [5/74]) isolates. However, among type A infections, case-fatality rates differed markedly between type A-east (14% [15/106]) and type A-west (0% [0/55]) ($p<0.002$).

Information on anatomic source was available for 280 (89%) isolates. Overall, more than half of the isolates were recovered from lymph nodes and a quarter from blood (Table 2). While type A and type B isolates did not differ significantly with respect to anatomic source, significant differences were observed between type A-west and both type A-east ($p<0.001$) and type B ($p<0.002$) isolates. Type A-east and type B isolates were more likely than type A-west isolates to be recovered from blood and lung (Table 2), whereas type A-west isolates were more likely to be recovered from lymph nodes.

The clinical form of the disease was known for only 104 (33%) source patients. Ulceroglandular and glandular were the most commonly reported clinical forms of tularemia, accounting for 68 (65%) of 104 cases with information available. Other clinical syndromes included pneumonic (17%, 18/104), typhoidal (12%, 12/104), oculoglandular (4%, 4/104), meningitic (1%, 1/104) and pharyngeal (1%, 1/104) forms. Patients with typhoidal or pneumonic disease were generally older (median age 48 years and 53 years, respectively) than those with either glandular or ulceroglandular disease (median age 11 years

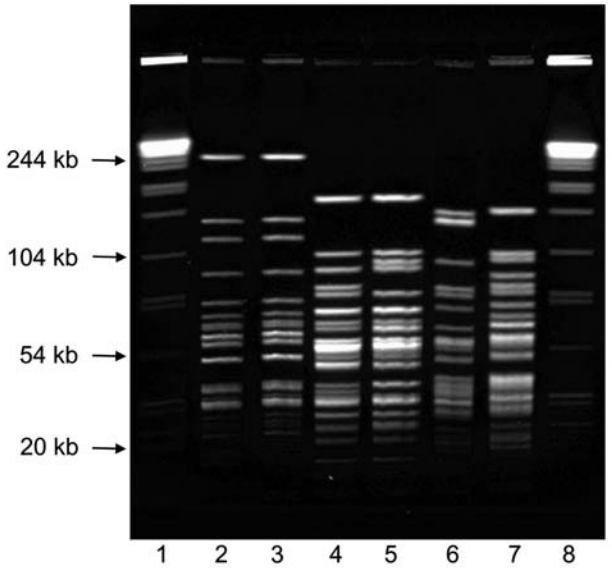

Figure 2. *Pme*I pulsed-field gel electrophoresis patterns for *Francisella tularensis* type A and type B. Lanes 1 and 8, *Salmonella enterica* serotype Braenderup standard; lane 2, Virginia 1997 type B; lane 3, Indiana 1999 type B; lane 4, New York 2004 type A-east; lane 5, Oklahoma 2001 type A-east; lane 6, Oregon 2004 type A-west; lane 7, California 2002 type A-west.

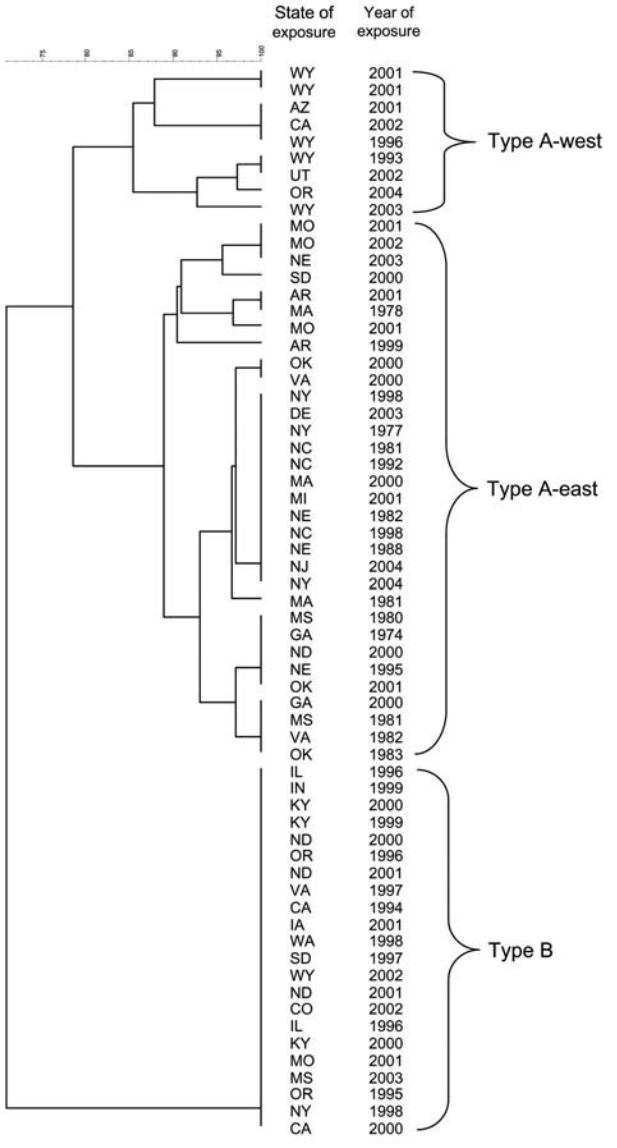

Figure 3. Dendrogram based on *Pme*I pulsed-field gel electrophoresis patterns of 41 *Francisella tularensis* type A and 22 type B isolates from humans. A 1.5% optimization and 1.5% tolerance were used to calculate Dice similarity coefficients. Isolate information is given with respect to exposure state and year.

and 37 years, respectively). Among the subset of patients for whom a clinical form of infection was reported, glandular or ulceroglandular disease was diagnosed more frequently in patients with type A infections (71% vs. 52% for type B) and pneumonic disease was diagnosed less frequently (12% vs. 30% for type B).

Among 292 source patients whose date of disease onset was known, 210 (72%) were infected in May through September; no difference in onset between type A and B infections was noted (Figure 4). A possible source of infections was reported for 133 (42%) of 316 patients. Direct animal contact accounted for 47 (47%) of 99 type A and 18 (53%) of 34 type B infections. Most type A infections were associated with exposure to either lagomorphs (53%, 25/47) or cats (30%, 14/47). Type B infections were most often associated with exposure to rodents (33%, 6/18) or cats (22%, 4/18); none were linked to lagomorph exposure. Arthropod bites accounted for 44 (44%) type A infections and 10 (29%) type B infections. Although the arthropod involved was not always identified, ticks were reported as the source for 21 (100%) of 21 type A-east, 9 (100%) of 9 type B, and 4 (44%) of 9 type A-west infections. Biting flies were only linked to type A-west infections and accounted for 5 (55%) of 9 type A-west infections attributed to a known arthropod bite. The remaining 8 (8%) type A and 6 (18%) type B infections were in patients with both animal and arthropod exposure or were attributed to environmental exposures such as landscaping.

Discussion

Categorization of *F. tularensis* type A and type B was first proposed by Olsufiev et al. in 1959 (*16*). These 2 subspecies have been suggested to differ in their ecology and possibly their virulence for humans (*17*). Our study, which includes isolates obtained during a 40-year period, further refines these views. By combining PFGE subtyping of isolates with geographic data, we found evidence that human type A infections can be further divided into at least 2 distinct subgroups (type A-east and type A-west). In addition, our data suggest that type A-west infections are less severe than type B or type A-east infections.

The most notable finding of this investigation concerns fatality rates. The case-fatality rate for type A-east infections was 14%, compared with 7% for type B infections and 0% for type A-west infections. Type A-east and type B isolates were more likely to have been recovered from patients' lungs or blood, whereas type A-west isolates were more often isolated from lymph nodes. The apparent reduced invasiveness of type A-west strains may be explained by the younger age of the patients infected. However, a review of the literature shows that few, if any, tularemia deaths have been reported in the Rocky Mountain region (*18–21*), regardless of patient age. We hypothesize that the milder form of clinical disease associated with type A-west may be due to differences in virulence factors or perhaps infectious doses associated with differing modes of transmission.

Infections caused by type A-west, type A-east, and type B appear to occur in distinct geographic foci, suggestive of different ecologic niches. Type B infections cluster along major waterways, such as the upper Mississippi River, and in areas with high rainfall, such as coastal areas of the Pacific Northwest. Type A-west infections predominate in

Table 2. Anatomic source of *Francisella tularensis* isolates, United States, 1964–2004

Site	Type A, no. (%)*	Type B, no. (%)	Type A-east, no. (%)	Type A-west, no. (%)
Blood	46 (25)	24 (24)	43 (36)	3 (5)
Cerebrospinal fluid	5 (3)	0 (0)	4(3)	1 (2)
Eye	2 (1)	5 (5)	1(1)	1 (2)
Lung	18 (10)	12 (12)	14 (12)	3 (5)
Lymph node	109 (60)	58 (59)	56 (48)	53 (87)

*One additional isolate was recovered from a penial exudate.

the arid region from the Rocky Mountains west to the Sierra Nevada Mountains. Type A-east infections occur in 2 main areas: 1) the central southeast states of Arkansas, Missouri, and Oklahoma and 2) along the Atlantic Coast, east of the Appalachians. The central southeast region is a major focus of human tularemia, accounting for 50% of all reported cases in the United States (*22*). The type A-east infections along the Atlantic Coast may be linked to the importation of rabbits from Arkansas, Oklahoma, Missouri, and Kansas to hunting clubs in Massachusetts, Pennsylvania, New Jersey, and Maryland in the 1920s and 1930s (*17*,*23*). Whether the geographic demarcation of type A-west and type A-east adheres strictly with the 100th meridian will require further PFGE analysis of more type A strains.

Animal studies have indicated that type A isolates are often associated with lagomorphs (rabbits and hares), while type B isolates are more often obtained from rodents (*12*,*17*). Consistent with these findings, our results show that human type A-east and type A-west infections were associated with exposure to lagomorphs, whereas human type B infections were associated with exposure to rodents. Both type A and type B infections were associated with exposure to cats. Ticks were implicated in transmission of both type A-east and type B infections to humans, whereas biting flies were only implicated in transmission of type A-west infections. The restriction of deerflies (*Chrysops* spp.) and associated human cases to western states was previously noted by Jellison (*24*).

This study is subject to several limitations, including record completeness, ascertainment bias, and number of *F. tularensis* isolates PFGE subtyped. Subspecies differentiation for *F. tularensis* has historically been dependent on the recovery of an isolate, and subtype information is not captured in the national disease reporting system. The overall case-fatality rate in this study was 9%, which is severalfold higher than the <2% previously reported (*25*). The higher rate suggests enhanced ascertainment of fatal cases. In addition, 36% of type A-east and 25% of type B isolates were recovered from blood, a much higher rate than previously reported. By analyzing only patients with culture-confirmed infections, we may have selected for patients with more fulminant disease.

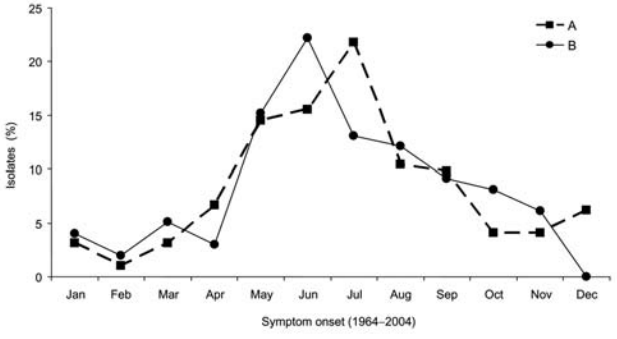

Figure 4. Seasonal distribution of type A and type B *Francisella tularensis* isolates from humans, United States, 1964–2004.

Our results demonstrate that *Pme*I PFGE subtyping is useful for dividing type A isolates into geographically and clinically meaningful subgroups. Nineteen type A isolates analyzed by PFGE in this study (12 type A-east, 7 type A-west) were previously analyzed by MLVA and divided into 2 subpopulations, A.I and A.II (*26*). Subpopulations independently identified by the 2 methods are in complete agreement, which suggests that type A-west is analogous to A.II and type A-east is analogous to A.I. With training and interlaboratory validation, the PFGE method described here could be adopted by PulseNet laboratories throughout the country that use the standardized PulseNet PFGE protocol for foodborne pathogens (*27*). The PulseNet network is an existing laboratory infrastructure with all of the necessary equipment and software to perform, normalize, and compare PFGE patterns. PFGE subtyping of *F. tularensis* isolates would allow states to determine the potential geographic origins of tularemia cases and also share and compare their PFGE patterns within the PulseNet network.

Although type A is often referred to as the more virulent subspecies of *F. tularensis* and of greatest concern with respect to bioterrorism, our comparative analysis suggests that this view should be reevaluated. We found that human type A-west infections are markedly less severe than type B infections. Further studies are warranted to determine the basis of the clinical, geographic, and ecologic differences between infections caused by type B, type A-west, and type A-east.

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Smallpox during Pregnancy and Maternal Outcomes

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A historical study evaluated maternal outcomes in pregnancy complicated by smallpox. The overall case fatality was estimated to be 34.3% (95% confidence interval [CI] 31.4–37.1), and the proportion of miscarriage or premature birth was estimated to be 39.9% (95% CI 36.5–43.2). Vaccination before pregnancy reduced the risk for death.

Pregnant women are at special risk for complications of smallpox vaccination (*1*); therefore, vaccination is not recommended for pregnant women in the absence of a reemergence of smallpox (*2*). Smallpox in pregnancy is believed to be more severe than in nonpregnant women or adult men (*3*), but this consensus is based on a limited number of studies conducted during the mid-20th century (*4–6*). This article examines the outcomes of pregnancy complicated by smallpox in historical records from the 19th and 20th centuries.

The Study

Since most large outbreaks were documented before the mid-20th century, I collected and reviewed the literature dating back to the 19th century. Technical details of the literature review are provided in online Appendix 1 (available at http://www.cdc.gov/ncidod/EID/vol12no07/05-1531_app1.htm). All selected publications were retrospective studies based on epidemiologic observations of outbreaks that reported case fatalities, miscarriages, or premature births. Because vaccination or advances in obstetrics over time could bias these outcomes, these factors were abstracted from each publication and considered separately, when possible. Outcomes were then stratified by gestation period at onset of smallpox (by trimester), clinical classification of smallpox, and vaccination history. Case fatalities were compared between pregnant and nonpregnant patients. Except in Rao's work in Madras (*4*), miscarriage and premature birth were not separated, so they are described together.

Nineteen outbreaks were identified from historical records (*4*,*7–20*), and of these, 16 allowed estimates to be made of case fatality, and 15 allowed estimates of the proportion of miscarriage or premature birth. Of 1,074 pregnant patients, 368 died; and of 830 pregnant patients, 331 miscarried or gave birth prematurely (Figure). Since these articles are from many years ago, the proportion of cases that were undetected or unreported cannot be determined nor can the length of time since vaccination in persons who were vaccinated. Descriptions of excluded literature are given in online Appendix 1; individual case records were provided in 3 outbreaks and are included in online Appendix 2 (available at http://www.cdc.gov/ncidod/EID/vol12no07/05-1531_app2.htm).

Figure, panel A, shows the distribution of estimated case fatalities for each outbreak with the corresponding 95% confidence intervals (CIs). Case fatalities varied widely among outbreaks. The earliest outbreak in 1830 (before compulsory vaccination) yielded the highest estimate (81.5%), while the 1913 outbreak in Australia had the lowest (4.3%). The overall crude case fatality was estimated to be 34.3% (95% CI 31.4–37.1). Case fatality, stratified by gestational age at onset of smallpox, is presented in Table 1; only 4 studies enabled stratification by gestational age. Case fatality was highest during the third trimester, except in Queirel's study, which included few cases (*18*). Case fatality, stratified by the clinical classification of smallpox, is shown in online Appendix 2. All patients with hemorrhagic cases died, but all patients without a rash (variola sine eruptione, VSE) survived.

Case fatalities among pregnant and nonpregnant patients are compared in Appendix 2. Case fatality was not

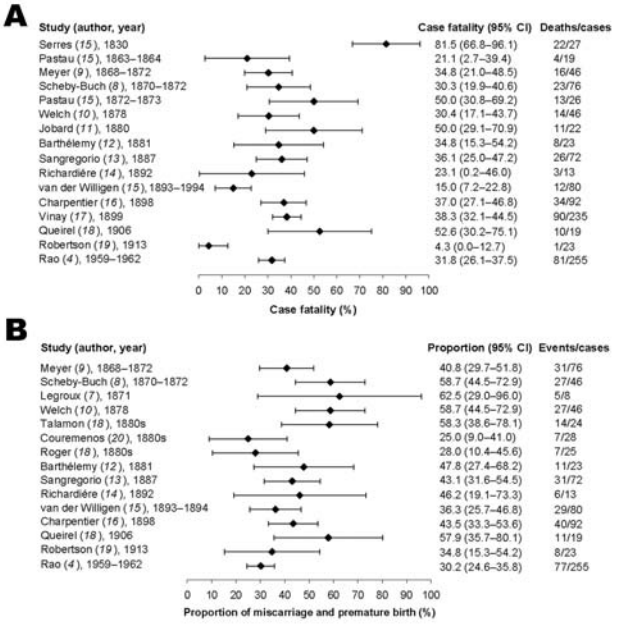

Figure. Maternal outcomes in pregnancies complicated by smallpox from data from 19th- and early 20th-century outbreaks. A) Case fatalities and B) proportions of miscarriage or premature birth are shown. Miscarriage and premature birth before maternal death are included.

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Table 1. Case fatality among pregnant women with smallpox by gestational age, according to data from 19th- and early 20th-century outbreaks*

	Gestational age ≤3 mo		Gestational age 4–6 mo		Gestational age 7–9 mo	
Reference	D/C	CF (95% CI)	D/C	CF (95% CI)	D/C	CF (95% CI)
Meyer (*9*), 1868–1872	3/33	9.0 (0.0–18.9)	11/33	33.3 (17.2–49.4)	8/10	80.0 (55.2–100.0)
Welch (*10*), 1878	4/12	33.3 (6.7–60.0)	4/22	18.2 (2.1–34.3)	6/12	50.0 (21.7–78.3)
Queirel (*18*), 1906	2/4	50.0 (1.0–99.0)	7/10	14.5 (41.6–98.4)	1/5	17.9 (0.0–55.1)
Rao (*5*), 1959–1962	7/21	33.3 (13.2–53.5)	16/65	24.6 (14.1–35.1)	34/94	36.2 (26.5–45.9)
Total	16/70	22.9 (2.3–43.4)	38/130	29.2 (14.8–43.7)	49/121	40.5 (26.8–54.2)

*D/C, smallpox deaths/cases; CF, case fatality; CI, confidence interval.

significantly higher in pregnant patients in the Rotterdam outbreak (p = 0.33), where many VSE cases apparently occurred. The risks for a fatal outcome among pregnant patients in Berlin and Madras were 2.5× and 4.2× higher than among nonpregnant patients (p<0.01 for each). I also compared vaccinated and unvaccinated pregnant patients, showing that the risk for death was significantly higher among unvaccinated women in these 3 outbreaks (7/7 vs. 7/39, p<0.01; 2/2 vs. 10/78, p = 0.02; and 9/12 vs. 17/82, p<0.01, respectively).

Crude proportions of miscarriage and premature birth, with 95% CI, are given in the Figure, panel B. The overall crude proportion of miscarriage or premature birth is estimated to be 39.9% (95% CI 36.5–43.2). Five outbreaks allowed stratification by gestational age at onset of smallpox (Table 2). The overall proportion of premature birth was highest during the last trimester of pregnancy, but no clear pattern was seen with regard to the frequency of miscarriage or premature birth. The proportion of miscarriage and premature birth, stratified by severity of smallpox, is shown in online Appendix 2. All hemorrhagic cases resulted in either miscarriage or premature birth before the mother's death. Even mild cases, those classified as discrete or VSE, tended to result in miscarriage or premature birth. Only the 1878 outbreak in Philadelphia (*10*) allowed a comparison between vaccinated and unvaccinated pregnant patients. Twenty-two of 39 vaccinated and 5 of 7 unvaccinated patients miscarried or delivered prematurely (p = 0.68).

These outcomes could only be compared by history of miscarriage in the 1913 outbreak in Australia (*19*). Two of 3 patients with no history and 6 of 20 with a history of miscarriage had a miscarriage or premature birth, but this difference was not significant (p = 0.27, odds ratio 4.7, 95% CI 0.4–61.8). Comparison by previous experience of normal delivery (primipara or multipara) could only be performed with the data from Rotterdam from 1893 and 1894 (*15*). Ten of 21 primipara patients and 18 of 53 multipara patients had a miscarriage or premature birth (p = 0.30), which suggests that delivery history did not greatly affect the outcome of pregnancy complicated by smallpox.

Conclusions

Since outbreaks have been limited since the mid-20th century by the successful smallpox eradication program, historical records are a useful tool to document common patterns of maternal outcomes in pregnancy complicated by smallpox. Such analysis may be limited by unknown numbers of missed or unreported cases or imperfect vaccination histories. My estimates of the overall crude case fatality and proportion of miscarriage or premature birth were high. This study and Rao's (*4*) improve our understanding of smallpox in pregnancy, highlighting 3 points. First, case fatality is highest during the last trimester of gestation, but miscarriage and premature birth do not vary by trimester. Physiologic changes in the third trimester could partly explain the higher case fatality (*21*). Second, even mild cases were at high risk of causing miscarriage or premature birth. Third, miscarriage and premature birth were not significantly associated with vaccination history or previous miscarriage or delivery. That is, vaccination may not prevent miscarriage and premature birth.

Although prior vaccination offers less protection to pregnant women than others (*22*), this study shows that vaccination might offer at least partial protection. Case fatality in the event of a bioterrorist attack could be

Table 2. Miscarriage or premature birth among pregnant women with smallpox by gestational age, according to data from 19th- and early 20th-century outbreaks*

	Gestational age ≤3 mo		Gestational age 4–6 mo		Gestational age 7–9 mo	
Reference	L/C	PL (95% CI)	L/C	PL (95% CI)	L/C	PL (95% CI)
Meyer (*9*), 1868–1872	7/33	21.2 (7.3–35.1)	16/33	48.5 (31.5–65.4)	8/10	80.0 (55.3–100.0)
Welch (*10*), 1878	8/12	66.7 (40.1–93.2)	9/22	40.9 (20.5–61.3)	10/12	83.3 (62.4–100.0)
Queirel (*18*), 1906	3/4	75.0 (32.8–100.0)	8/10	80.0 (55.3–100.0)	0/5	0 (NC)
Robertson (*19*), 1913	1/2	50.0 (0.0–100.0)	6/9	66.7 (36.0–97.3)	1/12	8.3 (0.0–23.9)
Rao (*5*), 1959–1962	10/21	47.6 (26.4–68.9)	16/65	24.6 (14.2–35.0)	41/94	43.6 (33.6–53.6)
Total	29/72	40.3 (29.0–51.5)	55/139	39.6 (31.5–47.7)	60/133	45.1 (36.7–53.5)

*L/C, miscarriage or premature birth/cases; PL, proportion of miscarriage and premature birth; CI, confidence interval; NC, not calculable.

lowered with vaccination before pregnancy and should be considered if the risk for such an attack is high.

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Japanese Spotted Fever, South Korea

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We describe the first case of Japanese spotted fever and the first isolate of spotted fever group rickettsia from a patient in South Korea. The isolated rickettsia from the patient was identified as *Rickettsia japonica* by analysis of the nucleotide sequences of 16S rRNA, *gltA, ompA, ompB*, and *sca4* genes.

The *Rickettsiaceae* family comprises obligate intracellular bacteria and contains 2 genera: *Rickettsia* (typhus group and spotted fever group[SFG]) and *Orientia*. Scrub typhus caused by *O. tsutsugamushi* is the most prevalent rickettsiosis in South Korea (*1*). SFG rickettsiae were first demonstrated to exist in South Korea by the isolation of *Rickettsia akari* from the Korean vole in 1957 (*2*). However, not a single case of rickettsialpox or other SFG rickettsiosis has been documented in South Korea. Recently, evidence for the existence of SFG rickettsiosis has been provided by serologic survey and DNA detection in South Korea (*3–5*). Moreover, SFG rickettsiae displaying homology with *R. japonica* and *R. rickettsii* were detected in *Haemaphysalis* ticks by polymerase chain reaction analysis of the citrate synthase (*gltA*) gene, 16S rRNA, and *ompA* genes (*6*). However, no human cases of SFG rickettsiosis have been reported, and no SFG strain has been isolated from a person so far.

In this report, we present the first documentation of Japanese spotted fever in South Korea and isolation of *R. japonica*. To our knowledge, this is the first report of an SFG rickettsia isolated from a patient in South Korea.

Case Report

A 65-year-old farmer was admitted to a hospital in Incheon, South Korea, on July 9, 2004; he had experienced fever, back pain, and myalgia for 5 days before admission. He lived in Mueui Island, ≈20 km east of Incheon. On physical examination, he had fever of 38.6°C, cervical and axillary lymphadenopathies, and a maculopapular rash. An eschar, which was smaller and more shallow than those of scrub typhus, was noticed on the chest wall (Figure 1). Laboratory studies showed a hemoglobin level of 7.7 mmol/L, a leukocyte count of 8×10^9/L, and a platelet count of 87×10^9/L. The patient was treated with oral doxycycline (200 mg/day), but the fever persisted during the treatment. On the third hospital day, petechiae developed on the trunk and extremities, including palms and soles (Figure 1). The leukocyte count increased to 11.2×10^9/L, and the platelet count decreased further to 32×10^9/L. He also showed confusion, irritability, and radiographic evidence of interstitial pneumonitis. The patient was then given azithromycin (500 mg/day intravenously) instead of oral doxycycline because of the possibility that he was infected with doxycycline-resistant *O. tsutsugamushi*. His fever resolved during next 5 days and he was discharged.

The serum samples were tested for antibody against *O. tsutsugamushi* (Boryong), *R. typhi* (Wilmington), and the isolated strain (Inha1) by using the indirect fluorescent-antibody (IFA) test. The serum specimen taken on the day of admission was negative for antibodies against all *Rickettsia* spp. by the IFA test. The serum sample taken after 18 days was sent to the national reference laboratory

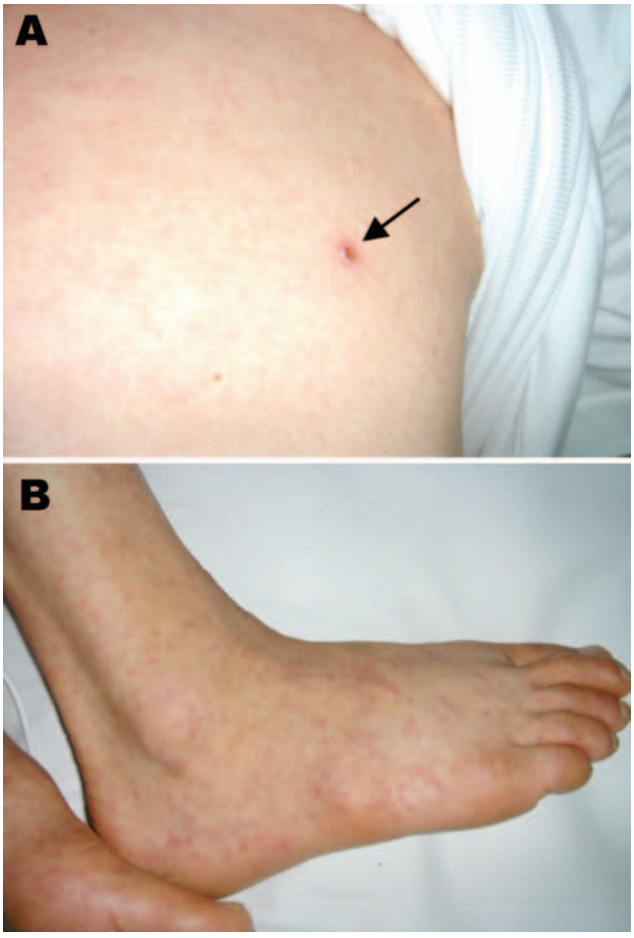

Figure 1. Skin findings of the patient. A small eschar (arrow) on the chest with erythematous rash (A) and petechiae (B) were observed.

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(Division of Rickettsial Diseases, Department of Bacteriology, National Institute of Health, Seoul, Republic of Korea) and was reported to be positive against *R. japonica* with a titer of 1,024. The convalescent-phase serum sample, taken on the 30th day after admission, was positive for antibodies against the isolated strain (Inha1) with a titer of 5,120.

To isolate the pathogen, a few drops of blood taken on the day of admission were added directly to the monolayer cultures of ECV304 cells, a spontaneously transformed cell line derived from human umbilical vein endothelial cells (obtained from S.Y. Kim) (*7*). After incubation for 24 h, monolayers of ECV304 were maintained in M199 medium containing 10% fetal calf serum and observed daily with an inverted microscope. The infected cells exhibited few cytopathologic changes, displaying only a few rounded cells. On day 28, IFA staining was done by using the convalescent-phase serum of the patient to visualize the rickettsiae. Many intracellular bacteria were observed inside the cells; they were not seen in the staining with control serum. From these results, we tentatively identified our isolated bacterium (strain Inha1), as a member of SFG rickettsiae.

Amplification and sequencing of the 16S rRNA, *gltA*, *ompA*, *ompB*, and *sca4* genes were as described by Lee et al. (*6*). Sequences were aligned by using the multiple-alignment algorithm in the MegAlign software package (Windows version 3.12e; DNASTAR, Madison, WI, USA), and phylogenetic trees were constructed by the neighbor-joining method with the MEGA program (*8*). Sequences were compared to the sequences with those of 16 reference strains of SFG rickettsiae (*9*–*11*). The nucleotide sequence (AY743328) of the 16S rRNA gene was identical to that of *R. japonica* YH. Inha1 demonstrated 16S rRNA sequence similarities of 95.9%–99.7% to the other strains of SFG rickettsiae. In the phylogenetic tree, Inha1 formed a cluster with *R. japonica* YH, separate from the other strains of SFG rickettsiae (Figure 2). The nucleotide sequence (AY743327) of the *gltA* gene of Inha1 showed a high similarity (99.8%) with that of *R. japonica* YH. The sequence similarities of Inha1 to the other strains of SFG rickettsiae were 93.6%–99.1%.

The nucleotide sequence of the *ompA*, *ompB*, and *sca4* of Inha1 strain also showed a high similarity to *R. japonica* YH and the sequence similarities to *R. japonica* YH were 100, 99.9, and 99.9%, respectively.

Conclusions

To identify the isolate at species level, we determined the sequence of 5 genes that have been used for the phylogenetic classification of rickettsiae (*9*–*14*). The sequences are identical or highly homologous to those of *R. japonica*. Although the sequence similarity of the *gltA* gene of Inha1 strain to *R. japonica* YH was 99.8%, the other 4 genes show sufficient similarity to fulfill the criteria suggested by Fournier et al. (*9*).

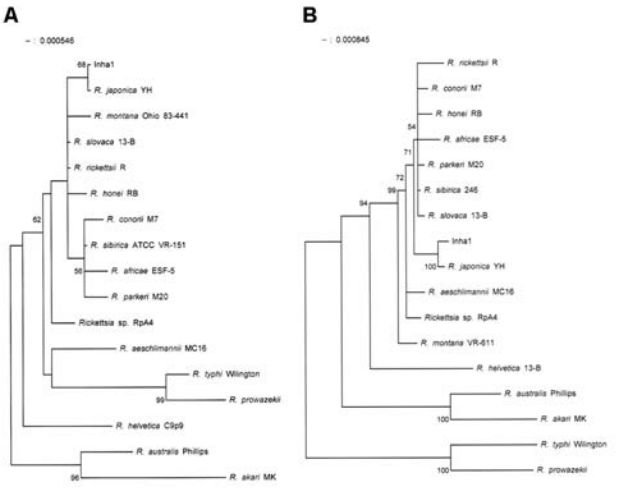

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences (A) and *gltA* gene sequences (B) of the isolated rickettsial strain (Inha1). The phylogenetic tree was constructed by the neighbor-joining method with MEGA software. Bootstrap analysis was performed with 100 replicates. The GenBank accession numbers for the 16S rRNA gene sequences included are as follows: *Rickettsia japonica*, L36213; *R. rickettsii*, L36217; *Rickettsia* sp. RpA4, AF120026; *R. aeschlimannii*, U74757; *R. africae*, L36098; *R. conorii*, AE008647; *R. honei*, AF060705; *R. montana*, U11016; *R. parkeri*, U12461; *R. sibirica*, D38628; *R. slovaca*, L36224; *R. helvetica*, L36212; *R. australis*, U12459; *R. akari*, L36099; *R. typhi,* U12463; and *R. prowazekii*, M21789. The GenBank accession numbers for the *gltA* gene sequences of these bacteria are U59724, U59729, AF120029, U59722, U59733, U59730, U59726, U74756, U59732, U59734, U59725, U59723, U59718, U59717, U59714, and M17149, respectively.

R. japonica was first isolated in Japan from human patients and ticks (*15*). The isolation of *R. japonica* in South Korea is not surprising because of the geographic proximity of South Korea to Japan. Furthermore, among 4 SFG rickettsiae detected in Korean ticks, 3 strains were highly homologous to *R. japonica* (*6*). Therefore, *R. japonica* may be the most dominant SFG rickettsiae distributed in South Korea, and the geographic distribution of *R. japonica* may be more widespread than previously known. However, other SFG rickettsiae, including *R. sibirica*, may be present in northeastern Asia. To clarify this issue, more strains of SFG rickettsiae must be isolated from other locations within Korea.

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Dr Chung is a member of the Korean Society of Infectious Diseases. His primary research interests are infections by

intracellular organisms, especially *Rickettsia* and *Plasmodium falciparum*.

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Transmission of New Bovine Prion to Mice

Thierry G.M. Baron,* Anne-Gaëlle Biacabe,* Anna Bencsik,* and Jan P.M. Langeveld†

We previously reported that cattle were affected by a prion disorder that differed from bovine spongiform encephalopathy (BSE) by showing distinct molecular features of disease-associated protease-resistant prion protein (PrP^{res}). We show that intracerebral injection of such isolates into C57BL/6 mice produces a disease with preservation of PrP^{res} molecular features distinct from BSE.

Until recently, transmissible spongiform encephalopathy (TSE) in cattle was believed to be caused by a single strain of infectious agent identified at the beginning of a foodborne epidemic of bovine spongiform encephalopathy (BSE). Characterization of the infectious agent associated with BSE showed unique features. These include defined incubation periods and distribution of brain lesions after transmission to wild-type mice, not only directly from cattle, but also after natural or experimentally induced cross-species transmission (*1*,*2*). The uniform features of the disease in cattle have also been shown by analysis of the distribution of neurodegenerative brain lesions at different places during the BSE epidemic (*3*,*4*).

Western blot analyses of protease-resistant prion protein (PrP^{res}) accumulating in the brains of animals and humans with BSE have demonstrated specific molecular features. These include a low molecular mass of unglycosylated PrP^{res} with high proportions of diglycosylated PrP^{res} (*5*,*6*). However, recent studies reported cases of prion abnormalities in cattle with different PrP^{res} features (*7*,*8*). Three cattle isolates from France have been reported, characterized by a higher apparent molecular mass of unglycosylated PrP^{res} (H-type isolates) and decreased levels of diglycosylated PrP^{res} when compared with BSE isolates (*7*). In addition. only PrP^{res} from H-type isolates were labeled by monoclonal antibody P4 with defined PrP^{res} N terminus epitope specificity, in contrast with PrP^{res} from BSE isolates, which suggests a different cleavage by proteinase K of the disease-associated protein (*9*).

Twenty years after identification of the BSE epidemic in cattle, the origin of the BSE agent remains controversial (*10*,*11*). Researchers have often considered the most likely source to be a recycled infectious agent derived from prion-associated diseases found in other species, such as scrapie in sheep and goats. The recent description of unusual phenotypes of bovine prion diseases distinct from BSE is therefore puzzling (*7*). This situation has been reinforced by a second bovine amyloidotic spongiform encephalopathy found in cattle in Italy (*8*). However, whether such cases of bovine prion disorders were transmissible, and to what extent the infectious agent caused specific features distinct from BSE, have not been demonstrated.

The Study

Experimental groups of 20 (4- to 6-week old) C57BL/6 female mice (Charles River, L'Arbresle, France) were injected intracerebrally with 20 µL of 10% (weight/volume) homogenates per mouse prepared from brain stem samples of 3 cattle TSE isolates. Two of the isolates were characterized, as previously described (*7*), by a higher molecular mass of unglycosylated PrP^{res} (H-type isolates) and labeling with P4 monoclonal antibody (Table). A typical cattle BSE isolate was also analyzed. Mice were housed and cared for in an appropriate biohazard prevention area (A3) according to European (directive 86/609/EEC) and French ethical committee (decree 87–848) guidelines. Mice were checked at least weekly for neurologic clinical signs and were killed when they exhibited signs of distress or confirmed evolution of clinical signs. The whole brain of every second mouse was frozen and stored at –80°C before Western blot analysis. The other brains were fixed in 4% paraformaldehyde for other histopathologic studies.

Frozen mouse brain tissues and fixed brain tissues were examined by Western blot analysis and immunohistochemical tests as previously described (*12*,*13*). PrP^{res} extracted from half of whole brain was detected with monoclonal antibodies Sha31 (1:10 from TeSeE sheep/goat Western blot, Bio-Rad, Hercules, CA, USA) (*14*) and (340 ng/mL) (*15*). These antibodies are directed against the 144-WEDRYYRE-151 and 88-WGQGG-92 murine amino acid PrP sequences, respectively. Antibody 12B2, which has an N-terminal specificity similar to that of monoclonal antibody P4, shows poor binding to BSE-derived PrP^{res}, but unlike P4, binds with high affinity to prion protein from most mammalian species, including mice and cattle. Bound antibodies were detected by using enhanced enzymatic chemiluminescence (Amersham, Little Chalfont, UK) or Supersignal (Pierce, Rockford, IL, USA) and visualized either on film (Biomax, Eastman Kodak, Rochester, NY, USA) or directly in an image analysis system (Versadoc, Bio-Rad). Molecular masses of PrP^{res} glycoforms were determined as the average of the center positions of the bands from at least 3 repeated electrophoretic

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Table. Cattle sources of transmissible spongiform encephalopathy (TSE) used for experimental infections of C57BL/6 mice and transmission results*

Cattle TSE isolate	Age, y	Breed	Molecular type	Survival periods (d) in C57BL/6 mice (mean ± SD)	Western blot results†
1	8	Charolais	H	702 ± 117	8/9
2	12	Crossbreed	H	652 ± 85	10/10
3	4	Prim'Holstein	Typical	511±- 89	8/9

*SD, standard deviation.
†No. mice positive for disease-associated prion protein/no. mice analyzed.

procedures, as measured by comparison with a biotinylated marker (B2787, Sigma, Saint Louis, MO, USA) included on each gel. Immunologic reactivities of antibodies 12B2 and Sha31 were compared in Western blots run in parallel with the same samples with both antibodies.

After intracerebral injection of cattle brain samples into C57BL/6 mice, disease was observed in mice with the 2 H-type isolates, as well as with the BSE sample. Survival periods of mice and results of PrP^{res} detection among mice analyzed by Western blot are shown in the Table.

Western blot analysis of PrP^{res} from H-type–infected mouse brains in comparison with BSE-infected mice is shown in Figure 1. All positive mice in the same experimental group showed the same Western blot pattern. This pattern showed higher molecular mass PrP^{res} glycoforms in mice infected with H-type isolates than in mice infected with a typical BSE agent (1.1- to 1.5-Da difference in the unglycosylated PrP^{res} (Figure 1A). Studies of PrP^{res} protease cleavage showed that only the PrP^{res} of mice infected with H-type isolates was recognized by antibody 12B2 (Figure 1B). This finding is in contrast to the result obtained with monoclonal antibody Sha31 directed against an epitope in the central region of the protein, which showed that the 12B2 epitope was preserved in H-type–infected mice. Thus, the molecular features of H-type cattle isolates, which are distinct from those of the BSE agent, were maintained after development of disease in mice.

Histopathologic analysis showed vacuolar lesions in the thalamus (Figure 2A) that were absent from the hypothalamus, cochlear nucleus, and superior collicules. These 3 neuroanatomic sites were severely affected in C57BL/6 mice brain after primary passage of the BSE agent, as we and others have reported (*1*). Abnormal PrP was detected only in amyloid plaques (Figure 2B), in contrast to what was reported after BSE transmission in C57BL/6 mice (*1*).

Conclusions

Our data show that the recently identified bovine H-type isolates involve an infectious agent that can induce development of a disease across a species barrier, while maintaining the specific associated PrP^{res} molecular signature. This evidence in favor of a new bovine prion strain in cattle suggests that BSE is not the only transmissible prion disease in cattle. The origin of such cases has not been determined (*7*). These cases suggest either the existence of alternative origins of such diseases in cattle or phenotypic changes of PrP^{res} after infection with the BSE agent. However, based on analysis of molecular features of prion diseases in cattle, this situation is similar to that in humans (*5*), in which different subtypes of sporadic Creutzfeldt-Jakob disease agents are found.

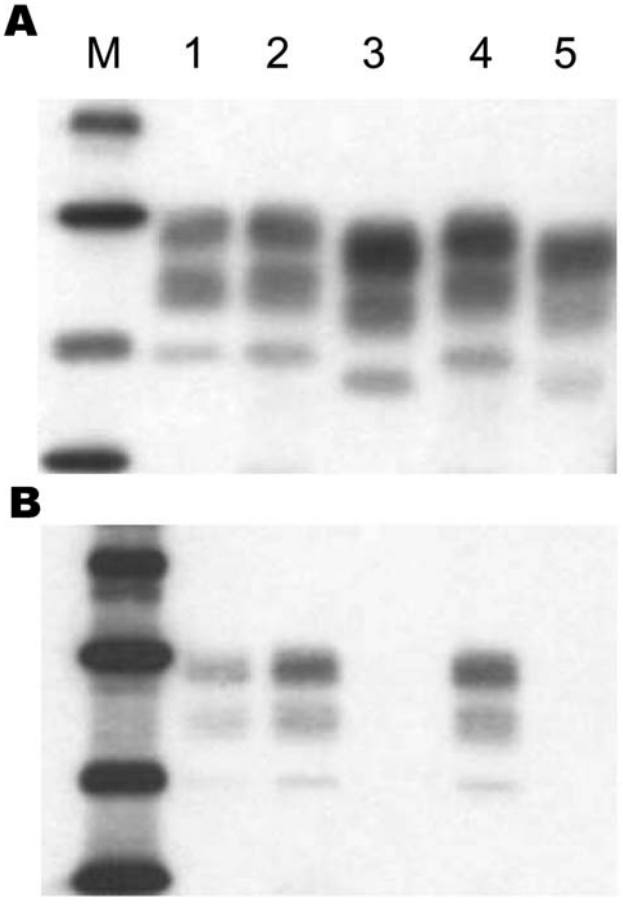

Figure 1. Western blot analysis of disease-associated prion protein (PrP^{res}) from proteinase K–treated brain homogenates of C57BL/6 mice infected with type H (lanes 2 and 4) or bovine spongiform encephalopathy isolates (lanes 3 and 5). PrP^{res} of mice infected with an experimental scrapie strain (C506M3) (*6*) was used as a control (lane 1). Monoclonal antibodies used for detection of PrP^{res} were Sha31 in panel A and 12B2 in panel B. Lane M, molecular mass markers: 39.8, 29, 20.1, and 14.3 kDa.

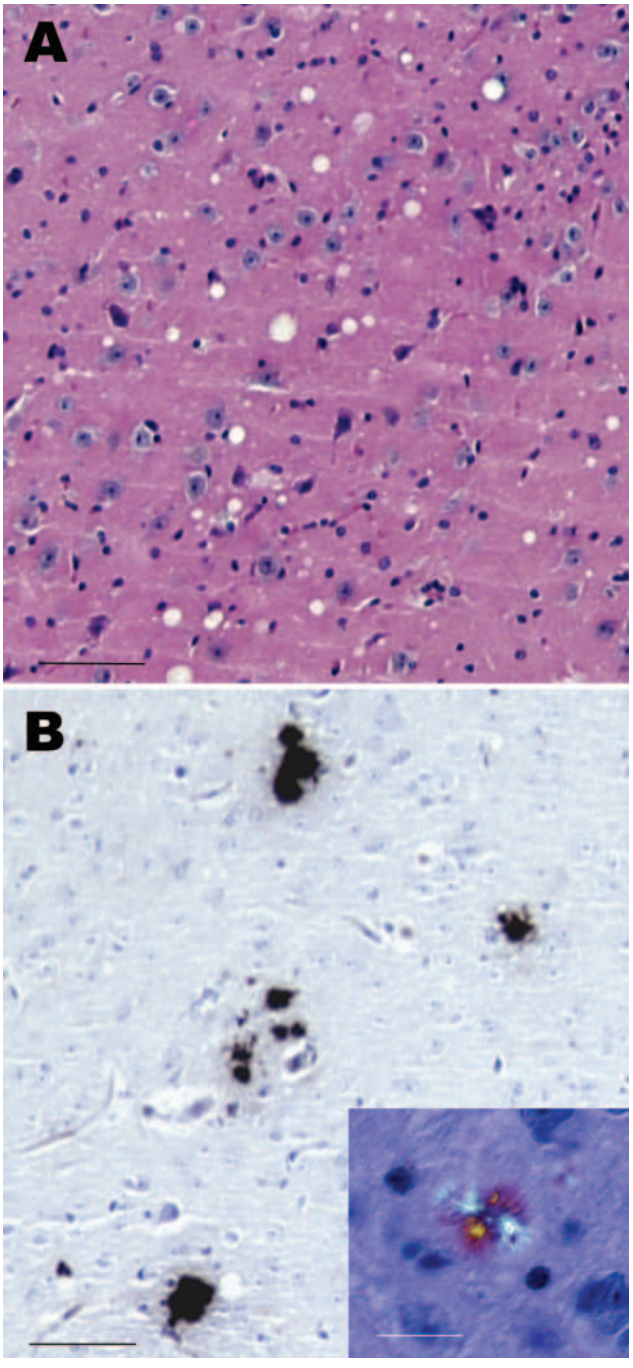

Figure 2. Histopathologic analysis of brain of a C57BL/6 mouse infected with a type H isolate. A) Characteristic vacuolar lesions in the thalamus (hematoxylin and eosin stained, scale bar = 60 μm). B) Immunohistochemical analysis of prion protein with monoclonal antibody 12B2 (diluted 1:200) shows the absence of granular deposition, but the presence of plaques in the thalamus. The inset shows that plaques are amyloids since they bind Congo red and show birefringence in polarized light (scale bar = 60 μm, scale bar in inset = 16 μm).

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Follow-up of 2003 Human West Nile Virus Infections, Denver, Colorado

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Tri-County Health Department and Boulder County Public Health conducted a follow-up study of all nonfatal West Nile virus (WNV) cases reported during 2003 in 4 metropolitan Denver, Colorado, counties. Self-reported patient information was obtained ≈6 months after onset. A total of 656 (81.2%) eligible WNV patients are included in this study.

In 2003, Colorado experienced a large West Nile virus (WNV) epidemic, which accounted for 29.9% of the nation's 9,862 reported WNV infections (*1*). Tri-County Health Department, which serves Adams, Arapahoe, and Douglas counties, and Boulder County Public Health collaborated to conduct a follow-up study of all WNV cases reported in these 4 counties in 2003. We conducted this follow-up study with 3 objectives: 1) to identify potential risk factors for developing neuroinvasive disease, 2) to describe the symptoms of patients 6 months after onset, and 3) to describe healthcare utilization and impact on daily activities associated with all types of WNV infection.

The Study

Since 2002, healthcare providers and laboratories have been required to report patients with laboratory evidence of acute WNV infection in Colorado. Patients were included in this study if WNV-specific immunoglobulin M (IgM) antibodies were found in either cerebrospinal fluid (CSF) or serum by enzyme-linked immunosorbent assay, or symptoms later developed in blood donors with a positive nucleic acid test result. In addition to laboratory confirmation, patients had to meet one of the following case definitions to be included in the study: 1) encephalitis cases required a physician's diagnosis and clinical manifestation of encephalitis, including mental status changes, delirium, disorientation, or coma; 2) meningitis cases required a physician's diagnosis or clinical exhibition of meningitis and abnormal CSF findings consistent with viral meningitis; and 3) fever cases required mild to moderate illness without clinical or laboratory evidence of central nervous system involvement. A compatible illness of WNV fever was defined as symptoms consisting of ≥2 of the following occurring within 90 days of testing: fever, headache, chills, myalgia, arthralgia, rash, lymphadenopathy, muscle weakness, or severe malaise. Any patient with a positive IgM test result on CSF was considered to have neuroinvasive disease.

Self-reported patient information was solicited through a standardized survey sent to 808 patients with nonfatal cases. Cases of meningitis and encephalitis were compared with cases of WNV fever. Measures of association between diagnosis and relevant patient characteristics were determined by Wald χ^2, odds ratios, and associated 95% confidence intervals for categorical variables and analysis of variance (ANOVA) testing for continuous variables. Multivariate logistic regression modeling was used to test for potential predictors of more severe disease at time of diagnosis. Variables were considered significant at the $p = 0.05$ level. Data were entered into EpiInfo 2002 (available from www.cdc.gov/epiinfo/) and analyzed with SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA).

A total of 656 (81.2%) patients completed the survey: 52.1% were female, 42.8% were ≥50 years of age, 80.9% had a diagnosis of fever, 12.8% had a diagnosis of meningitis, and 6.3% had a diagnosis of encephalitis. Nineteen cases were detected through blood donor screening, and all were categorized as uncomplicated fever cases. Nonrespondents were less likely to be female (42.1%, $p = 0.0259$) and ≥50 years of age (27.6%, $p = 0.0007$) but were similar by diagnosis category ($p = 0.5846$).

Mean ages by diagnosis were 60 years for encephalitis patients, 48 years for meningitis patients, and 46 years for fever patients. Encephalitis patients were significantly older than meningitis and fever patients ($p<0.0001$). The median period between onset of illness and completion of the follow-up survey was 178 days (range 102–299 days); 80% responded within 5–7 months after illness onset.

The overall prevalences of several chronic conditions and treatments are shown in Table 1. After adjustment for sex and age ≥50 years, encephalitis patients were significantly more likely than fever patients to report having several chronic conditions and to report having been on chemotherapy. Meningitis patients were more likely than fever patients to report having cancer and to have undergone chemotherapy.

Symptom duration was reported as >3 months for 48.7% of encephalitis patients, 26.2% of meningitis patients, and 20.3% of fever patients (Table 2). Muscle weakness and muscular pain at time of follow-up were reported by more than one third of encephalitis patients (Table 3). No notable differences in symptoms were reported based on the difference in the interval between onset date and date of completing the follow-up survey.

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Table 1. Prevalence of chronic health conditions and medication use among West Nile virus (WNV) study patients and multivariate model of predictors of more severe illness*

Condition	All WNV patients (n = 656); prevalence, %	Meningitis patients (n = 84); adjusted OR (95% CI)†	Encephalitis patients (n = 41); adjusted OR (95% CI)†
High blood pressure	12.2	1.0 (0.4–2.1)	2.1 (1.0–4.6)
Diabetes	6.1	0.8 (0.3–2.5)	2.6 (1.0–6.5)
Heart disease	3.8	1.4 (0.5–4.5)	2.7 (0.9–8.2)
Cancer	1.5	6.6 (1.6–27.5)	7.5 (1.2–45.4)
Kidney disease	1.2	2.3 (0.2–22.9)	24.9 (4.7–132.5)
Steroids	3.0	1.3 (0.4–4.6)	1.8 (0.4–8.5)
Chemotherapy	1.4	7.7 (1.5–40.0)	25.9 (4.2–159.7)

*CI, confidence interval; OR, odds ratio.
†Adjusted for sex and age ≥50 y.

Hospital admission was significantly more common among encephalitis (97.6%) and meningitis (91.7%) patients than fever patients (13.9%). The mean length of stay for all hospitalized patients was 11 days (range 1–165 days) and was significantly higher for encephalitis patients (20 days) than meningitis patients (10 days) and fever patients (7 days). Significantly more encephalitis and meningitis patients sought physical therapy (65.9% and 34.9%, respectively), occupational therapy (50.0% and 18.3%, respectively), and speech therapy (30.8% and 10.8%, respectively) than fever patients. Among fever patients, 6.6% reported receiving at least 1 of the 3 therapies.

Missing time from work was reported by most all categories of cases. For the 485 patients who were working at the time of illness onset, encephalitis patients and meningitis patients were significantly more likely to report missing work (100.0% and 98.3%, respectively) than fever patients (78.9%). The median number of work days missed was significantly higher among encephalitis patients (65 days) and meningitis patients (51 days) than fever patients (16 days). In addition, 91.0% of all patients reported that their routine daily activities were prevented by their WNV infection.

Conclusions

This study characterizes the severe impact that WNV infection had on all age groups and categories of WNV illness in a defined population-based cohort of 656 nonfatal infections. Our study results corroborated findings from previous studies that older age is predictive of more severe WNV illness, such as encephalitis (*2–4*) and death (*2*,*4–7*). In our study, the mean age of meningitis patients did not differ significantly from that of fever patients.

Additionally, we identified several preexisting medical conditions, as well as prior utilization of chemotherapy, that may predispose infected persons to the development of encephalitis or meningitis. The risk for encephalitis has been found to be higher among organ transplant recipients (*8*); however, the literature is inconsistent regarding whether preexisting medical conditions are predictive of neuroinvasive disease (*2*,*4*,*7*,*9*). The studies that did not detect such associations used different comparison groups than did our study and were limited by small sample size or low prevalence of these chronic medical conditions.

Only 1 other study has characterized the clinical spectrum of symptom duration among West Nile fever patients and missed work or school days (*10*). This study of 98 fever patients found that 39% had ongoing symptoms after an average of almost 6 months of follow-up, 82% reported limitations in household activities, and a median number of 10 missed work or school days (*10*). Our fever patients reported a higher number of missed work or school days with a median of 16. Additional studies with objective measures could better elucidate the long-lasting effects of WNV infection.

Because of the nature of self-reported data, both recall bias and misclassification of self-reported information are potential limitations of this study. However, we validated self-reporting of definitive fields such as sex and hospitalization because they were highly correlated with the initial data maintained in our statewide surveillance database.

Another limitation of our study was that a clinical diagnosis of flaccid paralysis or lack thereof was not confirmed in study cases. Estimated rates of flaccid paralysis are low (*2*,*11*) and therefore should not have had a large impact on our study findings. In addition, patients who had died were excluded from the study; therefore, we were not able to characterize this group for preexisting chronic conditions. Our study was limited to reported case-patients who

Table 2. Duration of symptoms for West Nile virus study patients

Duration of symptoms, d	Fever patients (n = 531); no. (%)	Meningitis patients (n = 84); no. (%)	Encephalitis patients (n = 41), no. (%)
≤30	241 (46.3)	20 (23.8)	8 (20.5)
31–90	174 (33.4)	42 (50.0)	12 (30.8)
>90	106 (20.3)	22 (26.2)*	19 (48.7)*

*Significantly different than among fever patients; p<0.05 (applies to overall distribution of 3 catagories).

Table 3. Symptoms ever experienced and still experiencing at time of follow-up for West Nile virus study patients

	Fever patients (n = 531)		Meningitis patients (n = 84)		Encephalitis patients (n = 41)	
Symptom	Ever, %	At follow-up, %	Ever, %	At follow-up, %	Ever, %	At follow-up, %
Muscle weakness	80.4	12.2	96.3	28.0*	92.7	46.3*
Muscle pain	85.9	12.1	92.6	14.8	76.3	39.5*
Headache	88.6	11.8	91.5	19.5	65.0*	12.5
Stiff neck	78.6	10.4	84.1	12.2	59.0*	12.8
Sensitivity to light	52.6	5.6	71.6*	11.1	63.4	12.2

*Significantly different than among fever patients; p<0.05.

sought medical attention and laboratory testing; therefore, our findings likely represent the more severe spectrum of infections.

Our study demonstrates that WNV infection caused considerable, long-lasting, severe illness during the 2003 Colorado epidemic and that the economic impact in terms of associated healthcare utilization and days of missed work was substantial. Public health officials should intensify prevention messages to help limit the severe manifestations of WNV infection and especially target those at greatest risk for severe disease.

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Triple Reassortant H3N2 Influenza A Viruses, Canada, 2005

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Since January 2005, H3N2 influenza viruses have been isolated from pigs and turkeys throughout Canada and from a swine farmer and pigs on the same farm in Ontario. These are human/classical swine/avian reassortants similar to viruses that emerged in US pigs in 1998 but with a distinct human-lineage neuraminidase gene.

Influenza viruses of the classical H1N1 lineage were the dominant cause of influenza among North American pigs for >60 years (*1*). However, in 1998, H3N2 viruses emerged and rapidly spread throughout the US swine population (*2*–*4*). These were unique triple reassortant genotype viruses, with hemagglutinin (HA), neuraminidase (NA), and RNA polymerase (PB1) genes of human influenza virus lineage; nucleoprotein (NP), matrix (M), and nonstructural (NS) genes of classical swine virus lineage; and RNA polymerase (PA and PB2) genes of North American avian virus lineage. Further reassortment between these viruses and classical H1N1 swine viruses led to the emergence of reassortant H1N2 and H1N1 viruses among pigs in the United States (*1*). The reassortant H3N2 and H1N2 viruses have also been isolated from turkeys and ducks in the United States (*5*–*8*). Despite geographic proximity and cross-boundary trade in pigs and turkeys between the United States and Canada (*9,* D. Harvey, pers. comm.), these reassortant viruses did not initially infect animals in Canada. However, beginning in approximately January 2005, H3N2 influenza viruses swept rapidly across Canada. We describe the genetic characterization of reassortant H3N2 viruses from pigs, turkeys, and a swine farm worker in contact with sick pigs during this outbreak.

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The Study

Influenza viruses were isolated in Madin-Darby canine kidney cells from lung tissue or nasal swab samples from pigs of various ages (young growers to adults) manifesting influenzalike illness (ILI) in Manitoba in January (A/Swine/Manitoba/12707/05), Alberta in February (A/Swine/Alberta/14722/05), British Columbia in May (A/Swine/British Columbia/28103/05), and Ontario in July (A/Swine/Ontario/33853/05). No clear epidemiologic links existed between these farms. A/Ontario/RV1273/05 was isolated in primary rhesus monkey kidney cells from a nasal swab specimen collected as part of a diagnostic workup from an otherwise healthy farm worker in Ontario in whom ILI developed 2–3 days after onset of ILI among pigs on his premises. Fourteen-day courses of oseltamivir therapy were prescribed for this patient beginning the day he saw his physician and for 11 other potentially exposed farm workers beginning 2 days later. The patient recovered uneventfully; no other respiratory viruses were identified from his samples. A/Turkey/Ontario/31232/05 was isolated in embryonated hen's eggs from a cloacal swab sample from turkeys showing a severe drop in egg production. The turkey farm was located across the road from a swine farm at which pigs concurrently exhibited ILI, although virus isolates were not available from those pigs.

Nucleotide sequences of the full-length coding regions of all 8 RNA segments from each virus were determined by direct cycle sequencing with previously described techniques and primers (*3*,*10*,*11*). Related reference viruses were identified by BLAST (basic local alignment search tool) analyses, sequence comparisons were conducted by using DNASTAR software, version 6.3 (DNASTAR Inc., Madison, WI, USA), and phylogenetic relationships were estimated from the nucleotide sequences by the method of maximum parsimony (fast heuristic search algorithm, PAUP software, version 4.0b10 [Sinauer Associates, Inc., Sunderland, MA, USA]) with a bootstrap resampling method (200 replications).

Pairwise nucleotide identities among the 2005 Canadian swine, turkey, and human isolates range from 94.0%–100% (NA) to 99.9%–100% (M), and amino acid identities range from 98.9%–100% (NA) to 100% (M, NP). The human and swine isolates recovered on a single farm in Ontario are 100% identical in nucleotide sequences across all 8 RNA segments. Phylogenetically, the 2005 Canadian viruses form single clusters on phylograms for each of the 8 viral RNA segments, confirming that this epizootic was caused by a single lineage of viruses. All of the viruses share the same human/classical swine/avian triple reassortant genotype as the H3N2 viruses that emerged in

pigs in the United States in 1998 (*2*–*4*). The HA genes (Figure 1) of the Canadian viruses are most closely related to the cluster III group of American viruses that were first isolated from pigs in 1999 (*4*) and subsequently from turkeys (*7*,*8*), though the HA phylogram topography suggests that these Canadian and related US viruses represent a new, separate cluster (IV) of viruses. The M, NP, NS, and polymerase genes of the Canadian viruses are also phylogenetically closely related to reassortant viruses dating back to 1998 in the United States (data not shown). In contrast, the NA genes (Figure 2) of the 2005 Canadian viruses, though still clearly of human lineage, are phylogenetically distinct from most of the US swine and turkey isolates. This lineage is represented by human H3N2 isolates from Asunción, Paraguay, (2001) and New York (2003). However, this lineage of NA genes has also been introduced into animal influenza viruses on 2 previous occasions. The first is represented by A/Turkey/Ohio/313053/04 (*8*), which phylogenetically is the most closely related virus to the Canadian viruses across all 8 RNA segments. Since this virus was isolated in February 2004 (*8*), nearly 1 year before the first isolations of viruses from Canada, one might conjecture that this or a closely related virus from the United States was the source of the Canadian viruses. However, this lineage of NA genes was also already present in human/classical swine reassortant H1N2 viruses (A/Swine/Ontario/48235/04, A/Swine/Ontario/55383/04) isolated from Canadian pigs in 2004 (*12*). Thus, it is neither possible nor prudent from the phylogenetic data alone to define the specific epidemiologic source(s) of the 2005 Canadian H3N2 viruses. However, the appearance of this lineage of NA genes among H3N2 viruses in turkeys in the United States and Canada and H3N2 and H1N2 viruses in pigs in Canada suggests that a complicated web of interspecies transmission, reassortment, and transboundary movement of viruses occurred in a relatively short period of time.

Conclusions

To our knowledge, this report describes the first isolation of a human/classical swine/avian triple reassortant H3N2 virus from a human. This isolation could not have occurred through cross-contamination in a laboratory since the animal and human virus isolations and sequencing were conducted in different locations. Hemagglutination-inhibition (HI) and virus neutralization (VN) assays of acute- and convalescent-phase (11 and 45 days after the acute-phase sample) sera did not show evidence of seroconversion against the patient's own isolate, A/Ontario/RV1273/05 (HI titer = 8 and VN titer = 16 on all 3 test dates). Thus, although this farm worker had a febrile respiratory illness and no other etiologic agent was identified, we cannot prove that he was actively infected with the triple reassortant virus; he may have simply been harboring the virus in his nasal passages. Nonetheless, this isolation shows that agricultural workers may be exposed to influenza viruses from livestock.

In summary, this report describes the emergence and rapid spread since January 2005 of reassortant H3N2 influenza A viruses among pigs and turkeys across Canada and isolation of a related virus from the nasal passages of a farm worker in Ontario. The 4 swine isolates chosen for our analyses provide a sampling of viruses from British Columbia to Ontario, but clinical reports indicate that the outbreak of ILI in pigs was much more extensive than this limited number of isolates might suggest. For example, H3N2 virus infections were confirmed on 22 swine farms in Ontario between late April and early July 2005.

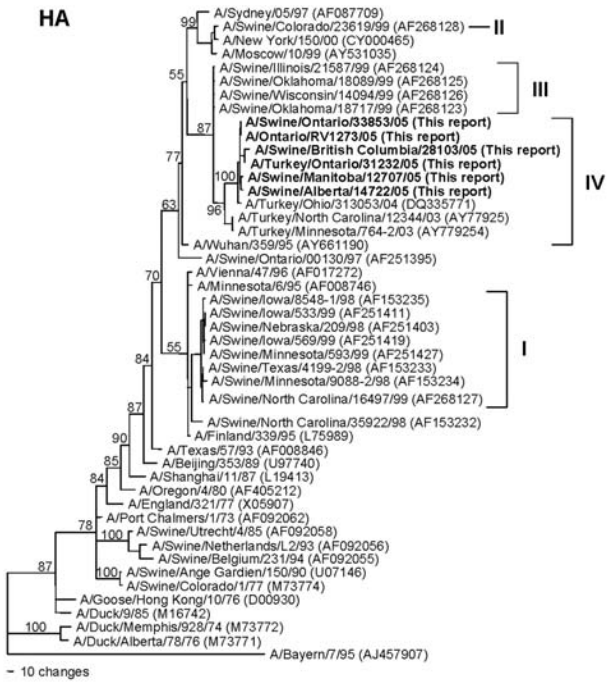

Figure 1. Nucleotide phylogram for the hemagglutinin (HA) genes of A/Swine/Manitoba/12707/05, A/Swine/Alberta/14722/05, A/Swine/British Columbia/28103/05, A/Swine/Ontario/33853/05, A/Turkey/Ontario/31232/05, A/Ontario/RV1273/05, and related reference viruses. The evolutionary relationships among these viruses were estimated by the method of maximum parsimony (PAUP software, version 4.0b10) with a bootstrap resampling method (200 replications) and a fast heuristic search algorithm. Numbers at the nodes of the phylograms indicate the bootstrap confidence levels. Horizontal-line distances are proportional to the minimum numbers of nucleotide changes needed to join nodes and gene sequences. Vertical lines are present to space the branches and labels. The designations I, II, and III identify clusters of viruses previously defined among triple reassortant swine viruses in the United States (*4*). The viruses described in this report and related viruses are proposed to represent a new cluster IV group of viruses. GenBank accession numbers for the sequences of all reference viruses are provided in parentheses after the virus names.

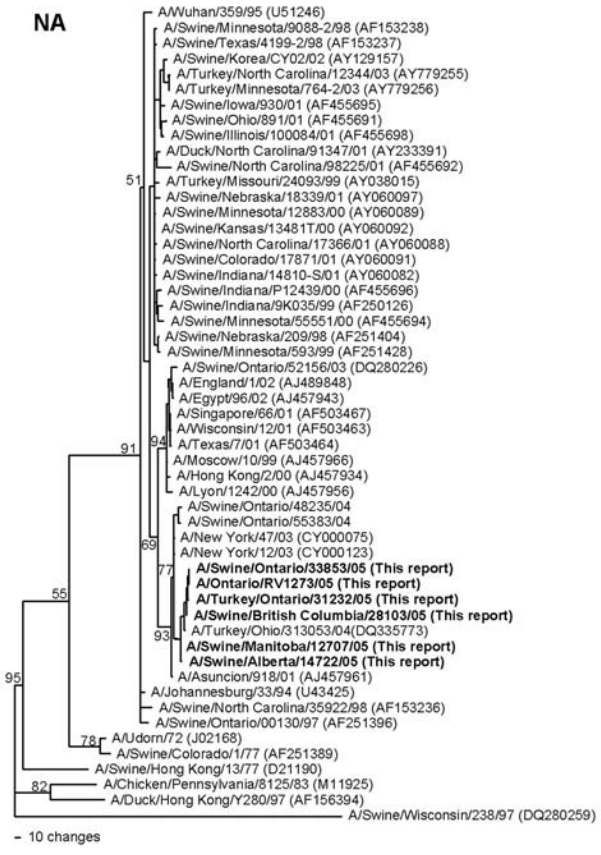

Figure 2. Nucleotide phylogram for the neuraminidase (NA) genes of A/Swine/Manitoba/12707/05, A/Swine/Alberta/14722/05, A/Swine/British Columbia/28103/05, A/Swine/Ontario/33853/05, A/Turkey/Ontario/31232/05, A/Ontario/RV1273/05, and related reference viruses. The evolutionary relationships among these viruses were estimated by the method of maximum parsimony (PAUP software, version 4.0b10) with a bootstrap resampling method (200 replications) and a fast heuristic search algorithm. Numbers at the nodes of the phylograms indicate the bootstrap confidence levels. Horizontal-line distances are proportional to the minimum numbers of nucleotide changes needed to join nodes and gene sequences. Vertical lines are present to space the branches and labels. GenBank accession numbers for the sequences of all reference viruses are provided in parentheses after the virus names.

Likewise, additional infections of turkeys with H3 viruses in 2005 were reported in British Columbia (on a farm that was near a swine farm where H3 virus was detected [*13*]) and in multiple flocks in Manitoba (A. Hamel and G. Nayar, pers. comm.). When this Canadian epizootic is considered together with the extensive spread of genotypically similar H3N2 and H1N2 viruses in pigs and turkeys seen in the United States since 1998, we see that viruses with this human/classical swine/avian triple reassortant genotype can efficiently infect both pigs and turkeys.

The GenBank numbers assigned to the gene sequences of viruses investigated in this study are as follows: A/Ontario/RV1273/05, DQ469955–DQ469962; A/Swine/Alberta/14722/05, DQ469963–DQ469970; A/Swine/British Columbia/28103/05, DQ469971–DQ469978; A/Swine/Manitoba/12707/05, DQ469979–DQ469986; A/Swine/Ontario/33853/05, DQ469987–DQ469994; and A/Turkey/Ontario/31232/05, DQ469995–DQ470002.

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Tickborne Encephalitis Virus, Norway and Denmark

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Serum from 2 Norwegians with tickborne encephalitis (TBE) (1 of whom was infected in Denmark) and 810 Norwegian ticks were tested for TBE virus (TBEV) RNA by reverse transcription–polymerase chain reaction. Sequencing and phylogenetic analysis were performed. This is the first genome detection of TBEV in serum from Norwegian patients.

Tickborne encephalitis (TBE) is a viral zoonotic disease caused by TBE flavivirus (TBEV). Three subtypes of TBEV have been reported: the European (TBEV-Eu) subtype, transmitted by *Ixodes ricinus* ticks and widely distributed in Europe, and the Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE) subtypes, carried by *I. persulcatus* ticks and present from the Far-East to Baltic countries (*1*). TBE is endemic in Scandinavia along the coastal areas of the Baltic Sea. The first reports of TBE from Sweden, Finland, and Denmark date back to 1954, 1956, and 1963, respectively. The disease was not diagnosed in Norway until 1997 (*2*). Since then, 11 serologically confirmed cases of indigenous human TBE have been reported. A related flavivirus has been isolated in Norway from sheep; it was subsequently analyzed as louping ill virus (LIV), not TBEV (*3*).

In neighboring Denmark, 14 human TBE cases on Bornholm Island were reported and serologically confirmed from 1994 to 2002 (*4*). Recently, both TBEV and LIV have been detected in ticks from Bornholm by reverse transcription–polymerase chain reaction (RT-PCR), although these viruses have not been further characterized genetically (*5*). Antibody tests suggest that human disease in Norway and Denmark is caused by TBEV, but virus has not been isolated from humans in these countries. The aim of this study was to identify and genetically characterize TBEV from Norway.

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The Study

Serum collected before the appearance of TBEV-specific immunoglobulin M (IgM) (acute-phase serum) was available from 2 of 11 TBE patients. The patients, both 38 years of age, included a man from Vest-Agder County, who had not been abroad during the last 4 weeks before disease, and a woman from Hordaland County, who was bitten by a tick on Bornholm Island. Both patients were hospitalized with intensive headache. Results of clinical and neurologic examinations were normal. Their leukocyte counts in cerebrospinal fluid were 87–100/mm^3. Both patients recovered.

High levels of TBEV IgM and moderate to high levels of TBEV IgG were detected in convalescent-phase sera from both patients by enzyme immunoassay (Enzygnost, Dade Behring, Marburg, Germany).

Ticks were collected by dragging a blanket in the field in areas where patients with TBE had been reported. All collected ticks were unfed. A total of 360 nymphs, adults, and larvae were collected in May and June 2003, and 450 nymphs, adults, and larvae were collected from August 27 to October 8, 2004. Ticks were pooled according to collection site. All pools were stored at –70°C until preparation of tick suspensions.

Acute-phase sera from the 2 patients and 810 ticks (*I. ricinus*) were examined for TBEV. RNA was extracted from serum samples and tick suspensions by using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA) and the TriPure RNA isolation system (Roche Diagnostics, Lewes, UK), respectively. For initial detection of TBEV RNA, the 5′ noncoding region was amplified by nested RT-PCR (*6*), and positive samples were amplified in the coding E protein region (nucleotides [nt] 1323–1765). RT was performed by using MMLV (Moloney murine leukemia virus) RT kit (Invitrogen, Carlsbad, CA, USA) and reverse primer 827R (nt 1777–1800) (Table) according to manufacturer's recommendations. PCR and nested PCR were performed by using primer pairs 283F1 (nt 1233–1255) to 827R1 (nt 1777–1800) and 349F2 (nt 1301–1322) to 814R2 (nt 1766–1787), respectively (Table). Additional details about PCR assays are available from the corresponding author upon request. PCR amplicons were purified and sequenced by using a DNA sequencing kit (ABI Prism, PE Biosystems, Foster City, CA, USA) on a 3100 genetic analyzer (PE Biosystems).

The Phylip program package (*7*) was used to analyze the E protein gene sequence data: 500 bootstrap replicates (Phylip's SeqBoot Program) were fed to the Dnadist program (with Kimura's 2-parameter option), distance matrixes were analyzed with the Fitch program (which used the Fitch-Margoliash algorithm), and the bootstrap support values for the tree were calculated with the Consense

Table. Primers used for reverse transcription–polymerase chain reaction

Primer	Sequence (5′→3′)
283F1	GAG AC/TC AGA GTG AC/TC GAG GCT GG
827R1	AGG TGG TAC TTG GTT CCA/C TCA AGT
349F2	GTC AAG GCG T/GCT TGT GAG GCA A
814R2	TTC CCT CAA TGT GTG CCA CAG G

program . The sequences obtained from GenBank for comparison are listed in the Figure.

From 360 ticks collected in 2003, only 1 pool (10 ticks) was positive by amplification of the 5′ noncoding region; similarly, only 1 pool of 450 ticks collected in 2004 was RT-PCR-positive. From the positive tick pool in 2004, we sequenced 179 nt of the highly conserved region in 5′ region of TBEV, enough to prove that the virus was TBEV, not LIV. No material from the tick pools was left to attempt virus isolation. Thus, the overall virus prevalence in ticks was 0.3% in 2003 and 0.2% in 2004, if we assume that only 1 tick in the positive pool was infected.

A partial E gene sequence (443 bp) was recovered from the 2 human samples. The corresponding TBEV strains were designated as Norway-1 and Denmark-1. The identity on the nucleotide level between these sequences was 98.6%, and they showed 97.2%–99.0% identity to other TBEV strains within the TBEV-Eu subtype. The levels of sequence identity to strains belonging to TBEV-FE and TBEV-Sib subtypes were 81.4%–83.0% and 83.5%–86.2%, respectively. Phylogenetic analysis of these sequences showed that they belong to the TBEV-Eu subtype (Figure), which does not show clear, separated lineages correlating to geographic regions. The Norwegian TBEV strain clustered together with strain Neudoerfl isolated in Austria, and the Denmark-1 strain clustered together with the group of strains from Latvia, Finland (Kumlinge), and Estonia, albeit bootstrap supports of these clusterings were below the widely accepted confidence limit, 70%. The sequence identity between strains Denmark-1 and Neudoerfl was 99.0%; between strains Norway-1 and Latvia9783, the sequence identity was 98.7%.

Conclusions

This is the first report of TBEV RNA in serum from Norwegian patients. One of the 2 patients was infected in Vest-Agder County in Norway, and the other on Bornholm Island, Denmark. Genetic analysis showed that the Norwegian and Danish strains belong to the TBEV-Eu subtype. Although the sequences of Norway-1 and Denmark-1 strains showed the highest level of identity to the corresponding sequences of TBEV-Eu subtype, they were distinguishable from each other and also from the sequences of TBEV-Eu strains characterized previously.

In TBE-endemic areas in Europe and on Bornholm Island, 0.5%–5% of ticks are infected with TBEV (*5*). In Norway, where TBE is a rare disease, the prevalence is lower (0.2%–0.3%). In Denmark TBEV has been detected in ticks by RT-PCR (*5*), but to our knowledge, no reports of TBEV findings in Danish patients exist.

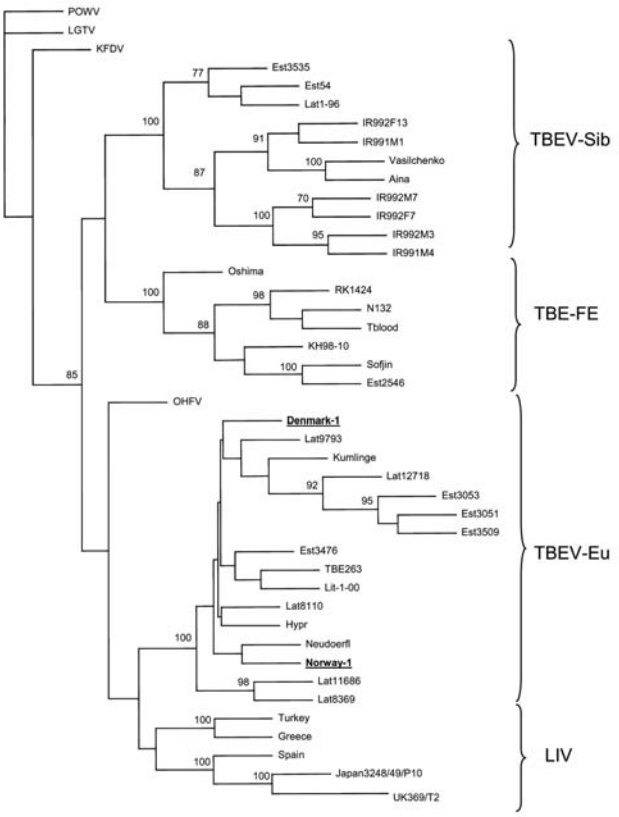

Figure. Consensus phylogenetic tree (Fitch-Margoliash) based on the E protein encoding sequence (nucleotides 1323-1765). Only bootstrap support values >70% are shown. Newly identified tickborne encephalitis virus (TBEV) strains from Norway and Denmark are underlined. Abbreviations and accession numbers for viruses and TBEV strains: Powassan virus, POWV, strain LB (L06436); Langat virus, LGTV, strain TP21 (AF253419); Kyasanur forest disease virus, KFDV, (X74111.1); Omsk hemorrhagic fever virus, OHFV, (X66694.1); TBEV, strain IR991M4 (AB049349); TBEV, strain IR992M3 (AB049350); TBEV, strain IR992M7 (AB049351); TBEV, strain IR992F7 (AB049352); TBEV, strain IR992F13 (AB049353); TBEV, strain IR991M1 (AB049348); TBEV, strain Aina (AF091006); TBEV, strain Vasilchenko (AF069066); TBEV, strain Latvia 1-96 (AJ415565); TBEV, strain Oshima (AB022292); TBEV, strain Sofjin (X03870.1); TBEV, strain KH98-10 (AB022297); TBEV, strain Tblood (AF091019); TBEV, strain RK1424 (AF091016); TBEV, strain N132 (AF091013); TBEV, strain 263 (U27491); TBEV, strain Latvia-12718-00 (AJ319586); TBEV, strain Hypr (U39292); TBEV, strain Neudoerfl (U27495); TBEV, strain Latvia-9793-00 (AJ319585); TBEV, strain Latvia-8110-00 (AJ319583); TBEV, strain Kumlinge-A52 (X60286); TBEV, strain Latvia-8369-00 (AJ19584); TBEV, strain Latvia-11686-00 (AJ319582); TBEV, strain Lithuania-1-00 (AJ414703); louping Ill virus (LIV), strain UK369/T2 (Y07863.1); LIV, strain Turkey (LO1265); LIV, strain Greece (X77732); LIV, strain Spain (X77470.1); LIV, strain Japan 3248/49/P10 (M94956.1).

The emergence of TBE in Norway in the 1990s poses the question of whether these new endemic foci have become truly established recently or have remained unnoticed because of underdiagnosis. Although the northern spread of TBEV due to climate changes has been predicted (*8*), other factors such as rates of contact between ticks and humans, abundance of ticks, and their amplifying hosts may play a role in TBE epidemiology. Further monitoring of the TBE situation in Norway both in patients and nature is needed to establish guidelines for preventive measures and vaccination programs in TBE-endemic areas.

We report the first genome detection and characterization of TBEV from persons with TBE in Norway and Denmark. Our results showed that the Norwegian and Danish strains clustered with earlier reported strains of the TBEV-Eu subtype.

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etymologia

malaria

[mə-lar′e-ə]

Malaria, "bad air" in Italian, was blamed for the deaths of >1,000 workers digging the Erie Canal in 1819. Work on the canal continued in winter, when the swamp was frozen over (and, although the vector was not known at the time, mosquitoes were dormant). Malaria, caused by parasites of the genus *Plasmodium* and usually transmitted by the bite of infected *Anopheles* mosquitoes, is endemic in many warm regions. Charles Louis Alphonse Laveran discovered the protozoan cause of malaria in 1880. The Office of Malaria Control in War Areas, which was established in 1942 to control malaria and other vectorborne diseases in the southern United States, evolved into what is today the Centers for Disease Control and Prevention.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; cdc.gov; and wikipedia.org

Detection of Infectious Poxvirus Particles

Andreas Nitsche,* Daniel Stern,* Heinz Ellerbrok,* and Georg Pauli*

To enable rapid and reliable detection of poxviruses in clinical and environmental specimens, a diagnostic approach was developed to detect ≤3 PFU of infectious poxvirus particles in <5 hours. This approach involved virus culture combined with real-time reverse transcription–polymerase chain reaction detection of 2 viral genes expressed immediately after infection.

After the attacks with anthrax spores in the fall of 2001 in the United States, the potential abuse of variola virus or genetically engineered orthopoxviruses in bioterrorist plots has been intensely discussed (*1*–*3*). To date, several diagnostic assays have been developed to rapidly and reliably detect poxvirus particles or poxvirus genomes in suspected samples. Electron microscopy (EM) can also identify poxvirus particles (*4*,*5*). However, it cannot differentiate between orthopoxvirus species and has limited sensitivity because reliable detection is only possible with particle concentrations >10^6/mL (*6*).

Molecular methods such as real-time polymerase chain reaction (PCR) are more sensitive, detecting <10 genome equivalents per PCR, but PCR can only identify short stretches of poxvirus DNA (*1*,*7*). Nevertheless, since EM and PCR cannot discriminate between infectious and noninfectious virus particles or nucleic acids, they are not satisfactory when an evaluation of the infectious capacity of viral particles is required.

Identifying viral particles by EM is usually sufficient to diagnose a poxvirus infection in clinical samples from patients with typical symptoms of this infection. Virus concentration should exceed 10^6 particles/mL; however, even at these concentrations only the virus family can be determined, and no additional classification is possible. Detection of poxvirus nucleic acids is sensitive and permits identification of virus-specific sequences and differentiation of a variola virus infection from an infection with other orthopoxviruses. Thus, a combination of both methods is recommended for frontline diagnostic procedures, and a positive result obtained by 1 of these methods would initiate a confirmation diagnosis.

If symptoms in clinical cases are unambiguous, they can usually be attributed to a replication-competent infectious virus. In contrast, in environmental samples, including samples from suspected parcels, a positive EM or PCR result would also require virus isolation to prove that particles could replicate to make a reasonable risk assessment (German Smallpox Preparedness Plan, available from www.rki.de).

With environmental samples, the unknown factor is to what extent the sample matrix influences the ability of the virus to replicate, and detecting particles by EM or DNA by PCR does not necessarily indicate infectious particles. The only diagnostic approach to identify replication-competent poxvirus particles is their propagation in a suitable cell culture system. With this system, it takes ≥1 day to reliably detect poxvirus proteins with specific antibodies.

We combined a cell culture approach that identifies virus replication with the speed and sensitivity of real-time PCR. To this end, we changed the target of real-time PCR from poxvirus DNA to poxvirus mRNA genes that are highly expressed during the first few hours of the infection cycle. Expression levels of these genes enable sensitive detection 1–2 hours after infection. The complete diagnostic approach can be performed in 96-well plates and provides results within 5 hours of receipt of a sample.

The Study

Briefly, 1.5×10^4 HEpG2 cells were infected with 150 PFU of vaccinia virus strain Lister Elstree. A 15-minute centrifugation step at $1{,}000 \times g$ increased the efficacy of infection by a factor of 10 compared with regular infection at 37°C (data not shown). Virus-containing supernatant was removed, and virus was allowed to replicate for 4 h. Every 30 minutes an aliquot of cells was harvested, and RNA and DNA were isolated by standard procedures (RNAeasy kit and Blood DNA kit, Qiagen, Hilden, Germany). RNA was subjected to 1-step real-time reverse transcription–polymerase chain reaction (RT-PCR) (QuantiTect Probe RT-PCR kit, Qiagen) in a real-time PCR 7700/7900/7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Amplification of fragments of the *F1L* gene, an apoptosis modulator, and the *rpo*18 gene, the small subunit of viral RNA polymerase (*1*) (both genes are encoded by all poxviruses including variola virus), was monitored by gene-specific 5′-nuclease probes.

Expression of the *F1L* and *rpo*18 genes could be detected 30 minutes and 1 hour after infection, respectively. The copy number of the transcripts was determined by comparison with in vitro translated RNA molecules that were generated according to standard procedures. Briefly, RNA was transcribed in vitro by T7 RNA polymerase (RiboMax RNA production system, Promega, Madison, WI, USA) from plasmids containing the respective PCR target region, and plasmid DNA was digested with DNase.

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During the first 4 hours after infection *F1L* mRNA increased 2.7 × 10^4-fold, indicating early expression of viral genes in the cells analyzed. The *rpo*18 mRNA showed a 410-fold increase after 4 hours. Quantification of viral DNA showed a slight decrease in DNA during the same period, and the ratios of RNA to DNA increased substantially, as shown in Figure 1. This high ratio of poxvirus RNA to poxvirus DNA demonstrates that a possible background of genomic viral DNA, which is derived from poxvirus particles that are noninfectious or from traces of poxvirus genomic DNA in the RNA preparation, does not result in false-positive results in real-time RT-PCR.

To evaluate the detection limit of our approach, a probit analysis was performed by repetition of the detection (N = 12) of vaccinia virus strain Lister Elstree. Vaccinia virus stocks were titrated according to standard procedures. The virus load used varied from 1.5 × 10^3 PFU to 0.1 PFU, which is equivalent to a multiplicity of infection of 0.15 to 1 × 10^{-5} (*8*). As shown in Figure 2, after 2 hours of incubation, real-time PCR analysis showed that the *F1L* assay detected 3 PFU of vaccinia virus, and the *rpo*18 detected 6 PFU of vaccinia virus with a confidence interval of 95%.

Conclusions

The extremely low detection limit of the new assay indicates that environmental samples, which may contain cell culture inhibitory substances and are routinely subjected to crude separation steps such as low-speed centrifugation before analyses, can be diluted by several orders of magnitude to dilute inhibitors while maintaining the viral load at detectable levels. The time frame required for the individual steps of the diagnostic approach is 15 minutes for sample infection, 2–4 hours for virus propagation, 30 minutes for RNA preparation, and 2 hours for real-time RT-PCR. Use of alternative, more rapid real-time PCR platforms further reduces the time required to complete an assay. For poxvirus-positive results, fluorescence melting curve analysis of the *rpo*18 PCR product allows rapid and reliable differentiation of variola virus (*1*). Under optimal conditions, results can be obtained <5 hours after the sample has arrived in the laboratory.

In summary, the combination of cell culture and real-time RT-PCR detection of early, highly expressed viral genes permits detection of minute quantities of infectious poxvirus particles in a suspected sample. Identification of variola virus can be performed by fluorescence melting curve analysis, therefore permitting a reliable risk assessment of a suspect parcel.

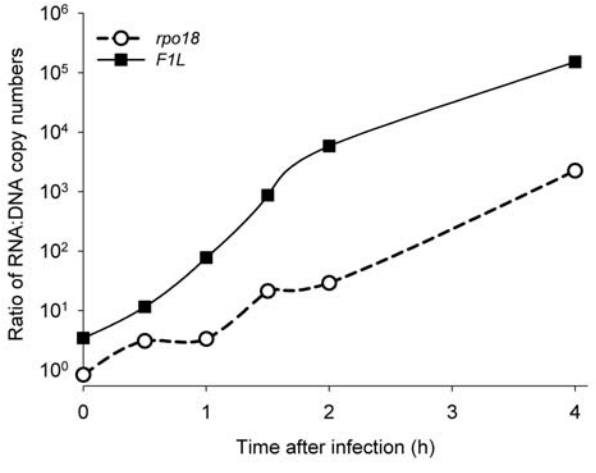

Figure 1. Comparison of RNA load and DNA load of the poxvirus *F1L* and *rpo*18 genes during the first 4 hours after infection with vaccinia virus strain Lister Elstree (multiplicity of infection 0.01). Cells were infected, and at the indicated time points RNA and DNA were prepared and quantified by real-time polymerase chain reaction. The ratio of RNA to DNA molecules is shown.

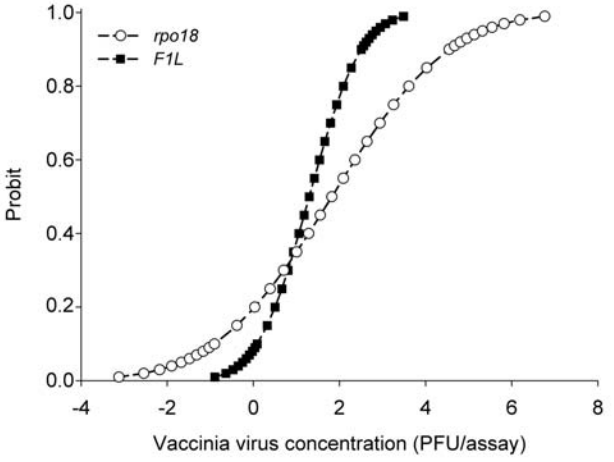

Figure 2. Comparison of probit (predicted proportion of replicates positive) regression curves for *rpo*18 (circles) and *F1L* (squares) genes calculated with SPSS software (SPSS Inc., Chicago, IL, USA). Probit versus vaccinia virus concentration was obtained from 12 replicates of 7 dilutions from 1,500 PFU to 0.1 PFU.

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European Bat Lyssavirus Type 2 RNA in *Myotis daubentonii*

Nicholas Johnson,* Philip R. Wakeley,* Sharon M. Brookes,* and Anthony R. Fooks*

Organ distribution of European bat lyssavirus type 2 viral RNA in its reservoir host, *Myotis daubentonii* (Daubenton's bat), was measured with a novel quantitative reverse transcription–polymerase chain reaction assay. High levels of genomic RNA were found in the brain and were also detectable in the tongue, bladder, and stomach.

Bat-mediated rabies has been reported in Europe for more than 50 years. Two variants or genotypes are now recognized that are distinct from rabies viruses of terrestrial mammals and new world bats (*1*). These are known as European bat lyssaviruses (EBLVs). A third lyssavirus, West Caucasian bat lyssavirus, has been isolated in eastern Europe (*2*). EBLV type 1 (EBLV-1) is found throughout mainland Europe and principally associated with the Serotine bat (*Eptesicus serotinus*) (*3*). EBLV-2 is found in *Myotis* bats (*Myotis daubentonii* [Daubenton's bat] and *Myotis dasycneme*) and has been identified in 3 locations in Europe: the Netherlands, the United Kingdom, and Switzerland (Table 1).

Two reports detail isolation of EBLV-2 from humans who died of rabies encephalitis in Finland and the United Kingdom (*4*,*5*). In addition, 4 isolations from Daubenton's bat have been reported in the United Kingdom since 1996. Seroprevalence studies suggest that EBLV-2 is maintained at certain sites in the United Kingdom at low levels (*6*). However, the small number of bats infected with EBLV-2 and the nocturnal habits of insectivorous bats have hampered attempts to understand the distribution, prevalence, and transmission of the virus. Biting by Daubenton's bats was suspected in the 2 human cases from Finland and the United Kingdom because both persons had handled this species before symptoms developed. However, like rabies virus, EBLV-2 does not persist in the environment outside of an infected host, and alternative routes of infection should be considered. Investigation of the second bat detected viable EBLV-2 in the brain, and genomic RNA in the heart, stomach, tongue, intestine, liver, and kidney by using a sensitive nested reverse transcription–polymerase chain reaction (RT-PCR) (*7*). This approach was unable to quantify viral RNA within particular tissues. Since this study, 2 additional cases have occurred in the United Kingdom (Table 1). The investigation and quantification of viral load within the infected host could provide evidence for release of virus and methods of transmission.

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The Study

In 2004, two EBLV-2 cases were identified in Daubenton's bats (Table 1). A diagnosis of EBLV-2 infection was confirmed on brain samples with a fluorescent-antibody test, the mouse inoculation test, and a rapid TaqMan assay (*8*). Attempts to culture EBLV-2 from organs in both cases failed because of cytotoxicity of the samples, which destroyed the cell monolayer. Sample dilution reduced the cytotoxic effects of the sample on the cell monolayer (used for virus isolation) and enabled the development of small foci of infection (bat 603/04). Heminested RT-PCR detected virus RNA in brain, tongue, thyroid gland, and bladder after the first round of amplification, and in salivary gland, heart, lung, intestine, and stomach after the second round of amplification. We suspect that inappropriate storage of bat 696/04 in a freezer with repeated freezing and thawing before submission resulted in inactivation of virus in this sample. Heminested RT-PCR detected virus RNA in samples of brain and stomach after the first round of PCR, and in samples of tongue, intestine, liver, and kidney after the second round of amplification.

An EBLV-2-specific real-time PCR was developed to measure virus genome to quantify the potential viral RNA load within organs. Analysis was only attempted on those organs with sufficient RNA within the sample (Figure). Primers EBLVNa (5′-CCTGGCAGATGATGGGAC-3′) and EBLVNb (5′-GCCTTTTATCTTGGATCACT-3′) are located within the nucleoprotein gene and amplify a 221-bp target. An amplified product from a previous case (*5*) was purified by using the RNeasy kit (Qiagen, Valencia, CA, USA) and quantified with a NanoDrop WD-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). This procedure enabled the absolute number of copies of the amplicon to be calculated by its approximate molecular weight and Avogadro's number, as previously described (*9*).

RNA was isolated from each organ with Trizol (Invitrogen, Carlsbad, CA, USA) and quantified. Dilutions were made to either 0.25 μg/μL (bat 603/04) or 1 μg/μL (bat 696/04) to standardize the quantity of RNA used for reverse transcription. Primer EBLVNa was used for cDNA synthesis from the genomic (negative) sense strand as previously described (*10*). All PCRs were performed by using SYBR Green JumpStart Taq ReadyMix (Sigma, Saint Louis, MO, USA) and an MX3000P real-time thermal

Table 1. Reports of European bat lyssavirus type 2 (EBLV-2) in Europe, 1985–2004*

Year	County	Description
1985†	Finland	30-year-old man admitted to department of neurology, Helsinki University Central Hospital, with ascending paralysis and radiating pain in right arm and neck; became agitated with hyperexcitability, hyperventilation, and spasms the following day; died 20 days after admission. Rabies diagnosis confirmed by FAT and MIT.
1986	Denmark	Rabies in pond bat (*Myotis dasycneme*).
1986	Denmark	Rabies in Daubenton's bat (*M. daubentonii*).
1986	Germany	Rabies in Daubenton's bat.
1987	Denmark	Rabies in Daubenton's bat.
1987†	The Netherlands	Virus isolated from pond bat in Wommels.
1987†	The Netherlands	Virus isolated from pond bat in Tjerkwerd.
1987	The Netherlands	Virus isolated from pond bat.
1989†	The Netherlands	Virus isolated from pond bat in Andijk.
1992†	Switzerland	Daubenton's bat found hanging on grill of ventilation shaft during daylight hours in Fribourg. Bat was weak, unable to fly, and died shortly afterwards. Rabies diagnosis confirmed by FAT and MIT.
1993†	The Netherlands	Virus isolated from pond bat in Roden.
1993†	Switzerland	Virus isolated from Daubenton's bat in Versoix.
1996†	United Kingdom	Sick Daubenton's bat found in cellar of public house in Newhaven bit a pregnant woman while it was being cared for in bat hospital. Bat deteriorated rapidly. Diagnosis confirmed by FAT, RTCIT, MIT, and RT-PCR.
2002†	United Kingdom	Juvenile female Daubenton's bat brought onto property adjoining Lancashire canal. Bat was in distress with wing damage; was treated for >7 weeks before signs of agitation and vocalization developed; became aggressive and tried to bite handler. Bat died 6 days after symptoms developed. Diagnosis confirmed by FAT, RTCIT, MIT, and RT-PCR.
2002†	Switzerland	Rabies in Daubenton's bat in Geneva.
2002†	United Kingdom	55-year-old man admitted to Dundee hospital with acute hematemesis and upper limb parasthesia; became aggressive and required sedation on day 5; died on day 14. Man had history of exposure to bats in United Kingdom; postmortem PCR on saliva detected EBLV-2. Virus recovered from brain tissue after autopsy.
2003† (bat 696/04)	United Kingdom	Adult male Daubenton's bat found Sep 2003 after flying into tree in daylight near Bury in Lancashire. Bat was cared for by volunteers and took water but attempted to bite carpet when placed there to feed. Bat died and was frozen until diagnosis was made Oct 2004.
2004† (bat 603/04)	United Kingdom	Grounded juvenile female Daubenton's bat was found in Staines and cared for by volunteers. Its condition was poor and it displayed signs of aggression and lethargy. Diagnosis confirmed by FAT, RTCIT, MIT, and RT-PCR.

*FAT, fluorescent-antibody test; MIT, mouse inoculation test; RTCIT; rabies tissue culture infection test; RT-PCR, reverse transcription–polymerase chain reaction.
†Virus identity confirmed by genomic sequence analysis.

cycler (Stratagene, La Jolla, CA, USA). A dilution series of the control amplicon was amplified simultaneously with the organ samples to create a standard curve for comparison of the threshold value (Ct) with target copy number (Figure, panel A).

A representative plot of amplification curves from organ samples taken from bat 696/04 is shown in the Figure, panel B, with 10 μL of product separated by electrophoresis on a 1% agarose gel included for comparison. The quantitative results for viral RNA load for both bats are shown in Table 2. In both cases, the brain had the highest viral genome load. Virus RNA was consistently detected in the tongue, intestine, and stomach. EBLV-2 was also found in the bladder of bat 603/04 but not in the kidney of bat 696/04 from which the bladder was not recovered because of carcass decomposition. Virus was not detected in the liver of either bat.

Conclusions

The detection and quantification of EBLV-2 RNA in bat organs by real-time PCR show the potential distribution of this virus. The choice of organ tested in both cases was severely limited by degradation of the carcass before investigation. Furthermore, live virus could not be recovered from many organs because of cytotoxicity of the samples and virus degradation caused by repeated freezing and thawing.

Viable virus was recovered from the brain of bat 603/04. Since the brain is the main site of EBLV-2 replication, this finding suggests that the virus displays a similar neurotropism to classical rabies virus. Rabies virus, especially in the late stages of disease, disseminates from the brain to other innervated sites within the host (*11*). For EBLV-2, the tongue was consistently found to contain detectable levels of viral RNA in this study and a previous study (*7*). Genomic RNA was also found in the stomach and intestines of 3 bats investigated (this study and [*7*]). All of these organs are highly innervated tissues, although virus RNA in the stomach could result from swallowing virus.

Dissemination of rabies virus to the salivary glands and subsequent virus shedding enables transmission through

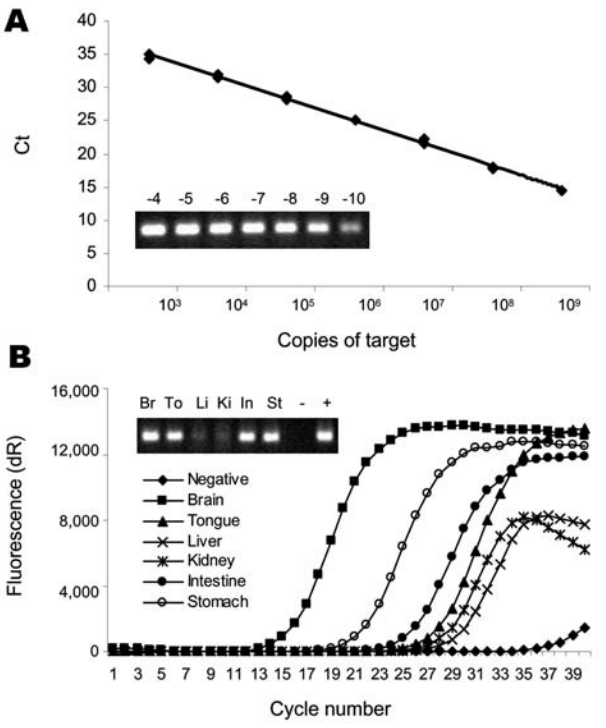

Figure. Results of quantitative polymerase chain reaction (PCR) of viral genome copies within organ samples taken from 2 Daubenton's bats infected with European bat lyssavirus type 2 (EBLV-2). A) Standard curve of duplicate dilutions of known quantities of EBLV-2 amplicon. A 20% (vol/vol) sample (10-fold dilution series) separated by electrophoresis on a 1% agarose gel is shown in the inset. Ct, threshold value. B) Real-time PCR amplification of EBLV-2 genomic RNA from organ samples from a bat (696/04) in Lancashire, United Kingdom infected with EBLV-2. dR, fluorescence change generated by a baseline-corrected algorithm calculated from every amplification cycle. A 20% (vol/vol) sample of each amplification separated by electrophoresis on a 1% agarose gel is shown in the inset. Br, brain; To, tongue; Li, liver; Ki, kidney; In, intestine; St, stomach; -, negative sample (no template); +, positive sample from a previous human case (*5*).

biting. Detection of EBLV-2 RNA in the tongue of infected bats leads us to conclude that transmission of EBLV-2 may occur through biting. However, since EBLV-2 genome was detected in a bladder sample, we cannot exclude the possibility of virus release from urine. In future cases, where possible, organs such as the salivary glands and lungs should be examined to provide further evidence for the route of virus transmission between bats.

Table 2. Quantification of European bat lyssavirus type 2 genome copies in organs of 2 naturally infected Daubenton's bats*

	Genome copies/μg total RNA	
Organ	Bat 603/04	Bat 696/04
Brain	204,000,000	640,000,000
Tongue	292,800	136,533
Liver	61,760	37,800
Bladder	839,680	ND
Kidney	ND	87,933
Intestine	277,067	680,667
Stomach	380,133	10,586,667

*ND, not done.

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Replicon Typing of Plasmids Encoding Resistance to Newer β-Lactams

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Polymerase chain reaction–based replicon typing represents a novel method to describe the dissemination and follow the evolution of resistance plasmids. We used this approach to study 26 epidemiologically unrelated *Enterobacteriaceae* and demonstrate the dominance of incompatibility (Inc) A/C or Inc N-related plasmids carrying some emerging resistance determinants to extended-spectrum cephalosporins and carbapenems.

Understanding the molecular epidemiology of resistance plasmids has been a major issue since scientists became aware of plasmids' role in the spread of antimicrobial drug resistance. However, understanding this epidemiology has been complex because of the diversity and promiscuity of these elements. The plasmid replication system, which dictates the plasmid's behavior (host range, copy number) is the major plasmid landmark from a biologic standpoint; it is used for plasmid classification and identification (*1*). Plasmids were originally classified in incompatibility (Inc) groups (*2*). Inc is a manifestation of plasmid relatedness based on commonality of replication controls. The standard procedure for determining Inc groups requires laborious hands-on work, multiple conjugation, transformation assays, or hybridization experiments (*1–3*).

Our objective of understanding the relationship among resistance plasmids prompted us to develop a polymerase chain reaction (PCR)–based replicon typing method (*4*). Our study has 2 aims: 1) to investigate phylogenetic relatedness among plasmids carrying extended-spectrum cephalosporin (ESC) and carbapenem resistance determinants emerging in 3 different countries (Greece, Italy, and the United States) and 2) to ascertain the sensitivity of the method.

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The Study

PCR-based replicon typing was applied to type the resistance plasmids carried by 26 *Escherichia coli* transconjugants or transformants obtained from epidemiologically unrelated clinical isolates of *Enterobacteriaceae* associated with community- or hospital-acquired infections in the United States or southern Europe (Italy and Greece). The resistance plasmids carried genes encoding β-lactamases of Ambler class A (SHV-12), B (VIM-1 or VIM-4), and C (CMY-2, CMY-4, or CMY-13) (Table), which represent key emerging resistance determinants to ESC and carbapenems.

Eighteen primer pairs were used to perform 5 multiplex and 3 simplex PCRs, which recognized FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, and FII replicons (*4*). All amplified replicons were sequenced by standard procedures and used as specific probes to confirm the replicon typing results by Southern blot hybridization on purified plasmid DNA (data not shown).

The plasmid donors from the United States consisted of 4 previously characterized ESC-resistant *Salmonella* isolates submitted to the National Antimicrobial Resistance Monitoring System (NARMS) from 1996 to 1998 (*12*) and 6 ESC-resistant *Escherichia coli* O157:H7 isolates collected by NARMS from 2000 to 2001 (*5*). During the study periods, participating state and local public health laboratories forwarded every tenth non-Typhi type *Salmonella* and every fifth *E. coli* O157 isolate they received to the Centers for Disease Control and Prevention for susceptibility testing. This collection includes representatives from sporadic and outbreak infections (*5,12*). The 6 *Salmonella* and 4 *E. coli* plasmid donors selected for this study were a small sample of epidemiologically unrelated isolates representative of those carrying a $bla_{\text{CMY-2}}$ β-lactamase gene on plasmids classified as type A or B on the basis of the $bla_{\text{CMY-2}}$ hybridization pattern (*6,13*). The PCR-based replicon typing method assigned the A/C and I1 replicons to type A and type B plasmids, respectively (Table), which was confirmed by DNA sequencing. The I1-type amplicon sequences were identical to the R64 IncI1 reference plasmid (no. AP005147), whereas the A/C-type amplicon sequences exhibited 26 nucleotide (nt) substitutions with respect to the RA1 IncA/C reference plasmid (no. X73674), which caused 3 amino acid variations. Therefore, the A/C-replicon from the US plasmids may represent a new replicon variant, which we designated repA/C_2 (DNA sequence released under EMBL accession no. AM087198). The Figure shows conserved *Pst*I restriction profiles obtained for the A/C_2 plasmids that are different from those exhibited by the I1 plasmids.

The plasmid donors from Italy consisted of 7 multidrug-resistant isolates of various species of *Enterobacteriaceae* carrying either $bla_{\text{SHV-12}}$ or $bla_{\text{CMY-4}}$

Table. Phenotypic and genetic characteristics of plasmids and transformant/transconjugant strains analyzed in this study

Original strain	Species and serovar	Transferred resistance traits in transconjugants or transformants*	*bla* genes identified on transferred plasmids	Replicons detected by PCR†
USA-4204	*Salmonella enterica* serovar Typhimurium	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-2039	*S.* Typhimurium	AmpCazCroCtxFoxGmTo	CMY-2-type A	A/C_2
USA-3977	*S.* Typhimurium	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-8401	*Escherichia coli* O157:H7	AmpCazCroCtxFox	CMY-2-typeA	A/C_2
USA-8749	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-8868	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-1091	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-7546	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-11371	*E. coli* O157:H7	AmpAtmCazCroCtxFox	CMY-2-type B	I1
USA-1358	*S.* Thompson	AmpAtmCazCroCtxFox	CMY-2-type B	I1
IT-VA416/02	*Klebsiella pneumoniae*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-4, CMY-4	A/C_2
IT-VA417/02	*Enterobacter cloacae*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-4, CMY-4	A/C_2
IT-FI045T	*Enterobacter aerogenes*	AmpAtmCaz	SHV-12	FII
IT-FI008T	*E. coli*	AmpAtmCazTo	SHV-12	FII
IT-BG003T	*Serratia marcescens*	AmpAtmCazTo	SHV-12	FII
IT-NO003T	*Klebsiella oxytoca*	AmpAtmCaz	SHV-12	A/C_1
IT-BG017T	*K. pneumoniae*	AmpAtmCazCroCtx	SHV-12	I1
GR-541	*E. coli*	AmpAtmCazCtxCroFoxIpmTo	VIM-1, CMY13	N
GR-116	*E. coli*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-1, CMY13	N
GR-700	*K. pneumoniae*	AmpCazCroCtxFoxIpmGmTo	VIM-1	N
GR-2564	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-1943	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-1955	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-5866	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-51395	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-6/100	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N

*Transconjugants or transformants were obtained by conjugation on solid medium or electroporation, respectively, with *E. coli* (strains DH5-α or DH10B or 26R793 as recipients), as described previously (*5*–*10*). Antimicrobial drug susceptibility of the transconjugants or transformants was determined by disk diffusion as recommended by the Committee on Clinical and Laboratory Standards (*11*) with the following drugs: Amp, ampicillin; Atm, aztreonam; Caz, ceftazidime; Cro, ceftriaxone; Ctx, cefotaxime; Fox, cefoxitin; Ipm, imipenem; Gm, gentamicin; To, tobramycin. Transferred resistance traits were inferred by a detectable reduction of susceptibility to the drug in the transconjugant or transformant, compared to the *E. coli* recipient.
†Discrimination between A/C_1 and A/C_2 replicons was performed by DNA sequencing of the amplicon obtained by replicon typing.

and bla_{VIM-4} plasmidborne β-lactamase genes (Table). These isolates had been collected from 2002 to 2003 at 4 different hospitals in northern or central Italy (*7*,*8*) and were epidemiologically unrelated, except for IT-VA416/02 and IT-VA417/02, which were from the same patient (*7*). PCR replicon typing of the 5 bla_{SHV-12}–carrying plasmids detected 3 repFII (100% identical to the reference sequence no. M33752), 1 repI1 (100% identical to the R64 plasmid), and 1 repA/C_1 (99% homologous to the RA1 plasmid) (Table), suggesting mobilization of this gene among different plasmid scaffolds. The bla_{SHV-12} plasmids showed different *Pst*I restriction patterns, which confirmed their diversity (Figure). The 2 plasmids carrying bla_{VIM-4} and bla_{CMY-4} were assigned by PCR replicon typing to the A/C type. The sequence of these replicons showed the same 26 characteristic nucleotide substitutions of the A/C_2-replicon identified in the US plasmids. These 2 A/C_2-plasmids showed an apparently identical *Pst*I restriction profile (data not shown), which was also very similar to that of some USA bla_{CMY-2} plasmids (see the 2039 and 3977 US plasmids and the Italian VA416/02 plasmid in the Figure). The 2 Italian A/C_2 plasmids, in addition to bla_{CMY-4} (which is a bla_{CMY-2} variant different by only a single nucleotide substitution), also carried the bla_{VIM-4} carbapenemase gene, which has not been reported on bla_{CMY-2}–carrying plasmids from the United States and may represent a novel acquisition. These findings indicate intercontinental spread of these plasmids and novel acquisition of resistance genes.

The plasmid donors from Greece consisted of a collection of 7 *Klebsiella pneumoniae* isolates carrying the bla_{VIM-1} gene (*9*) and 2 *E. coli* isolates carrying bla_{VIM-1} and bla_{CMY-13} genes (*10*). These isolates, randomly collected from 5 different hospitals in Athens and Piraeus from 2001 to 2003, are representative of the VIM-1–producing isolates circulating in Greece. No repetitive samples were taken from patients. All isolates exhibited decreased susceptibility to carbapenems. Restriction analysis of these plasmids classified them into 6 different groups on the basis of their restriction profiles (Figure). By replicon typing, all of these plasmids were assigned to the same repN-type replicon, which exhibited 2-nt point mutations (99%

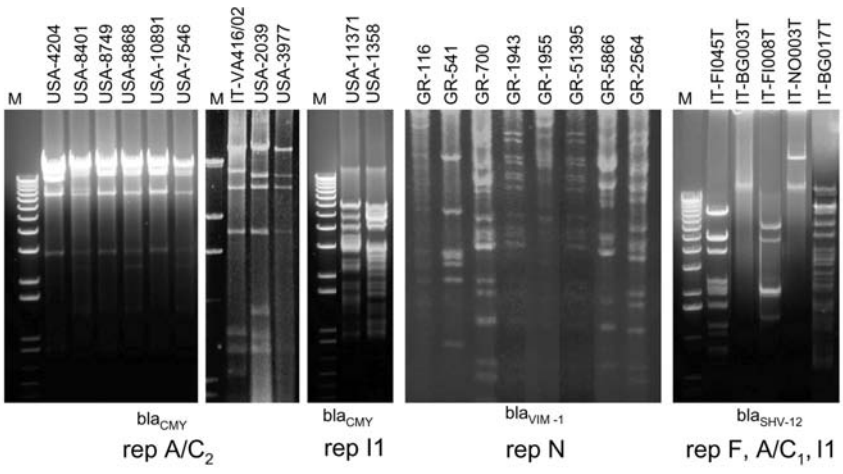

Figure. Restriction enzyme analysis of plasmid analyzed in this study. Numbers and letters above each lane indicate strain reference names as defined in the Table. M is the1-kb Plus DNA ladder (Gibco-BRL, Gaithersburg, MD, USA). Plasmids were transferred by conjugation or transformation in *Escherichia coli* K12 strains and purified by Midi-prep purification kit. Plasmids were digested with restriction endonuclease *Pst*I and separated by agarose (1%) gel electrophoresis in 1× TBE buffer. DNA was stained with ethidium bromide and visualized under UV light. The replicons and the β-lactamase genes carried by each group of plasmids are indicated below the figure.

homology) in respect to the R46 IncN reference plasmid (no. NC_003292), an indication that they were phylogenetically related and probably evolved from a common ancestor. Although one might expect similar plasmid scaffolds to exist among isolates in Greece and Italy because of geographic proximity, this was not the case. This finding explains the great variability of resistance plasmids carrying different combinations of resistance genes.

Since the origin of replication is a constant and conserved part of a plasmid, replicon typing focused on this portion of the plasmid is a more sensitive and specific method for identifying phylogenetically related plasmids than restriction-based analysis of the entire plasmid. This fact is probably due to the presence of multiple mobile elements (IS elements, transposons, integrons) that can mediate rearrangements of the plasmid scaffolds, which leads to the formation of apparently divergent plasmids. In fact, this phenomenon was demonstrated for the GR-541 plasmid that contains multiple copies of insertion sequences and other mobile genetic elements within its scaffold (*14*).

Conclusions

A PCR-based replicon typing approach was successfully applied to relevant resistance plasmids. Coupled with sequencing, the approach allowed high-resolution typing of the plasmid replicons. Typing results provided original insights into the molecular epidemiology of resistance plasmids. For instance, the $bla_{\text{CMY-2}}$–carrying plasmid circulating in the United States was also detected in Europe in the form of a derivative that also carries the VIM-4 carbapenemase determinant. This finding demonstrates that plasmids carrying resistance to clinically relevant antimicrobial agents can spread worldwide among bacteria responsible for both nosocomial and community-acquired infections. The heterogeneity among Italian plasmids encoding SHV-12 (the most prevalent SHV-type extended-spectrum β-lactamase in this country) (*15*) suggests a notable potential for this determinant to spread among different plasmid replicons. On the other hand, replicon typing indicated that the VIM-1–encoding plasmids from Greece were all related despite their different restriction profiles, which points out the common origin of these plasmids. The $bla_{\text{CMY-13}}$ gene from Greece is located on the repN plasmid, whereas Italy and the United States share the A/C_2 plasmid as a vehicle of the bla_{CMY} gene, despite their geographic distance. Further research is necessary to determine the influences on plasmid trafficking as well as further similarities and differences. Replicon identification may provide useful clues to the evolution of these resistant plasmids. The ability to trace and screen plasmids by PCR may facilitate further understanding of the horizontal transfer of antimicrobial drug resistance.

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Azithromycin Failure in *Mycoplasma genitalium* Urethritis

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We report significant failure rates (28%, 95% confidence interval 15%–45%) after administering 1 g azithromycin to men with *Mycoplasma genitalium*–positive nongonococcal urethritis. In vitro evidence supported reduced susceptibility of *M. genitalium* to macrolides. Moxifloxacin administration resulted in rapid symptom resolution and eradication of infection in all cases. These findings have implications for management of urethritis.

Mycoplasma genitalium has been well described as a pathogen in men with acute and chronic nongonococcal urethritis (NGU) and has been associated with cervicitis in women (*1*). Since culturing the organism is difficult, limited information has been available regarding its antimicrobial drug susceptibility. In vitro studies suggest it is susceptible to tetracyclines, macrolides, and fluoroquinolones (*2–4*), although reduced susceptibility to tetracyclines (*5*) and specific fluoroquinolones has been reported (*4*,*6*). In clinical studies, doxycycline and levofloxacin (*4*,*7–11*) have substantial failure rates, whereas early reports suggest single-dose azithromycin may be more efficacious (*10*,*11*). Treatment guidelines for acute NGU include 1 g single dose of azithromycin or doxycycline for 7 days, but no evidence-based guidelines exist for treatment of *M. genitalium*–positive NGU.

We report treatment failure of single-dose and multidose azithromycin therapy in *M. genitalium*–positive NGU and provide in vitro evidence of macrolide resistance in clinical isolates. Persistent infection was eradicated with moxifloxacin.

The Study

Cases were derived from a case-control study of acute NGU conducted from March 2004 to March 2005 at Melbourne Sexual Health Centre (MSHC), Australia (*12*). Participants completed a questionnaire, underwent examination, and had first-void urine samples analyzed by strand-displacement amplification (ProbeTec-ET CT-Amplified-DNA-Assay, Becton, Dickinson and Company, Sparks, MD, NJ, USA) for *Chlamydia trachomatis* and by polymerase chain reaction (PCR) for *M. genitalium* (*13*), herpes simplex viruses (HSV-1 and -2), *Trichomonas vaginalis, Ureaplasma urealyticum* and *parvum, Gardnerella vaginalis,* and adenoviruses (*12*). Culture of urethral samples in modified-Thayer-Martin medium was performed for *Neisseria gonorrhoeae*.

Men with *M. genitalium* infection were instructed regarding partner notification and reinfection and were asked to return for a test of cure (TOC) 1 month posttreatment. Men with persistent *M. genitalium* infection were given 1 g single dose of azithromycin or 1 g weekly for 3 doses, but after apparent failure of azithromycin therapy in 3 men without reinfection, participants with persistent infection were offered moxifloxacin, 400 mg daily for 10 days. Four urethral specimens from men for whom azithromycin therapy failed were inoculated into SP4 medium, frozen (–80°C), and shipped on dry ice to Statens Serum Institut, Denmark, for culture in Vero cells and antimicrobial drug susceptibility testing (*6*). *M. genitalium* strains in Vero cell culture were grown in the presence of different concentrations of antimicrobial drugs, and growth of *M. genitalium* was monitored by quantitative PCR for determination of MIC (*6*).

The Human Research and Ethics Committee of the Alfred Hospital, Victoria, approved the study. Data were stored in Microsoft Access and analyzed by using SPSS version 12 (SPSS Inc., Chicago, IL, USA). Ninety-five percent confidence intervals (CIs) were calculated for proportions, which were compared by using the Fisher exact test. Patients were excluded from the analysis when information or specimens were not available.

M. genitalium was detected in 31(9.4%) of 329 patients (95% CI 6.6%–12.9%) and 3 of 307 controls. No patients with *M. genitalium* infection had other pathogens detected (*12*). Men with *M. genitalium* had a median age of 33 years (range 22–54 years); 25 were heterosexual, and 9 were homosexual (behavioral and clinical data are presented elsewhere [*12*]). Six female and 4 male asymptomatic sexual contacts of *M. genitalium*–infected men were tested; a throat and anal sample in 1 man and a cervical sample in 1 woman were positive for *M. genitalium*. Contacts were presumptively treated with 1 g single dose of azithromycin; however, the infected male contact required moxifloxacin after azithromycin treatment failed in this patient and in the index patient.

Thirty-two men (94%) completed their TOC, a median of 31 days (range 17–59 days) after receiving azithromycin.

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Twenty-three (72%) men had a negative TOC (95% CI 55%–85%) and were asymptomatic; however, 9 (28%, 95% CI 15%–45%) were positive for *M. genitalium* by PCR. No treatment failures reported unprotected sexual contact posttreatment or previous antimicrobial drugs. Azithromycin treatment failed in 4 (44%, 95% CI 16%–76%) homosexual males, compared to 5 (22%, 95% CI 8%–42%) heterosexual males (p = 0.23). Five men for whom azithromycin treatment failed reported sexual contact with partners from Asia before symptom onset (56%, 95% CI 24%–84%) compared to 6 azithromycin responders (27%, 12%–48%), p = 0.22. Eight patients for whom azithromycin treatment failed reported an initial reduction or resolution of symptoms following azithromycin and then experienced recurrent urethral symptoms; 1 male was persistently asymptomatic. The Table outlines urethral Gram stain findings and treatment of men with persistent infection; all 8 men became asymptomatic after receiving moxifloxacin.

The 4 TOC specimens from men with azithromycin failure available for culture yielded growth of *M. genitalium*. Antimicrobial drug susceptibility testing showed increased MICs to macrolides: azithromycin >8 mg/L, erythromycin >32 mg/L, and clarithromycin >32 mg/L. All isolates were susceptible to moxifloxacin (MIC range 0.031–0.125 mg/L) and could be considered susceptible to doxycycline (MIC range 0.125–0.25 mg/L). However, correlates between in vitro MICs and treatment efficacy have not yet been established.

Conclusions

The azithromycin failure rate in *M. genitalium*–positive NGU was 28% (15%–45%) in this study and was associated with recurrent urethral symptoms in 8 of 9 cases. Longer course azithromycin ameliorated but did not resolve symptoms or eradicate infection, whereas moxifloxacin resulted in rapid symptom resolution and eradicated infection. Symptom improvement followed by recrudescence has been reported after levofloxacin failure (*9*). Culture of *M. genitalium* from all 4 specimens, and reduced susceptibility to azithromycin in vitro, demonstrates that azithromycin-resistance rather than reinfection caused treatment failure and that nonviable DNA was not the reason for a persistently positive PCR. The availability of strains in pure culture will enable investigation into resistance mechanisms, and work in progress indicates that mutations in region-V of the 23S-rDNA explain the azithromycin resistance (J.S. Jensen, unpub. data).

M. genitalium has been associated with persistent NGU (*1*). Recent data indicate that sequence variation in the gene mediating adhesion to epithelial cells coincides with the immune response in patients and that changes in this gene occur rapidly with persistent infection (*14*). In vitro studies also suggest that macrolide-resistant mutants can

Table. Case-patients experiencing single-dose azithromycin treatment failure*

		TOC-1			TOC-2			TOC-3			TOC-4
Patient†	Pretreatment PMN/HPF	PMN/ HPF	Mg PCR	Treatment-2	PMN/ HPF	Mg PCR	Treatment-3	PMN/ HPF	Mg PCR	Treatment-4	Mg PCR
1‡	≥5	≥5	Pos	1 g AZI	≥5	Pos	1 g AZI weekly, 3 doses	<5	Pos	MOX, 400 mg bd 10 d	Neg
2‡	≥5	<5	Pos	1 g AZI	§	Pos	1 g AZI weekly, 3 doses	§	Pos	MOX, 400 mg bd 10 d	Neg
3	<5	§	Pos	1 g AZI weekly 3 doses	<5	Pos	MOX, 400 mg bd 10 d	§	Neg		
4‡¶	≥5	≥5	Pos	MOX, 400 mg bd 10 d		Neg					
5‡	≥5	≥5	Pos	MOX, 400 mg bd 10 d		Neg					
6	<5	§	Pos	MOX, 400 mg bd 10 d		Neg					
7#	≥5	<5	Pos	MOX, 400 mg bd 10 d		Neg					
8	≥5	<5	Pos	MOX, 400 mg bd 10 d		Neg					
9	≥5	≥5	Pos	MOX, 400 mg bd 10 d		Neg					

*PMN/HPF, polymorphonuclear count per high power field (×1,000 magnification); Mg, *Mycoplasma genitalium*; PCR, polymerase chain reaction; Pos, positive; Neg, negative; AZI, azithromycin; MOX, moxifloxacin; d, days; bd, twice daily; tests of cure (TOCs) were performed 1 month after commencement of each therapy; TOC-1, first test of cure 1 month after treatment.
†All men treated with 1 g of azithromycin at first examination.
‡Patients with specimens cultured, MIC data available and presented for all 4 isolates.
§Urethral PMN count not available.
¶Patient 4 saw his general practitioner 3 weeks after receiving 1 g azithromycin with recurrent urethral discharge and dysuria and was retreated with 1 g azithromycin before his TOC-1.
#Only patient who was asymptomatic with persistent infection.

be selected by serial passage of mycoplasmas in subinhibitory concentrations of macrolide (*15*). Macrolide resistance in our study could have been induced by single-dose azithromycin, which may be suboptimal for eradication of a slow-growing bacterium such as *M. genitalium.* Studies are ongoing to establish whether resistance in our isolates was present pretreatment or emerged after azithromycin-exposure. It is possible that initial use of higher doses or longer durations of azithromycin in *M. genitalium*-positive NGU could avoid selection of resistant mutants. The association between azithromycin failure and sexual partners from Asia may be clinically relevant, given the high levels of antimicrobial drug resistance reported in other sexually transmitted infections such as *Neisseria gonorrhoeae* infections in Asia, and the higher failure rates seen in homosexual men, while not statistically significant, may represent a core-group effect.

Azithromycin or doxycycline is recommended treatment for NGU. While treatment-failure in *M. genitalium*–positive NGU appears common with doxycycline (*4*,*7*–*11*), early reports suggest 1 g azithromycin is more effective, with cure rates of 85% (*10*,*11*), and that prolonged azithromycin treatment (500 mg on day 1 and 250 mg on days 2–5) eradicates *M. genitalium* in 95% of cases (*10*). However, if treatment-failure after 1 g azithromycin is as prevalent as indicated by our study in *M. genitalium*–positive NGU, this has implications for the use of single-dose azithromycin as first-line treatment for NGU and leaves few evidence-based treatment options. Information regarding sensitivity of *M. genitalium* to fluoroquinolones has been limited, but reports suggest differential activity against *M. genitalium,* with levofloxacin (*4*,*9*) less active than gatifloxacin, sparfloxacin, and tosufloxacin in vitro and in vivo and moxifloxacin more active than levofloxacin and ciprofloxacin in vitro (*6*).

We report significant failure rates of azithromycin in *M. genitalium*–positive NGU that is supported by in vitro evidence of reduced susceptibility to macrolides. Recurrent urethral symptoms following azithromycin therapy only occurred in persons with persistent *M. genitalium* infection and resolved with moxifloxacin.

Because single-dose azithromycin is recommended treatment for NGU, these findings have implications for treatment guidelines and highlight the need for randomized studies to determine optimal treatment for *M. genitalium*–positive NGU and *M. genitalium* infection in women, who are at high risk for sequelae.

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Leptospirosis in Squirrels Imported from United States to Japan

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We diagnosed leptospirosis in 2 patients exposed to southern flying squirrels imported from the United States to Japan. Patients worked with exotic animals in their company. *Leptospira* isolates from 1 patient and 5 of 10 squirrels at the company were genetically and serologically identical and were identified as *Leptospira kirschneri*.

Leptospirosis is a worldwide zoonosis caused by infection with *Leptospira interrogans* sensu lato species. *Leptospira* is mostly transmitted to humans through contaminated water or soil and by direct contact with a variety of infected animals (*1–3*). To date, a variety of wild animals have been imported from foreign countries to Japan. In this study, 2 men working at an animal trading company were infected with *Leptospira* spp. To determine the source of infection, *Leptospira* spp. were isolated from animals in their company and sequenced.

The Cases

An animal trading company in Shizuoka, Japan, imported 106 southern flying squirrels from Miami, Florida, on March 27, 2005. Three workers handled these animals, which were housed 10 animals to a cage. Before patient 1 became ill, the workers dressed casually and touched the animals with bare hands in their routine work. Wild rats (such as *Rattus norvegicus* or *R. rattus*) had not invaded the animal house.

On April 22, 2005, patient 1, a 29-year-old man who handled a variety of exotic animals at the company, was hospitalized in Shizuoka Saisei-kai General Hospital with fever (temperature 40°C), headache, chills, nausea, vomiting, jaundice, and uremia, symptoms similar to those of locally acquired leptospirosis. Leptospirosis was diagnosed by polymerase chain reaction (PCR) targeted to the flagellin gene (*flaB*) and confirmed serologically with convalescent-phase serum by microscopic agglutination test. The patient was seronegative and PCR-negative for hantavirus, which causes symptoms similar to those observed in the patient. He was treated with an intramuscular injection of streptomycin (2 mg/day) for 7 days, which is the recommended treatment for leptospirosis in Japan (*4*); he consequently recovered.

On June 1, 2005, patient 2, a 28-year-old man who worked at the same company, was hospitalized in Shizuoka Saisei-kai General Hospital with fever (temperature 39°C), headache, chills, nausea, vomiting, jaundice, and uremia. The patient had been in contact with imported animals. He recovered with intramuscular injections of streptomycin (2 mg/day) for 3 days, followed by treatment with oral amoxicillin for 3 days.

Leptospira DNA was detected in serum samples from patient 1 and whole blood from patient 2 by *flaB* PCR (*5*). Sequences were determined by Prism 3130-avant DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences of *flaB* detected from both patients were identical and showed a high degree of similarity to *L. kirschneri*.

Diagnosis was performed serologically by microscopic agglutination test with a panel of *Leptospira* reference strains (*3*). Convalescent-phase serum samples from both patients reacted to *L. kirschneri* strain Moskva V and strains isolated from southern flying squirrels, although serum collected on the day of hospitalization was negative in both patients (Table 1). To cultivate *Leptospira*, a few drops of blood from patient 2 were placed in several tubes of Ellinghausen-McCullough-Johnson-Harris medium supplemented with 2.5% rabbit serum. After 7 days of incubation at 30°C, *Leptospira* was detected from the culture (isolates P5.4, P10.1, P10.2).

To determine the validity of the association between animals held by the company and the illness, exotic animals (75 animals, 7 species) housed in the company were tested. *Leptospira* was isolated from 5 of 10 kidney cultures (isolates AM1, AM2, AM3, AM7, AM8) from southern flying squirrels. DNA from the urinary bladders, including the animals' urine, was extracted by using proprietary DNA extraction kits (Quick gene, Fuji Film Co., Tokyo, Japan). Five of 10 southern flying squirrels were *flaB* PCR-positive (Table 2). Species of the isolates were identified by using *flaB* and DNA gyrase B subunit gene (*gyrB*) sequencing analysis. We amplified 1.2-kb partial sequences of *gyrB* by using primers UP1TL (5′-CAyGCnGGnGGnAArTTyGA-3′; n: A, G, T, or C; r: A or G; y: C or T) and UP2rTL (5′-TCnACrTCnGCrTCnGTC AT-3′; n: A, G, T, or C; r: A or G) (*6*). The isolates obtained from patient 2 and southern flying squirrels had identical

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Table 1. Microscopic agglutination titer of patients' sera collected while hospitalized and during the convalescent phase

Patient	*Leptospira* strain used as antigen*	Patient serum	
		Hospitalized	Convalescent-phase
1	Serovar Grippotyphosa Moskva V	<50	100
	Animal isolate AM3	<50	800
	Animal isolate AM1	<50	800
2	Serovar Grippotyphosa Moskva V	<50	200
	Animal isolate AM3	<50	200

*Samples were not reactive to a panel of representative serovars, Australis, Autumnalis, Carlos, Bataviae, Cynopteri, Hebdomadis, Copenhageni, Icterohemorrhagiae, Javanica, Pomona, Pyrogenes, Hardjo, Sejroe, Wolffi, and Tarassovi; serovars Canalzonae, Huanuco, Muelleri, and Valbuzzi belong to serogroup Grippotyphosa.

flaB (data not shown) and *gyrB* (Figure 1) DNA sequences and were identified as *L. kirschneri.* The *flaB* sequences from the serum of patient 1 and whole blood of patient 2 were identical to those of isolates from patient 2 and animals. Additionally, restriction fragment length polymorphism (RFLP) analysis based on pulse-field gel electrophoresis was conducted (*7*). These isolates showed identical RFLP patterns (Figure 2), which suggests that patients were infected with *L. kirschneri* from southern flying squirrels.

To determine serovar of the isolates, a cross-agglutination test was performed with a panel of hyperimmune rabbit serum raised to representative serovars Icterohemorrhagiae, Copenhageni, Autumnalis, Hebdomadis, Australia, Grippotyphosa, Javanica, and Castellonis, which are present in Japan. These isolates reacted with anti-Grippotyphosa serum but not with the others (data not shown). Convalescent-phase sera from patients reacted with *Leptospira* isolates from the squirrels and also with serovar Grippotyphosa strain Moskva V (Table 1).

On April 24, the local health government prohibited the company from trading animals and directed them to use protection, such as latex gloves and disinfection of the floor with sodium hypochlorite, against infection. On June 2, all southern flying squirrels were euthanized by carbon dioxide, and the animal house was disinfected by the local health government. PCR detected *flaB* DNA on the surface of the squirrels' bodies and in urine on the soaked paper in the cages; the sequences were identical to those of the isolates. Before the first case was detected, 27 southern flying squirrels had been distributed to retail pet shops. Sixteen were returned, 2 died, 7 remained at pet shops, and 2 had been sold. The 2 sold animals and 7 remaining at the pet shops were recovered and euthanized. No illness was reported among persons in contact with these animals.

Table 2. Detection and isolation of *Leptospira* from imported animals in the company

Animal	No. samples positive/ no. samples tested	
	Kidney culture	*flaB* PCR
Spiny mouse (*Acomys cahirinus*)	0/9	0/9
House mouse (species unknown)	0/4	0/4
Golden spiny mouse (*Acomys russatus*)	0/13	0/13
Mongolian gerbil (*Meriones unguiculatus*)	0/9	0/9
Southern flying squirrel (*Graucomys volans*)	5/10*	5/10*
Baluchistan pygmy jerboa (*Salpingotulus michaelis*)	0/20	0/20
Siberian chipmunk (*Tamias sibiricus*)	0/10	0/10

*Four of 5 culture-positive animals were positive by polymerase chain reaction (PCR). Remaining culture-positive animal was PCR negative, whereas 1 culture-negative animal was PCR positive.

Conclusions

Serovar Grippotyphosa commonly causes canine leptospirosis (*8*,*9*) and infects a variety of domestic and wild animals in the United States (*10*–*13*). In Japan, serovar Grippotyphosa is distributed in the southernmost islands, the Okinawa archipelago (*14*), but not on Honshu Island, the main island. Patients did not travel to Okinawa or foreign countries before disease onset. Our findings support the conclusion that the patients were infected with *L. kirschneri* serovar Grippotyphosa by contact with southern flying squirrels. Similarly, in the United States, humans have acquired monkeypox infection from pet prairie dogs, which had themselves been infected by exotic African

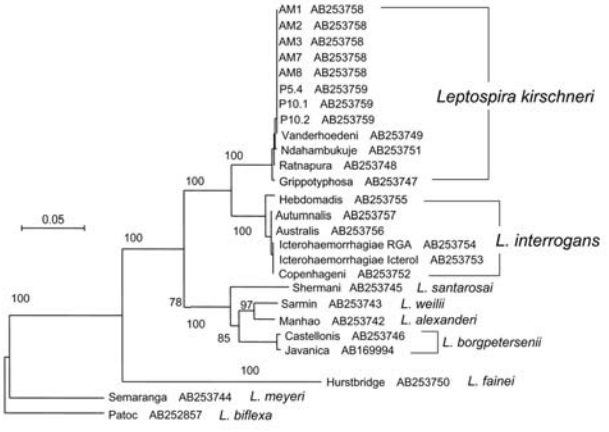

Figure 1. Phylogenetic tree based on the *Leptospira* DNA gyrase B subunit gene (*gyr*B) sequence. The sequences obtained have been deposited in DDBJ/GenBank/EMBL with accession numbers indicated.

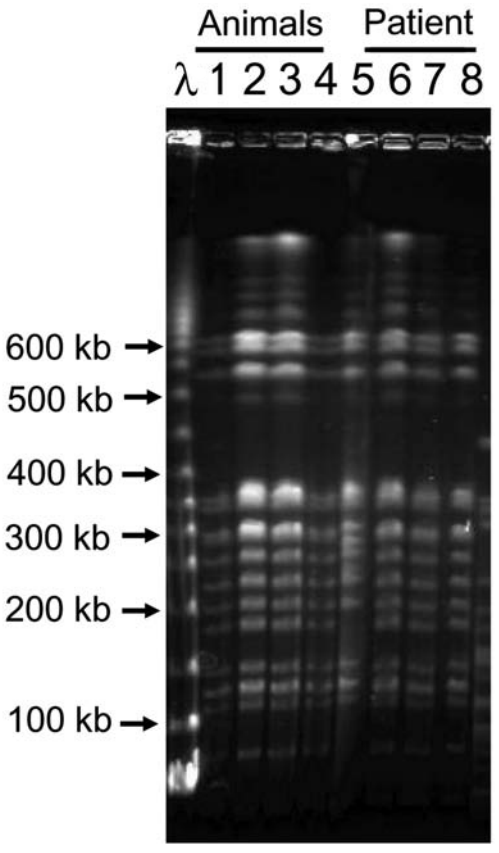

Figure 2. Pulsed-field gel electrophoresis analysis of *Not*I restriction fragment of *Leptospira* isolates from patient 2 and southern flying squirrels. *Leptospira* cells were lysed, and DNA was digested with restriction enzyme *Not*I in agarose gels. DNA in the gel was electrophoresed with 1% pulsed-field certified agarose in 0.5× Tris-borate-EDTA buffer under a pulse time of 10 s for 5 h and 30 s for 12 h, followed by 60 s for 7 h at 200 V. Lane 1, AM1; lane 2, AM2; lane 3, AM3; lane 4, AM7; lane 5, serovar Grippotyphosa strain Moskva V; lane 6, P10.2; lane 7, P10.1; lane 8, P5.4; λ phage DNA concatemer is used as a DNA size marker. Isolate AM8 showed an identical restriction fragment length polymorphism pattern to that of others.

rodents (*15*); these findings show that exotic pets represent a substantial hazard. The outbreak demonstrated how new infectious diseases could be emerging because of importation from overseas. If, during shipping and housing of the animals, the infection were to have expanded among southern flying squirrels, the infection rates and risk for humans would have increased. The leptospirosis cases reported here warn against importing exotic animals.

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Tuberculosis Outbreak in Marijuana Users, Seattle, Washington, 2004

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Matching *Mycobacterium tuberculosis* isolates were noted among 11 young tuberculosis patients socially linked through illicit drug–related activities. A large proportion of their friends, 14 (64%) of 22, had positive tuberculin skin-test results. The behavior of "hotboxing" (smoking marijuana inside a closed car with friends to repeatedly inhale exhaled smoke) fueled transmission.

Although overall US tuberculosis (TB) rates are declining, certain populations such as the foreign-born (*1*,*2*), homeless persons (*3*,*4*), and those who use illicit drugs (*5*,*6*) continue to challenge TB control efforts. A cluster of TB cases was recognized in Seattle from February to April 2004 among 4 young East-African immigrants with histories of incarceration and illicit drug use. Because patients resisted revealing names of contacts, traditional TB control efforts were hampered. We describe an outbreak fueled by illicit drug use and characterized by accelerated progression of disease.

The Study

Mycobacterium tuberculosis isolates from all culture-positive TB patients in Seattle and King County, Washington, during 2003–2004 were genotyped by spacer oligonucleotide typing and mycobacterial interspersed repetitive unit methods. We included patients who had an isolate that matched the outbreak strain or who had a social link to an already included patient.

Patient medical records were reviewed, and infectious periods were calculated. For sputum smear–positive patients, the infectious period extended from 3 months before symptom onset or the first positive smear (whichever was earlier) until 2 weeks after the start of appropriate TB treatment or until the patient was placed into isolation or produced consecutively negative smears. For sputum smear–negative patients, the infectious period extended from 1 month before symptom onset, the start of appropriate TB treatment, or the date that the patient was isolated (whichever was earlier), until 2 weeks after the start of appropriate TB treatment or until patient isolation (*7*).

We interviewed patients to learn their contacts, activities, and locations frequented while they were contagious. Additional contacts were found by outreach workers and a disease intervention specialist from the East-African community who was hired to work in the neighborhoods frequented by the patients. While in these neighborhoods, outreach workers and the disease intervention specialist recruited persons seen with patients or their contacts to be evaluated for TB and latent TB infection. Contact activities, specifically those related to illicit drugs, were observed or self-reported.

We categorized contacts as friends or others. Friends were defined as contacts of patients who spent time within a close-knit network of young men who exhibited similar marijuana-using behavior. Other contacts were defined as the families and relatives of patients and those who were named but were not closely associated with this network. Contacts received a TB evaluation including a tuberculin skin test (TST) to detect infection. Infection rates for friends and others were compared to guide contact prioritization for screening.

Patient 1 was first evaluated in December 2003, when a chest radiograph suggested pulmonary TB (i.e., upper lobe cavitary infiltrate). However, only clarithromycin was prescribed, and the patient was lost to follow-up. He was again seen in an emergency room in April 2004 after the infection evolved into bilateral extensive pulmonary TB. His sputum tested smear-positive for acid-fast bacilli. He was reluctant to name contacts.

Ten additional patients were found from February to October 2004 (Table 1). Isolates from all patients had matching TB genotypes. In Washington State, this genotype has only been identified among the patients in this outbreak. Patients' median age was 22 years (range 18–41). Eight patients were born in East Africa; a median of 13 years (range 6–22) had passed since their arrival in the United States. All but 1 patient were of East-African origin. Patient 5 was a white woman who received illicit drugs from patient 1.

Patients were symptomatic and had findings indicating infectiousness: all had pulmonary TB, 7 had cavitary disease, and 8 had sputum that tested smear-positive for acid-fast bacilli. One patient was HIV infected. Consecutive chest radiographs indicated progression to cavitary disease in ≤75 days in 3 patients and ≤121 days weeks in another patient. Table 2 shows the dates of clear chest radiographs

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Table 1. Tuberculosis outbreak patient and disease characteristics, N = 11

Characteristic	n
Patient	
East African origin	10
Foreign birth	8
Male	9
Incarceration history	11
Recent victim of assault	7
Illicit drug use	11
Hotboxing	11
Unemployed	11
Disease	
Pulmonary disease	11
Cavitary	7
Culture-confirmed	11
Sputum smear-positive for acid-fast bacilli	8
Symptomatic at diagnosis	9
HIV infection*	1

*Unknown for 1 patient.

interpreted as normal and the first chest radiographs showing disease.

While contagious, patients stayed in various locations, including cars, for most of the day. A single-bedroom apartment occupied by at least 1 patient while he was contagious was regularly visited by 2 other patients. Numerous members of the friend network slept there on any given night, and many others would regularly visit during a 10-week period beginning in April 2004 (Figure). The occupants nailed boards over the apartment windows to conceal activities, primarily marijuana use, from outsiders.

All patients were unemployed and had histories of incarceration and illicit drug use. No patients spent time together while incarcerated. All reported frequent "hotboxing," the practice of smoking marijuana with others in a vehicle with the windows closed so that exhaled smoke is repeatedly inhaled.

The Figure illustrates patients' infectious periods. Considerable overlap in infectious periods was noted, which highlights the potential for simultaneous contact with multiple contagious patients. We found 121 potentially exposed contacts. Fifty-four were friends, and the remaining were other contacts. At least 31 (57%) friend contacts spent time at the 1-bedroom apartment. After those with a past positive TST result were removed, 14 (64%) of 22 screened friends and 6 (23%) of 26 other contacts had a positive TST result. The risk for a positive TST result was 2.8× greater among friends than among other contacts (95% confidence interval = 1.3–6.0). Twenty-nine (54%) friend contacts self-reported or were observed hotboxing. Among the friends who reported or were observed hotboxing, 11 (79%) of 14 who received a TST had a positive result. Twelve friend contacts began treatment for latent TB infection, and 8 completed treatment.

Conclusions

Risk factors for TB include birth in a country with high TB prevalence (*2*) and incarceration (*8*). Although most patients in this outbreak were foreign-born and had histories of incarceration, genotyping results and epidemiologic findings suggest that TB was transmitted recently in the community rather than before immigration or during incarceration.

Frequent marijuana use has been reported among TB outbreak patients (*9*) and was the behavior linking these patients together. Creative sharing of marijuana has been described recently as a factor for *M. tuberculosis* transmission. In Australia, sharing a water pipe (i.e., "bong") was linked to transmission (*10*). "Shotgunning" refers to inhaling smoke from illicit drugs then exhaling it directly into another's mouth (*11*) and was associated with *M. tuberculosis* transmission among a group of exotic dancers and their contacts (*12*).

This investigation noted that a similar activity, hotboxing, might have contributed to transmission. As with shotgunning, hotboxing promotes the sharing of exhaled smoke and air. One patient with smear-positive cavitary

Table 2. Chest radiograph dates and results, N = 11

Patient	HIV infection	Date of normal chest radiograph before TB diagnosis	Date of first abnormal chest radiograph consistent with TB	No. days between normal and abnormal chest radiographs	Cavitary disease
1*	Declined	Undocumented	12/24/2003		Yes
2	Negative	Undocumented	2/22/2004		No
3	Negative	2/7/2004	4/19/2004	72	Yes
4	Negative	2/10/2004	4/25/2004	75	Yes
5	Positive	1/13/2004	5/13/2004	121	No
6	Negative	Undocumented	6/18/2004		Yes
7	Negative	5/15/2004	6/24/2004	40	Yes
8	Negative	Undocumented	7/9/2004		No
9	Negative	8/17/2003	7/23/2004	341	Yes
10	Negative	5/14/2003	8/30/2004	474	Yes
11	Negative	Undocumented	8/26/2004		No

*Source case.

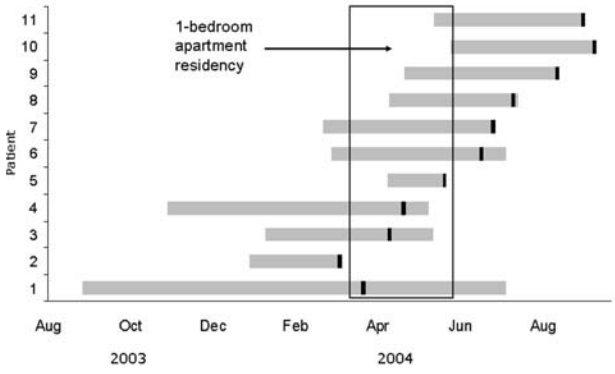

Figure. Infectious periods of tuberculosis patients. Vertical black bars indicate treatment start dates.

disease reported daily hotboxing with friends, often for most of the day. In addition, marijuana smoking might induce cough, creating an ideal environment for transmission. Many friends stayed and used marijuana at the single-bedroom apartment during the height of the outbreak. Furthermore, by nailing boards over the windows, ventilation was limited, creating an environment similar to that of hotboxing.

Disease rapidly progressed in HIV-negative patients in this outbreak. Seven patients had cavitary pulmonary TB. Three had chest radiographs interpreted as normal ≤75 days before TB diagnosis. Although progressive primary TB by nature is thought to be due to recent transmission, progressive primary TB with cavitation is uncommon (*13*). The pathogenesis of progressive primary TB with cavitation is not clear. However, frequent marijuana use and the setting of intense exposure may have played a role. In addition, poor nutrition and unhealthy lifestyles might have predisposed these young men to more rapid progression of disease. While no laboratory investigation to assess genetic susceptibility or strain virulence was conducted, these factors might have also contributed to the development of cases.

This outbreak resembles an outbreak reported among regular patrons of a neighborhood bar (*14*). Both were fueled by a highly infectious source patient who spent extended amounts of time indoors with 1 group of persons who regularly used substances (i.e., alcohol or marijuana). The result in both situations was a higher than expected incidence of TB disease and latent TB infection. In the outbreak reported in this article, however, the substance of choice was illicit and further complicated the control of this outbreak.

Patients' illicit drug activities promoted a reluctance to name contacts at risk and locations frequented. Traditional name- or location-based contact investigations did not work. Efforts had to revolve around meeting these young patients at times and locations convenient to the group. Then after gaining the groups' trust, outreach workers successfully found and screened contacts. Many successful screenings took place on street corners and in parking spaces throughout the community. Often outreach workers were successful only after spending hours driving throughout the community searching for patients and contacts. Four patients were originally screened as unnamed contacts located in the field. Alternative strategies to name-based contact investigations may become increasingly critical to TB control as TB recedes further from the general population, yet persists within smaller guarded groups (*15*).

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Dr Oeltmann is a senior epidemiologist in the Division of Tuberculosis Elimination, Centers for Disease Control and Prevention. His research interests include examining the effectiveness of methods used during TB contact and outbreak investigations such as case-control studies, social network analysis, geographic information systems, and TB genotyping.

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60 Years of Progress—CDC and Infectious Diseases

Tanja Popovic* and Dixie E. Snider, Jr.*

Malaria Control in War Areas was formed in 1942 to ensure that the areas around military bases in the southern United States remained malaria-free. Initial facilities were modest, a few rooms on the sixth floor of the Volunteer Building on Peachtree Street in Atlanta. Hardly anyone could have foreseen the future of this small organization. But Joseph W. Mountin, who was charged with setting it up, was not just anyone. An architect of modern public health, Mountin quickly realized that malaria control operations serving the needs of the states (response to state calls for help, laboratory and epidemiologic investigations, training) could become the foundation for improving the health of the nation.

Indeed, in 1946 the Public Health Service established the Communicable Disease Center to work not only on malaria but on typhus and other infectious diseases. The following year, a token payment of $10 was made for a 15-acre area on Clifton Road to house the operations. In the next 60 years, minor changes were made to the name (Center for Disease Control, Centers for Disease Control, Centers for Disease Control and Prevention), but the initials, CDC, remained the same. The campus on Clifton Road grew to include 2 biosafety level 4 laboratories and other state-of-the-art facilities; operations were established in Morgantown, Cincinnati, Fort Collins, and overseas; and the work expanded to include all infectious diseases, as well as occupational health, toxic chemicals, injury, chronic diseases, health statistics, and birth defects.

A magnet for gifted scientists and other professionals looking to serve in public health, CDC has attracted an exceptional cadre of talent over the years. Mountin was succeeded by leaders who pushed the agency to new levels of achievement, constantly probing new challenges and seeking new public health solutions. The thousands who work in laboratories and offices or trot the globe on epidemiologic investigations; the physicians, veterinarians, microbiologists, statisticians, economists, social scientists, other scholars, and support personnel; the many volunteers who serve on institutional review and other boards and committees; and CDC's many partners in academia, industry, clinical practice, and state and local governments all share unequivocal dedication to public health.

In this climate of idealism and dedication, the achievements have been many and span all areas. CDC scientists, typically working with like-minded colleagues, identified and characterized several infectious agents and emerging infectious diseases; invented devices, tools, and stains for diagnoses and systems for surveillance; demonstrated the value of combining laboratory practices and epidemiology; and through vision and leadership, worked closely with state and local health departments to increase their effectiveness as public health organizations. Some in its midst made such major contributions that microorganisms were named after them (Lee Ajello, *Ajellomyces* spp.; Dannie Hollis, *Vibrio hollisiae*; Don Brenner, *Neisseria brenneri*; Robert Weaver, *Neisseria weaveri*; Joseph McDade, *Legionella micdadei*).

CDC led the US campaign to immunize all children against vaccine-preventable infectious diseases; efforts to "link" states in search of foodborne disease outbreak causes by using molecular approaches to trace the causative organisms (PulseNet); efforts to translate science to practice, protecting women and children from such emerging infection-related conditions as toxic shock syndrome and aspirin-associated Reye syndrome.

Achievements in international health have been major benchmarks. CDC contributions range from support for and leadership of the global effort to eradicate smallpox to the establishment of Projet SIDA in Africa to initiate scientific research on the HIV/AIDS epidemic.

Science has changed in the past 60 years. Laboratory techniques used to detect, identify, and characterize microorganisms have moved from Petri dish and viral culture to real-time polymerase chain reaction and genome sequencing. During the 1976 Christmas holidays, a CDC

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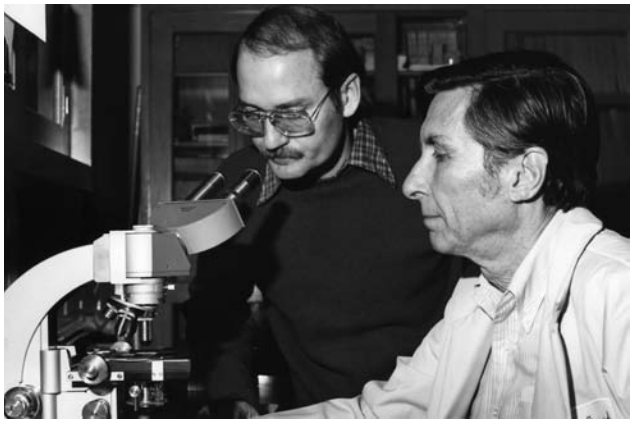

Figure 1. On January 14, 1977, the director of the Center for Disease Control's laboratory division, Charles C. Shepard (right), and microbiologist Joseph E. McDade isolated the agent that caused an outbreak of respiratory disease among members of the American Legion in July 1976. (Photo courtesy of Public Health Image Library, Centers for Disease Control and Prevention)

laboratory scientist, using simple microbiologic methods, injected guinea pigs with material from persons who died of Legionnaires' disease. When some guinea pigs died, he injected their spleen into chicken eggs. He saw what was later confirmed to be the cause of this disease by looking under the light microscope (Figure 1). Thirty years later, others at CDC are able to identify all of almost 200,000 nucleotides that compose the genome of the smallpox virus.

Figure 2. Terrence Tumpey recreated the 1918 influenza A (H1N1) virus to identify characteristics that made it such a deadly pathogen. (Photo by James Gathany, courtesy of Public Health Image Library, Centers for Disease Control and Prevention)

But science moves on. Recently, CDC scientists and colleagues have been able to recreate and reconstruct the 1918 influenza virus that caused the death of 40 to 50 million people (Figure 2). Information technology advances have enabled modeling to predict illness and death under specific circumstances, facilitate advance planning, and improve preparations for natural and human-made disasters.

Infectious diseases have changed in the past 60 years. All but hailed as being under control, they have found new virulence, emerging and reemerging globally without end. The new landscape of disease has required changes in management and control. The spectrum of science expertise has broadened, from entomologists and parasitologists (at Malaria Control in War Areas and the 1950s) to epidemiologists, microbiologists, and immunologists (predominating in the 1960s to 1980s). Over the past 2 decades, the CDC community has become increasingly multidisciplinary, embracing molecular biologists, geneticists, bioinformatics specialists, statisticians/mathematicians, behavioral and social scientists, modelers, economists, and other scholars.

What have not changed are the unique links between epidemiology and multiple other disciplines and between science and practice that keep CDC on the "speed dial" of every state and local public health official, every World Health Organization representative, and every minister of health worldwide. What has not changed is CDC's passion for science and public health. CDC scientists are proud to have served with so many colleagues and partners around the world on some of the greatest challenges to public health over the past 60 years. Nothing tells us we can rest on our collective laurels, impressive though they may be. Indeed, the most important lesson we have learned is that working together in research, applied public health, and preventive action is paramount because the emerging infectious disease and microbiologic challenges of the next 60 years may be even tougher than those we have already faced.

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Influenzalike Illness Among Homeless Persons

To the Editor: We report rates of influenzalike illness (ILI) and influenza vaccination among homeless persons at 3 shelter clinics in New York City examined from 1997 through 2004. Little is understood regarding the prevalence and transmission of influenza among the homeless (*1*). Further inquiry on this topic is timely because of concern over a possible influenza pandemic, because of US goals to increase vaccination rates among high-risk groups (*2*), and because of the potential threat to persons who live and work in shelters. Homeless shelters are paradigmatic congregate settings and thus likely sites for transmission of airborne pathogens such as influenza viruses and tubercle bacilli, shown in part by numerous tuberculosis outbreaks among the homeless (*3*).

Homeless persons experience high rates of pneumonia (*4*) and related death (*5*,*6*). This outcome indicates that the homeless also have high rates of influenza because pneumonia is a common complication of influenza. Depending upon patient's age and sex, death rates attributed to pneumonia or influenza among homeless adults ranged from 1.6 to 6.3 (95% confidence interval 0.4–24.1) in one study (*7*). The New York City Departments of Health and Mental Hygiene and Homeless Services reported in December 2005 that 1% of hospitalizations and 3.4% of deaths of homeless adults in New York City from 2001 to 2003 were caused by influenza or pneumonia (*8*).

We analyzed 4,319 medical charts of persons who received medical services in 3 New York City homeless shelter clinics during influenza seasons (i.e., October 1 through May 30) from 1997 through 2004. This study was approved by the St. Vincent's Hospital Research Committee and Institutional Review Board. This analysis identified 59 recorded cases of ILI, defined as temperature ≥100°F (37.8°C) and cough, sore throat, or both (Table). ILI is accepted as an indicator of influenza by the Centers for Disease Control and Prevention and others (*9*).

The overall medical chart review also showed that less than one fourth of all persons examined and one third of those ≥65 years of age had evidence of influenza vaccination noted in their charts. Vaccinations are available from many sources, but those given at shelter clinics accounted for a large percentage, and vaccination rates varied widely by homeless shelter clinic site.

This study has some limitations. Because vaccinations are offered at numerous health centers, rates of vaccination based on the medical charts we studied may be underestimated. Moreover, since only those homeless persons at shelters who attended the medical clinic provided data, the findings cannot be used to make generalizations regarding ILI or influenza vaccination rates among the general population of the shelters. Nonetheless, these numbers can serve as a basis for more rigorous inquiry.

The implementation of an appropriate public health response is critical in maintaining the health of homeless persons. Controlling influenza transmission within shelters may benefit the broader public in the same way that reducing the rates of tuberculosis among homeless persons is regarded as essential in preventing transmission to the general population.

The decision to receive an influenza vaccination is influenced by many factors. These factors include concern with related side effects, belief that the vaccine is not required, previous bad reactions, dislike of injections, and doubts about vaccine efficacy (*10*). Understanding how these factors affect vaccination rates among the homeless would be valuable in planning healthcare interactions and quality improvements. Similarly, since the New York City Departments of Health and Mental Hygiene and Homeless Services recommend that influenza immunizations be provided to all sheltered homeless adults and shelter staff (*8*), further inquiry would help determine the risk-benefit balance of such an approach.

Table. Cases of influenzalike illness (ILI) among homeless persons by influenza season, New York City, 1997–2004

Season	Shelter 1			Shelter 2			Shelter 3			Total		
	No. cases	No. patients seen	% patients seen with ILI	No. cases	No. patients seen	% patients seen with ILI	No. cases	No. patients seen	% patients seen with ILI	No. cases	No. patients seen	% patients seen with ILI
1997–98	5	284	1.8	3	221	1.4	3	202	1.5	11	707	1.6
1998–99	4	363	1.1	5	197	2.5	5	240	2.1	14	800	1.8
1999–00	2	170	1.2	1	186	0.5	4	248	1.6	7	604	1.2
2000–01	1	198	0.5	2	206	1.0	4	227	1.8	7	631	1.1
2001–02	2	202	1.0	2	122	1.6	1	258	0.4	5	582	0.9
2002–03	2	196	1.0	1	136	0.7	1	218	0.5	4	550	0.7
2003–04	6	152	3.9	1	157	0.6	4	235	1.7	11	544	2.0
Total	22	1,565	1.4	15	1,225	1.2	22	1,628	1.4	59	4,418	1.3

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Human West Nile Virus Infection, Catalonia, Spain

To the Editor: West Nile virus (WNV) is a mosquitoborne flavivirus that is widespread in Africa, the Middle East, Asia, and southern Europe, where it causes outbreaks and sporadic cases of the disease. It has become an emergent disease in North America, where it was detected for the first time in 1999 and became epidemic shortly thereafter (*1*). Although WNV was initially considered to have a minor health effect in the Mediterranean basin, human and equine outbreaks reported in the last decade in different countries (*2–5*) have made WNV infections a public health concern.

The epidemiology of WNV in Europe differs from that in America and has only been associated with nonrecurrent, sporadic outbreaks. The reasons for this difference are controversial; it may be due to environmental factors, reservoirs, or even mosquito vectors. In Spain, neither equine nor human WNV cases have been reported. However, some human serosurveys that used hemagglutination inhibition suggested that WNV or closely related flaviviruses circulated during the 1970s in the Ebro delta and areas in Spain (*6*,*7*). The Ebro delta, a wetland in Catalonia, in the northeast of Spain, is a stopping-off point for birds migrating between regions of Africa and Europe where different WNV vectors and reservoirs have been identified. The delta could be considered a high-risk area for WNV and other arthropodborne virus infections.

To evaluate WNV seroprevalence in the human population of the Ebro delta, a survey was conducted in 2001. After obtaining informed consent, 992 serum samples were obtained from inhabitants of the area. The population studied was representative of the whole area and was stratified by sex and age.

Anti-WNV immunoglobulin G (IgG) antibodies were determined by using an in-house indirect enzyme-linked immunosorbent assay (ELISA), as previously described (*8*). Results were classified as the sample absorbance/positive control absorbance ratio. Samples showing ratio values >0.2 were tested for WNV IgG and IgM by using an indirect and a μ-chain capture ELISA, respectively (Focus Technologies, Cypress, CA, USA), and an in-house microneutralization test.

For the microneutralization test, samples were tested in duplicate and assayed twice. Twofold dilutions (25 μL) of the samples (1:16–1:256 dilutions) were assayed by using 100 $TCID_{50}$ (50% tissue culture infectious dose) of West Nile Eg-101 reference strain in 96-well tissue culture plates with Vero cells and after 7 days of incubation at 37°C and 5% CO_2.

Thirty-eight samples showed IgG ratios >0.2 by the in-house ELISA. Of these, 12 showed WNV IgG, and 1 was positive for WNV IgM and IgG, according to the Focus assays. Two samples showed positive neutralizing activity, with titers of 32 and 256. The highest titer was shown by the sample that yielded positive levels of both IgM and IgG in the ELISA, which suggests recent WNV infection.

Anti-WNV IgG was more often detected in participants in the 20- to 29-year age group (odds ratio [OR] 4.23, 95% confidence interval [CI] 1.04–16.02, p = 0.03) and in persons who reported frequent mosquito bites (OR 8.62, 95% CI 0.44–169, p = 0.08). IgG-positive persons were equally divided by sex. No significant differences were found between antibody-positive or antibody-negative persons with respect to their profession, place of occupation, current residence, time in current residence, outdoor activities, use of insecticides and repellents, or symptoms related to WNV infection.

No symptoms related to WNV infection were reported by the IgM/IgG-positive participant, who was 31 years of age, was born in the area, worked outdoors, and was frequently bitten by mosquitoes. He also reported travel to Cuba 1 year earlier, but he had not been vaccinated against flavivirus, and serologic test results for dengue were negative.

The other IgG- and neutralizing antibody–positive participant was 45 years of age and was born and works in the area. He had never traveled abroad or been vaccinated against flavivirus. He reported a 4-day fever of unknown origin during the summer 1 or 2 years before the study. He often fishes in the areas and is frequently bitten by mosquitoes.

In conclusion, the study found evidence of recent WNV infections in humans living in the Ebro delta, where previous flavivirus circulation has been suggested by Lozano and Filipe (*6*). IgG-positive results not confirmed by neutralization could be due to cross-reactive antibodies induced by other flavivirus infections or vaccinations (*9*,*10*). The probable WNV infection described was asymptomatic, as occurs in ≈20% of cases. Other WNV infections in the area may have remained undetected, including neuroinvasive cases. Intensified research and surveillance in this area will help determine and refine thresholds for public health interventions.

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Shigellosis and Cryptosporidiosis, Baltimore, Maryland

To the Editor: Floret et al. argue convincingly that natural disasters, including severe floods and windstorms, tend not to result in epidemics of infectious disease (*1*). This conclusion is consistent with the lack of epidemics of shigellosis and cryptosporidiosis after hurricane rains in Baltimore, Maryland.

Shigellosis and cryptosporidiosis are associated with waterborne and foodborne transmission (*2*,*3*). We examined Baltimore shigellosis and cryptosporidiosis incidence to assess whether disease risk was related to temperature or rainfall from January 1, 1998, to December 31, 2004. Maryland FoodNet supplied case data; population estimates were acquired from the Maryland Department of Planning State Data Center; and meteorologic data for Baltimore Washington International airport (≈10 miles from the city center) were obtained from the National

Atmospheric and Oceanic Administration (*4*).

During the study period, 38 cases of cryptosporidiosis and 943 cases of shigellosis were reported in Baltimore. Temperature was strongly seasonal; precipitation was not. A dry period during 1999 was observed. No seasonal cryptosporidiosis patterns were identifiable. Two outbreaks of shigellosis occurred; in 2000 (≈50 cases) and 2002–2004 (≈870 cases). Sporadic cases of shigellosis were not seasonal.

Two hurricanes resulted in heavy rainfall in Baltimore during the study period (*5*). Hurricane Floyd inundated the city with rain on September 16, 1999, and on September 19, 2003, Hurricane Isabel produced heavy rains and storm surge in Baltimore (which is located near the northern end of Chesapeake Bay). Approximately 4 other named tropical storms or depressions directly affected Baltimore rainfall during the study. However, collectively, none of these events had distinguishable signatures in the incidence of shigellosis or cryptosporidiosis in this urban environment.

The institutional review boards of the University of Maryland School of Medicine, The George Washington University Medical Center, and the Maryland Department of Health and Mental Hygiene approved this study. Dr Hartley is supported by a National Institutes of Health Career Development Award (K25 AI-58956).

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Human Hantavirus Infection, Brazilian Amazon

To the Editor: Since hantavirus pulmonary syndrome (HPS) caused by Sin Nombre virus (SNV) was identified in the southwestern United States in 1993, cases have been diagnosed in many Latin American countries, and an increasing number of hantaviruses and their rodent reservoirs have been reported (*1*). The first evidence of hantavirus circulation in the western Brazilian Amazon region was documented in 1991 (*2*). Vasconcelos et al., by using antigens from the Old World hantavirus, found evidence of hantavirus antibodies in 45.2% of serum samples acquired from contacts of patients who died with undiagnosed hemorrhagic fever in Manaus.

The first human cases of symptomatic infection by hantaviruses were reported from Brazil in 1993, in Juquitiba (São Paulo State). HPS developed in 3 young brothers, who lived in a forested region along the Atlantic Coast, after they had cleared trees on their land, and 2 of them died. These patients were living in poor conditions, without appropriate storage spaces for human food or for animal feed, and their dwelling was constantly invaded by wild rodents who were looking for food (*3*). Since then, many other HPS cases have been reported, especially from the southern and southeastern regions of Brazil where agricultural activities are prominent; the mean case-fatality ratio is 48% (*3*). In the Brazilian Amazon, HPS has been frequently reported in Mato Grosso and sporadically in Maranhão and Pará states, which indicates an endemic circulation of hantaviruses (*4*,*5*)

We report here the first human cases of HPS in the state of Amazonas in the western part of the Brazilian Amazon. All 4 patients belonged to the same family cluster and came from a rural area near the town of Itacoatiara, on the edge of an important industrial waterway for soybean transport (the Itacoatiara soybean terminal). This family (patients 1, 2, and 3) had cleared a forested area on their farm and killed many rodents found in the bases of trees and near the house from May 25 to June 5, 2004. They also reported that wild rodents were inside their house.

All serologic tests were performed in the Arbovirology and Hemorrhagic Fever Department, at the Evandro Chagas Institute (Pará, Brazil), with

antigens provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA). An enzyme-linked immunosorbent assay (ELISA) was performed by using cellular fluid and Laguna Negra virus antigens for immunoglobulin M (IgM) detection (MAC-ELISA), and recombinant SNV antigens for IgG detection. Samples were considered positive with an optical density ≥0.2 in 1:100 (IgM) and 1:400 (IgG) dilutions (*6*,*7*). Viral isolation or polymerase chain reaction (PCR) for hantavirus were not attempted in human or rodent samples.

In the index patient, symptoms developed 15 days after she had killed 20 rodents with hot water during the tree-clearing process on the farm. She was a 25-year-old woman who sought treatment with an acute syndrome of high fever, dry cough, and dyspnea. She was admitted to the Itacoatiara general hospital; her condition was diagnosed as bacterial pneumonia and treated with intravenous penicillin. She died within 5 days because of respiratory failure; since no laboratory tests were conducted, she does not fulfill the case definition criteria for HPS. This was the only case in this series not confirmed with laboratory tests.

The second case was in the first patient's 31-year-old husband. Symptoms developed 2 weeks after the wife's death, starting with a 5-day febrile syndrome, which progressed to a dry cough and then respiratory distress, with a petechial rash, hemoconcentration, and thrombocytopenia (53,000 platelets/μL) over the next 2 days. He exhibited a diffuse, alveolar infiltrate on chest radiograph and a mild cardiomyopathy on echocardiogram. He was admitted to an intensive care unit and required mechanical ventilation for 10 days; he made a gradual recovery. Results of his laboratory tests ruled out malaria, dengue fever, and leptospirosis. Three consecutive blood culture samples were negative for bacterial growth. The IgG and IgM ELISA results for hantavirus were positive in both acute- and convalescent-phase serum samples.

The third case was in the second patient's brother, a 43-year-old man, who exhibited a self-limited, acute febrile syndrome 1 month after the index patient. He did not live on the same farm but visited there often and had actively participated in removing the trees on his brother's farm. He had no respiratory complaints, and results of his chest radiographs were normal, but the complete blood count showed hemoconcentration and mild thrombocytopenia (130,000 platelets/μL). He was hospitalized for 3 days and recovered completely. An IgM ELISA result was positive for hantavirus in 2 consecutive blood samples, and an IgG ELISA result was positive in convalescent-phase serum.

The fourth patient was a 67-year-old farmer, the uncle of the last 2 patients. He visited his nephew's farm regularly and was present during the deforestation process. He presented for medical assistance after a 15-day febrile syndrome, with a dry cough and mild dyspnea, 5 weeks after the index patient. He was hospitalized for 3 days and also had an uneventful recovery. The IgM ELISA result in this patient was also positive for hantavirus in 2 consecutive blood samples as was the IgG ELISA result for convalescent-phase serum.

Shortly after the report of the first 3 cases, the Brazilian Health Surveillance Secretary (Ministry of Health) performed an epidemiologic field study to seek the probable site of infection, collect sylvatic rodents, and conduct a serologic survey of human contacts. No areas for soybean cultivation or seed storage were found, but local farmers commonly store dry corn for feeding their domestic fowl. Eighty-two blood samples were collected from asymptomatic persons and were all negative for hantavirus IgG antibodies by ELISA. Eleven sylvatic rodents were captured in 3 days of trapping (270 traps/night) on the farm, on neighboring farms, and in the nearby forest. Two species were identified, *Proechimys* sp. (2 animals) and *Oligoryzomys microtis* (9 animals). This finding is very similar to reports of rodents in other regions of Brazil (*8*). Four *Oligoryzomys microtis* had positive IgG results for hantavirus (*9*) .

Identification of human and rodent hantavirus infection in the Amazonas State adds this emergent disease to our differential diagnoses of febrile tropical diseases and to our syndromic surveillance approach for febrile respiratory diseases. Further research is needed to identify the viral genotype that circulates in this area and to determine the real prevalence of human infection and the epidemiologic scenario of HPS in the western Brazilian Amazon region.

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H5N1 Influenza Virus, Domestic Birds, Western Siberia, Russia

To the Editor: Highly pathogenic H5N1 avian influenza virus caused disease outbreaks in poultry and wild birds in several Asian, European, and African countries from 2003 to 2006. This virus caused >90 human deaths in Vietnam, Thailand, People's Republic of China, Indonesia, Turkey, Iraq, and Cambodia (*1*–*3*). Hemagglutinin (HA) and neuraminidase (NA) genes of this virus were derived from the Gs/Gd/1/96-like lineage, and 6 genes that encode internal viral proteins were derived from other lineages (*1*).

Highly pathogenic H5N1 virus genetically related to the A/Chicken/Shantou/4231/03 (People's Republic of China) isolate caused disease outbreaks in poultry in Japan from the end of December 2003 to March 2004 (*4*). In May and June 2005, highly pathogenic H5N1 virus was isolated from migratory birds during disease outbreaks near Lake Qinghai in western People's Republic of China. HA, NA, and nucleoprotein genes of the Qinghai virus were closely related to H5N1 virus A/Chicken/Shantou/4231/03 isolated in People's Republic of China in 2003. Five other viral genes (matrix, PA, PB1, PB2, and nonstructural protein) were closely related to an H5N1 Hong Kong Special Administrative Region, People's Republic of China 2004 isolate (A/Peregrin falcon/HK/D0028/04) and H5N1 virus A/Chicken/Shantou/810/05 isolated in People's Republic of China in 2005 (*5*,*6*).

In July 2005, domestic poultry began to die in the village of Suzdalka in western Siberia, Russia (Dovolnoe County, Novosibirsk region). Autopsies showed serious alterations in all internal organs tested. Approximately 95%–100% of the lungs were affected, and all serous membranes showed petechial and confluent hemorrhages. The highest concentration of hemorrhages was in the pericardium.

Organs from 3 birds (1 turkey and 2 chickens) that had died during this outbreak were further analyzed. Homogenates of lungs, kidneys, and spleens were tested by hemagglutination inhibition (HI) assay. The highest titers, 32 and 16, were observed in the spleen of the turkey and kidneys of the chickens, respectively. H5 influenza A virus was identified in a homogenate of turkey spleen by conventional HI assay (*7*) with a panel of reference antisera.

For the identification of NA subtype, RNA was isolated from turkey spleen homogenate and synthesis of viral cDNA was performed as previously described (*7*). Amplification by polymerase chain reaction (PCR) and sequencing of an NA gene fragment were performed with in-house primers (sequences of primers are available on request). The nucleotide sequence obtained (547 bp, GenBank accession no. DQ231243) showed 100% identity with the NA gene of H5N1 viruses isolated in People's Republic of China in 2005 (e.g., A/Great black-headed gull/Qinghai/1/05) (*5*,*6*).

Homogenates of bird organs (turkey spleen and chicken kidneys) were injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Three hemagglutinating agents were isolated (titers 1,024–2,048) and identified as H5 influenza A virus (A/Turkey/Suzdalka/Nov-1/05, A/Chicken/Suzdalka/Nov-11/05, and A/Chicken/Suzdalka/Nov-12/05) by reverse transcription–PCR and sequencing (isolation of RNA from allantoic fluid and synthesis of virus cDNA were performed as previously described [*7*]). PCR amplification and sequencing of a fragment of the HA gene were performed with an in-house primer set for the H5 gene (available on request). Phylogenetic

analysis of nucleotide sequences obtained (GenBank accession nos. DQ231242, DQ231241, and DQ23-1240) indicated that western Siberian 2005 isolates belong to the Gs/Gd/1/96-like lineage and form a cluster with H5N1 viruses isolated from migratory birds in the People's Republic of China in 2005 (*5*), from poultry in Japan in 2004 (*4*), and from poultry and humans in Asian countries in 2003 and 2004 (*1*) (Figure). Deduced amino acid HA cleavage site sequences of all isolates (PQGER-RRKKR/GL) corresponded to highly pathogenic Asian H5N1 influenza virus variants (*5*,*6*).

To test virulence, 10 six-week-old chickens were intravenously infected with isolate A/Turkey/Suzdalka/Nov-1/05 as previously described (*7*). All viruses isolated were highly pathogenic (all chickens died within a day of infection).

We isolated H5N1 influenza virus from the spleen of a turkey that died during an outbreak in poultry in western Siberia in July 2005. HA and NA genes of this virus were closely related to those of H5N1 avian influenza viruses that caused outbreaks in birds in Asian countries from 2003 to 2005 and in Japan in 2003 and 2004. The corresponding isolate, A/Turkey/Suzdalka/Nov-1/05, from turkey spleen was highly pathogenic for chickens in the laboratory intravenous pathogenicity index test. The origin of this H5N1 virus in western Siberia is not known. Migratory birds could have introduced this virus because western Siberia is located on a flyway of wild birds that migrate in the spring from southeastern Asia. Highly pathogenic Asian H5N1 influenza virus in western Siberia demonstrates spread of these Asian viruses into new areas and suggests a larger geographic distribution.

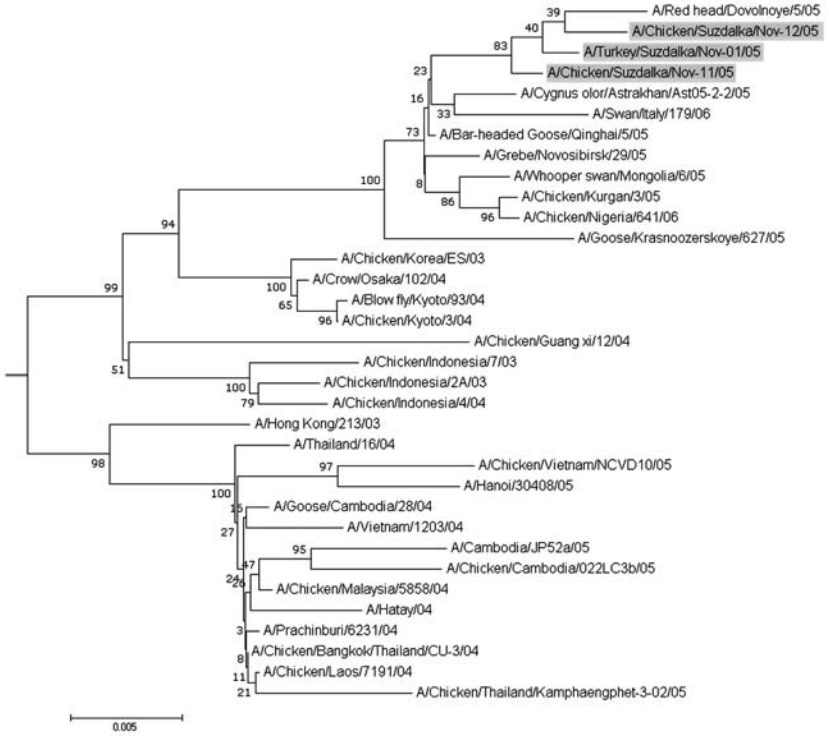

Figure. Phylogenetic tree of H5 hemagglutinin genes of influenza A viruses. The 3 H5 western Siberian 2005 viruses isolated in this study are shaded. Phylogenetic analysis was performed by the neighbor-joining method with the Molecular Evolutionary Genetic Analysis 2 program (Center for Evolutionary Functional Genomics, Tempe, AZ, USA). Scale bar indicates relative value of distance in matrix normalized units. Numbers indicate tree divarication.

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Leishmaniasis among Gold Miners, French Guiana

To the Editor: In 2004, the Cayenne General Hospital and public health centers recorded 348 new cases of cutaneous leishmaniasis (CL) in French Guiana (*1*). A case of CL was considered confirmed if cutaneous lesions were present for ≥2 weeks; the patient had a compatible epidemiologic history; and microscopic examination of dermal scrapings, parasite cultivation, or both showed positive results for *Leishmania*. According to the population estimate given by the French National Institute for Statistics and Economical Studies (INSEE, Cayenne), the incidence of CL in 2004 was 0.2%–0.4% and has been relatively stable since 1979 (*2*,*3*). However, when the annual number of cases per village were examined, new CL cases were heterogeneously distributed. Saint Elie, a gold-mining village in the inland neotropical forest, had an apparent incidence rate of 25.9% in 2004 and 28.9% in 2005 (Figure); risk for infection in this village was, on average, 65× higher than anywhere else in French Guiana. We tested samples from 12 random CL patients with a *Leishmania*-specific polymerase chain reaction–restriction fragment length polymorphism test that targeted the internal transcribed spacer 1 of ribosomal RNA genes with primers SSU-12103-D (5′-GGGAATATCCTCAGCACGT-3′) and 5.8S-13333-R (5′-CGACACTGAGAATATGGCATG-3′) (*4*). All these patients were infected with *Leishmania guyanensis.*

Isolated in dense rainforest (no road or airport) and with 239 inhabitants (INSEE, Cayenne), Saint Elie is situated on a gold seam; miners illegally create trails from the village to deposits in a 10-km circumference in the dense forest around the village. Compared to other French Guianan villages, such as Saül and Régina, which are similarly isolated in the rainforest and have 160 and 765 inhabitants (INSEE, Cayenne), respectively, and Iracoubo, the village closest to Saint Elie with 1,430 inhabitants (INSEE, Cayenne), substantially more new CL cases have been observed in Saint Elie since 2003. Since 2000, medical rounds have been undertaken every 15 days in the villages of Saint Elie and Saül, whereas people from Régina and Iracoubo have doctors at their disposal every day.

Official records indicate that the population of Saint Elie has doubled in the past 10 years, reaching 239 inhabitants in 1999 (INSEE, Cayenne). However, 860 new medical files have been registered in the Saint Elie Health Centre since 2000. This finding could be explained by the high number of illegal workers in this area. Patient interviews showed that most of these workers (≈90%) originated from the poorest northern Brazilian states (Pará, Amapá, Roraima, and especially Maranhão). Thus, the incidence rate of 25.9%, calculated on the basis of 239 inhabitants, was likely overestimated. Taking into account a substantial turnover in migrant populations, the denominator could be 500–1,000 inhabitants, and the incidence rate would be 6.2%–12.4%. All patients worked in the small-scale gold mines surrounding Saint Elie, and CL cases were recorded without seasonal fluctuations. Imported cases are possible, but reports are likely to be anecdotal because clinical observations, estimated dates of infection, and duration of patient stay in Saint Elie were congruent and because all genotyped strains were Guianan *L. guyanensis* (*1*).

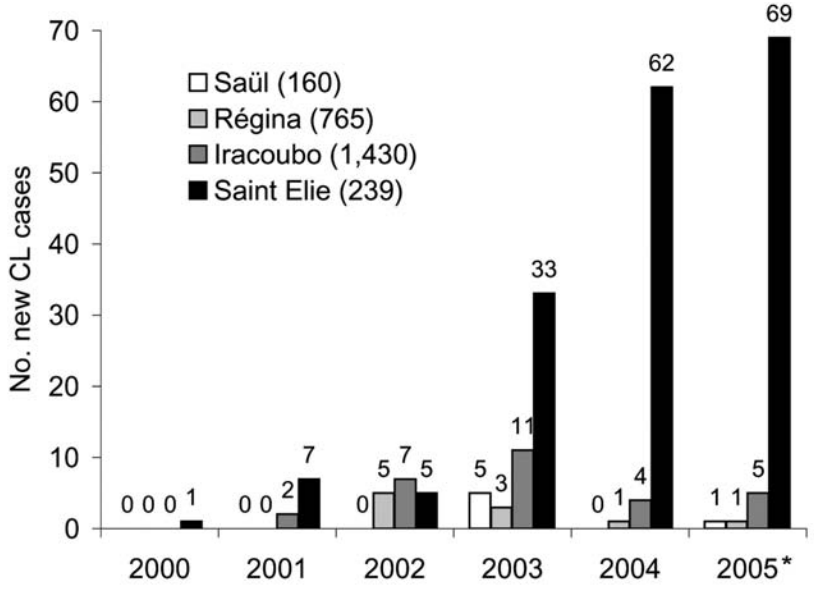

Figure. Number of new cutaneous leishmaniasis (CL) cases registered in health centers of 4 villages of French Guiana (Iracoubo, Régina, Saül, and Saint Elie) from 2000 to 2005. For each village, the 1999 population estimate (French National Institute for Statistics and Economical Studies, Cayenne) is given in parentheses. *Cases Jan–Aug 2005.

Several infection risk factors exist simultaneously in this situation. In a CL-endemic area, immigrant populations, who are mostly nonimmune, exert pressure on the environment (deforestation) that directly increases their risk for exposure to infected vectors, in the absence of prophylactic measures. The initial short-term effect of deforestation is the mobilization of aggressive adult sandflies, which have been disturbed while resting. However, the ability of zoophilic vectors to adapt to peridomestic environments has also already greatly influenced the distribution of leishmaniases in South America (*5*–*7*).

Considering the uncertainty of the population estimate, turnover, and immunity status, we assume that incidence rates should be considered cautiously. Nevertheless, we found that gold mining in forested areas constitutes a risk factor for CL, at least in French Guiana and probably in all Amazonian rainforests. This risk could be a public health concern. Larger studies in other gold-mining areas are required to quantify the incidence of CL among workers to effectively focus prophylactic and preventive campaigns.

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Mycobacterium tuberculosis Drug Resistance, Ghana

To the Editor: The directly observed treatment strategy (DOTS) for tuberculosis (TB) treatment has been implemented in Ghana since 1994. Before then, TB was treated without adherence to any concerted guidelines. The 2003 report of the Ghanaian National Tuberculosis Programme (NTP) stated a TB incidence of 281/100,000 (*1*). NTP ensures treatment of all patients with an 8-month course of streptomycin, isoniazid, rifampin, and pyrazinamide (for 2 months), followed by thiacetazone and isoniazid (6 months). The cure rate for 2003 was >50% (*1*), and >75% is anticipated for 2005.

To determine the extent of drug resistance and to make suggestions for future Ghanaian NTP strategies, we assessed resistance against anti-TB drugs used in Ghana. A total of 2,064 patients with new cases of pulmonary TB were recruited at Korle Bu Teaching Hospital, Accra; Komfo Anokye Teaching Hospital, Kumasi; 15 periurban hospitals; and hospitals in the Ashanti, Eastern, and Central Regions of Ghana. These patients were consecutively enrolled in a cross-sectional study from September 2001 to December 2004. On all patients' clinical examinations, chest radiographs, sputum smears for staining of acid-fast bacteria, HIV testing, and culturing of *Mycobacterium tuberculosis* complex strains were performed. Samples were taken only after informed consent was given. The study was approved by the appropriate ethics committees.

A total of 2,064 *Mycobacterium* isolates were cultured at the Kumasi Centre for Collaborative Research. After decontamination of sputum samples (N-acetyl-L-cysteine/NaOH) and centrifugation, sediments were transferred onto Lowenstein-Jensen (LJ) media, incubated (37°C), and read weekly for 10 weeks for mycobacterial growth. Subsequently, cultures were sent to the German National Reference Centre for Mycobacteria in Borstel, Germany, a reference laboratory of the World Health Organization, for drug sensitivity testing (DST; proportion method on LJ media). Sensitivity to isoniazid, rifampin, pyrazinamide,

ethambutol, and streptomycin was determined for 2,064 isolates and to thiacetazone for 1,288 isolates. For ambiguous results and DST of thiacetazone, the modified proportion method (Bactec 460TB; Becton Dickinson, Cockeys-ville, MD, USA) was performed. Data were analyzed with EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Fourth Dimension (ACI Group, San Jose, CA, USA) software programs.

Of the isolates, 32.8% were from female patients, and 67.8% were from male patients. The mean age of participants (33 years, range 10–60) did not differ by sex. HIV prevalence was 14.3% (males, n = 179, females, n = 117).

A total of 1,578 (76.5%) isolates were susceptible to all drugs tested, whereas 304 (14.7%) were monodrug resistant, and 177 (8.7%) were multi- or polydrug resistant to combinations (multidrug resistance meant resistance to at least isoniazid and rifampin (2.2%); polydrug resistance meant resistance to several drugs, excluding combined resistance to isoniazid and rifampin (6.5%). The overall prevalence of any drug resistance was 23.5% (486 isolates) (Table). No differences were observed between HIV-negative and HIV-positive patients. The highest level of resistance was against streptomycin, followed by isoniazid. Resistance to rifampin, pyrazinamide, and thiacetazone was lower. Monoresistance to ethambutol was not observed; resistance to ethambutol combined with other drugs occurred in 0.9% of isolates.

In all, 6.5% of isolates were polydrug resistant and virtually always included resistance to isoniazid. Among isolates with double- and triple drug resistance, combinations of resistance to isoniazid and streptomycin and to isoniazid-thiacetazone-streptomycin occurred most frequently. Other combinations were relatively rare.

Table. Resistance to first-line antituberculosis drugs, Ghana*

	Isolates from HIV-negative patients, n (%)	Isolates from HIV-positive patients, n (%)
Resistance	1,768† (85.7)	296† (14.3)
Any resistance	415 (23.4)	71 (24.0)
Monoresistance	255 (14.3)	49 (16.6)
H only	74 (4.2)	15 (5.1)
R only	12 (0.7)	4 (1.4)
S only	160 (9.0)	25 (8.4)
Z only	7 (<0.5)	5 (1.7)
T only	2 (<0.5)	–
E only	–	–
HR resistance (MDR)	39 (2.2)	4 (1.4)
HR	3 (<0.5)	1 (<0.5)
HRE	1 (<0.5)	–
HRS	11 (0.6)	2 (0.7)
HRZ	1 (<0.5)	–
HRES	6 (<0.5)	–
HREZ	1 (<0.5)	–
HRTS	3 (<0.5)	–
HRZS	4 (<0.5)	1 (<0.5)
HRETS	4 (<0.5)	–
HRESZ	5 (<0.5)	–
H + other resistance	116 (6.6)	15 (0.5)
HE	1 (<0.5)	–
HT	5 (<0.5)	–
HS	88 (5.0)	11 (3.7)
HES	–	1 (<0.5)
HTS	15 (0.8)	3 (1.0)
HSZ	6 (<0.5)	1 (<0.5)
HTSZ	1 (<0.5)	–
R + other resistance	1 (<0.5)	1 (<0.5)
RS only	1 (<0.5)	1 (<0.5)
Any drug resistance		
Any H	232 (13.1)	36 (12.2)
Any R	54 (3.1)	10 (3.4)
Any S	306 (17.3)	45 (15.2)
Any Z	26 (1.5)	7 (2.4)
Any T	33 (3.0)	4 (2.2)
Any E	18 (1.0)	1 (<0.5)

*H, isoniazid; R, rifampin; S, streptomycin; Z, pyrazinamide; T, thiacetazone; E, ethambutol; MDR, multidrug-resistance.
†Resistance to T tested in only 1,108 isolates and 180 isolates from HIV-negative and HIV-positive persons, respectively.

In 1989, an initial drug resistance rate of 54.5% in pulmonary TB was observed in Ghana (*2*); 27% were resistant to isoniazid, 23% to streptomycin, 29% to thiacetazone, 16% to streptomycin-isoniazid, and 5% to thiacetazone-streptomycin-isoniazid. A later study reported a high prevalence of primary drug resistance to isoniazid (23%), while sensitivity to rifampicin, pyrazinamide, ethambutol, streptomycin, and ciprofloxacin was maintained (*3*). However, the number of isolates tested was fewer in both studies (n = 99 and 25, respectively) than in ours. This report supplements data from patients in Ghana whose conditions were newly diagnosed as HIV-negative and HIV-positive. Samples were collected in 2 large regions of Ghana, the Greater Accra and the Ashanti Regions, and were supplemented by samples from additional regions. Thus, these results are likely representative of the entire country.

The overall primary drug resistance rate of 23.5% in Ghanaian TB patients ranks Ghana among those

African countries with a high prevalence of drug-resistant TB. The high degree of mono-, multi- and polyresistance to streptomycin may be the result of selective pressure exerted by treatment of other infections with streptomycin and to incomplete treatment courses. Drug resistance to streptomycin and isoniazid are of concern, since these drugs are core components of the NTP. The relative ineffectiveness of streptomycin and the low level of resistance to ethambutol justify the most recent replacement of streptomycin by ethambutol by the Ghanaian NTP.

Low rates of initial drug resistance have been reported in countries in which the DOTS strategy has been successfully implemented. Adequate use of standardized treatment regimens under DOTS will limit further emergence of drug resistance but not substantially reduce the current degree of resistance (*4*). Although the levels of drug resistance in Africa are lower than in several other countries (*5*), measures to provide controlled application of second-line drugs, supervision of drug distribution and compliance, enforcement of DOTS protocols, and sustained training of all personnel involved in TB management are crucial.

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Avian Influenza Risk Communication, Thailand

To the Editor: Twenty-two human cases of H5N1 highly pathogenic avian influenza (HPAI) have been reported in Thailand since 2003, with 14 deaths (*1*). From July to December 2005, I investigated Thai consumers' food safety practices by conducting an oral survey prepared in the Thai language. Interviews were conducted in 3 areas that have not had cases of H5N1 avian influenza, Bangkok (urban, n = 126), Rangsit (suburban, n = 125), and Phetchabun (rural, n = 50). Of the 301 Thai consumers surveyed, 92% thought that Thailand has ≥1 food safety problems, such as pesticide residues (62%), poor personal hygiene of food vendors (39%), and microbiologic/viral contamination of food (26%). Although the Thai Ministry of Public Health has conducted an aggressive public education campaign regarding HPAI (*2*), only 6% named bird flu as their primary concern. Most participants had some knowledge of avian influenza; 88% of participants knew the name of the disease, and of those, all knew that infections can be deadly, and 97% knew that interacting with and slaughtering infected birds are the most risky activities.

In the rural area, 72% of participants had backyard chickens (almost no one had them in urban and suburban areas). Of those, only 6% were aware of the symptoms of HPAI in poultry. Most villagers knew that minimizing contact with birds could reduce their risk for infection; however, they were not sure how they could minimize contact. None of the owners of backyard chickens had tested them for HPAI. The reporting system for HPAI was not easily accessible for home poultry producers.

The findings of this study are similar to those of Olsen et al., who

reported that widespread knowledge of avian influenza had not resulted in behavior change (*2*). Behavior change is a complex process; both motivators and barriers contribute to change. One participant said that the household chickens were a very important economic source, not only for the household but also for her entire village. Eggs were usually consumed within the household or sold at the local market. This villager also said that government educators told villagers not to directly interact with or slaughter chickens at home. Although she was well aware of the danger of HPAI, she thought the recommendations would be impossible to follow since feeding and egg collection involve direct interaction with chickens. When a chicken is no longer able to produce eggs, the participant slaughters the hen and either eats or sells the meat. No facility that could safely slaughter chickens is available in the village, so she does it at home.

The pattern of the villagers' risk perception was interesting. They were very aware of the risk backyard chickens present in the mid-northern area of Thailand, where many HPAI infected poultry have been reported, but they simply thought it would not happen to their chickens. The villagers' lack of concern is compatible with Slovik's theory of risk perception, whereby familiar, naturally occurring risks elicit much less concern than unfamiliar, human-made risks (*3*). The complacency among these villagers indicates that behavior changes will not occur unless villagers are provided with practical recommendations.

Many organizations, such as the Food and Agricultural Organization of the United Nations, the World Health Organization, and the Centers for Disease Control and Prevention, have determined that risk communication is one of the most important strategies to respond to an influenza pandemic. The Thai Ministry of Public Health is conducting a national public awareness campaign to stop the spread of HPAI. Thailand has a rapidly developing metropolitan area and many traditional village areas, and the campaign targets people in all areas. The campaign must provide highly practical recommendations for persons who own backyard chickens.

Three practical items should be included in the campaign: 1) a list of detailed symptoms of HPAI in poultry and humans; 2) guidelines on raising and slaughtering home-raised poultry, with a list of protective equipment such as boots, masks, and goggles, as well as cleaning materials; 3) instructions on how to report sick birds or persons to the Thai Ministry of Health.

Many obstacles prevent Thai consumers from following recommendations to reduce their risk for HPAI, primarily their economic status. Reporting sick birds voluntarily could lead to the destruction of their source of income unless they are compensated for depopulated flocks. To encourage persons to report or test sick birds, home poultry producers should be informed that the Thai government has initiated a system to compensate them for culled birds. Purchasing protective equipment for home slaughter may be cost-prohibitive, however. Therefore, a successful campaign must address economic considerations.

Conducting a risk communication program with consumers can be a tremendous challenge. However, considering the high literacy level of Thai consumers (98%) (*4*), written information is well accepted; therefore, increasing the awareness of HPAI and providing practical recommendations could be achieved in Thailand, if planned carefully.

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Panton-Valentine Leukocidin Genes in *Staphylococcus aureus*

To the Editor: The pathogenicity of *Staphylococcus aureus* depends on various bacterial surface components and extracellular proteins. However, the precise role of single virulence determinants in relation to infection is hard to establish. The frequent recovery of staphylococcal isolates that produce leukocidal toxins from patients with deep skin and soft tissue infections, particularly furunculosis, cutaneous abscesses, and severe necrotizing pneumonia, suggests that the Panton-Valentine leukocidin (PVL) is 1 such virulence factor that has a major role in pathogenicity (*1–3*).

In 1932, Panton and Valentine described PVL as a virulence factor belonging to the family of synergohymenotropic toxins (*4*). These toxins form pores in the membrane of host defense cells by synergistic action of 2 secretory proteins, designated LukS-PV and LukF-PV, which are encoded by 2 cotranscribed genes of a prophage integrated in the *S. aureus* chromosome (*5*). PVL is mostly associated with community-acquired methicillin-resistant *S. aureus* (MRSA) infections and distinguishable from nosocomial MRSA by nonmultidrug resistance and carriage of the type IV staphylococcal chromosome cassette element (SCC*mec* type IV) (*6,7*).

Despite the presumed importance of PVL as a virulence factor, few data are available on its prevalence among *S. aureus* isolates from the nares of healthy persons compared with stains isolated from infections. This lack of data led us to investigate the frequency of PVL gene–positive *S. aureus* strains obtained from the nares of healthy carriers in the community. For this purpose, a single polymerase chain reaction method was used to detect both *lukS-PV* and *lukF-PV* genes (*2*).

In a previous study, the population structure of *S. aureus*, isolated from the nares of healthy persons in the Rotterdam area, the Netherlands, was elucidated (*8*). Strains were obtained from healthy children (<19 years) and elderly persons (>55 years). Invasive strains (blood culture, skin and soft tissue infections, and impetigo isolates) were included in this study (Table). All carriage and clinical isolates (n = 1,033) were *mecA* negative. We used the same strain collection to study the PVL prevalence in carriage and invasive isolates of *S. aureus* from a single geographic region.

Five PVL-positive *S. aureus* strains (0.6%) were found in the carriage group (n = 829), and 3 (2.1%) of 146 blood-culture isolates carried the PVL gene (Table). This finding is in agreement with previously reported low PVL prevalences by Prevost et al. (0% in 31 carriage isolates and 1.4% in 69 blood-culture isolates) and Von Eiff et al. (1.4% in 210 carriage isolates and 0.9% in 219 blood-culture isolates) (*9,10*). However, a higher prevalence of PVL (38.9%) was found in *S. aureus* strains causing abscesses and arthritis (Fisher exact test, p <0.0001) (*8*). This finding is also in agreement with the proposed involvement of PVL in severe and invasive (soft tissue) staphylococcal infections (*1–3*). No significant differences were found in the presence of PVL when carriage isolates were compared with invasive blood-culture isolates. PVL was found in each major genomic amplified fragment length polymorphism (AFLP) cluster, indicating that PVL has been introduced in distinct phylogenetic subpopulations of *S. aureus* (online Figure; available from http://www.cdc.gov/ncidod/EID/vol12no07/05-0865-G.htm). Multilocus sequence typing analysis of a subset of the strain collection showed that the 15 PVL-positive strains were within clonal complex (CC) 30 (n = 7), CC 121 (n = 3), CC 1 (n = 2), CC 8 (n = 1), CC 22 (n = 1), and CC 45 (n = 1) (Table) (*8*). Although PVL was found among several staphylococcal

Table. Panton-Valentine Leukocidin (PVL) distribution among carriage and invasive isolates per genetic cluster of *Staphylococcus aureus*

	Amplified fragment length polymorphism cluster					
	I (n = 462)	II (n= 261)	III (n = 208)	IVa (n = 62)	IVb (n = 40)	Total (N = 1,033)
	PVL positive, n (%)					
Carriage isolates (n = 829)	1	1	1	0	2	5 (0.6)*
Bacteremia isolates (n = 146)	1	1	0	0	1	3 (2.1)†
Soft tissue infection isolates(n = 18)	1	5	0	1	0	7 (38.9)‡
Impetigo isolates (n = 40)	0	0	0	0	0	0 (0.0)
Total (N = 1,033)	3 (0.6)§	7 (2.7)	1 (0.5)¶	1 (1.6)	3 (7.5)#	15 (1.5)
MLST** data of PVL-positive isolates	CC 1, n = 2 CC 8, n = 1	CC 30, n = 7	CC 45, n = 1	CC 22, n = 1	CC 121, n = 3	

‡ versus * Fisher exact test (2-sided); p<0.0001.
‡ versus † Fisher exact test (2-sided); p<0.0001.
# versus § Fisher exact test (2-sided); p = 0.0079.
# versus ¶ Fisher exact test (2-sided); p = 0.0140.
**MLST, multilocus sequence typing; CC, clonal complex.

genotypes, it was slightly overrepresented in AFLP cluster IVb (CC 121) compared with major clusters I and III. Whether the prevalence of PVL in carriage- and blood-culture isolates is higher and differs among distinct genetic clusters of *S. aureus* in countries with endemic CA-MRSA has to be investigated further.

In conclusion, we have shown that the PVL-encoding phage has entered distinct staphylococcal lineages, although its prevalence differs per clonal group. PVL is associated with skin and soft tissue infections but not with bacteremia, which suggests that PVL is not likely to be involved in the pathogenesis of bacteremia. Infections caused by PVL-positive *S. aureus* strains have been documented since the 1930s. Expansion and increased incidence of such infections, however, are more recent, and further epidemiologic studies for tracking this phenomenon are still warranted.

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Small Anellovirus in Hepatitis C Patients and Healthy Controls

To the Editor: Torquetenovirus (TTV) and torquetenominivirus (TTMV) are characterized by a small, negative-sense, circular, single-stranded DNA genome and by an extraordinary ability to produce chronic plasma viremia. Indeed, >80% of humans harbor variably high viral loads of TTV, TTMV, or both, in plasma, regardless of geographic provenance, age, sex, and health conditions (*1*). Currently, TTV and TTMV are classified as distinct species in the floating (although closely linked to the family *Circoviridae*) genus *Anellovirus*, but their extreme genetic heterogeneity and some distinctive features in genomic organization have led some to suggest that they should be classified as an independent family (*2*,*3*). Most recently, after examining serum specimens from patients with symptoms of an acute viral infection by using DNase sequence-independent single-primer amplification, Jones et al. (*4*) identified, among other viruses, 2 novel TTV- and TTMV-like agents. Because of their even smaller genomes (≈2.4 and 2.6 kb vs. 3.6–3.8 kb for TTV and 2.8–2.9 kb for TTMV), these agents were named small anelloviruses (SAVs).

Because tissue culture and serologic methods are not yet available, diagnosis of anellovirus infection relies exclusively on viral DNA detection. We tested 55 Italian hepatitis C patients (mean age 56 ± 14 years, male/female ratio 30/25, 53 TTV positive) and, for comparison, 35 healthy donors (mean age 36 ± 12 years, male/female ratio 17/18, 33 TTV positive) for SAV in plasma by using the polymerase chain reaction (PCR) primers described by Jones et al. (*4*), followed by direct amplicon

sequencing. To increase assay sensitivity, a heminested PCR format was adopted that used a sense primer designed in a segment of the untranslated region that is highly conserved among all anelloviruses (5′-TCAAGGGGCA ATTCGGGCT-3′). We found 5 positive results among the hepatitis C patients (9.1%, all of whom were TTV positive) and 3 positive results among healthy controls (8.6%); and all were confirmed by sequence data.

The amino acid sequences inferred from the coding segment of the amplicon of SAV in this study and the corresponding sequences of the 10 SAV in GenBank at the time of this writing were then aligned with representative TTV and TTMV sequences (online Appendix Figure 1, available from http://www.cdc.gov/ncidod/EID/vol12no07/06-0234-G1.htm). This method allowed us to identify the motif $WX_7HX_3CXCX_5H$, which is highly characteristic of the open reading frame 2 (ORF2) of anelloviruses (*5*), in all SAVs. SAV sequences, as well as a large number of TTVs and all TTMVs, were then used to construct a phylogenetic tree and to calculate the extent of genetic divergence within SAV, TTV, and TTMV. Although a precise phylogenetic description will require the analysis of full-length ORF2, the SAV sequences clustered quite separately from those of TTV and TTMV, and the extent of divergence observed among SAV was huge and in the same range as among TTV or TTMV. Furthermore, SAVs obtained from hepatitis C patients and healthy participants were intermingled (online Appendix Figure 2, available from http://www.cdc.gov/ncidod/EID/vol12no07/06-0234-G2.htm).

While this study was under way, Biagini et al. reported a 12% prevalence of SAV viremia in French blood donors (*6*). Our results confirm the high prevalence of SAV viremia in healthy persons and extend the finding to hepatitis C patients. Our data, combined with those of Biagini et al., indicate that, since SAV clusters separately from previously identified anelloviruses, it should be considered a distinct species (or possibly genus). This would increase the already high genetic diversity of anelloviruses, further arguing for the appropriateness of creating a separate viral family.

Because the clinical and viral parameters of hepatitis C in SAV-positive patients were not significantly different from those in the SAV-negative patients (data not shown), our results suggest that, similar to TTV (*7*), SAV has little or no effect on the course of hepatitis C. Although anelloviruses have not yet been definitely linked to any specific disease, evidence is growing that they might be involved in acute respiratory diseases in children (*8*,*9*). Furthermore, a florid TTV replication in the respiratory tract correlated with severity of lung impairment in children with asthma (*10*). A precise appreciation of the wide range of viruses classified within the anelloviruses is a prerequisite to understanding such disease associations and the disease-inducing potential of these viruses in general.

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Lyme Borreliosis and *Borrelia spielmanii*

To the Editor: A report on erythema migrans (EM) caused by *Borrelia spielmanii* in a recent issue of Emerging Infectious Diseases (*1*) was a stimulus for a review of data on this *Borrelia* species in patients with early Lyme borreliosis (LB). We report a patient with EM, examined at our LB outpatient clinic, from whom *B. spielmanii* was isolated from the skin lesion. The presence of this species was ascertained by using a 5S–23S spacer amplicon after digestion with *Mse*I and demonstration of fragments having sizes typical for *B. spielmanii* (106, 68, and 51 bp) (*2*).

A 69-year-old woman was examined on October 30, 1996, for a skin lesion on her left thigh. Her medical history indicated arterial hypertension, intermittent pain in the cervical and lumbar region due to spondylosis, frequent headaches and myalgias, and treatment of typical EM skin lesions at our LB outpatient clinic in 1992 and 1994; the latter lesions were culture positive for *Borrelia*. Fourteen days before examination, she noticed a small area of redness, accompanied by mild local itching, burning, and pain on her left knee. On examination, a 24 × 20–cm ringlike lesion was found on her left thigh. Basic blood tests did not show abnormal results, and a serum sample was negative for borrelial antibodies (immunofluorescence test using a *B. afzelii* skin isolate as antigen) (*3*). However, *B. spielmanii* was isolated from an EM skin biopsy specimen. The patient was treated with amoxicillin, 500 mg 3 times a day for 15 days. The skin lesion disappeared within 3 weeks, and a culture of a repeat skin biopsy specimen was negative for *Borrelia* 2 months after the first biopsy. Her clinical course during a 1-year follow-up was uneventful.

B. spielmanii was detected in the patient by a general approach we have used for several years. In all consenting patients, a skin specimen from an EM lesion is cultured for borreliae in modified Kelly medium before and, in case of a positive result, ≈2 months after antimicrobial drug treatment is started. Isolated strains are typed by using the 5S–23S spacer amplicon.

The findings in this report are generally consistent with those in other reports of adult patients with EM (*4–8*). One difference was that the patient did not report a tick bite at the site of the EM. Approximately two thirds of our patients with EM recalled a tick bite and ≈10% of patients treated for early LB had previously had EM (*4–8*).

Previous reports indicate several differences in patients with EM caused by *B. burgdorferi* and *B. afzelii* (*7*) and patients with EM caused by *B. afzelii* and *B. garinii* (*8,9*). Some of the findings in our patient are unusual and rarely found in those with early LB. However, the small number of patients infected with *B. spielmanii* (1 reported herein and 4 previously reported) does not allow any reliable conclusion to be made on differences in clinical manifestations of LB caused by *B. spielmanii* compared with those of other species.

Our results corroborate previous findings that *B. spielmanii* is a cause of LB in Europe. Thus, in addition to the Netherlands (*2*), Germany (*10*), and Hungary (*1*), LB caused by *B. spielmanii* is also present in Slovenia.

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Feral Cats and Risk for Nipah Virus Transmission

To the Editor: Nipah virus (NiV) emerged in peninsular Malaysia in 1998 and 1999 as a respiratory and neurologic disease of domestic pigs and an acute febrile encephalitic disease in humans (*1*). Nipah virus infection is associated with a case-fatality ratio of 40% to 76% in humans (*1*,*2*). Cats (*Felis catus*) were infected with NiV at the site of the outbreak in northern Malaysia (*3*). Experimental studies have shown that cats are susceptible to Hendra virus and NiV (*4*,*5*). Infected cats shed NiV through the nasopharynx and in urine while viremic, and 1 (of 2) recovered from experimental NiV infection with a high neutralizing antibody titer (>256) within 21 days (*5*).

Fruit bats of the genus *Pteropus* are believed to be the reservoir for NiV in Malaysia (*6*). In June 2000, NiV was isolated from partially eaten fruit and from the urine of *Pteropus hypomelanus* in the village of Air Batang on Tioman Island, Peninsular Malaysia (*7*). Although humans live in close proximity to these bats, no evidence for local human exposure to NiV has been seen (*8*). In contrast, epidemiologic evidence from recent NiV outbreaks in Bangladesh suggests that direct infection from pteropid bats may occur, possibly when bats are pregnant (*2*,*9*).

Despite limited contact with bats, residents and visitors to Air Batang have ample opportunity for close contact with feral cats, which are often fed and sometimes housed by residents. Cats have been observed under trees that are occupied by roosting fruit bats in Air Batang. NiV could be transmitted from bats to cats through urine and then among cats oronasally, given their gregarious nature, which frequently includes mutual grooming. Cats are also frequently seen in close contact with humans in restaurants, on the tables, and in food preparation areas, where they are fed. If NiV is also present in bat fetal tissues, cats could become infected through contact with or by eating these tissues after mass births among bats.

We tested feral cats from Air Batang for neutralizing antibodies to NiV to determine whether cats might play a role in the zoonotic transmission of Nipah virus. Fifty bats were captured from Air Batang and tested for NiV and neutralizing antibodies to NiV as part of a long-term NiV surveillance study (A. Rahman, unpub. data).

Thirty-two cats were caught July 12–19, 2004, in a 200-m radius of a bat colony. Cats were anesthetized, and 3.0 mL blood was collected from the jugular vein or medial saphenous vein. Serum was allowed to separate at 4°C for 24 hours and was then further separated and frozen in liquid nitrogen. Serum was tested by serum neutralization test (SNT), which is considered the reference standard for serologic assays, at the Australian Animal Health Laboratory, Geelong, Australia, as described (*5*,*10*).

The time of year was similar to the time when NiV was isolated from bats in 2000; however, none of the 32 cats (18 males, 14 females; 25 adults, 7 juveniles [<1 year of age]) had detectable antibodies to NiV on SNT. All cats appeared healthy except for 1 adult that was markedly jaundiced. The period of the study did not overlap the seasonal gestation period of *P. hypomelanus*, and none of the adult female bats tested (n = 20) were pregnant. Although attempts to isolate virus from bat urine and saliva were unsuccessful (A. Rahman, unpub. data), 7 (14%) of 50 bats, including 1 (8%) of 13 post-weaning juveniles (4 months to 2 years of age) had neutralizing antibodies (all >32) to NiV on SNT, which suggests that virus had circulated in the colony since 2000.

Our finding of no seropositive cats may be explained in 3 ways: 1) feral cats are rarely, if at all, exposed to NiV in nature; 2) the death rate from NiV infection in cats is so high that few or none survive with immunity; or 3) our sample size was too small to detect a seropositive cat. We believe that the first hypothesis is most likely. A low incidence of NiV infection in this population of bats (95% confidence interval for 0 of 50 bats, 0.00–0.71), combined with a short viremic period, would make transmission between bats and cats unlikely. However, if transmission occurred, we would expect to find some cats with a detectable titer (*5*). While the exact age of the cats in this survey was unknown, 25 (78%) of 32 were adults (>1 year of age) and may have been in Air Batang either in 2000, when NiV was isolated from bats, or during a more recent outbreak. We conclude that exposure of feral or peridomestic cats to Nipah virus on Tioman Island is rare and that the risk for zoonotic transmission is low.

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Gastroenteritis at a University in Texas: An Epidemiologic Case Study

Centers for Disease Control and Prevention

The Centers, Atlanta, GA, 2005

Format: CD-Rom. Price: $30 from the Public Health Foundation or download at no charge from http://www.cdc.gov/epicasestudies

This CD-ROM is an important addition to case exercises in field epidemiology that serve to educate when actual participation in a field investigation is not possible or practical. The authors have prepared a case exercise based on an actual field investigation with real data that have been put together in a meaningful and effective way. The use of an epidemic of gastroenteritis is a cutting-edge element, since foodborne disease is a major public health problem today. The epidemic occurs on a college campus, which lends an air of verisimilitude, and the causative agent, norovirus, is a genuine public health threat.

This reviewer had a number of specific editorial recommendations for the authors that could enhance forthcoming versions. These suggestions included inserting a case definition in the investigation outline; adding the role of the state laboratory; consistently labeling outbreak, epidemic, and epidemic curve throughout the program; clarifying the rationale for limiting the outbreak to the university; further refining methods for the study and controls; using 2×2 tables to illustrate epidemiologic ratios; and expanding the employee training plan.

Overall, these types of training aids are needed as we attempt to further expose public health workers to field investigations so that they can conduct investigations effectively. The reference to additional educational material throughout the steps is a well-conceived and appropriate aspect of the investigation. The narrative information, questions, and explanations are appropriate and flow smoothly.

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Antimicrobial Resistance in Bacteria of Animal Origin

Frank M. Aarestrup, editor

ASM Press, Washington DC, 2006
ISBN: 9781555813062
Pages: 442; Price US $115.95

Resistance to antimicrobial agents develops soon after these life-saving drugs are introduced into human and animal medicine. The role of veterinary and animal use of antimicrobial agents has been debated for years. Frank Aarestrup and colleagues attempt to summarize information concerning this topic in their new book, Antimicrobial Resistance in Bacteria of Animal Origin. This book has 51 contributors, who have written 25 chapters on the public health, clinical, and regulatory importance of antimicrobial drug resistance in bacteria of animal origin. The editor recognizes the complexity of this subject and makes no claims to cover all the issues but rather highlights what he and the contributors believe to be the most important topics.

The first 6 chapters highlight modes of action and resistance for antimicrobial agents, history of usage, susceptibility testing, antimicrobial-drug resistance detection methods, dosing schedules, and mechanisms that lead to the spread of bacterial resistance. These chapters provide the reader with very detailed molecular and genetic information on resistance mechanisms in bacteria of animal origin. Knowing the pharmacodynamics and pharmacokinetics of antimicrobial agents is essential for these drugs to be used correctly, and a good overview of these mechanisms is also provided in these beginning chapters. The book also stresses the urgent need for establishing veterinary-validated breakpoints for species-specific host-pathogen combinations that are clinically relevant. Some of the tables and diagrams in these chapters contain a large amount of material and need to be read carefully to understand the total wealth of information.

The 12 middle chapters provide an in-depth review of the known resistance mechanisms found in most of the pathogenic bacteria and bacteria of public health importance in animals. Each chapter takes a closer look at a particular family, genus, or species of bacteria and, when possible, attempts to estimate the prevalence of resistance to key antimicrobial agents. The information provided in these chapters is useful to clinicians, researchers, public health officials, and regulators. For some zoonotic agents, the animal health consequences of resistance are not known. For future editions, expanding on this topic would be helpful.

The last 7 chapters attempt to tie all of the previous information together by providing an overview of the

licensing and approval procedures for veterinary antimicrobial agents, surveillance systems that monitor resistance and usage, and the use of risk assessments to guide industry and government in decision making. These chapters take a global approach. When possible, side-by-side comparisons of resistance data or surveillance systems are discussed.

This book is the first of its kind to provide a comprehensive overview of resistance mechanism in bacteria of animal origin rather than concentrating solely on zoonotic or foodborne bacteria. All uses of antimicrobial agents contribute to resistance, and each use must be examined in an attempt to understand its part in encouraging further dissemination of resistance in bacteria, including bacteria of animal origin. This book will serve as a valuable reference for persons who treat, research, or monitor resistance in bacteria of animal origin.

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Alfons Mucha (1860–1939). Zodiac (1896) (detail). Color lithograph (46 cm × 35 cm)

Fine Art and Good Health for the Masses

Polyxeni Potter*

Art is eternal, Alfons Mucha maintained, so it could never be merely "nouveau" (*1*). Contradicting those who saw him as part of a larger art movement, he insisted that his work was his alone. He followed his own creative impulses, was inspired by Czech folk traditions, and sought the spiritual in art. Yet, his palette so expressed the aesthetics of art nouveau that the movement was dubbed *le style Mucha*.

In cities across Europe and North America, amidst sweeping modernization and mechanization in the 1870s to the 1900s, art nouveau embraced all forms and designs, encompassed diverse styles and media, and remained influential well into the 20th century (*2*). Born of discontent with existing notions and styles, the movement fueled experimentation and reform and shattered the barriers between fine arts (painting, sculpture) and applied arts (ceramics, glassware, furniture, textiles, metalwork). Revolt against convention at times went hand in hand with political revolt against oppressive regimes, as was the case in Mucha's native Moravia, now Czech Republic, then still part of the Austrian and Austro-Hungarian Empire.

Folklore was plentiful in Ivancice, Mucha's hometown near Brno, as were Eastern religious traditions and Slav nationalism, all of which colored his work. Although he reputedly started to draw before he could walk, his talent for music was recognized earlier than his gift for art. As a child and throughout his youth, he sang in the cathedral choir in Brno. Religious images on the walls of the cathedral and other churches awakened his interest in art, particularly drawing. But when he applied for admission to Prague's Academy of Fine Arts, he was rejected: "Find yourself another profession where you'll be more useful" was the academy's recommendation (*3*). He left for Vienna, starting his career as scene painter in the theater. In Munich and then Paris, he received formal art training and worked on magazine and theater designs.

He earned a modest living from illustrations and lithographs and knew Gauguin before his legendary trip to Tahiti, when they briefly shared a studio above the crémerie on Rue de la Grande Chaumière. When they met

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again 2 years later, "The poor insignificant painter whom Gauguin had known at Madame Charlotte's in 1891 was safely on the way to success…" (*4*). Mucha's career took off when he stepped in at the last minute to design an advertising poster for Sarah Bernhardt, then the most famous actress in Paris. The poster (*1*), created in 2 weeks as an advertisement for Gismonda, became an instant sensation. Its distinctive elongated shape, muted colors, and elaborate decor became an icon and launched a lucrative association between Bernhardt and Mucha. His innovative designs complemented the actress' striking persona.

Some of Mucha's best work (advertising posters, jewelry, theater sets and costumes, book illustrations, carpet and wallpaper designs), on fine paper or fabric, was created at this time. Originals were translated into popular reproductions on matchboxes, postcards, calendars, and home designs, all illustrated with a richness reminiscent of the Byzantine icons he loved and collected. Accessible and dynamic, they became part of the vernacular, their figures harmoniously integrated with the surroundings in complex linear compositions. Curves, spirals, and intricate ornamentation spilled over into architectural and folk designs and dominated graphic art.

"We can't allow a split… ranking one art above the others," wrote artist Henry van de Velde in 1895, articulating a doctrine of art nouveau: art should affect the lives of all people, should enter their homes and influence their furnishings, uniting beauty and utility (*2*). Even mass-produced machine-made objects (stamps, money, lottery tickets, police uniforms) should be guided by sound design. To emphasize the social character of art, such projects as an International Exhibition of Art and Popular Hygiene sought to bring art to public facilities, public houses, and railway stations. The graceful organic shapes of Paris Métro entrances (Hector Guimard, 1867–1942) exemplified this principle (*5*).

Nature, a main source of inspiration, stood for modernity. With publication in 1859 of The Origin of Species and the development of evolutionary theory, progress in culture began to be viewed as analogous to evolution in nature. Rare and exotic plants and animal forms seen under the microscope found themselves in home and other designs as many artists became versed in natural history and biology and published in academic journals of those fields. Undulating, nongeometric "whiplash" curves, hyperbolas and parabolas, and intertwined organic forms dominated everything from jewelry design (René Lalique in Paris) to glassware (Louis Comfort Tiffany in New York).

For Mucha the astonishing success of his popular designs was only a prelude to what he considered his best work, The Slav Epic, a monumental painting inspired by his devotion to the Czech people. He died of pneumonia before the work was finished. Reaction to the painting was mixed, and his fame, particularly among his compatriots, diminished, to be revived again during the 1960s and remain strong to this day.

Zodiac, on this month's cover, shows why Mucha's work was instantly popular. The image exudes comfortable familiarity even as it invites contemplation. The human figure and its surroundings, harmonious and integrated, are evocative of nature. Floral and celestial elements are arranged symmetrically around a portrait, the focus of the intricate composition. Gaze and posture show directness, innate confidence, a sense of self. Filled with energy and movement, she is the dominant star in the celestial sphere. And "flowering" into the complex botanical frame, she captivates with poise and modesty. An exotic gypsy queen, she holds the mysteries of the zodiac, the flow of time, the riddles of nature, the fortunes of the world, deriving her power and magnetism from the symbols surrounding her, in perfect harmony and balance with the content of the universe.

A common motif in Mucha's work, the zodiac alludes to the birth of life and tries to identify and define it, predict its course, and control its outcome. Derived from the artist's faith in the spiritual aspect of art and the power of tradition as source of inspiration, it does what art nouveau sought to do, elevate folk elements to fine art accessible to everyone.

The desire near the end of the 19th century to beautify and advance the world culturally as it was advancing and evolving scientifically is understandable. Social purpose in art, which graces the mundane for the common people, is no different from social purpose in medicine, which improves and extends their lives. Public service for the greater good is like fine art for the masses. Mucha's Zodiac seems a fitting astrologic birthday card, as the Centers for Disease Control and Prevention celebrates 60 years of identifying, describing, explaining, and preventing unknown elements for the benefit of humanity.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the August issue for the following topics:

Venezuelan Equine Encephalitis Virus Transmission and Effect on Pathogenesis

Streptococcus suis Sequence Type 7 Outbreak, Sichuan, China

Macrolide Resistance in Adults with Bacteremic Pneumococcal Pneumonia

Antibody Response to *Pneumocystis jirovecii* Major Surface Glycoprotein

Human and Canine Pulmonary Blastomycosis, North Carolina, 2001–2002

O'nyong-nyong Virus, Chad

Human Bocavirus in French Children

Bocavirus Infection in Hospitalized Children, South Korea

Changing Pattern of Visceral Leishmaniasis, United Kingdom

Mental Status Deficits after West Nile Virus Infection

Human Metapneumovirus, Australia, 2001–2004

Community-acquired Methicillin-resistant *Staphylococcus aureus* in Children, Taiwan

Rickettsia felis in *Xenopsylla cheopis*, Java, Indonesia

Complete list of articles in the August issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

July 24–August 4, 2006
Diagnostic Parasitology Course
Uniformed Services University of the Health Sciences
Bethesda, MD, USA
Contact: 301-295-3139 or jcross@usuhs.mil
http://www.usuhs.mil/pmb/TPH/dpcourse.html

August 6–10, 2006
Advancing Global Health: Facing Disease Issues at the Wildlife, Human, and Livestock Interface
55th Annual Meeting, Wildlife Disease Association with American Association of Wildlife Veterinarians
University of Connecticut
Storrs, CT, USA
Contact: wda.2006@gmail.com
http://www.conferences.uconn.edu/wildlife/

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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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.

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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.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

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