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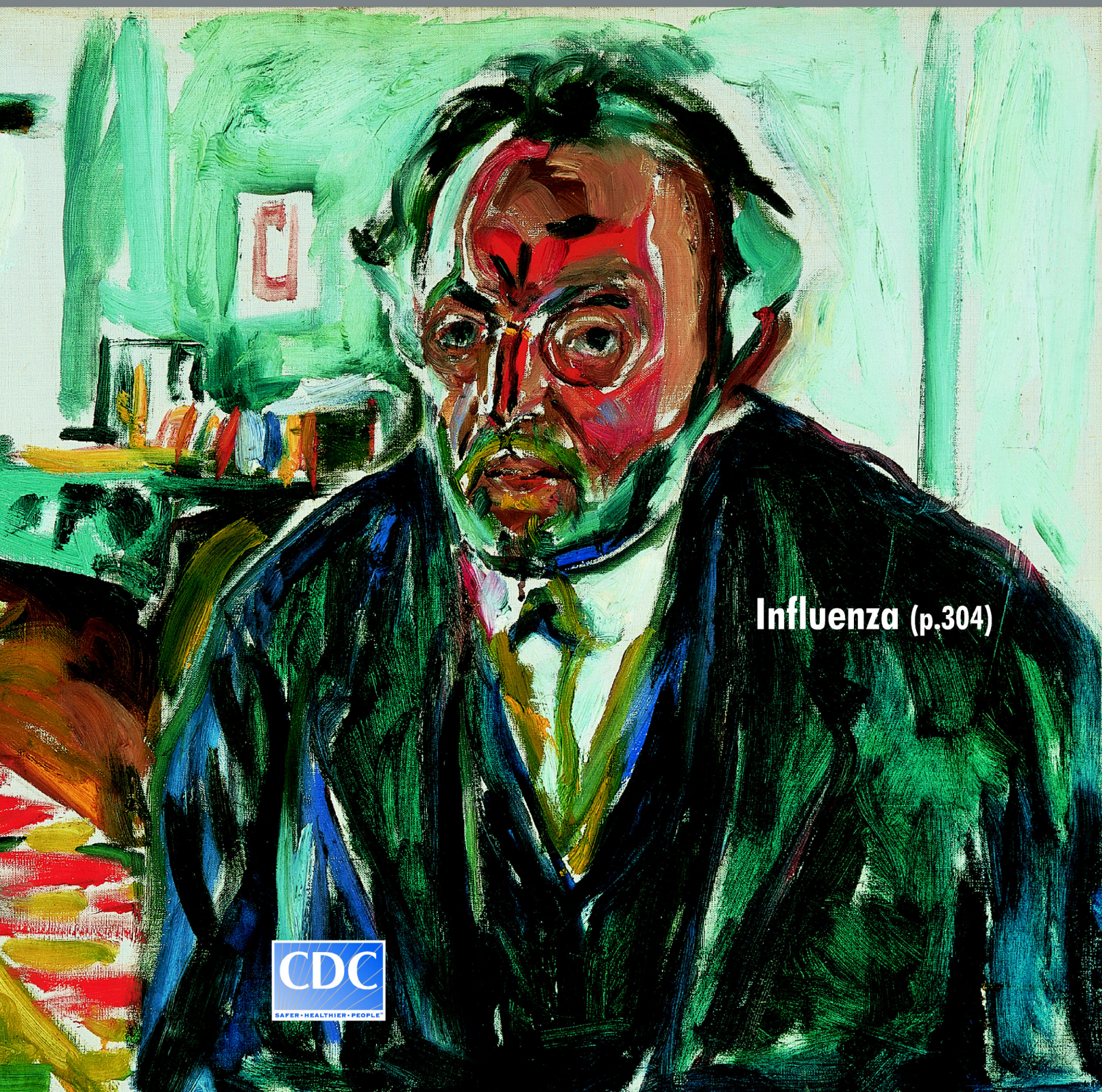

Influenza (p.304)

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Edvard Munch (1863-1944)
Self-Portrait After the Spanish Flu (1919-20)
Oil on canvas, 59 cm x 73 cm
Munch Museum, Oslo, Norway

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Emerging Trends in International Law Concerning Global Infectious Disease Control[1]

David P. Fidler*

International cooperation has become critical in controlling infectious diseases. In this article, I examine emerging trends in international law concerning global infectious disease control. The role of international law in horizontal and vertical governance responses to infectious disease control is conceptualized; the historical development of international law regarding infectious diseases is described; and important shifts in how states, international institutions, and nonstate organizations use international law in the context of infectious disease control today are analyzed. The growing importance of international trade law and the development of global governance mechanisms, most prominently in connection with increasing access to drugs and other medicines in unindustrialized countries, are emphasized. Traditional international legal approaches to infectious disease control—embodied in the International Health Regulations—may be moribund.

Globalization creates challenges for infectious disease policy (1–3). These challenges are horizontal and vertical in nature. Horizontal challenges constitute problems that arise between states from global microbial traffic (4). Vertical challenges, such as inadequate surveillance capacity (5), are problems countries face inside their territories that require responses within states. States cannot handle horizontal or vertical challenges without cooperating with each other. Unilateral efforts have limited impact when the source of the problem is beyond national jurisdiction (6). Similarly, unindustrialized countries need assistance to improve domestic public health (7). International cooperation mechanisms, including international law, are crucial to respond to both types of challenges. I examine the role international law plays in responses to horizontal and vertical challenges, analyze the historical development of international law in this area, and explore emerging trends in international law on infectious diseases that depart from traditional patterns.

Governance Responses to Globalization

The challenges globalization presents for infectious disease policy require governance responses. For horizontal challenges, the response of the government focuses on interstate cooperation to minimize disease exportation and importation. Vertical challenges require strategies that reduce disease prevalence through improved domestic public health.

The state constitutes the key actor in infectious disease governance. Public health is a "public good," which the public sector must produce because private actors lack sufficient incentives or resources (7). Governance responses to globalization occur at national, international, and global levels (Figure). National governance occurs when a state acts within its own territory to respond to globalization. International governance involves states cooperating to confront globalization challenges and often creates norms, rules, and institutions (i.e., regimes) to facilitate cooperation. Global governance involves not only states and international organizations but also nonstate actors, such as multinational corporations and nongovernment organizations (8,9), whose participation becomes critical to the success of governance efforts.

International law has different functions within vertical and horizontal strategies on infectious diseases and the three governance frameworks (Table 1). An historical overview of infectious disease governance delineates the strategic emphases in the governance frameworks and the functions of international law.

Horizontal International Regimes and Infectious Diseases, 1851–1951

Before the mid-19th century, states contended with infectious diseases through national governance. States adopted policies to manage infectious disease threats without international cooperation (10). The increased volume and speed of international trade and travel moved states from national to international governance in the mid-19th century, and the 1851 International Sanitary Conference marked the beginning of international governance on infectious diseases (11). International governance focuses primarily on horizontal strategies concerning the exportation and importation of infectious diseases. In the first century of international health governance, three horizontal international legal regimes relating to infectious diseases appeared—the classical, organizational, and trade regimes.

International sanitary conventions adopted from the late 19th century until World War II (12) and the World Health Organization (WHO)'s International Sanitary Regulations (1951) (later renamed the International Health Regulations

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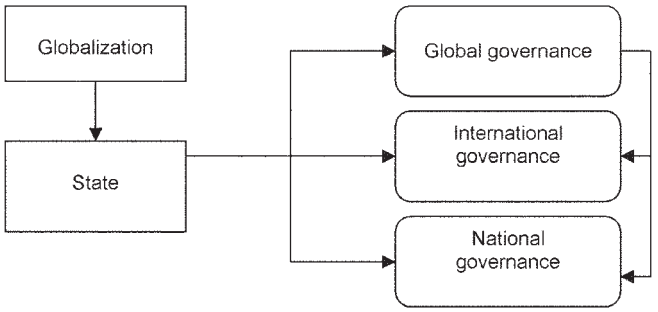

Figure. Governance responses to globalization challenges.

[IHR]) (13) represent the classical regime. The IHR's purpose—"to ensure the maximum protection against the international spread of disease with minimum interference with world traffic" (13)—captures the classical regime's objectives. This set of rules focuses on transmission of diseases across borders by requiring that 1) states notify other countries about outbreaks of specified diseases in their territories and maintain adequate public health capabilities at points of disease exit and entry; and 2) disease-prevention measures restricting international trade and travel be based on scientific evidence and public health principles (13).

The second horizontal international legal regime is organizational, that is, international health organizations created to deal with infectious diseases and other public health problems (10). WHO serves as the leading representative of this governance framework. Although international law was central to the creation of international health organizations, the treaties establishing them did not impose specific duties regarding infectious disease control (14). States created international health organizations to facilitate horizontal cooperation in public health; however, unlike the classical regime, the organizational regime's legal duties in regard to infectious disease control are few (14).

The third horizontal regime created in the 1851–1951 period was the trade regime, represented by the General Agreement on Tariffs and Trade (GATT, 1947), which liberalized trade but recognized that states may restrict trade to protect health (GATT, Article XX[b]). Trade-restricting health measures are legitimate if the measures conform to GATT rules. Thus, the trade regime contributes to horizontal international governance on infectious diseases.

Globalization, Infectious Diseases, and Governance, 1951–2002

Transition from the Classical to the Trade Regime

The classical regime's effectiveness has long been an issue. From Koch's criticism of late 19th-century international sanitary conventions (15) to analysis in the 1960s and 1970s of the IHR's problems (16–19), the classical regime's contribution to international governance on infectious diseases has been questionable. In the past half-century, the classical regime's importance has diminished, while the trade regime's influence has grown. Two events reflect this shift. First, in 1995 WHO recognized that the IHR did not achieve their twin goals of maximum protection from the spread of international diseases while incurring minimum interference with world traffic (20). WHO launched an effort to revise the regulations to update the classical regime for new globalization challenges (21).

Second, the World Trade Organization (WTO) became the central horizontal regime for international law on infectious diseases after its creation in 1995. The Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS), the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), and the WTO's powerful dispute settlement mechanism made WTO more important for infectious disease control policy than the discredited IHR. The trade regime's ascendancy over the classical regime is apparent in the contrast between the public health attention and controversy generated by WTO agreements and the IHR's obscurity in global public health discourse.

IHR Revision: Rejuvenation or Death of the Classical Regime?

The shift from the classical to the trade regime raises questions about the IHR revision process. WHO seeks to rejuvenate the regulations to make the classical regime more effective against contemporary disease threats (20–22). The IHR revision process may, however, signal the classical regime's death.

The revised IHR would have the same objectives as the original regulations: maximum protection against the international spread of disease while incurring minimum interference with world traffic (21). To date, the revision process has moved away from binding legal rules on disease notifications—one of the classical regime's pillars—to reliance on global information networks, represented by WHO's Global Outbreak Alert and Response Network (hereafter, the Global Network). WHO argues that this network has helped the organization identify, verify, and investigate hundreds of outbreaks since 1998, including outbreaks of cholera, meningitis, hemorrhagic fevers, viral encephalitis, and anthrax (23). WHO's Executive Director of Communicable Diseases claims the Global Network operates "within the framework" of the IHR (23).

The claim that the IHR support the Global Network is not correct under international law. First, the Global Network collects data from government as well as nongovernment sources. The IHR only authorize WHO to use information provided by member states (13,21). WHO's proposals to include in the revised IHR an ability to collect data from nongovernment sources (21) demonstrate that the IHR cannot provide the legal foundation for the Global Network's incorporation of nongovernment information. Second, the IHR only address three diseases—cholera, yellow fever, and plague (13). This limited coverage was one reason WHO wanted to revise the regulations (21). The IHR cannot support WHO's ability to manage, through the Global Network, meningitis, hemorrhagic fevers, viral encephalitis, anthrax, and other diseases not subject to the IHR.

Table 1. Governance frameworks, public health strategies, and international law on infectious diseases

Governance framework	Primary strategic emphasis	Function of international law	Infectious disease example
National	Vertical public health strategies	None	National sanitary reform, 19th century
International	Horizontal public health strategies	Provides architecture for horizontal public health strategies	International Health Regulations
Global	Vertical public health strategies	Provides norms informing vertical public health strategies	Global Fund to Fight AIDS, Tuberculosis, and Malaria

In addition, the Global Network operates without the revised IHR being in place. From July 1998 to August 2001, WHO used this network to verify "578 outbreaks of potential international importance in 132 countries, and investigated many hundreds more" (23). These statistics suggest that WHO's global surveillance strategy operates without the IHR revision process being completed. Revising the IHR to support global surveillance—the first raison d'être of the classical regime—does not appear urgently required given WHO's claims of its Global Network's success.

WHO's ideas for strengthening global surveillance under the revised IHR center on requiring member states to report all "public health emergencies of international concern" (21) to WHO. Member states would use WHO-developed criteria to assess whether an outbreak constitutes such an emergency. The criteria would include whether the event is serious, unexpected, and likely to involve international spread and to trigger trade and travel restrictions (21). The problem is that member states will want to determine for themselves whether a disease event constitutes a "public health emergency of international concern," no matter how many useful criteria WHO provides.

Further, the criteria are subjective rather than objective (e.g., is a disease event serious?), leaving member states with the discretion to argue that they did not report an event because they did not believe the event met the criteria. The fact that WHO reaches a different conclusion would not trigger legal consequences for the member state that failed to report an event. WHO's proposals on reporting "public health emergencies of international concern" do not indicate whether the criteria will be exclusive and legally binding or whether member states can use different criteria, which they develop.

The move from specific disease reporting to reporting "public health emergencies of international concern" indicates that WHO seeks to improve surveillance on major disease events rather than routine outbreaks. As the Global Network suggests, WHO will likely initially learn about major infectious disease events through sources other than member-state notifications. The Global Network approach reduces the legal importance of official member-state reports concerning major disease events.

WHO has also proposed that the revised IHR allow member states to make confidential, provisional notifications (21). WHO claims that the IHR do not allow such notifications (21). Concerning those diseases subject to the regulations, WHO's claim is accurate; however, the IHR cover only three diseases. WHO member states have always been free to consult with WHO staff about diseases not subject to the IHR. This suggested change only affects outbreaks of cholera, plague, and yellow fever, not the majority of infectious diseases considered global threats.

WHO has also proposed improving maximum protection against the spread of international diseases by requiring member states to have national surveillance systems that meet minimum requirements, including the ability to identify "public health emergencies of international concern" (21). Given the history of IHR violations and member-state reluctance to limit sovereignty, member states are unlikely to bind themselves to minimum standards. Further, WHO's proposals contain no discussion of what happens when member states fail to meet those standards.

Even if WHO member states agreed to minimum standards for national surveillance, the requirements would be empty without the commitment of industrialized countries to fund surveillance improvements in unindustrialized countries. Unindustrialized countries would oppose a legal requirement to improve national surveillance without financial commitment from industrialized countries. The end result would likely be authorization for WHO to issue recommendations on how member states should organize national surveillance. WHO already possesses, however, the power to issue recommendations (WHO Constitution, Article 23).

The IHR's second objective seeks maximum protection against international disease spread with minimum interference with world traffic (13). The existing IHR contain maximum measures that member states may take against trade and travel with respect to cholera, plague, and yellow fever (13). The IHR did not prevent WHO member states from imposing excessive and irrational travel- and trade-restricting health measures. Under WHO's proposals, the revised IHR would empower WHO to make recommendations about how member states should handle "public health emergencies of international concern" (21).

WHO's proposals mention "recommendations," which presumably would not be legally binding. WHO also proposes, however, that a core obligation of the revised IHR be that member states apply measures recommended by WHO during public health emergencies of international concern (21). Why WHO confuses obligations with recommendations is not clear. If history is any guide, member states will not allow WHO to issue binding regulations on an ad hoc basis.

Constitutionally, World Health Assembly approval would be required before any rule becomes binding (WHO Constitution, Article 21). This requirement, supported by member states' guarding of sovereignty, means the revised IHR would only allow WHO to make recommendations about how member states should handle public health emergencies of

international concern. Such authority would be redundant because WHO already has the power to issue recommendations (WHO Constitution, Article 23).

Thus, in connection with minimum interference with world traffic, the revised IHR would replace legally binding requirements with the authority to issue nonbinding recommendations, a power WHO already has. This shift does not address what happens when member states ignore WHO recommendations. The existing problem of member states' behaving in irrational, unjustified ways against outbreaks in other countries is left unresolved.

Today, WTO provides the more important forum for states concerned about irrational trade-restricting health measures because of the SPS Agreement and the WTO dispute settlement mechanism. Thus, the second raison d'être of the classical regime—disciplines against irrational health measures—has weakened for travel-related measures and migrated to the trade regime for health measures that restrict trade in goods.

Vertical International Governance

In addition to the shift from the classical to the trade regime, new international governance frameworks focusing on vertical public health strategies were developed in the post-1945 period. These approaches seek to reform how a government deals with its health inside the state's territory.

The "soft-law regime" represents guidelines, practices, and policies generated by international health organizations for adoption by states. Such norms are not legally binding, which is why they constitute soft rather than hard law. WHO has generated many soft-law norms. In fact, WHO has preferred soft law to the creation of binding legal commitments (24). The horizontal organizational regime has thus proved more valuable for creating vertical public health strategies than for providing discipline in interstate public health relations.

The "environmental regime" encompasses international environmental law, much of which seeks to reduce environmental threats to human health (25,26). Environmental treaties often require states to reduce environmental degradation within their territories and through cross-border transmission of harmful products. International environmental law supports both horizontal and vertical strategies. Such law is, however, weakest in connection with vertical public health strategies because it does not address local air and water pollution, the major environmental cause of illnesses and deaths due to infectious diseases (27).

The "human rights regime" imposes obligations on governments for treatment of persons in their territories. International human rights law is almost entirely vertical in orientation. Although such law has long incorporated public health, the HIV/AIDS pandemic brought international human rights law to bear more prominently on public health (28). Public health experts argue that international human rights law protects persons living with HIV/AIDS from discrimination and imposes obligations on governments to respect, protect, and fulfill their citizens' human right to health by making prevention and treatment programs universally available (29). International human rights law contributes to vertical strategies that seek to control infectious diseases within states rather than address their cross-border movement.

Global Governance Mechanisms

The third major change of the post-1945 period is development of global governance mechanisms. Global governance involves states, international organizations, and nonstate entities, such as multinational corporations and nongovernment organizations. Nonstate actor involvement distinguishes global from international governance (8). Nonstate organizations have long been involved in national and international governance (30), but globalization has stimulated new forms of governance in which such organizations have heightened roles. The effectiveness of horizontal international governance through the organizational regime became a major issue in the late 20th century. Although WHO eradicated smallpox, the organization has been ineffective in handling the increasing global devastation wrought by emerging and reemerging infectious diseases, especially HIV/AIDS, malaria, and tuberculosis. The organizational regime's infectious disease problems have encouraged exploration of new approaches.

Public health experts increasingly focus on global health governance (8,31–34), with emphasis on the role of nonstate organizations. The best examples of this trend are public-private partnerships, which have proliferated in global public health (35). Nonstate actor participation in global governance efforts ranges from the formal to the informal. Nongovernment organizations' presence on the governing body of the Global Fund to Fight AIDS, Tuberculosis, and Malaria (Global Fund) represents formal nonstate participation in global governance (36). WHO's use of information from nongovernment sources in the Global Network informally incorporates nonstate entities. Global governance mechanisms seek to provide "global public goods" for health that states, especially unindustrialized countries, can use within their territories to reduce the prevalence of infectious disease. Such global governance mechanisms primarily support vertical public health strategies.

The Access Regime

Many global governance initiatives work to increase access to drugs, vaccines, and other medicines. Public-private partnerships—such as Global Alliance for Vaccines and Immunization, Global Alliance on TB Drug Development, and Medicines for Malaria Venture —seek to develop and/or deliver more effectively new or existing drugs and vaccines in unindustrialized countries. The Global Fund hopes, for example, to increase access to antiretrovirals in unindustrialized countries (36). The global movement to increase access to essential medicines involves the evolution of a new governance framework—the so-called access regime—that has become the most prominent development in international law on infectious diseases.

The access regime arose from the clash of the horizontal trade regime and the vertical human rights regime. The core of

this clash was the collision of the TRIPS-led movement for greater protection for patented pharmaceutical products with the human rights-inspired effort to increase access to essential medicines. The "TRIPS versus public health" battle produced a dramatic moment in November 2001, when WTO adopted the Declaration on the TRIPS Agreement and Public Health (37). The Declaration places public health objectives, especially access to medicines, above the trade-related goal of increasing pharmaceutical patent protection. Experts see the Declaration as a victory for the human right to health and for global health governance (38).

The involvement of nonstate entities, namely multinational pharmaceutical corporations and nongovernment organizations (e.g., Médecins Sans Frontières) characterizes the development of the access regime. Nonstate organizations play important roles in all aspects of the access regime, particularly through public-private partnerships for developing new drugs (e.g., Medicines for Malaria Venture, Global Alliance on TB Drug Development) or improving access to existing drugs (e.g., Global Alliance for Vaccines and Immunization, Green Light Committee on second-line tuberculosis drugs [39]). Nongovernment organizations' formal governance role in the Global Fund provides another indication that the access regime represents an important development in global governance on infectious diseases. Finally, governance efforts to improve access to drugs and vaccines are found within each governance framework (Table 2).

The access regime takes the human right to health, developed originally as vertical international governance, into the realm of global governance through the leadership of nonstate organizations. The access regime aims to develop not only governance conducive to improved access but also new pharmaceutical products that governments, international institutions, and nonstate organizations can use to reduce illness and death caused by infectious diseases.

The access regime captures how international law's function differs in global governance from its role in international governance. Norms found in international law, principally the human right to health, inspire global governance on access; however, international law does not provide the architecture for such governance. Public-private partnerships are not based on treaties. The participation of states and international organizations in these global governance efforts is nonbinding under international law. The access regime uses international law differently than states and international organizations have used it for public health since international health diplomacy began in 1851.

Although many view the access regime as progressive, concerns exist about its impact on public health. The emphasis on access may divert attention and resources away from strengthening overall public health and infectious disease control infrastructures. The human rights focus may heighten concern for individual healthcare at the expense of protecting population health. Finally, increasing drug access may exacerbate antimicrobial resistance if proper attention to rational use is lacking (40).

Conclusion

Globalization creates infectious disease challenges that force states to cooperate. Historically, international law has been important in facilitating such cooperation. International law's use in infectious disease control has to address the governance challenges that globalization presents public health. The last 50 years, and the 1990s in particular, have witnessed shifts in how international law factors into global infectious disease policy. Within the traditional realm of horizontal international governance, attention has moved from the classical to the trade regime, changing the structure and substance of international law's role in infectious disease control. In addition, revision of the IHR signifies the death of the classical regime rather than its rejuvenation. Such a death would mark the end of the traditional use of international law for infectious disease control. Attention has also shifted from horizontal international governance to vertical global governance. This shift finds international law's traditional function in international governance supplemented by a context in which international law informs global governance endeavors that are not legally binding.

These developments mean that international law's role in infectious disease control today has never been more important and uncertain. International law remains important to horizontal international governance, as indicated by WTO's international legal role in public health. International law also informs vertical global governance even though these regimes find no formal expression in international law. Uncertainty looms,

Table 2. The access regime and governance frameworks[a]

National governance	International governance	Global governance
NGO lawsuits filed in national court systems to force national governments to increase access to HIV/AIDS therapies under the human right to health (e.g., South African case of *Treatment Action Campaign v. Minister of Health* (July 2002))	Unindustrialized-country and WHO advocacy to strengthen the public health safeguards in TRIPS to ensure access to affordable drugs and medicines (e.g., Declaration on the TRIPS Agreement and Public Health)	NGO activism directed at MNCs, international organizations, and national governments (e.g., MSF's global campaign opposing pharmaceutical MNCs' lawsuit against South Africa) Involvement of MNCs and NGOs in drug-development PPPs Formal governance roles for nonstate actors in new institutions (e.g., Global Fund)

[a]NGOs, nongovernment organizations; WHO, World Health Organization; TRIPS, the Agreement on Trade-Related Aspects of Intellectual Property Rights; MNCs, multinational corporations; MSF, Médecins Sans Frontières; PPPs, public-private partnerships.

however, in the IHR revision process, questions about WTO's impact on public health, and concerns that vertical global governance will not deliver global public goods for health in an important and sustainable manner.

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Enterovirus 71 Outbreaks, Taiwan: Occurrence and Recognition

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Enterovirus 71 (EV71) caused a large outbreak in Taiwan in 1998 with 78 deaths, and smaller outbreaks recurred in 2000 and 2001. The outbreak was recognized because of a large number of hand, foot, and mouth disease cases and the rapid deaths of children with the disease. Virologic and pathologic studies indicated that EV71 was the most important agent related to severe and fatal cases and that a neurogenic inflammatory response was involved in the pathogenesis of cardiopulmonary collapse resulting from fulminant EV71 infection. Seroepidemiologic study suggested that EV71 had circulated for at least 16 years and that the accumulation of susceptible hosts might have triggered the 1998 outbreak. However, a change in EV71 neurovirulence and host genetic susceptibility may also have affected the clinical outcome. The Taiwan outbreak shows that worldwide attention should be paid to such outbreaks, new antiviral drugs should be developed, and that vaccination of children under 5 years of age may be warranted.

Enteroviruses consist of 68 serotypes and usually cause self-limited infections in children. Enterovirus 71 (EV71) was first isolated in California in 1969 (1). Since then, EV71 has been isolated in many parts of the world. Two patterns of EV71 outbreaks have been observed: small outbreaks associated with occasional patient death and severe outbreaks associated with a high case-fatality rate. The latter pattern occurred in Bulgaria in 1975 with 44 deaths (2) and Hungary in 1978 with 45 deaths (3). During the past 5 years severe outbreaks have occurred: in Malaysia in 1997 with 30 deaths and in Taiwan with 78 deaths in 1998, 25 deaths in 2000, and 26 deaths in 2001 (4).

How Did the Taiwan Outbreaks Occur?

Sentinel Surveillance Systems in Taiwan

In 1989, a physician-based sentinel surveillance system for infectious diseases was established in Taiwan and operated by the Ministry of Health. This system included 850 physicians, representing 8.7% of primary physicians in Taiwan. Hand, foot, and mouth disease (HFMD) and herpangina were included in the system after an outbreak of HFMD in Malaysia in 1997. When the case incidence after 1997 was compared with the case incidence of HFMD in 1997, the number of cases in Taiwan markedly increased after March 1998 (5). Because the numbers of severe and fatal cases of HFMD were increasing rapidly, a hospital-based reporting system for monitoring such cases was added in May 1998. Since June 1998, both physician-based and hospital-based surveillance systems have been maintained simultaneously (5).

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1998 Outbreak

The 1998 outbreak occurred in two waves. The first and bigger wave peaked during the week of June 7 and encompassed all four regions of Taiwan. The second and smaller wave occurred during the week of October 4, mainly in southern Taiwan. The total number of HFMD and herpangina cases reported was 129,106 (6).

The severe cases of EV71 infection peaked in early June as well: 405 severe cases were reported with 78 deaths (6). Ninety one percent of patients with fatal cases were <5 years old (6). Most of these patients died of fulminant infection within 1 or 2 days of hospitalization. Of 96 patients with severe EV71 cases, 67 (70%) had encephalitis (6).

Virologic and Pathologic Studies

Of 78 patients with fatal cases, 37 had a positive viral culture in which 34 (92%) yielded EV71 (4). In 1998, 177 strains (42%) of EV71, 73 strains (18%) of coxsackievirus A16, and 168 strains (40%) of other enteroviruses (4) were isolated in Chang Gung Children's Hospital; only EV71 was isolated from patients who died or survived with chronic health problems (4). Thus, EV71 was the most important agent among fatal cases, although other enteroviruses also circulated during the outbreaks.

Pathologic studies showed extensive inflammation in the central nervous system (CNS), with predominant lesions in the brain stem and spinal cord (7). Marked pulmonary edema with focal hemorrhage occurred without evidence of myocarditis. EV71 was isolated from CNS tissues but not from other tissues. The autopsy findings were similar to those reported in Malaysia (8).

Clinical, virologic, and pathologic findings suggested that the CNS, especially the brain stem, is the primary area involved in severe EV71. We hypothesized that a neurogenic and systemic inflammatory response may be involved in the pathogenesis of cardiopulmonary collapse that occurs in severe EV71 infections (Figure). EV71 may invade the CNS through its presence in the blood or directly through the cranial nerves (facial or hypopharyngeal nerve) on day 2 to day 5 of illness. A

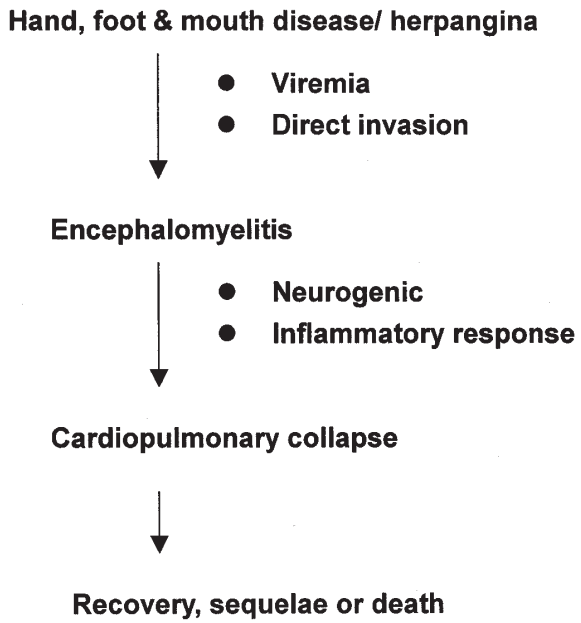

Figure. Pathogenesis of severe EV71 infections.

severe inflammatory response occurs in the CNS, and we found a high interleukin 6 level (1326+/-389 pg/mL) in the cerebrospinal fluid on the first 2 days of CNS involvement (9), when cardiopulmonary collapse with pulmonary edema usually occurs. If appropriate cardiopulmonary supports are provided, patients may survive but usually have sequelae: for example, central hypoventilation; cranial nerve palsy such as dysphagia, abducens palsy, and facial palsy; and shoulder weakness and atrophy. These sequelae are compatible with magnetic resonance imaging findings, which usually reveal high signal intensity from the pons to the cervical spinal cord (10).

Did EV71 Circulate in Taiwan Before 1998?

Chang et al. initiated a seropidemiologic study before and after the 1998 outbreak and found that in 1997 EV71 seroprevalence rates in adults and children >6 years of age were 57% to 67% (11). Lu et al. examined serial serum antibody titers to EV71 in 81 children born in 1988 who had yearly blood samples saved from 1989 to 1994 and in 1997 and 1999 (12). These researchers discovered that the incidence of EV71 seroconversion increased yearly from 3% to 11% between 1989 and 1997 and that 68% of these children had serologic evidence of EV71 infections by 1997 (12). In addition, EV71 had been isolated from patients with HFMD and poliomyelitis-like paralysis in Taiwan as early as 1981 and 1986 (6,13).

How Was the 1998 Outbreak Recognized?

The evidence presented above indicated that EV71 had circulated in Taiwan for at least 16 years before 1998. However, its clinical significance in Taiwan was not investigated before 1998. Severe cases of EV71 probably had occurred but had not been recognized. In 1998, however, with the availability of specific EV71 monoclonal antibody, observant pediatricians in Taiwan linked the association of EV71 with young children dying of an unexplained acute illness. Both the physician-based and hospital-based surveillance systems supplied critical information leading to the recognition of the EV71 outbreaks and provided the opportunity for the study of EV71 infections.

Why Did the 1998 Outbreak Occur?

The 1998 EV71 outbreak in Taiwan may have occurred for the following reasons: l) mutation of the virus to a form with increased virulence; 2) presence of host factors—the accumulation of susceptible populations and individual genetic susceptibility. The outreak was recognized because of the establishment of a sentinel surveillance system (clinical and laboratory) and the awareness of health care workers.

Outbreaks after 1998

One year after the large outbreaks in 1998, cases of EV71 infection decreased dramatically with only one fatal EV71 case in 1999. However, severe and fatal EV71 cases recurred, and 25 children died in 2000 and 26 in 2001 (Table). EV71 outbreaks likely will continue to occur for the next several years in Taiwan.

Virus Studies

Wang et al. showed that most EV71 strains isolated in the 1998 outbreaks belonged to genotype C (14). In 1999 and 2000, however, genotype B was the most common strain. Recombination strains did not develop between the two genotypes, and particular EV71 genotypes did not affect clinical outcome (14).

Shih et al. analyzed the complete sequence of two selected EV71 strains (15), one from a fatal case and the other from an uncomplicated HFMD case. They found the identity to be similar (from 97% to 100%) both in amino acid and nucleotide sequence throughout the whole genome. Although the genetic identity of EV71 in the fatal case and the uncomplicated case was highly similar, their clinical outcomes were contrary.

Currently available genetic techniques cannot detect the EV71 genetic difference except the difference in genotype. As with polioviruses (16), a minor yet critical genetic change may have led to unusual neurovirulence and caused this outbreak.

Table. Number of confirmed severe/fatal enterovirus infections and viral isolation results from patients with fatal cases, Taiwan, 1998–2000[a]

Case/enterovirus serotype	1998	1999	2000	2001
Severe cases	405	35	291	389
Fatal cases	78	9	41	55
Enterovirus 71	34	1	25	26
Coxsackievirus B3	0	3	1	0
Echovirus 4	0	0	0	3
Other enteroviruses	3	4	12	7
Negative	31	1	3	13
Specimens not available	10	0	0	6

[a]Data provided by the Center for Disease Control, Ministry of Health, Taiwan (1998–2001).

Host Factors

Chang et al. showed that preepidemic EV71 seroprevalence rates were inversely correlated with death and disease (11). Among a cohort of 81 children, Lu et al. found that the annual EV71 seroconversion rates (3% to 4 %) between 1994 and 1997 were significantly lower than the rates (7% to 11%) before 1994 (12). The incidence of EV71 infection was likely lower between 1994 and 1997, and the accumulation of susceptible hosts over the threshold density of EV71 caused the 1998 outbreak.

Our preliminary data did not show a difference in tumor necrosis factor-a promoter polymorphism between uncomplicated and serious HFMD cases (unpub. data). We still believe that individual host genetic factors may affect clinical severity.

Surveillance System

Pediatricians with a high index of suspicion and timely autopsy findings were the major factors that caused the outbreak to be recognized. Otherwise, this fulminant cardiopulmonary collapse would have been regarded as unexplained death. At present, no single factor can fully explain why a fairly common enterovirus can cause outbreaks of severe disease in selected countries of the world.

Relationship between EV71 and HFMD and the Implications

In addition to the Bulgarian, Hungarian, Malaysian, and Taiwanese EV71 outbreaks, a study in Singapore also showed that the age-specific EV71 seroprevalence rate remained steady at approximately 50% among children >5 years old in 1996–1997 (17); EV71 also caused a polio-like syndrome after poliovirus was eradicated in Brazil (18), and the EV71 seropositive rate among 12- to 15-year-old Brazilian children was 69.2% (19). Given the lack of adequate viral diagnostic laboratories and the difficulty in isolating some strains of the virus, reports of EV71 infection may represent only the tip of the iceberg. Therefore, outbreaks of EV71 infection could spread to other parts of the world and cause substantial illness and death.

In conclusion, EV71 infection may have a fulminant clinical course, and particularly because approximately 90% of the fatal cases were in children <5 years of age, effective control measures must be implemented: new antiviral drugs should be developed and mass active vaccination of children <5 years old in disease-endemic areas should be considered.

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Dr. Lin is the superintendent of Chang Gung Children's Hospital, Taoyuan, Taiwan. His research interests include vaccines, the pathogenesis and management of meningitis, and enterovirus infection.

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Electron Microscopy for Rapid Diagnosis of Infectious Agents in Emergent Situations[1]

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Diagnostic electron microscopy has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative stain preparation, the undirected, "open view" of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen. Details for efficient sample collection, preparation, and particle enrichment are given. Applications of diagnostic electron microscopy in clinically or epidemiologically critical situations as well as in bioterrorist events are discussed. Electron microscopy can be applied to many body samples and can also hasten routine cell culture diagnosis. To exploit the potential of diagnostic electron microscopy fully, it should be quality controlled, applied as a frontline method, and be coordinated and run in parallel with other diagnostic techniques.

In late September 2001, a letter containing spores of *Bacillus anthracis* arrived at a publishing house in Palm Beach, Florida, and resulted in the death of one employee from inhalation anthrax. Over the next 6 weeks, similar letters were delivered to television news centers in New York City and government offices in Washington, D.C. Ultimately >32,000 suspected exposures and five deaths were recorded in the United States. The collateral spread of exposure to spores was a sobering reminder of the bioterrorism attack scenario hypothesized by O'Toole (1).

Today, technology allows genetic engineering of potentially devastating agents such as modified ectromelia virus (2), the weaponizing variola virus (former USSR) (3), the long distance dispersal of yellow fever–infested mosquitos (United States) (4), and the weaponizing of anthrax spores by many nations. The ease with which the recent anthrax attacks were delivered indicates that unsophisticated methods are still effective. Thus, the most potent defenses remain rapid identification of the event and agent, treatment of the victims, and containment of infection. Successful outbreak management depends on early recognition of a suspected infectious case by the primary care physician and obtaining an accurate, timely laboratory diagnosis. An unexpected temporal or geographic cluster of illness of apparently infectious nature or an unusual age distribution of pneumonia with respiratory failure, intradermal hemorrhage, or chickenpox-like illnesses may indicate infection caused by a novel agent or a bioterrorist act. Similarly, the sudden appearance of vesicular lesions or respiratory illness in farm animals may be evidence of an emerging disease, a possible zoonosis, or an agriterrorist act. While recent studies suggest that healthcare systems are ill prepared to treat victims and contain the spread of an infectious agent (5), the performance of physicians, epidemiologists, and diagnostic specialists in identifying outbreak-associated agents as diverse as Nipah virus (6) and gastroenteric agents (7) indicate that identification of an outbreak and its associated agent may be done rapidly and successfully.

Electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory (8). We describe the role of transmission electron microscopy in viral diagnosis and outbreak management; methods for specimen collection, preparation, and examination; laboratory safety and quality control; and the differential morphologic diagnosis of infectious agents. In addition, an online appendix lists facilities that provide electron microscopic diagnostic support (available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0327-app.htm).

Role of Electron Microscopy in Virus Identification

The first electron micrograph of poxvirus was published in 1938. In 1941, immunologic procedures were first used in electron microscopic studies of tobacco mosaic virus (9), and electron microscopy was introduced successfully in the differential diagnosis of smallpox and chickenpox infections in the late 1940s (10,11). With the introduction of negative staining in the late 1950s (12) and the wider availability of electron microscopes, electron microscopy (as a catchall method) became essential in characterizing many new isolates detected in diagnostic cell cultures and clinical samples, e.g., stool, urine, and biopsied specimens (7,13–16). Pattern recognition, i.e., information on size and particle morphology, leads to rapid identification of infectious agents. The initial classification of many agents was therefore based on a combination of morphology and genome structure. Currently, >30,000 different viruses comprising 56 separate families have been identified, and humans have been found to host 21 of the 26 families specific for vertebrates (17). The distinct morphology of members of

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different viral families usually allows an agent to be assigned to a particular family. This morpho-diagnosis, combined with clinical information is, in most cases, sufficient to permit a provisional diagnosis or rule out a more serious infection and to initiate treatment and containment protocols without waiting for other test results.

Because electron microscopy is not suitable for screening large numbers of samples, many alternate immunologic and molecular methods have been developed on the basis of nucleic acid amplification techniques. While immunologic tests have almost unlimited throughput, the high specificity of these assays may result in failure to identify etiologic agents with different antigenic determinants. Further, reagents may not currently exist that would permit complete immunologic testing (18,19). Even when an immunologic test is appropriate for the etiologic agent, the sensitivity may only equal that of electron microscopy (20,21). Nucleic acid amplification techniques have similar limitations. They are more sensitive but are only capable of identifying the presence of genomic material for previously identified agents. Although primers exist that will permit amplification of most enteroviruses (22,23), few multiplex systems can identify all genotypes and serotypes within, or between, the different families of viruses that infect humans (22,24,25). Further, mutations in the primer target region may negate the effectiveness of primers. Because nucleic acid amplification techniques will not identify subviral components such as empty virions, which may be produced late in an infection, some studies suggest that their practical level of sensitivity does not always exceed that of electron microscopy (19,25,26). Because this modern armament has taken over most routine diagnostics, with the exception of gastroenteric viral infections, electron microscopy may be concentrated on infectious disease emergencies. The "open view" of electron microscopic testing allows an unbiased, rapid detection of viruses and other agents if sufficiently high particle concentrations exist (Figure 1). Because of this capability, electron microscopic testing must be a frontline method, applied either to samples directly from a suspected lesion, bodily fluids, or biopsies after cell-culture augmentation of a cultivable agent or from letters and environmental samples.

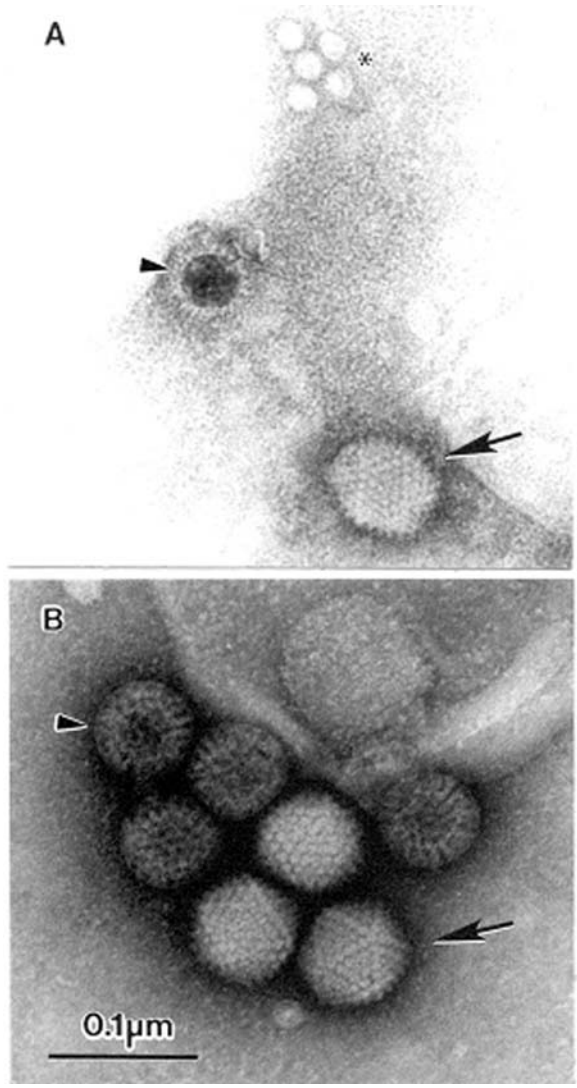

Figure 1. The open view of diagnostic electron microscopy. A. Multiple agents observed in a fecal sample from a pediatric patient with diarrhea. A 10% suspension was prepared in distilled water, cleared by low-speed centrifugation followed by 5 minutes at 15,000 x *g* in a bench top centrifuge, and centrifuged directly to the grid using an Airfuge EM-90 rotor (Beckman, Palo Alto, CA): adenovirus-(→), incomplete rotavirus-particle (▶), and small round featureless particles, probably adeno-associated virus (.) phosphotungstic acid stained. B. Double infection with adenovirus (→) and complete rotavirus particles (▶), in the stool of a 1-year-old child. The sample was suspended 1:3 in distilled water, cleared by low-speed centrifugation, and prepared for examination by the two-step method. Aqueous uranyl acetate stained. Bar = 100 nm.

Specimen Collection

Successful investigation of any outbreak or novel case starts with specimen collection. Insufficient, improper, or inadequate sampling may delay or prevent identification of a causative agent. Sufficient sampling requires identification of, and sampling from, all areas where infection may have been established. Fecal samples are ideal for investigating gastroenteric episodes, as are lesion fluids or smears from skin lesions of possible viral origin.

A major cause of insufficient sampling can be failure to collect acute-phase sera from affected case-patients and more importantly, their contacts, who might well be asymptomatic. First, the existence of a blood-borne pathogen may not be evident when examining unexplained cases, as demonstrated by the difficulty identifying HIV (27) and hepatitis C virus infections, and associating human parvovirus B-19 with Fifth disease (16). Second, acute-phase sera are essential for demonstrating seroconversion to a suspected agent. Third, clinical symptoms may be caused by an immune response to an infection that has resolved by the time they appear. However, specimens from apparently uninfected contacts of patients with acute cases may contain the agent involved (16). Convalescent-phase sera collected from case-patients 4–6 weeks after onset of illness are also powerful diagnostic reagents. If no agent has

been identified by standard virus detection procedures (e.g., electron microscopy, tissue culture, immunoassay, or nucleic acid amplification techniques), these serum samples may be used to detect the causative agent (28), while matched acute:convalescent-phase serum pairs collected at least 2 weeks apart may be used to demonstrate a significant rise in specific antibody among cases by immuno-electron microscopy (Figure 2) (7). Infectious agents may also be identified in cerebrospinal fluid, lesion crusts, nasopharyngeal washes, saliva, tears, urine, and biopsied tissue specimens (29). However, low viral load, sampling difficulties, or both may reduce the effectiveness of rapid electron microscopic diagnosis on these later types of specimens without initial tissue culture amplification, as observed in Nipah virus studies (6).

Safety concerns, miscommunication between infectious diseases specialists and staff who collect samples, or inadequate training may result in improper sample collection. Although swab samples placed into viral transport media may allow nucleic acid amplification techniques or culture of nonfastidious agents to be carried out, such specimens are not very conducive to successful rapid electron microscopy diagnosis of lesion exudates because of dilution effects and interfering components. Several effective ways of collecting lesion fluids exist (8). A method readily available to the physician or in a hospital ward is collection into the barrel of a 26-gauge needle attached to a tuberculin syringe. A fresh lesion is unroofed or the beveled surface of the needle is placed against the base of an open lesion, and fluid is aspirated into the barrel. After capping, the sample may be transported directly for rapid electron microscope diagnosis (Figure 3A). Alternatively, coated electron microscope specimen grids may be lightly touched directly to the vesicle fluid, lesion base, or both; allowed to air dry; and transported directly for examination (direct touch preparation) (Figure 3B). Because repreparing the sample with direct touch preparations may not be possible, at least two grids should be obtained when the specimen is collected. For safety and containment of hazardous infectious materials, the syringe or grid should be placed in a rigid sterile container, e.g., conical 15-mL centrifuge tube or Beem capsule (Beem Co., Bronx, NY), sealed with Parafilm (American National Can Co., Greenwich, CT), and the outside of the tube washed with 0.5% sodium hypochlorite (10% household bleach) before transport (Figure 3). Safety regulations usually require further packaging of the sample inside a second container.

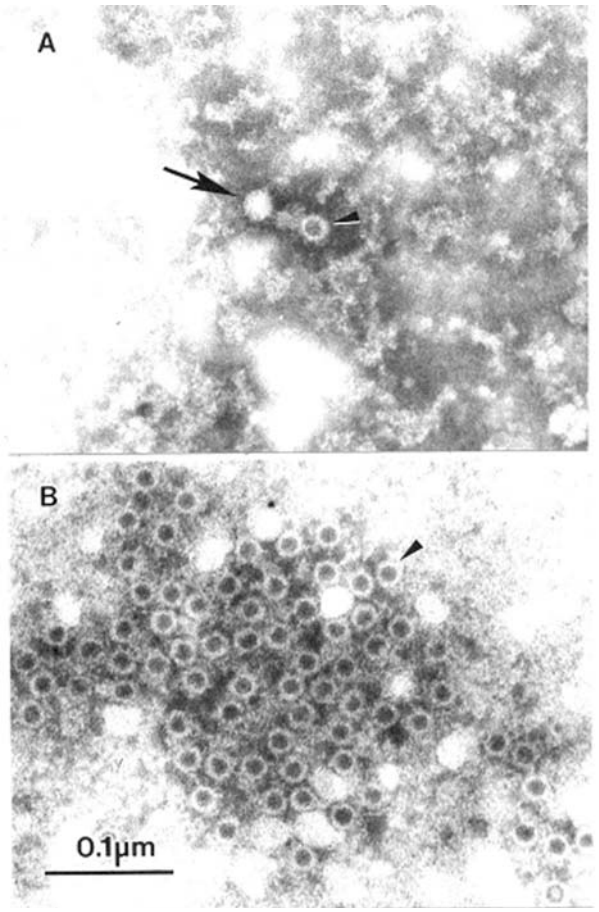

Figure 2. Association of human parvovirus B-19 with erythema infectiosum by immunoelectron microscopy. A. Airfuge EM-90 rotor (Beckman, Palo Alto, CA) preparation of human serum prospectively collected at time of contact with case of erythema infectiosum. Erythema infectiosum-like rash developed 1 week after collection of serum. B. Immunoelectron microscopy preparation of the serum in panel A. The serum was mixed with matched convalescent-phase serum (final dilution convalescent-phase serum 1:100), incubated for 90 min at 37°C, and virions/immune complexes centrifuged directly to a specimen grid with the EM-90 rotor. Arrow, complete virion; arrowhead, genome-defective virion; phosphotungstic acid. Bar = 100 nm. For study details, see Plummer et al., 1985 (16).

In the late 1940s, direct touch preparations from skin lesions were prepared in North Africa and sent to Toronto, Canada, where they were examined successfully for smallpox virus for up to 4 months after collection (11). In another comparative study in Winnipeg, Canada, which used matched lesions, we observed an average increase of 10.2:1 in the number of virions visualized by direct touch as opposed to needle aspirate preparations, and the ratio was >1.0 in 92% of total cases examined ($n=12$; $p<0.02$; [Wilcoxon signed-rank test]). We observed no difference in the number of positive identifications or homogeneity of virion distribution on the grid between these two methods. Lesion smears on glass slides may also be used effectively for both electron microscopy and immunofluorescent microscopy examination (Figure 3C). Smears, i.e., dried down vesicle fluids, are especially effective when syringes and electron microscopic grids are not available. Both direct touch and smear preparations are useful when specimens must be transported some distance for electron microscopic examination.

The collection of lesion exudates as swab samples placed in viral transport medium is less effective. A change in specimen collection protocols in 1995, from direct touch/lesion aspirates to swab specimens in transport medium, has resulted in a decline in successful identification of virions in lesion specimens in Winnipeg from 62% to 75% to approximately 10% (Hazelton, unpub. data). While complete fecal samples are preferable, collecting rectal swab samples for diagnosis of gas-

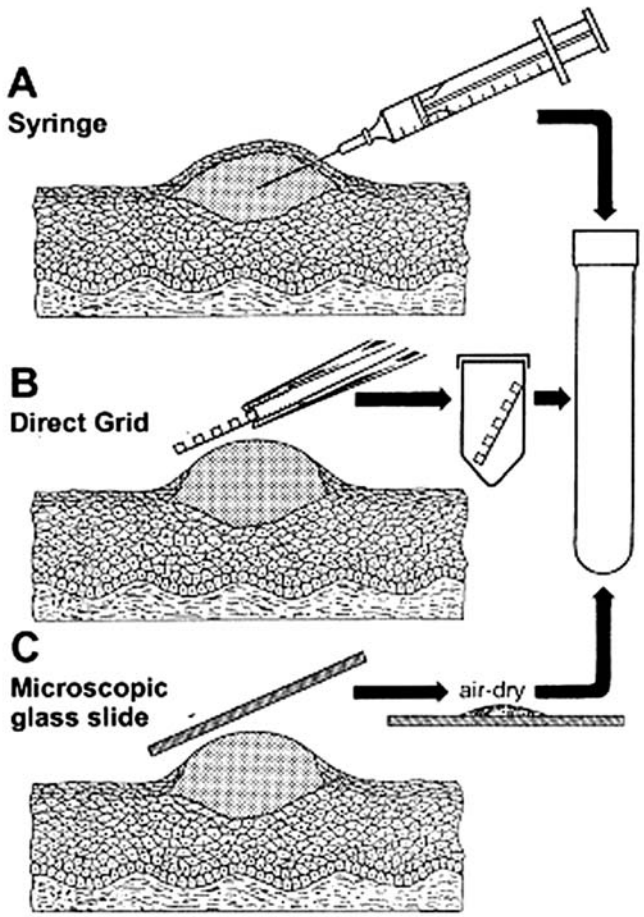

Figure 3. Three methods for efficient collection of vesicular and blister fluids for diagnostic electron microscopy. A. The contents of a vesicle are collected into the barrel of a needle. B. After the blister is opened, a coated electron microscope grid is touched to the fluid and air-dried (direct electron microscopy). C. A glass microscope slide is touched directly to an unroofed lesion and a smear prepared. Samples are then placed in rigid containers for transport to the electron microscopy laboratory.

troenteric agents may be necessary. These swab samples should be placed in capped conical centrifuge tubes with 0.2 mL sterile, distilled water, sealed with Parafilm, and sent for electron microscopic diagnosis. Lesion crusts should also be placed in sterile conical tubes. The addition of any liquid medium to lesion crusts, cerebrospinal fluid, nasopharyngeal washes, saliva, tears, and urine will not assist the electron microscope laboratory. Tissue biopsy samples in buffer without fixatives should be stored at 4°C and sent directly to both an electron microscope facility and a viral identification laboratory for rapid electron microscopy and other diagnostic testing. Fixation may interfere with antibody binding and thus preclude infectivity tests and successful application of any immunoelectron microscopy.

Finally, failure to collect an adequate volume of sample will limit the tests that may be used and the ability to successfully identify causative agents. Lesion fluids are deceiving. For example, samples containing poxvirus or varicella zoster virus that appear to have no material drawn into a needle barrel (Figure 3A) or attached to a grid may still contain numerous virions. When possible, at least 1 g of fecal material should be collected into a commercial stool collection vessel. A minimum of 5.0 mL of blood should be collected into tubes without anticoagulants. When a special interest in the case or outbreak occurs, large samples may provide reagents for later testing. All samples should be immediately sent for rapid electron microscopic diagnosis, with storage at 4°C if possible. Dried smears may be stored and transported at ambient temperature. Under no condition should samples be frozen for storage and transport before receipt at the diagnostic facility (30).

Containment of Biological Hazards and Laboratory Safety

Protecting staff and containing infectious agents are important considerations in the handling of all clinical specimens. Samples may contain agents that are highly infectious or associated with a high mortality rate. In consideration of the possibility of bioterrorism and agriterrorism, delivering samples to a central facility at biological safety level (BSL) 3 or higher may be necessary for inactivation before electron microscopic examination. Regardless, preparation must be done in a laminar flow hood with BSL-2 or greater containment capability. Most infectious agents may be inactivated in suspension by adding formaldehyde or glutaraldehyde (20 min, final concentration 2% and 0.5%, respectively). Alternatively, hazardous samples may be inactivated after they are mounted on the grid by treating the grid with fixative, by subjecting stained preparations to ultraviolet irradiation (UV) for 5 min before removing them from the biological safety cabinet, or both. UV treatment may, however, affect both virion morphology and grid stability. Prolonged treatment with glutaraldehyde or formaldehyde has little effect on morphology while inactivating most agents (31). Both formaldehyde and glutaraldehyde immobilize structures by Schiff reactions involving aldehyde side groups. As a di-aldehyde with a 5-carbon backbone, glutaraldehyde is more effective than formaldehyde at intermolecular crosslinking. Glutaraldehyde may, therefore, cause aggregation and obscure some fine structural detail. Samples suspected of containing spores should be inactivated with 10% formaldehyde final concentration because spores are more resistant to chemical inactivation (32). Specimens that may contain prions require more harsh treatment, such as the addition of 1 M NaOH, to inactivate the samples. However, treatment with NaOH will degrade most biologic structures to an indecipherable tangle of artefacts, and is, therefore, not conducive to electron microscopic examination.

Specimens that have not been inactivated must still be treated as potentially infectious after electron microscopic examination. For example, no decrease was observed in a 50% tissue culture infective dose ($TCID_{50}$) of poliovirus samples after they were mounted on the grid and stained with 2.5 mM (1.6%) phosphotungstic acid, pH 7.0. Subsequent exposure to vacuum and the electron beam for 1 min reduced $TCID_{50}$ by at

least $10^{6.5}$ and $10^{7.5}$ for adenoviruses and polioviruses, respectively. More importantly, 10-min vacuum and electron beam exposure of grids containing sporulating B. subtilis preparations permitted colony recovery in 60% of tests and reduced colony-forming units 500-fold, and exposure to either vacuum or phosphotungstic acid-negative stain alone had little effect on the viability of adenovirus, poliovirus, or spore preparations (33). These observations underline the extreme resistance of spores in different weapons delivery systems. Because of the risk for residual infectivity, all grids must be disposed of as infectious waste, and equipment used to handle samples and grids, e.g., forceps, must be decontaminated by treatment with 5% glutaraldehyde for 20 min. Alternatively, equipment may be disinfected with 1 M NaOH. Cleaning is also necessary to prevent false-positive results caused by crossover contamination between specimens. Staff involved in rapid electron microscopy should be vaccinated for multiple agents, including smallpox and hepatitis B.

Specimen Preparation

While rapid electron microscopy may be performed with any type of specimen, the requirement for truly rapid electron microscopic diagnosis is not common. Indicators include limiting exposure in clinically threatening situations in which an infectious cause is not ruled out, as may occur if a patient has suspected herpetic lesions in a ward for immunocompromised, newborn, or transplant patients; new clinical symptoms are observed with immunocompromised patients; the need to initiate early treatment; or the risk of passing infection during birth. Since a viral agent may be found by rapid electron microscopy in over 90% of poxvirus (34) and other skin lesions of viral etiology (Gelderblom and Hazelton, unpub. data), this method is ideal for investigating outbreaks of rash-like illness and suspected cases of bioterrorism.

A morphologic diagnosis may be obtained within 10 min of specimen arrival in the electron microscope facility. The standard two-step drop method, i.e., adsorption followed by negative staining, is used for preparation (Figure 4A). Viral load is usually more than sufficient to allow successful diagnosis of herpesvirus, poxvirus, and some gastroenteric infections. Negative-stain examination is simple and may be conducted in any electron microscope facility. The first item needed is a 400-mesh electron microscope grid coated with either a single plastic layer or a plastic film reinforced with carbon (32,35,36). Carbon-coated plastic films have higher thermal stability and are less prone to specimen movement during examination. However, they may be more hydrophobic than plain plastic films. Electron microscope units that specialize in virus preparative or diagnostic techniques prepare their own plastic-coated, carbon-stabilized films, and glow discharge the films to improve hydrophilicity, particle adherence, and distribution of both sample and stain (36,37). Coated grids may also be purchased through most electron microscopy suppliers. Clinical samples with high concentrations of protein often do not require glow discharge pretreatment to reduce hydrophobicity.

Lesion fluids received in the barrel of a needle or capillary tube are expelled onto a hydrophobic surface such as Parafilm. If the sample has dried, a small drop of redistilled water (15 µL), sterilized through a 0.2-µm–pore filter, is drawn into the specimen container and washed back out. If required, an aliquot of suspension should immediately be transferred to viral transport medium and submitted for cell culture, nucleic acid amplification techniques, and other virologic procedures. Lesion crusts and biopsy material may be soaked in 3 volumes buffer and solubilized by 10–12 pestle strokes in a Dounce homogenizer, while fecal material may be suspended by vortexing with glass beads in 3–9 volumes of distilled water. Heavy debris is allowed to settle, and the suspension cleared by low-speed centrifugation (1,000 x *g* for 5 min). Liquid samples (cerebrospinal fluid, nasopharyngeal washes, saliva, tears, and urine) may be used directly. If required, an equal volume of double concentration fixative may be mixed with the suspension to inactivate any infectious agents present before mounting the sample on the coated grid. A grid is floated with the coated surface on a drop of fixed suspension for 0.5–2 min and excess material wicked away with an edge of filter paper (Figure 4A,B). If bacteria are to be negatively stained, higher

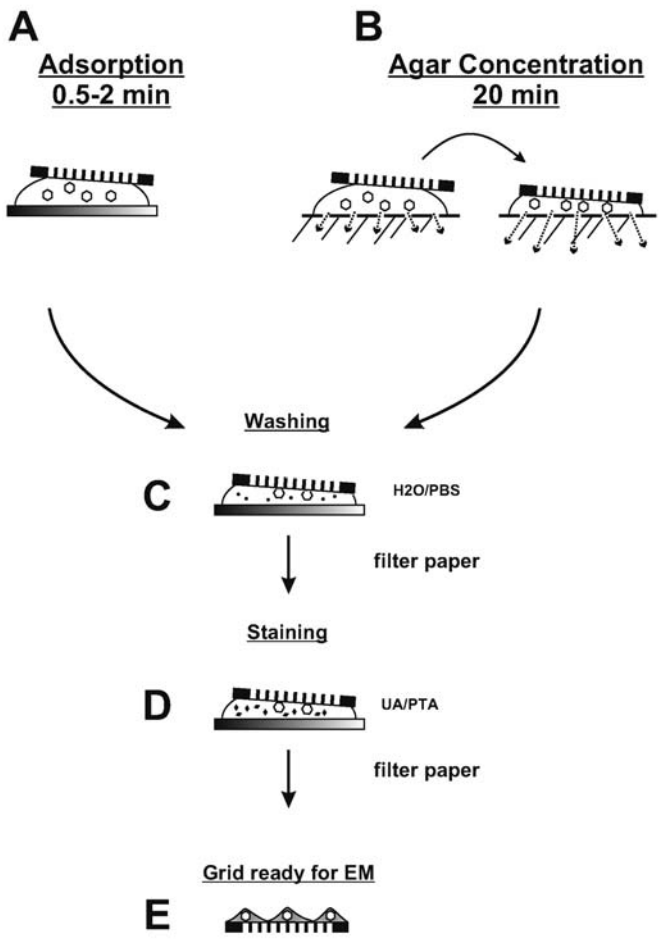

Figure 4. Two-step staining preparation of suspension samples. See text for details.

numbers of microorganisms will attach to the grid because of sedimentation when the drop is placed on the grid. Adsorption is not an absolute process. Any extra manipulations, such as washing the grid, may reduce the number of adsorbed particles. Pretreating the carbon-reinforced grids by glow discharge, poly-L-lysine, alcian blue, or UV light may also help for tighter binding (32,35) and is particularly useful when staining aldehyde-inactivated samples. Direct touch lesion fluid preparations, which are already mounted on the grid, may be rehydrated and inactivated before staining by floating the grid on a drop of fresh 2% formaldehyde.

Rapid immunologic methods that improve sensitivity when searching for unknown agents include solid-phase immunoelectron microscopy (SPIEM) (38) and serum in agar (SIA) (39), both of which may use either pooled human immunoglobulins (HuIgG) or specific antibodies. HuIgG may be obtained from most immunologic suppliers or hospital pharmacies. SPIEM concentrates antigens on the grid by immune capture, thereby improving the probability of observing an etiologic agent. The coated surface of a grid is floated on a drop of pooled HuIgG (100 µg/mL and 20 µg/mL in phosphate-buffered saline [PBS] B) or antiserum (1/100 and 1/500 in PBS) for 10 min, washed on 6 sequential drops PBS, and floated on the specimen for 30–60 min at 37°C. The sample may be stabilized after SPIEM with 0.1% glutaraldehyde to ensure tight binding of the captured antigens, washed on 6 drops of PBS, negative stained, and examined (38). SIA uses immunoaggregation to identify antigens. In addition, type-specific antisera may be used in SIA to serotype the agent present. Antibody (1/100 for antisera and 100 µg/mL for HuIgG) is prepared in cooled 1% agar. A grid is placed on the solidified agar, and a drop of sample placed over the grid. Diluent diffuses into the agar while antibody diffuses into the suspension and antigen:antibody complexes form, which then adsorb to the grid as diluent volume is reduced (Figure 4B) (39).

Negative Staining

Biologic structures, because of low mass density, interact weakly with electrons used for imaging, and therefore, show little contrast or detail. Several ways exist to generate sufficient image contrast and resolution; the most versatile is positive and negative staining with heavy metal ions, e.g., lead, tungsten, and uranium ions. Positive staining depends on chemical reactivity with the components of the object and involves fixation, postfixation, embedding in resins, ultrathin sectioning, and multiple staining incubations. These procedures may take 4–5 days before a sample is ready for examination. Rapid embedding protocols can reduce the time to approximately 1 day but with a loss in specimen quality (32). In contrast, negative staining is simple, rapid, and well suited for examination of small particulate suspensions. A coated grid with sample adsorbed to the surface is floated on a drop of negative stain for 0.5–2 min, excess stain wicked away with a piece of filter paper, air dried for 1–3 min, and examined by electron microscopy (Figure 4D). Structures on the grid are surrounded and stabilized by the drying stain. Thus, they appear as transparent, highly detailed negative images within a dark halo of stain (Figure 5B).

The most common negative stains are 1% (60 mM) aqueous uranyl acetate, pH 2-4.5, and 1% (2.5 mM) phosphotungstic acid, pH adjusted to 7.0 with NaOH. Aqueous uranyl acetate is unstable at higher pH values. Because aqueous uranyl acetate and phosphotungstic acid differ in staining properties, both stains should be applied in parallel in case of unknown

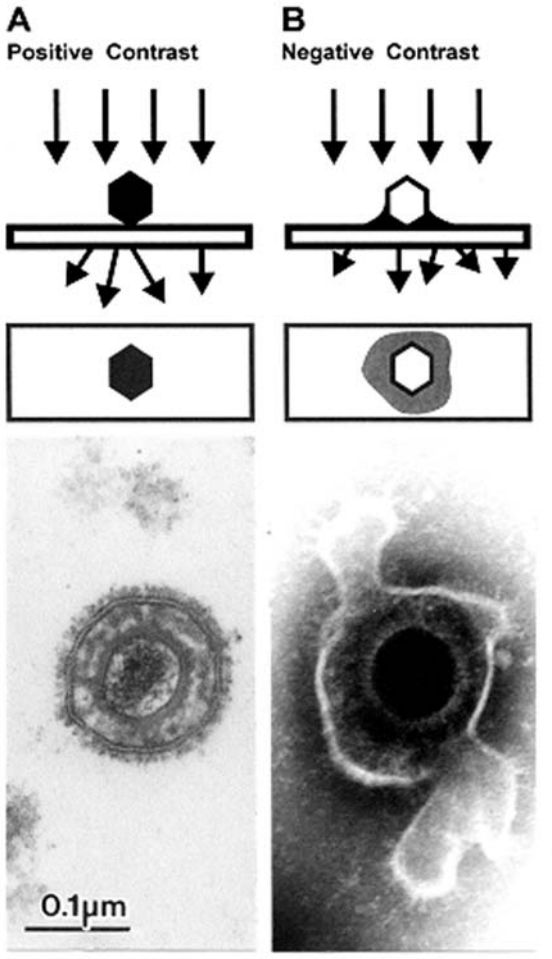

Figure 5. Comparison of herpesvirus appearance after positive and negative stain electron microscopic. A. Positive staining. Samples undergo a lengthy process of fixation, incubation with heavy metal ions (osmium, uranyl), dehydration, embedment, ultrathin sectioning, and staining. Chemical moieties in the object show differential affinities for the heavy metal stains, resulting in a clear outline of the viral bilayer envelope, viral envelope proteins, nucleocapsid, and the dense nucleic acid containing core. B. Negative staining. After a brief fixation, samples are mounted directly on electron microscopic grids and stained as in Figure 4. The electron-dense stain (phosphotungstic acid [phosphotungstic acid], uranyl acetate, and the like) penetrates the virion and embeds the particle in a matrix of stain. Due to density differences between the stain and weakly scattering biological components of the virion, the virion appears as a transparent and detailed reverse (negative) image. Penetration of stain into the nucleocapsid provides a dense core with the crenellated appearance presented by the central channel of capsomers on the nucleocapsid surface. Viral surface proteins appear as projections from the labile envelope. Phosphotungstic acid stained. Bar = 100 nm.

samples. Stains should be relatively fresh and stored in brown glass bottles at 4°C (32,35,36). While the stained grid is being examined, additional grids may be left floating on the sample droplet, protected from dust and drying. This method reduces preparation time in the event additional grids must be prepared for electron microscopic inspection.

Particle Enrichment

If no virus has been identified after 20 min or after the examination of 10 grid squares, the result may be considered to be "no etiologic agent identified." Routine two-step drop preparations for electron microscopic diagnostic procedures require particle concentrations of 10^6 to 10^8/mL. Therefore, negative evidence is not an absolute diagnosis. A number of effective concentration or immunologic procedures exist that markedly increase sensitivity of electron microscopic diagnostics for samples with lower particle concentrations (32,40). These procedures take from 0.5 to 16 hours and are labor and training intensive. Viral research or diagnostic facilities generally have access to at least one advanced procedure. Nonimmunologic procedures include: a) ultracentrifuge concentration—the material from cleared suspensions is sedimented by ultracentrifugation, resuspended in a smaller volume and then prepared by the standard two-step drop method (32); b) agar diffusion—a 20–50 [20- to 50-µL drop of suspension is placed on 1% agar. As the fluid is absorbed the virus is concentrated. After 15–20 min, a grid is placed on the remaining suspension and then stained as with the two-step method above (Figure 4D). This procedure will result in an enrichment factor of approximately 5x (32); and c) direct centrifugation to the electron microscopic grid with the Beckman Airfuge (Beckman, Palo Alto, CA) EM-90 rotor or A-100 rotor, a procedure that increases sensitivity up to 1,000 fold (40–42). Immunoaggregation and immunodecoration with type- and genus-specific antibody may be used to concentrate material or to specifically identify the agent, e.g., herpes simplex 1 and 2 and varicella zoster. Also, convalescent-phase serum samples may be used to identify infectious agents or provide evidence of seroconversion to the agent when paired with acute-phase sera. For standard immunoelectron microscopy, the suspension is incubated for 1 h at 37°C with serum samples diluted in PBS, and then mounted on the grid by using either the drop method or direct centrifugation to the grid. Immunoaggregation may be very powerful in the identification of a suspected or novel agent or with small, dispersed virions (7,13,16). Immunoelectron microscopy was particularly useful in the initial identification of noncultivable agents such as hepatitis C, Norwalk virus, and Winnipeg virus (7,13,43). Detailed methods may be found in references (29,32,35,44).

As with all diagnostic laboratory procedures, diagnostic electron microscopy should be performed in a quality-controlled manner. For routine external quality control, the Konsiliarlaboratorium für EM-Erregerdiagnostik at the Robert Koch-Institut in Berlin has conducted an External Quality Assurance-EM Virus Program, which provides panels of specimens containing different agents, since 1994 (www.rki.de/INFEKT/CONSULLAB/EM-DIAG). More than 95 laboratories from 27 countries participated in EQA-EMV 11 during August and September 2001. Each laboratory used its preferred method for preparation (45). A review of results submitted from participating facilities indicated that 27 of 69 laboratories correctly identified all test samples, while an additional 28 successfully identified four of five positive specimens. A trend towards higher success existed among laboratories that used enrichment procedures (35 of 55) when compared with those that were less successful (4 of 14) ($p=0.055$). However, experience, as defined by years of service and number of samples examined annually, was another important success factor.

Identification of Viral Agents

Several major pitfalls exist in the identification of viral agents by negative stain electron microscopy. First, the failure to detect and identify an agent does not mean that it is not there. Second, if you look long enough and hard enough, you will eventually find something that resembles what you wish to find. Third, the presence of a single picture cannot validate the interpretation of morphology. While the diagnostician must not be afraid to find something novel, the finding must be real. One example is the observation of multiple particles with similar morphology. In addition, photographic records must be made for all possible positive identifications and reviewed to confirm the accuracy of the initial diagnosis. Further, when a particle is assigned to a proper virus family, reviewing the case may be necessary to identify the genus or strain. For example, not all samples with orthopoxvirus morphology will be smallpox (Figure 6). While natural infections of variola virus have been eradicated, many other orthopoxvirus continue to be found and identified, e.g., camel-, cow-, monkey-, mouse-, and vaccinia pox viruses (17,46). In addition, the molluscipoxvirus Molluscum contagiosum is morphologically indistinguishable from orthopoxviruses. Identification of Molluscum contagiosum was essentially non-existent in Winnipeg before 1983. With the growth of the immunocompromised sector of the population, the number of identifications increased to 6–10 cases per year until 1995, when the change in sampling methods from lesion aspirates to swab collection in transport medium resulted in a reduction to 1–2 Molluscum contagiosum identifications per year (Hazelton, unpub. data). Further differentiation of poxviruses into variola, vaccinia, cowpoxviruses, or molluscipoxvirus may be performed by immuno electron microscopy with type-specific antibodies. Appropriate antibodies and the latest nucleic acid amplification techniques are also available for this determination at the World Health Organization Collaborating Centers at the Centers for Disease Control and Prevention, and VECTOR, Koltsovo, Novosibirsk Region, Russia.

Future Impact of Diagnostic Electron Microscopy

Compared with other laboratory diagnostic methods, electron microscopy excels with respect to rapidity and the open

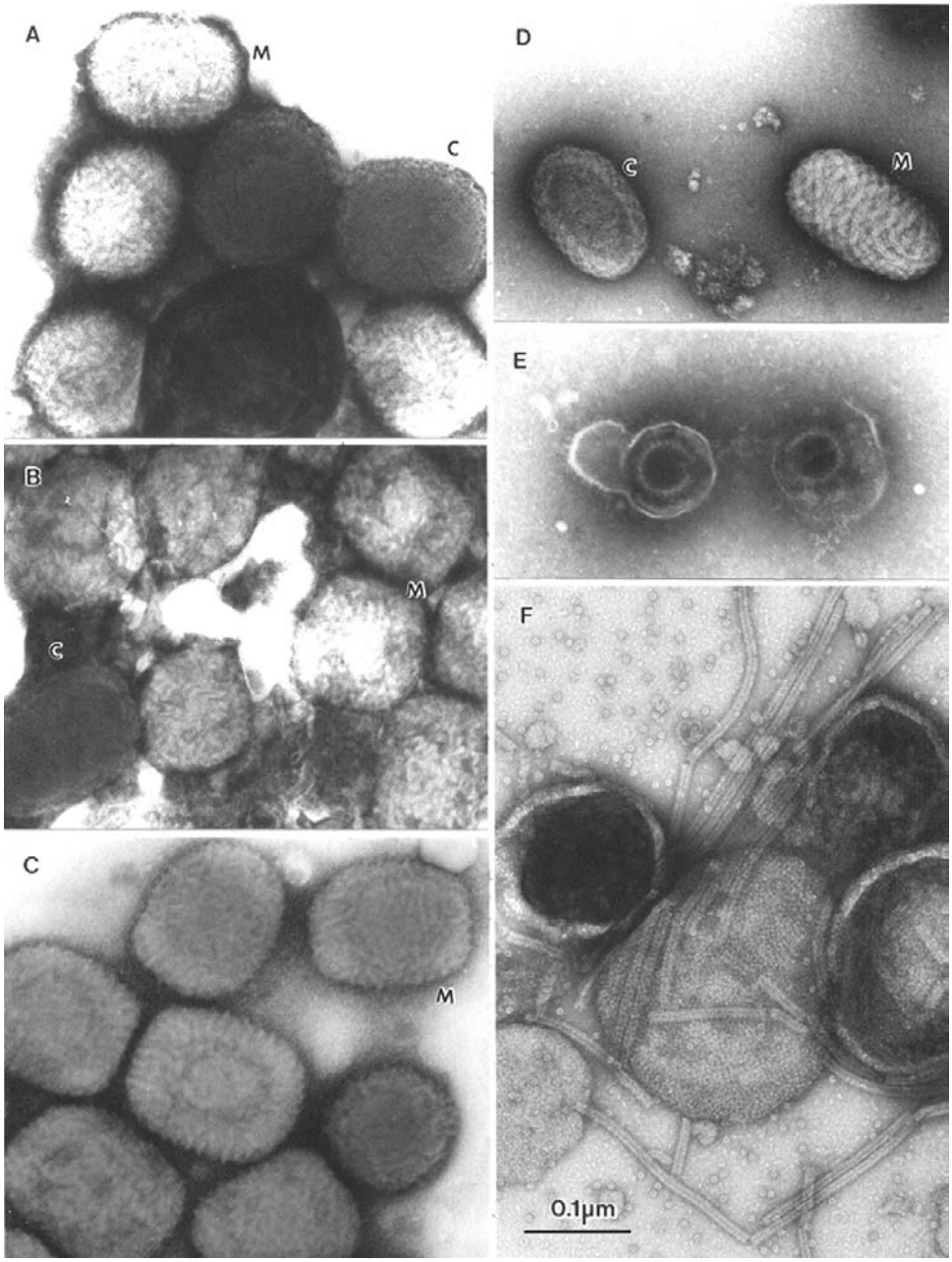

Figure 6. A–E. Comparison of clinically relevant viral agents associated with skin lesions. A–C show poxviruses indistinguishable in appearance from variola virus, the agent of smallpox. The slightly rounded, brick-shaped virions measure about 270 by 350 nm. Two types of particles may be seen. M, or mulberry forms show a 10- to 20-nm diameter short-tubular or beaded surface (M). Capsular, or C forms, partly penetrated by the stain, are recognized by a 30-nm membrane (C): A. Molluscum contagiosum (molluscipoxvirus) virions from skin lesions observed in an adult; B. Vaccinia virus vaccine strain WR (orthopoxvirus) from cell culture; C. Ectromelia virus (orthopoxvirus) from culture material. D. Parapox viruses measure up to190 by 300 nm and are more distinctly ovoid. Tubules, 10 to 20 nm wide and approximately 1,000-nm long, spiral around the virion, giving a distinctive crosshatched appearance. E. Herpesvirus particles from a skin lesion of a primary varicella zoster infection observed in an adult. Direct electron microscopy shows two virions. The envelopes are broken, liberating the 100-nm nucleocapsid. F. Cell culture supernatant from a patient with an infantile respiratory tract infection. The enveloped virions are studded with tiny surface spikes. The 18-nm helical nucleocapsids have been released from disintegrating virions. The nucleocapsids and envelope details are typical of paramyxoviruses. A–B, phosphotungstic acid, C–F, uranyl acetate. All prints at the same magnification, bar = 100 nm.

view that permits detection and identification of both novel agents and those not considered by the clinician. However, full exploitation of this potential requires early and coordinated application of electron microscopy with other frontline diagnostic procedures. The use of electron microscopy to examine diagnostic cultures of Hendra virus provided evidence of a paramyxovirus 3 days before any other results were available. Thus, focusing further characterization on the proper virus family was possible, and a novel pathogenic agent, which became the prototype strain for the henipah viruses, a proposed new genus of paramyxoviruses, was found (17,47). Diagnostic electron microscopy does not need to be either expensive or difficult to perform if executed in a diagnostic network, i.e., by recruiting instruments and electron microscopists working in other departments, e.g., cell biology or pathology (48). Respective arrangements are facilitated by using inactivated samples and implementing new technologies, such as automated pattern recognition (49) and telemicroscopy by using digital image acquisition and remote operation of the instrument or review of micrographs through the Internet (50).

As with smallpox diagnosis from the 1940s to the 1970s, electron microscopy differential diagnosis has often ruled out the occurrence of dangerous pathogens. The power to rapidly identify agents of bioterrorism has now been demonstrated convincingly by Tom Geisbert and Peter Jahrling, U.S. Army Medical Research Institute of Infectious Disease, when they identified and quantified spores in the B. anthracis bioterrorist letter attack upon U.S. Senate Majority Leader Daschle

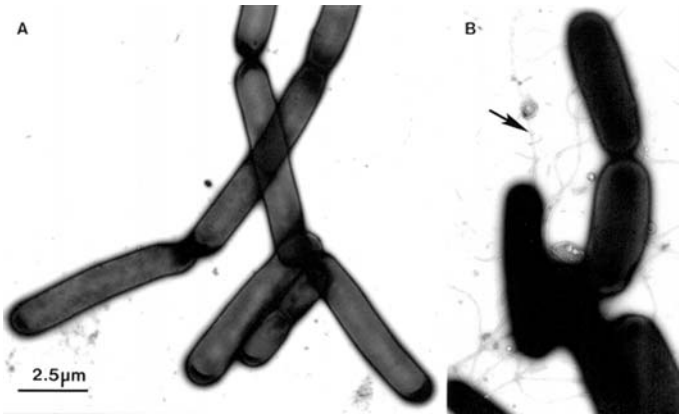

Figure 7. A. A colony of *Bacillus anthracis* was suspended, inactivated, and negatively contrasted with aqueous uranyl acetate, as described for Figure 4. The microorganisms, which grow in long chains, do not have flagella. B. The ubiquitous *B. subtilis* may also grow as long chains. However, in contrast to *B. anthracis*, the *B. subtilis* cells show distinct flagella (arrow). Bar = 2.5 µm.

(Jahrling, pers. commun.) (Figure 7). Because the unusual and unexpected can be rapidly identified, electron microscopy must remain a frontline method for rapid diagnostic virology, investigation of potential bioterrorist events, and investigation of new and unusual cases of suspected infectious origin.

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Influenza AH1N2 Viruses, United Kingdom, 2001–02 Influenza Season

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During the winter of 2001–02, influenza AH1N2 viruses were detected for the first time in humans in the U.K. The H1N2 viruses co-circulated with H3N2 viruses and a very small number of H1N1 viruses and were isolated in the community and hospitalized patients, predominantly from children <15 years of age. Characterization of H1N2 viruses indicated that they were antigenically and genetically homogeneous, deriving the hemagglutinin (HA) gene from recently circulating A/New Caledonia/20/99-like H1N1 viruses, whereas the other seven genes originated from recently circulating H3N2 viruses. Retrospective reverse transcription-polymerase chain reaction analysis of influenza A H1 viruses isolated in the U.K. during the previous winter identified a single H1N2 virus, isolated in March 2001, indicating that H1N2 viruses did not widely circulate in the U.K. before September 2001. The reassortment event is estimated to have occurred between 1999 and early 2001, and the emergence of H1N2 viruses in humans reinforces the need for frequent surveillance of circulating viruses.

The influenza A virus genome consists of eight single-stranded RNA segments of negative sense. The segmented nature of the genome allows reassortment of genes between different influenza A strains infecting one host, which may generate novel antigenic variants and give rise to pandemics of disease in humans. Although the influenza pandemic of 1918 appears to have followed the introduction of an avian-like H1N1 virus into the human population (1), the H2N2 and H3N2 viruses responsible for the 1957 and 1968 human pandemics, respectively, were generated by reassortment between human and avian viruses (2–4). Since the last influenza pandemic of 1977, which was caused by the reemergence of the H1N1 subtype, two subtypes of influenza A (H1N1 and H3N2) have been co-circulating in humans together with influenza B viruses.

The co-circulation of influenza A H1N1 and H3N2 viruses in humans has led to sporadic reports of the isolation of H1N1-H3N2 reassortant viruses in humans (5–10). In contrast, after their isolation from pigs in 1994 (11,12), influenza A H1N2 reassortant viruses, derived from human and avian viruses, have become established in swine throughout the U.K. Influenza viruses of H1N2 subtype, derived from genetic reassortment of strains endemic in pigs, have also been established in pigs in Japan since 1978 (13,14) and, more recently, in France (15) and North America (16).

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During 1988–89, several H1N2 viruses were isolated from humans in China (9,10). Genetic analysis of these reassortant viruses indicated that only the hemagglutinin (HA) gene was derived from the prevailing human H1N1 virus, whereas all other genes, including the neuraminidase (NA) gene, were introduced from the prevailing human H3N2 strain. These reassortant viruses did not spread to other countries until 2001; further isolations of influenza A H1N2 viruses in humans have not been documented.

Influenza AH1N2 viruses were detected for the first time in humans in the U.K. during the winter of 2001–02. We examine the diversity of H1N2 viruses emerging in the U.K. over a 12-month period in 2001–02. The antigenic and genetic properties of these viruses are described, as well as the relationship of reassortant viruses to parental H1N1 and H3N2 strains. Furthermore, the impact of the circulation of H1N2 viruses on disease in the community is considered, as well as the implications for diagnostic testing of influenza strains.

Materials and Methods

Epidemiologic Data

Data on the rates of community cases of influenza and influenzalike illness were collected by the Birmingham Unit of the Royal College of General Practitioners and are based on weekly returns from approximately 69 sentinel practices throughout England and Wales. The Communicable Disease Surveillance Centres in Wales and Northern Ireland and the Scottish Centre for Infection and Environmental Health provided additional data. These three mechanisms for monitoring clinical incidence of influenzalike illness cover a total population of 800,000 persons. Incidence data were recorded as new physician consultations per 100,000 persons.

Source of Isolates

Influenza viruses isolated in hospital laboratories throughout the U.K. were sent to the Enteric, Respiratory and Neurological Laboratory at the Central Public Health Laboratory for antigenic characterization. Throughout the influenza season (October–March), a subset of the clinical practices group in the Birmingham Unit of the Royal College of General Practitioners network obtained nose and throat swabs from patients who had influenzalike illness (17), which were sent directly by mail to Enteric, Respiratory and Neurological Laboratory for virus isolation and subsequent antigenic and genetic characterization. All specimens were given a laboratory sample number upon arrival.

Clinical Sample Inoculation

Combined nose and throat swab specimens in virus transport medium were treated as previously described and inoculated onto confluent Madin-Darby canine kidney cells (18). The cells were incubated at 33°C, and the medium was tested at day 7 for HA of turkey red cells.

Virus Typing

Influenza viruses were typed by using ferret antisera in hemagglutination inhibition tests as described (19). All HI tests were carried out by using 8 HA U of virus and 0.5% (v/v) turkey red blood cells. All ferret sera were treated with receptor-destroying enzyme. After typing, virus isolates were given a unique strain designation number in strict chronologic order.

Amantadine Sensitivity Assays

Susceptibility of influenza virus replication to inhibition by amantadine was determined by virus infectivity assays (20). Madin-Darby canine kidney cells were overlaid with medium containing amantadine (SigmaAldrich, Poole, Dorset, England) at a concentration of 0.1, 1.0, or 10.0 μg/mL.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Nucleotide Sequencing

Viral RNA was extracted from 150-μL samples by using the MagNA Pure LC total nucleic acid isolation kit, on a MagNA Pure LC extraction robot (Roche Molecular Biochemicals, Mannheim, Germany) (21). Reverse transcription was performed as previously described (20). PCR to detect N1 and N2 neuraminidase genes was performed with nested primer sets (primer sequences available on request), modified from previously described assays (22).

Viruses selected for genetic analysis are shown in Table 1. Amplification of the HA1 domain of the HA gene, the complete coding region of the NA gene and portions of the PB2, PB1, PA, NP, NS, and M genes required primers specific for the eight genes (primer sequences available by request). The regions amplified for sequence analysis were PB2, 46-487; PB1, 970-1427; PA, 499-817; HA, 84-1058; NP, 1045-1428; NA, 20-1426; M, 249-612; and NS, 146-843. PCR products were purified by using agarose gel electrophoresis and a QIAquick gel extraction kit (Qiagen Ltd, Crawley, West Sussex, England) and sequenced by using a Beckman Coulter CEQ 2000 capillary sequencer and CEQ 2000 Dye Terminator cycle sequencing Quick Start kit (Beckman Coulter, Fullerton, CA). The fragment lengths compared were PB2, 442; PB1, 458; PA, 319; HA, 975; NP, 384; NA, 1407; M, 364; and NS, 698 nucleotides. Nucleotide sequences determined in this study are available from the European Molecular Biology Laboratory database (accession nos. AJ489485–AJ489502, AJ489530–AJ489559, and AJ489846–AJ489862). The nucleotide sequences for the NA, M1, and NP of A/Panama/2007/99 and A/Moscow/10/99 viruses are available in the European Molecular Biology Laboratory database under accession numbers AJ457937, AJ457966, AJ458298, AJ458297, AJ458268, and AJ458267 (23).

Phylogenetic Analysis

Sequences were analyzed by neighbor joining with the Phylip (version 3.57) suite of programs (DNADist and Fitch) and bootstrapping by Seqboot (24). Bootstrap values >70 were regarded as statistically significant.

Table 1. Influenza A H1N1 and H1N2 U.K. viruses selected for genetic analysis

Virus	Sample date	Region	Source	Gene analyzed
H1N1				
A/England161/2002	9/2/02	South	Hospital	HA
H1N2				
A/England/627/2001	19/3/01	South	Hospital	PB2, PB1, HA, NP, NA, M, NS
A/Scotland/122/2001	27/9/01	Scotland	Hospital	PB2, PB1, HA, NP, M
A/England/689/2001	24/12/01	Not known	Hospital	HA
A/England/691/2001	21/12/01	Central	Hospital	PB2, PB1, PA, HA, NP, NA, M, NS
A/England/1/2002	4/1/02	Central	Hospital	NA
A/England/3/2002	8/1/02	Central	Hospital	PB2, PB1, NP, M, NS
A/England/5/2002	9/1/02	Central	Community	PB2, PB1, PA, NP, M, NS
A/England18/2002	15/1/02	Central	Community	HA
A/England/57/2002	16/1/02	South	School	PB2, PB1, PA, NP, NA, M, NS
A/England/73/2002	30/1/02	South	School	PB2, PA, NP, M, NS
A/England/90/2002	24/1/02	North	School	PB2, PB1, PA, NP, M, NS
A/England/97/2002	1/2/02	North	Hospital	PB2, PB1, NP, NA, M, NS
A/Scotland/15/2002	19/2/02	Scotland	Hospital	HA
575/2001[a]	13/12/01	Central	Hospital	HA
576/2001[a]	13/12/01	Central	Hospital	HA
1352/2002[a]	24/1/02	North	School	HA
1496/2002[a]	30/1/02	North	Community	NA
1660/2002[a]	6/2/02	Central	Hospital	HA

[a]Indicates where virus was not isolated by culture from polymerase chain reaction--positive material.

Results

Epidemiology

During the period September 2001–March 2002, clinical death indices for the measurement of influenzalike illness in England and Wales did not rise above baseline levels, indicating low influenza activity (Figure 1). Very low levels of influenza activity were also recorded in Scotland (data not shown). The clinical indices of influenzalike illness activity peaked in week 6 and correlated well with the peak of influenza viruses detected and typed by Central Public Health Laboratory (data not shown; available from: URL: http://www.phls.co.uk).

In September 2001, the first isolate of the influenza season (A/Scotland/122/2001), an H1N2 virus, was isolated from a 1-year-old child. H1N2 viruses continued to be isolated from the patients in the community and in hospitalized patients throughout the U.K. until the end of March 2002. H1N2 reassortant viruses co-circulated with a relatively equal proportion of H3N2 viruses throughout the season and a small number of H1N1 viruses. Of 420 influenza A viruses isolated and characterized in the winter of 2001–02, 54.0% were influenza A H1N2, 45.2% H3N2, and 0.7% A H1N1 subtype.

During the previous influenza season (2000–01), H1N1 and influenza B viruses co-circulated in the U.K., and no H3N2 viruses were detected. In comparison, only a small number of influenza B viruses were isolated during the winter of 2001–02 (data not shown; available from: URL: http://www.phls.co.uk).

Retrospective analysis using RT-PCR to determine the NA subtype, on 198 of 323 influenza A H1 viruses isolated during 2000–01, identified only a single H1N2 subtype virus isolated from a 9-year-old child in March 2001. Therefore, before September 2001, H1N2 viruses were not widely circulating in the U.K.

Clinical Impact of H1N2 Viruses

Despite the fact that H1N2 viruses emerged and were as frequently isolated as H3N2 viruses during the winter of 2001–02 in England, the levels of clinical influenzalike illness activity were among the lowest in the last 15 years (Figure 1). This low level indicates that the new H1N2 strain was not associated with particularly severe influenzalike illness activity. The low levels of influenzalike illness activity were also associated with correspondingly low levels of excess death rates (death from all causes), (available from: URL: http://www.phls.co.uk). More than 75% of H1N2 virus isolates were obtained from children <15 years of age (Figure 2), indicating that the major age group affected by the H1N2 viruses was young persons, possibly undergoing a primary infection. A similar proportion of H3N2 viruses isolated were also from children <15 years of age. Few H1N2 virus isolates were obtained from adults, and only a limited number were obtained from adults >65 years old. These facts suggest that the young are most susceptible to H1N2; adults and vaccinated elderly appear to have adequate protective immunity to the new subtype. Age-specific consultation data for influenzalike illness in 2001–02 confirm that the age range most severely affected by influenzalike illness was the 5–14 age group (data not shown; available from: URL: http://www.phls.co.uk).

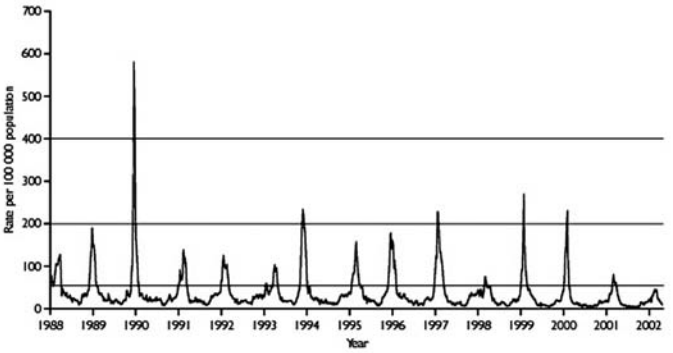

Figure 1. Consultation rate (per 100,000 population) for influenzalike illnesses with sentinel physicians in England in 1988–2002 (from the Royal College of General Practitioners Weekly Returns Service). Baseline activity is defined by a consultation rate <50/100,000; normal seasonal activity, 50–200/100,000; higher than seasonal activity, 200–400/100,000, and epidemic activity is defined as >400/100,000 population.

Antigenic Analysis of H1N2 Viruses

Antigenic characterization of the 228 H1N2 viruses isolated during 2001–02 was performed by using hemagglutination inhibition tests with postinfection ferret antisera to influenza A H1N1 and H3N2 reference and vaccine strains (Table 2). The HA gene of the H1N2 viruses was antigenically related to that of the H1N1 strain used in vaccines in 2001–02, A/NewCaledonia/20/99, and was antigenically indistinguishable from that of co-circulating A/NewCaledonia/20/99-like H1N1 viruses. No significant indication of antigenic drift in the

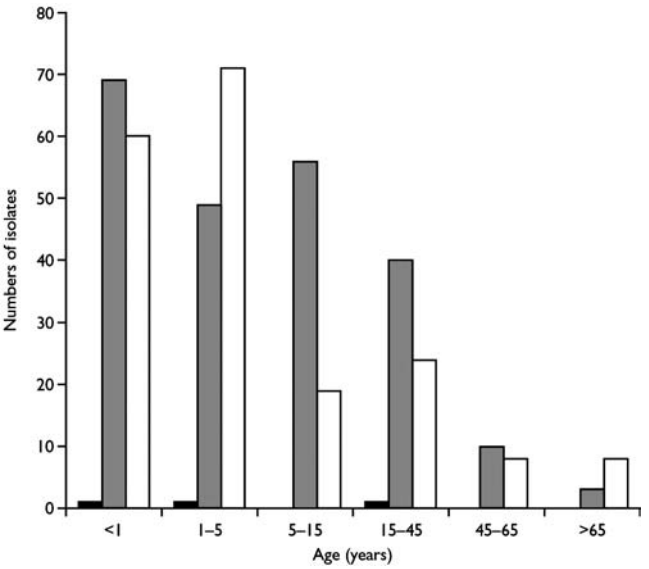

Figure 2. Age distribution of patients from whom influenza A viruses were isolated during 2001–02 in the U.K . ■H1N1 isolates; ■H1N2 isolates □H3N2 isolates.

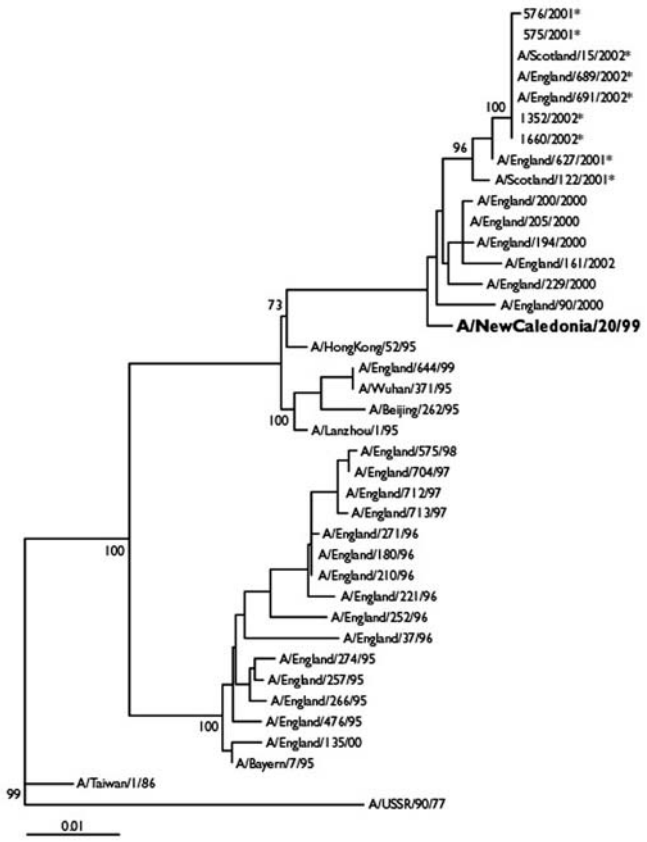

Figure 3. Phylogenetic tree of influenza A H1N1 and H1N2 virus HA1 nucleotide sequences. The tree was generated by using neighbor-joining analysis. The lengths of the horizontal lines are proportional to the number of nucleotide substitutions per site. Trees were bootstrapped x100. H1N2 viruses are indicated with an asterisk. The current H1N1 vaccine strain is in bold typeface.

HA genes of these viruses was observed over the period the viruses were isolated (March 2001–March 2002) (Table 2).

Sequence Analysis of HA and NA Genes

Phylogenetic analysis indicated that the HA1 sequences of the H1N2 viruses analyzed (Table 1) were most closely related (sequence similarity of 98.7% to 99.1%) to those of the H1N1 vaccine strain, A/NewCaledonia/20/99 (Figure 3). The HA1 sequences of the H1N2 viruses analyzed were very similar, exhibiting only 0%–1.2% nucleotide divergence between sequences. This divergence is in contrast to that of A/NewCaledonia/20/99-like H1N1 viruses isolated in the U.K. during 2000–01, which exhibited 0.3%–3.1% nucleotide divergence in the HA1.

Of the four amino acid differences previously observed in the HA1 sequences of H1N2 viruses isolated from various countries and compared to A/NewCaledonia/20/99 (23), all U.K. H1N2 viruses analyzed had the substitutions V169A and A193T. All but the earliest U.K. H1N2 viruses had the substitutions V178I and A218T in the HA1.

The NA genes of six H1N2 viruses analyzed (Table 1) were closely related genetically to the NA genes of recently circulating H3N2 viruses, represented by A/Moscow/10/99 (99.1% nucleotide sequence homology). All analyzed H1N2 NA gene sequences from the U.K. differed from those of A/Moscow/10/99 at the three amino acid positions observed in the NA of H1N2 viruses isolated from other parts of the world (23). In addition, with the exception of the earliest U.K. H1N2 isolate (A/England/627/2001), all analyzed H1N2 NA sequences from the U.K. had the substitution M24T, which is located in the transmembrane region of the protein (25).

We observed no mutations in the NA or HA genes that have been reported to confer resistance to NA inhibitors (26,27). None of the amino acid substitutions in the HA or NA genes resulted in the loss or creation of potential glycosylation sites in the HA or NA surface proteins of the H1N2 viruses.

Genetic Variation in the Internal Protein Genes

A region of the M1 gene of nine H1N2 isolates (Table 1) was analyzed and compared with sequences of prototype H1N1, H2N2, and H3N2 viruses, in addition to M1 gene sequence data obtained from influenza viruses isolated in the U.K. since 1951. Little sequence divergence was observed between the M1 sequences of the H1N2 viruses (0% to 0.9%), and they were most closely related to those of recent H3N2 viruses. No amino acid differences were seen between the partial M1 sequences of H1N2 viruses and those of A/Panama/2007/99, whereas one amino acid difference (R174K) was found from the M1 of A/Moscow/10/99. This substitution was first observed in H3N2 strains isolated in England in 1996 and was fixed in H3N2 viruses isolated after 1998 in the U.K.

Partial gene sequencing of the PB2, PB1, PA, NP, and NS genes indicated that the analyzed H1N2 viruses (Table 1) also derived each of these genes from a virus of the H3N2 subtype. Very little sequence divergence was observed in these five genes of the H1N2 viruses analyzed.

Amantadine Sensitivity

The susceptibility of H1N2 virus replication to inhibition by amantadine was determined. All of the analyzed H1N2 viruses (A/Scotland/122/2001, A/England/45/2002, and A/England/63/2002) were susceptible to inhibition of virus growth by amantadine, with MIC 50 <0.1μg/mL of drug.

Discussion

In the winter of 2001–02, which was a mild influenza season, influenza A H1N2 viruses were detected for the first time in the U.K. Analysis of the diversity of >200 H1N2 isolates from the U.K indicated that they were closely related antigenically and genetically and derived the HA gene from A/NewCaledonia/20/99-like H1N1 viruses, whereas the other seven genes originated from recently circulating H3N2 viruses. The H1N2 viruses were isolated throughout the influenza season and co-circulated with H3N2 viruses. In contrast to the previous winter's results, few influenza A H1N1 viruses were detected. Retrospective RT-PCR analysis of 61.4% of influenza

Table 2. Antigenic analysis of influenza A H1N2 by hemagglutination inhibition assays

		Post infection ferret antisera				
		A/Wuhan	A/Bay	A/NewCal	A/Moscow	A/Pan
Virus	Sample date	371/95	7/95	20/99	10/99	2007/99
H1N1						
A/Wuhan/371/95		1,280	320	80	<40	<40
A/Bayern7/95		80	5,120	1,280	<40	<40
A/NewCaled/20/99		160	80	5,120	<40	<40
A/England/70/2002	29/1/02	160	40	1,280	<40	<40
A/England/47/2002	29/1/02	80	40	320	<40	<40
H1N2						
A/England/627/2001	19/3/01	160	80	640	<40	<40
A/Scotland/122/2001	27/9/01	160	160	2,560	<40	<40
A/England/691/2001	18/12/01	80	40	1,280	<40	<40
A/England/46/2002	29/1/02	40	<40	320	<40	<40
A/England/334/2002	6/3/02	80	<40	1,280	<40	<40
H3N2						
A/Moscow/10/99		<40	<40	<40	640	320
A/Panama/2007/99		<40	<40	<40	80	640
A/England/687/2001	29/11/01	<40	<40	<40	2,560	1,280
A/England/12/2002	16/1/02	<40	<40	<40	2,560	320
A/Scotland/2/2002	23/1/02	<40	<40	<40	5,120	1,280

AH1 viruses isolated during 2000–01 in the U.K. identified one H1N2 virus that had been isolated in March 2001, indicating that H1N2 viruses had not circulated widely in the U.K. before becoming established in autumn of 2001. H1N2 viruses have also been identified during 2001–02 from outbreaks of influenza in different countries in Africa, America, Asia, and Europe (28). The earliest H1N2 viruses identified worldwide retrospectively were also isolated in March 2001 in Saudi Arabia (23).

During the past decade, influenza A H1N1 viruses have circulated intermittently in the U.K. (29). Between October 2000 and April 2001, a season of low influenza activity in the U.K., influenza A H1N1 viruses were the predominate influenza A strain circulating. Most H1N1 viruses were antigenically closely related to the vaccine strain used in the 2000–01 influenza season, A/NewCaledonia/20/99. The circulation of H1N2 reassortant viruses appears to have displaced A/NewCaledonia/20/99-like H1N1 viruses, although circulation of H1N2 does not appear to be associated with the generation of viruses with antigenically different surface proteins since the HA and NA of H1N2 viruses are antigenically similar to those of recently circulating H1N1 and H3N2 viruses. The observation that H1N2 viruses were isolated mainly from children <15 years of age suggests a preexisting immunity to H1 and N2 in the population (Figure 2). Furthermore, a similar proportion of the H3N2 viruses isolated during 2001–02 (79%) were also isolated from children <15 years old, with A/Panama/2007/99-like H3N2 viruses circulating during the same period of time as the A/NewCaledonia/20/99-like H1N1 viruses in the U.K. This conclusion is supported by the observational disease data in which the age group most severely affected by the influenza viruses circulating was the 5–14 age group and suggests that disease associated with both H1N2 and H3N2 was mainly in children acquiring a primary infection. In contrast, during the 2000–01 winter season, when influenza A H1N1 and influenza B viruses co-circulated, the disease data demonstrated that the age groups most affected by influenza viruses were the 5–14 years of age group and 15–44 years of age group (available from: URL: http://www.phls.co.uk). The age distribution of the disease data correlates with the finding that most H1N1 viruses isolated during 2000–01 were from the 5–14 years of age and 15–44 years of age groups.

Our analysis of genetic variation in the HA gene showed that seven amino acid substitutions have accumulated in the HA1 gene of influenza A H1N1 viruses between the circulation of A/Beijing/252/95-like H1N1 viruses in the U.K. and the emergence of viruses similar to the new variant, A/NewCaledonia/20/99, in October 2000. Of these, four mutations (T136S, E156G, S186P, and I194L) are located in two of the five antigenic sites (A and B) of HA1. The emergence of A/NewCaledonia/20/99–like viruses correlates with the finding that new drift variants of epidemiologic importance typically have four or more amino acid substitutions located in two or more of the antigenic sites (30). Additionally, the substitutions A193T and A218T are located in antigenic sites B and D, respectively, of HA1, indicating the potential for further drift in the HA gene of H1N2 viruses and the possible subsequent generation of a new epidemic variant.

Although H1N1 and H3N2 subtypes have co-circulated in the human population since 1977, reassortant combinations of HA and NA subtypes are rare. Reassortant H1N2 viruses have previously been isolated from sporadic cases in humans and were not maintained in circulation in humans (9). The H1 HA–N2 NA combination may provide a better functional match between receptor-binding and receptor-destroying activities of HA and NA, respectively, perhaps providing H1N2 viruses with a fitness advantage over contemporary H1N1, H3N2, or both

viruses. Postreassortment mutations in the HA gene located in the vicinity of the receptor-binding pocket have previously been shown to compensate for any imbalance between HA and NA activities and may be a factor in influenza virus evolution (31,32). Analysis of the HA1 sequences of H1N1 and H1N2 viruses isolated between December 2000 and February 2002 in the U.K. showed three substitutions at positions 178, 193, and 218 that were present only in the HA gene of the H1N2 viruses analyzed; residue 193 situated near to the HA receptor binding pocket. As these three mutations were not observed in the HA1 of recent H1N1 viruses analyzed, the substitutions may have occurred in H1N2 viruses after reassortment.

The NA genes of the H1N2 viruses are closely related genetically to the NA genes of A/Moscow/10/99-like H3N2 viruses. Of the three substitutions observed in the NA genes of H1N2 viruses compared to those of A/Moscow/10/99, residues 199 and 431 are located in antigenic sites on surface of the NA gene (33,34). Residue 199 has also been assigned to one of the 12 phylogenetically important regions of the NA gene (35). All of the amino acid residues that form the sialic acid–binding site of the NA gene were conserved in the NA of H1N2 viruses. Although changes in receptor binding and the interaction between the HA and NA proteins of H1N2 reassortants may have facilitated the emergence of the H1N2 viruses, the contribution of the internal proteins to the replicative efficiency and transmission of these viruses and their interaction with the surface glycoproteins is unknown.

The early detection and characterization of newly emerging influenza variants are two of the primary aims of the World Health Organization global influenza surveillance network. Reassortment between circulating influenza viruses leading to the emergence of a novel subtype, such as H1N2, highlights the need for subtyping of the influenza A viruses isolated. Most current diagnostic tests rely on the detection and typing of the HA of influenza viruses. Only a few laboratories perform analysis of the NA gene (as NA inhibition assays are difficult to perform) or analyze the internal proteins and the genes encoding them. The use of molecular techniques provides a rapid means for the detection and subtyping of influenza viruses, although current PCR assays target only N1 and N2 subtypes (22,36). In addition, heteroduplex mobility assays can be used to genetically characterize the HA and internal genes of influenza viruses (20,37,38). However, although molecular methods aid the rapid detection and identification of influenza viruses, virus isolation by culture is still required for antigenic characterization of influenza viruses. How influenza H1N2 reassortants will evolve and whether H1N2 viruses will be maintained in circulation in humans remain to be seen.

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Dr. Ellis is a clinical scientist in the Enteric, Respiratory and Neurological Virus Laboratory, in the Public Health Laboratory Service. Her current research interests include the development of improved molecular techniques for the diagnosis of influenza infections, and molecular epidemiology of influenza viruses.

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Experimental Infection of North American Birds with the New York 1999 Strain of West Nile Virus

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To evaluate transmission dynamics, we exposed 25 bird species to West Nile virus (WNV) by infectious mosquito bite. We monitored viremia titers, clinical outcome, WNV shedding (cloacal and oral), seroconversion, virus persistence in organs, and susceptibility to oral and contact transmission. Passeriform and charadriiform birds were more reservoir competent (a derivation of viremia data) than other species tested. The five most competent species were passerines: Blue Jay (*Cyanocitta cristata*), Common Grackle (*Quiscalus quiscula*), House Finch (*Carpodacus mexicanus*), American Crow (*Corvus brachyrhynchos*), and House Sparrow (*Passer domesticus*). Death occurred in eight species. Cloacal shedding of WNV was observed in 17 of 24 species, and oral shedding in 12 of 14 species. We observed contact transmission among four species and oral in five species. Persistent WNV infections were found in tissues of 16 surviving birds. Our observations shed light on transmission ecology of WNV and will benefit surveillance and control programs.

West Nile virus (WNV) is a mosquito-borne flavivirus (family: *Flaviviridae*) that uses birds as primary vertebrate reservoir hosts (1). WNV emerged in North America in New York City in 1999 (2,3) and has since spread throughout much of the North American continent (4). The virus affects the health of the public as well as domestic animals and wildlife. In 1999–2001, WNV was associated with 149 cases of clinical neurologic disease in humans (e.g., encephalitis and meningitis) (2,4,5), 814 cases of equine encephalitis (4–6), and 11,932 deaths in birds in the United States (4,5,7). Most reported fatal infections in birds occurred in crows. The American Crow (see Table 1 for scientific names of birds) has been proposed as the basis for a national surveillance system for avian deaths attributed to WNV (7,8). Since 1999, >150 species of dead birds have been reported as WNV positive to the Centers for Disease Control and Prevention (CDC) ArboNET surveillance program (unpub. data). Although the precise cause of death in these birds may not be proven, WNV has been isolated from the carcasses or WNV-specific RNA sequences have been detected. However, not all birds die from infection with the New York 1999 strain of WNV. Many birds sampled in 1999 and 2000 in New York City survived natural WNV infection and developed humoral immunity (9,10).

Although crows are commonly reported as infected with WNV (11), the identity of the avian reservoirs for WNV remains unknown. Surveillance data on avian deaths and seroprevalence studies suggest hypotheses about reservoir host species but do not indicate the competence of a particular species to infect a culicine vector. Furthermore, birds may be involved in transmission by means other than mosquito bites, yet little is known about contact or oral transmission among birds.

To better understand the role of birds in WNV transmission, we exposed 25 species of birds, representing a wide range of avian orders and families, to infectious mosquito bites. We then monitored viremia titers, clinical outcomes, viral shedding in cloacal and oral cavities, persistence of viral infections in organs, and development of neutralizing antibodies. The viremia data generated were used to quantitate reservoir competence. We also evaluated susceptibility to oral and direct contact transmission when possible.

Methods

Source of Birds

Birds were obtained commercially when possible or as nonreleasable injured birds (raptors only), or from the wild (Table 1). Only seronegative birds were used. Information on the plaque-reduction neutralization assay used is available in Appendix A (online only; available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0628-appA.htm).

Source and Infection of Mosquitoes

We used colonized mosquitoes (*Culex tritaeniorhynchus*) originally obtained from Taiwan in 1997. Adult female mosquitoes (<10 days old), used for infecting birds, were inactivated by chilling at approximately 4°C and inoculated intrathoracically with 1 µL of an aqueous solution containing 10^7 PFU WNV (NY99-6480) per 1 mL. Mosquitoes were then incubated at 16:8 h light:dark, 28°C, 80% relative humidity for 6–10 days before they were exposed to birds. Successful infection of mosquitoes was confirmed by plaque assay of whole mosquito homogenates (after incubation).

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Table 1. Classification, sample sizes, types of transmission studies and sources for 25 species of birds infected experimentally with West Nile virus

Common name	Latin name	Family	Order	No. used	Transmission trials[a]	Source type
Canada Goose	*Branta canadensis*	Anatidae	Anseriformes	3	M	Wild
Mallard	*Anas platyrhynchos*	Anatidae	Anseriformes	3	M, C	Commercial
American Kestrel	*Falco sparverius*	Falconidae	Falconiformes	5	M, O	Rehabilitator
Northern Bobwhite	*Colinus virginianus*	Odontophoridae	Galliformes	6	M, C, O	Commercial
Japanese Quail	*Coturnix japonicus*	Odontophoridae	Galliformes	6	M, C, O	Commercial
Ring-necked Pheasant	*Phasianus colchicus*	Phasianidae	Galliformes	3	M	Commercial
American Coot	*Fulica americana*	Rallidae	Gruiformes	2	M, C	Wild
Killdeer	*Charadrius vociferus*	Charadriidae	Charadriiformes	2	M	Wild
Ring-billed Gull	*Larus delawarensis*	Laridae	Charadriiformes	7	M, C	Wild
Mourning Dove	*Zenaida macroura*	Columbidae	Columbiformes	6	M, C, O	Wild
Rock Dove	*Columba livia*	Columbidae	Columbiformes	12	M, C	Commercial
Monk Parakeet	*Myiopsitta monachus*	Psittacidae	Psittaciformes	6	M, C, O	Commercial
Budgerigar	*Melopsittacus undulatus*	Psittacidae	Psittaciformes	6	M, C, O	Commercial
Great Horned Owl	*Bubo virginianus*	Strigidae	Strigiformes	2	M, O	Rehabilitator
Northern Flicker	*Colaptes auratus*	Picidae	Piciformes	5	M, O	Wild
Blue Jay	*Cyanocitta cristata*	Corvidae	Passeriformes	6	M, C	Wild
Black-billed Magpie	*Pica hudsonia*	Corvidae	Passeriformes	8	M, C, O	Wild
American Crow	*Corvus brachyrhynchos*	Corvidae	Passeriformes	22	M, C, O	Wild
Fish Crow	*Corvus ossifragus*	Corvidae	Passeriformes	20	M, C, O	Wild
American Robin	*Turdus migratorius*	Turdidae	Passeriformes	6	M, C, O	Wild
European Starling	*Sturnus vulgaris*	Sturnidae	Passeriformes	8	M, C	Wild
Red-winged Blackbird	*Agelaius phoeniceus*	Icteridae	Passeriformes	4	M	Wild
Common Grackle	*Quiscalus quiscula*	Icteridae	Passeriformes	12	M, C, O	Wild
House Finch	*Carpodacus mexicanus*	Fringillidae	Passeriformes	3	M, C, O	Wild
House Sparrow	*Passer domesticus*	Passeridae	Passeriformes	15	M, C, O	Wild

[a]M, mosquito-exposed; C, contact-exposed; O, orally exposed.

Source of Virus

Two isolates of WNV (New York 1999) were used. The NY99-6480 strain was isolated from mosquitoes (*C. pipiens*) and passed once in Vero cell culture. The NY99-4132 strain was isolated from brain of an American Crow and passed one to three times in Vero cell culture. The TBH-28 strain of St. Louis encephalitis virus (SLEV; family: *Flaviviridae*) was obtained from the CDC reference collection.

Experimental Infection

We exposed birds to WNV-infectious mosquito bites by holding their exposed skin (usually of the breast) against a screened carton containing 5–15 mosquitoes. Birds were considered sufficiently exposed when one mosquito had engorged to repletion. In the few cases when birds were probed extensively by mosquitoes but no visible blood was imbibed, we considered them infected if viremia developed. When possible, at least one uninfected conspecific bird (contact-exposed group) was placed in a cage with a mosquito-exposed bird as a control for direct transmission (in the absence of mosquito-borne transmission). Some birds (orally exposed group) were exposed to per os infections by using a variety of techniques; our objective was to show that per os transmission is possible. Techniques used included placing 200 uL water (containing a suspension of WNV [NY99-4132]) in the back of the oral cavity to stimulate the swallow reflex; placing a dead infected mosquito (containing approximately 10^7 PFU) in the bird's oral cavity and stimulating the swallow reflex with 200 uL of water; and placing a dead infected adult House Mouse (*Mus musculus*) or House Sparrow (euthanized 3–5 days after subcutaneous injection of 2,000–8,000 PFU) in the cage. Viral loads in the mice and House Sparrows were inferred from infected cohorts and estimated at $>10^5$ PFU per animal. Information about methods for venipuncture is available in Appendix B (online only; available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0628-appB.htm).

Collection of Oral and Cloacal Samples

For some birds, daily cloacal or nasopharyngeal (oral) swabs were collected concurrently with blood samples during the first 7 days postinoculation (dpi). Cotton- or Dacron-tipped applicators were used, and contaminated swabs were dipped in cryovials containing 0.5-mL BA1 to transfer any virus to the cryovial. These cryovials were placed immediately on wet ice (temporarily) and stored at –70°C for subsequent titration by Vero plaque assay (described in Appendix C, online only; available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0628-appC.htm).

Illnesses, Deaths, and Euthanasia

Exposed birds were observed twice a day for signs of severe illness, such as neurologic irregularities and recumbency. Birds unable to ambulate or consume food and water were euthanized by CO_2 asphyxiation or intravenous inoculation of sodium pentobarbitol at a dose of approximately 80 mg/kg. We recorded fatal cases to determine estimates of mortality rates for each species.

Necropsy

At the close of each infection study (in most cases 14 dpi), surviving birds were euthanized. Necropsies were performed immediately or after carcasses were stored at –70°C. Eleven organs were sampled for each bird by removing approximately 0.5 cm^3 to a sterile TenBroeck tissue homogenizer containing alundum grinding crystals and 0.2 mL BA1, 20% fetal calf serum. After grinding, 1.8 mL BA1, 20% fetal calf serum was added to each homogenate, and then each homogenate was transferred to 1.7-mL Eppendorf tubes for clarification by centrifugation at 7,500 rpm for 3 min. Supernatants were transferred to cryovials for storage at –70°C until titrated by plaque assay.

Calculation of Reservoir Competence Values

An index of reservoir competence (C_i) was derived as the product of three factors: susceptibility (*s*), the proportion of birds that become infected as a result of exposure; mean daily infectiousness (*i*), the proportion of exposed vectors that become infectious per day; and duration (*d*) of infectiousness, the number of days that a bird maintains an infectious viremia (12). This simple equation can be expressed as $C_i = s * i * d$. Thus, the competence index indicates the relative number of infectious vectors that derive from a particular bird species and is calculated as a function of the viremia that develops after mosquito-borne infection. To produce these data, we used a threshold level of infectious viremia of $10^{5.0}$ PFU/mL serum and estimated infectiousness of each bird's viremia levels from a standard curve for infection of *C. pipiens* as a function of viremic titer derived from Turell et al. (13) (Appendix D, online only; available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0628-appD.htm).

Results

Viremia Profiles

We determined WNV viremia profiles for 25 species of birds representing 17 families and 10 orders (Figure 1). Four (a Budgerigar, a Monk Parakeet, and two Japanese Quail) of 87 birds did not develop a detectable viremia (threshold of detection 50 PFU/mL serum) (Table 2). Four birds sustained detectable viremias of 7 days (a Ring-billed Gull, a House Finch, and two Fish Crows). Fish Crows were bled daily after 7 dpi for an additional 4 days to investigate whether viremias may endure >7 days (Table 3). They did not, although one moribund Fish Crow became viremic at 11 dpi, shortly before dying. Generally, viremias averaged greater in magnitude and duration in passerine and charadriiform birds than in other orders. Psittacine and gallinaceous birds had the lowest titered and shortest duration viremias.

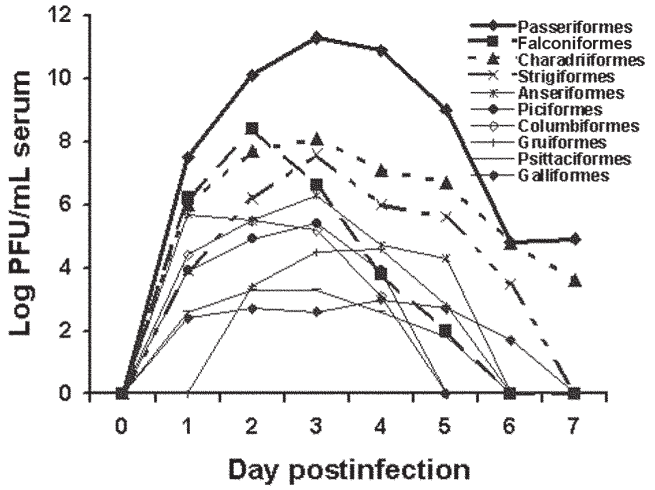

Figure 1. Comparative West Nile virus viremia profiles for 10 orders of birds.

Illness and Death

Of the 87 mosquito-exposed birds, we observed obvious signs of illness in 28 birds, including members of certain passerine species (in particular, the corvids) and the Ring-billed Gull. Signs of illness included generalized lethargy, ruffled feathers, unusual posture (Blue Jay), inability to hold head upright (Ring-billed Gull), and ataxia (Ring-billed Gull). In most cases, clinical signs were followed by death within 24 h. Moribund birds were euthanized, although ill birds were rarely found moribund because death occurred rapidly. External hemorrhage, either from the mouth or from the cloaca, was noted in a small number of American Crows that died. Although our sample sizes and controls were insufficient to generate accurate estimates of mortality rates, our observations can be used to generate preliminary estimates (Table 4).

Oral Transmission

We evaluated oral susceptibility to WNV infection for 15 species of birds representing 11 families and seven orders (Table 1). We confirmed susceptibility to orally acquired WNV infection in Great Horned Owl, American Crow, Common Grackle, House Finch, and House Sparrow. The owl that ingested infected mice developed viremia and seroconverted. American Crows also became infected after consuming a WNV-infected House Sparrow carcass (83% susceptibility, n=6); three Black-billed Magpies and a Fish Crow did not become infected after consuming infected House Sparrows or infected mice. American Crows and House Sparrows became infected after ingesting an aqueous solution containing $10^{7.4}$ PFU (100% susceptibility; n=6 and n=3, respectively). Grackles became infected after ingesting an aqueous solution

Table 2. Mean West Nile virus viremia levels (shown as log_{10} PFU/mL serum, with ranges) for each of 7 days postinoculation by mosquito bite, and mean duration of detectable viremia (days, with ranges)[a,b]

Species	n	Day postinoculation							Duration of viremia
		1	2	3	4	5	6	7	
Canada Goose	3	2.8 (<1.7–3.0)	5.3 (3.2–5.8)	4.5 (3.5–4.8)	3.4 (<1.7–3.8)	1.9 (<1.7–2.0)	<1.7	<1.7	4.0 (3–5)
Mallard	2	6.1 (<1.7–6.4)	5.7 (5.5–5.9)	6.7 (3.4–7.0)	5.1 (1.7–5.4)	4.7 (<1.7–5.0)	<1.7	<1.7	4.0 (4–4)
American Kestrel	2	6.2 (5.5–6.4)	8.4 (5.8–8.7)	6.6 (6.1–6.8)	3.8 (3.6–4.0)	2 (<1.7–2.3)	<1.7	<1.7	4.5 (4–5)
Northern Bobwhite	3	2.8 (1.7–3.1)	2.9 (2.3–3.3)	2.0 (1.7–2.2)	1.9 (<1.7–2.4)	1.2 (<1.7–1.7)	<1.7	<1.7	4.0 (3–5)
Japanese Quail	3	<1.7	<1.7	2.8 (<1.7–3.3)	3.4 (<1.7–3.9)	3.1 (<1.7–3.6)	2.2 (<1.7-2.7)	<1.7	1.3 (0–4)
Ring-necked Pheasant	3	2.1 (<1.7–2.3)	2.8 (<1.7–3.2)	2.6 (1.7–3.0)	1.8 (1.7–2.0)	1.2 (<1.7–1.7)	<1.7	<1.7	3.7 (3–4)
American Coot	1	<1.7	3.4	4.5	4.6	2.8	<1.7	<1.7	4.0
Killdeer	2	6.2 (5.9–6.4)	7.5 (6.5–7.8)	8.1 (4.3–8.4)	4.9 (2.1–5.2)	2.6 (<1.7–2.9)	<1.7	<1.7	4.5 (4–5)
Ring-billed Gull	2	5.4 (5.4–5.4)	7.8 (6.5–8.1)	8.0 (5.4–8.3)	7.4 (2.9–7.7)	7.2	5.3	4.1	5.5 (4–7)
Mourning Dove	3	4.8 (3.0–5.3)	5.9 (3.9–6.3)	5.6 (3.4–5.9)	3.3 (<1.7–3.6)	<1.7	<1.7	<1.7	3.7 (3–4)
Rock Dove	6	3.5 (<1.7–4.0)	4.3 (3.5–4.8)	4.2 (3.5–4.5)	2.9 (<1.7–3.7)	NT	NT	NT	3.2 (3–4)
Monk Parakeet	3	2.8 (<1.7–3.0)	3.6 (<1.7–4.0)	3.5 (<1.7–3.9)	1.7 (<1.7–2.0)	<1.7	<1.7	<1.7	2.7 (0–4)
Budgerigar	3	2.3 (<1.7–2.8)	1.9 (<1.7–2.4)	2.8 (<1.7–3.3)	2.9 (<1.7–3.4)	2.1 (<1.7–2.6)	<1.7	<1.7	1.7 (0–4)
Great Horned Owl	1	3.9	6.2	7.6	6.0	5.6	3.5	<1.7	6.0
Northern Flicker	1	3.9	4.9	5.4	3.9	<1.7	<1.7	<1.7	4.0
Blue Jay	4	8.5 (5.6–8.8)	11.1 (7.8–11.6)	12.1 (7.5–12.6)	10.5 (5.0–11.0)	2.2	<1.7	<1.7	4.0 (3–5)
Black-billed Magpie	3	5.3 (3.7–5.7)	8.3 (7.7–8.6)	8.8 (8.4–9.1)	4.9 (4.8–5.0)	4.0 (3.9–4.0)	–	–	5.0 (5–5)
American Crow	8	5.8 (<1.7–6.6)	8.7 (<1.7–9.6)	9.9 (6.7–10.6)	10.2 (9.2–10.8)	10.0 (8.2–10.4)	–	–	3.8 (3–5)
Fish Crow	9	5.4 (3.0–6.2)	6.8 (5.6–7.4)	7.8 (5.5–8.7)	8.9 (<1.7–9.9)	8.5 (<1.7–9.5)	4.0 (0-4.9)	1.3 (<1.7-2.0)	5.0 (4–7)
American Robin	2	5.8 (5.6–5.9)	8.9 (7.8–9.2)	7.3 (6.8–7.5)	4.6 (3.7–4.9)	2.0 (<1.7–2.3)	<1.7	<1.7	4.5 (4–5)
European Starling	6	5.3 (3.5–6.0)	6.1 (5.3–6.5)	4.9 (2.0–5.4)	2.3 (<1.7–3.1)	<1.7	<1.7	<1.7	3.2 (3–4)
Red-winged Blackbird	3	5.9 (5.5–6.1)	8.6 (7.5–9.0)	6.0 (5.5–6.3)	<1.7	<1.7	<1.7	<1.7	3.0 (3–3)
Common Grackle	6	6.1 (3.3–6.8)	10.2 (5.4–11.0)	11.8 (4.7–12.5)	11.8 (<1.7–12.5)	<1.7	<1.7	<1.7	3.3 (3–4)
House Finch	2	5.4 (2.3–5.7)	5.8 (5.6–6.0)	8.8 (8.6–8.9)	6.6 (6.0–6.8)	6.0 (5.9–6.1)	6.2	6.3	6.0 (5–7)
House Sparrow	6	7.8 (3.9–8.6)	9.8 (7.6–10.5)	10.3 (4.8–11.0)	10.3 (2.4–11.0)	8.4 (<1.7–9.0)	1.8 (<1.7-2.1)	<1.7	4.5 (2–6)

[a]NT, not tested; –, no birds survived to be sampled.

[b]Log_{10}-transformed mean peak viremias ranged from 3.0 for Ring-necked Pheasants (range 2.0–3.2) and Budgerigars (range <1.7–3.4) to 12.1 for Blue Jays (range 7.8–12.6). Mean duration (in days) of detectable viremias ranged from 1.3 in Japanese Quail to 6.0 in House Finches and a Great Horned Owl.

containing 1,000 PFU (100% susceptibility; n=4) but were resistant to a dose of 100 PFU (n=2). One of two House Finches that ate an infected mosquito, representing a dose of about 10^7 PFU, became viremic. Three each of Mourning Doves and Budgerigars did not become infected after ingesting an infected mosquito; three each of Japanese Quail and Monk Parakeet and two Bobwhite, did not become infected after ingesting an aqueous suspension containing about 3,400 PFU. Viremias generated from oral infection were similar to those from mosquito bite–derived infection, although the onset of detectable viremia was consistently delayed by at least a day (Figure 2), except for the one House Finch and the Great Horned Owl. Viremia profiles of these birds were similar to their mosquito-exposed counterparts, with no delay in the onset of viremia.

Contact Transmission

We monitored for direct transmission between mosquito-exposed birds and their cage mates among 18 species of bird representing 12 families and seven orders (Table 1). Transmission to cage mates was detected only in Ring-billed Gulls, Blue Jays, Black-billed Magpies, and American Crows

Table 3. Daily viremia determinations for nine Fish Crows infected with West Nile virus by mosquito bite[a]

Bird no.	Day postinoculation										
	1	2	3	4	5	6	7	8	9	10	11
015	3.6	5.6	5.7	5.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7
016	5.4	6.5	5.9	3.4	<1.7	<1.7	<1.7	<1.7	<1.7[b]		
036	4.6	5.6	5.6	3.9	4.7	4.9	2.0	<1.7	<1.7	Dead	
038	4.3	5.9	5.5	4.6	3.0	2.8	1.7	<1.7	<1.7	<1.7	3.3[c]
049	4.7	7.0	6.9	4.7	2.4	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7
050	3.0	7.4	8.7	9.9	9.5	Dead					
058	5.0	6.7	6.2	5.3	3.3	2.3	<1.7	<1.7	Dead		
403	6.2	6.8	5.7	3.0	<1.7	<1.7	<1.7	NT	NT	NT	NT
404	3.1	5.7	7.0	<1.7	3.3	<1.7	<1.7	NT	NT	NT	NT

[a]Values shown are log_{10} transformed and represent the number of PFU/mL serum.

[b]Moribund/euthanized.

[c]Dead at 12 days postinoculation.

Table 4. Deaths observed in eight species of birds exposed to West Nile virus (WNV) by mosquito bite

Species	No. exposed	No. unexposed[b]	No. fatal infections (% exposed)	Days postinoculation that death occurred	Mean no. days to death (range)
Ring-billed gull	2	0	2 (100)	5, 13[c]	9.0 (5–13)
Blue Jay	4	0	3 (75)	4, 5, 5	4.7 (4–5)
Black-billed Magpie	3	0	3 (100)	6, 6, 6	6.0 (6–6)
American Crow	8	8	8 (100)	4, 4, 5, 5, 5, 6, 6, 6	5.1 (4–6)
Fish Crow	9	0	5 (55)	6, 9, 10, 10,[c] 13	9.6 (6–13)
Common Grackle	6	6	2 (33)	4, 5	4.5 (4–5)
House Finch	2	3	2 (100)	6, 8	7.0 (6–8)
House Sparrow	6	5	3 (50)	3, 5, 6	4.7 (3–6)

[a]Preliminary mortality rates were highest in Passerines, especially the corvids. No signs of clinical illness were observed among species of the following orders: Anseriformes, Falconiiformes, Galliformes, Gruiformes, Columbiformes, Psittaciformes, Strigiformes, and Piciformes. No obvious differences in mortality rates were observed among birds exposed to WNV by means other than mosquito bite (orally exposed and contact-exposed groups; data not shown).

[b]Unexposed controls were blood sampled daily for the same period as the exposed birds, with no resulting illness.

[c]Euthanized.

(Table 5). The viremia profile of contact-exposed American Crows was similar to that of mosquito-exposed and orally exposed crows (Figure 2). In contact-exposed American Crows and Black-billed Magpies, onset of viremia occurred subsequent to death of their mosquito-exposed cage mates, suggesting that infection occurred near the time of death of the mosquito-exposed birds. The two contact-exposed Blue Jays both became infected while their mosquito-exposed cage mates were still viremic and apparently healthy. The one contact-exposed Ring-billed Gull that became infected did not develop viremia within 7 days of the inoculation of its two mosquito-exposed companions but was viremic at 14 dpi.

Development of Neutralizing Antibodies

Most mosquito-exposed birds that survived WNV infection were euthanized at 14 dpi (House Finches were held until 21 dpi, and Rock Doves were held for 64 dpi). We evaluated final serum samples for neutralizing antibodies. Only two birds, both Budgerigars, did not produce at least 70% neutralization activity in the final serum sample (tested at a 1:10 dilution). One of these also did not develop detectable viremia. The other had a detectable viremia only at 24 h postinoculation (log_{10} titer 2.8 PFU/mL serum), which may have represented residual virus from the injection rather than viral multiplica-

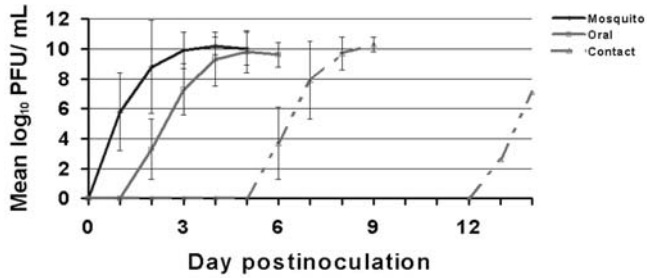

Figure 2. West Nile virus viremia profiles in American Crows that were mosquito-exposed (n=8), orally exposed by ingestion of sparrow carcasses (n=5), or contact-exposed (n=4). A fifth contact-exposed crow developed an ephemeral low-titered viremia ($10^{2.2}$/mL serum) and was treated as an outlier in this analysis. Error bars show standard deviation of log_{10}-transformed viremia titers.

Table 5. West Nile virus cage mate transmission trials[a]

Species	No. of cages	No. of mosquito-exposed birds	No. of contact-exposed cage mates	No. of transmissions (individuals)	No. of transmissions (cages)	Cage transmission rate
Mallard	1	2	1	0	0	0
Northern Bobwhite	1	3	3	0	0	0
Japanese Quail	1	3	3	0	0	0
American Coot	1	1	1	0	0	0
Ring-billed Gull	1	2	1	1	1	1.0
Mourning Dove	3	3	3	0	0	0
Rock Dove	6	6	6	0	0	0
Monk Parakeet	3	3	3	0	0	0
Budgerigar	3	3	3	0	0	0
Blue Jay	2	2	2	2	2	1.0
Black-billed Magpie	3	3	3	2	2	0.7
American Crow	4	8	5	5	4	1.0
Fish Crow	4	8	9	0	0	0
American Robin	1	2	1	0	0	0
European Starling	2	6	2	0	0	0
Common Grackle	6	6	6	0	0	0
House Finch	1	2	3	0	0	0
House Sparrow	2	6	5	0	0	0

[a]Uninfected birds (contact-exposed group) were placed within cages containing birds (of the same species) that were infected by mosquito bite (mosquito-exposed group). Transmission to uninfected cage mates was determined by development of viremia or seroconversion.

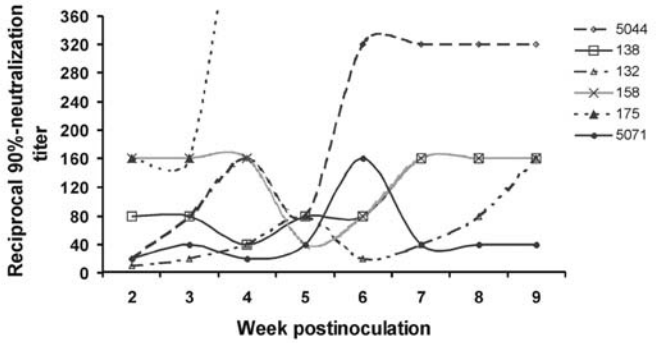

Figure 3. West Nile virus-neutralizing antibody response of six mosquito-exposed Rock Doves (pigeons). Rock Dove 175 reached a titer of 1:640 at 4 weeks postinoculation and then died of other causes.

tion. The neutralizing antibody response of Rock Doves was tracked weekly for 9 weeks postinoculation (Figure 3). Between weeks 2–9 postinoculation, reciprocal 90%-neutralization titers ranged from 10 to 640 and tended to rise early, then fall, and then rise again between weeks 3–7 postinoculation.

Viral Shedding

Cloacal and in some cases oral (nasopharyngeal) swabs were collected from 24 species of birds that were exposed to mosquitoes for subsequent virus isolation attempts. By swabbing the cloaca, we documented that most birds shed WNV in feces (17 [71%] of 24 species; 46 [59%] of 78 individual birds). Some passerine birds shed large quantities of WNV through the cloaca (Table 6). Cloacal shedding was generally first detected after several days of viremia and persisted longest in Fish Crows (>9 days), with peak cloacal swab titers occurring at 4–5 dpi. Although the highest cloacal swab titers were detected in American Crows and Blue Jays, these did not persist beyond 4 days because the birds died. By swabbing the oral cavity, we documented that most birds shed WNV in oral exudates (11 [85%] of 13 species; 29 [69%] of 42 individuals birds); the highest titers were observed in Great Horned Owl, American Crow, and American Kestrel (Table 7). Shedding per os persisted longest in the American Kestrel (up to 10 days). Shedding (either per cloaca or per os) was observed in representatives of 8 of the 10 orders, with the exceptions of Psittaciformes (n=6

Table 6. West Nile virus shedding in living birds, as determined by daily cloacal swabbing of 24 species of birds exposed by mosquito bite[a,b]

Species	n	Day postinoculation 1	2	3	4	5	6	7	No. birds Shedding (%)
Canada Goose	3	<0.2	<0.2	<0.2	<0.2	2.3 (<0.7–2.7)	0.6 (<0.7–1.0)	2.1 (<0.7–2.6)	2 (67)
Mallard	2	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	0
American Kestrel[c]	2	1.6 (1.6–1.6)	2.8 (2.0–3.1)	4.2 (1.9–4.5)	4.9 (4.0–5.2)	4.2 (3.6–4.4)	2.3 (2.2–2.4)	3.1 (<0.4–3.4)	2 (100)
Northern Bobwhite	3	<–0.2	<–0.2	0.2 (<0.4–0.7)	<–0.2	<–0.2	<–0.2	<–0.2	1 (33)
Japanese Quail	3	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	0
Ring-necked Pheasant	3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0
American Coot	1	<0.7	<0.7	<0.7	<0.7	2.7	<0.7	NT	1 (100)
Killdeer	2	<0.1	0.5 (<0.4–0.8)	<0.1	1.5 (<0.4–1.8)	<0.1	<0.1	<0.1	1 (50)
Ring-billed Gull	2	<0.1	<0.1	2.4 (0.4–2.7)	2.3 (2.2–2.5)	<0.4	1.8	<0.4	2 (100)
Mourning Dove	3	<–0.2	1.1 (0.8–1.5)	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	2 (67)
Rock Dove	6	<–0.5	<–0.5	<–0.5	<–0.5	1.2 (<0.4–1.7)	NT	NT	4 (67)
Monk Parakeet	3	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	0
Budgerigar	3	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	0
Great Horned Owl[c]	1	<0.4	2.3	2.1	3.1	2.1	3.3	2.0[c]	1 (100)
Northern Flicker	1	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	0
Blue Jay	4	<–0.3	3.0 (<0.4–3.6)	4.3 (2.1–4.8)	6.0 (2.0–6.4)	2.0	1.0	<0.4	4 (100)
Black-billed Magpie	3	<–0.2	1.8 (<0.4–2.3)	2.4 (2.1–2.5)	1.9 (<0.4–2.3)	2.9 (<0.4–3.4)	–	–	3 (100)
American Crow	6	0.8 (<0.4–1.5)	3.3 (<0.4–4.0)	5.2 (2.7–5.8)	5.0 (3.8–5.5)	5.7	–	–	6 (100)
Fish Crow[d]	8	–0.2 (<0.4–0.7)	1.5 (<0.4–2.1)	3.0 (1.0–3.8)	2.5 (<0.4–3.0)	3.3 (<0.4–4.1)	3.9 (<0.4–4.8)	3.6 (<0.4–4.4)	8 (100)
American Robin	2	<0.4	0.8 (<0.7–1.1)	2.2 (<0.7–2.5)	<0.4	<0.4	<0.4	<0.4	1 (50)
European Starling	6	<–0.5	<-0.5	-0.5 (<0.4–0.4)	<–0.5	–0.5 (<0.4–0.4)	0.8 (<0.4–1.5)	<–0.5	3 (50)
Red-winged blackbird	3	–0.2	0.4 (<–0.4–0.8)	<–0.2	–0.2 (<0.4–0.7)	<0.2	<–0.2	<–0.2	1 (33)
Common Grackle	6	<–0.5	>2.0 (<0.4–>2.7)	4.5 (0.7–5.3)	5.6 (<0.4–6.4)	5.2 (<0.4–5.9)	0.8 (<0.4–1.4)	0.1 (<0.4–0.7)	6 (100)
House Finch	2	<0.4	<0.4	<0.4	<0.4	<0.4	<0.7	<0.7	0
House Sparrow[e]	6	NT	<–0.5	NT	>1.9 (<0.4–>2.7)	1.3 (<0.4–2.1)	NT	NT	2 (33)

[a]Presented as mean $\log_{10}$ PFU/swab, with ranges.

[b]NT, not tested; –, no birds survived the infection to be sampled.

[c]American Kestrels and the Great Horned Owl were tested at 9–11 days postinoculation, with no detectable virus in swabs.

[d]Surviving Fish Crows were tested at 8 and 9 days postinoculation with the following results for day 8 and day 9, respectively: 1.2 (<0.4–1.8); 1.5 (<0.4–2.1).

Table 7. West Nile virus shedding in living birds, as determined by plaque assay of oral swabs collected daily from 14 species of birds exposed by mosquito bite[a,b]

Species	n	Day postinoculation							No. birds shedding (%)
		1	2	3	4	5	6	7	
American Kestrel[c]	2	1.2 (1.0–1.3)	1.9 (1.6–2.1)	3.9 (3.0–4.2)	4.1 (2.8–4.4)	4.9 (4.6–5.1)	5.1 (4.6–5.3)	3.3 (3.1–3.4)	2 (100)
Northern Bobwhite	3	<–0.2	<–0.2	0.2 (<0.4–0.3)	<–0.2	<–0.2	<–0.2	<–0.2	1 (33)
Japanese Quail	3	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	2.5 (<0.4–3.0)	1.9 (<0.4–2.4)	1 (33)
Killdeer	2	NT	<0.1	0.4 (<0.4–0.7)	0.1 (<0.4–0.4)	0.8 (<0.4–1.1)	0.1 (<0.4–0.4)	0.4 (<0.4–0.7)	1 (50)
Ring-billed Gull[d]	2	NT	3.1 (2.0–3.3)	2.8 (1.8–3.0)	3.3 (2.6–3.6)	3.5	3.5	3.4	2 (100)
Mourning Dove	3	NT	<–0.2	0.8 (<0.4–1.2)	0.5 (<0.4–0.8)	0.5 (<0.4–0.8)	<–0.2	<–0.2	2 (67)
Monk Parakeet	3	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	0 (0)
Budgerigar	3	NT	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	<–0.2	0 (0)
Great Horned Owl	1	<0.4	1.3	3.1	<0.4	5.8	4.9	2.8	1 (100)
Northern Flicker	1	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	0 (0)
Blue Jay[e]	2	0.9 (<0.4–1.2)	4.0 (1.4–4.3)	3.9 (2.5–4.2)	3.6	3.7	2.2	1.2	2 (100)
Black-billed Magpie	3	<–0.2	0.8 (<0.4–1.0)	2.1 (1.4–2.5)	3.1 (2.5–3.3)	4.0 (<0.4–4.4)	–	–	3 (100)
American Crow	6	<–0.5	2.3 (<0.4–2.5)	5.0 (1.6–5.5)	4.3 (3.1–4.7)	5.7	–	–	6 (100)
Fish Crow[f]	8	<–0.6	1.0 (<0.4–1.7)	3.4 (1.3–4.2)	3.6 (<0.4–4.1)	3.8 (1.8–4.4)	3.8 (1.6–4.6)	3.5 (1.9–4.1)	8 (100)

[a]Presented as mean $\log_{10}$ PFU/swab, with ranges.

[b]NT, not tested; –, no birds survived the infection to be sampled.

[c]American Kestrels were tested at 9, 10, and 11 days postinoculation, with the following results for days 9 and 10, respectively: 2.3 (1.8–2.5); 1.8 (<0.4–2.1). WNV was not isolated from oral swabs collected on day 11.

[d]One Ring-billed Gull developed signs of illness after 7 days postinoculation and was euthanized at 13 days postinoculation, at which time an oral swab contained 10 PFU WNV.

[e]One Blue Jay was sampled at 8 and 9 days postinoculation. No virus was isolated from oral swabs.

[f]Surviving Fish Crows were tested at 8 and 9 days postinoculation with the following results for days 8 and 9, respectively: 3.6 (2.0–4.3); 2.1 (<–0.5–2.5).

individual birds) and Piciformes species (n=1). Although environmental sampling was not undertaken rigorously, we did detect infectious WNV in a water dish that had been contaminated with Blue Jay feces and from bloody oral effusion collected underneath a dead American Crow.

Viral Load and Viral Persistence in Organs

Some birds that died acutely were necropsied to determine viral load in different organs (Table 8). Almost all organs evaluated were infected, although certain organs harbored consistently more virus. Among the four species of corvids evaluated, titers were higher in American Crows and Blue Jays than in Fish Crows and Black-billed Magpies. Titers were lowest in Ring-billed Gulls, but most tissues were still infected.

Most surviving birds were necropsied after euthanization to determine whether infectious WNV could be detected in any of 11 organs, including brain, eye, kidney, heart, spleen, liver, lung, intestines, gonads, esophagus, and skin. This analysis determined that 18 of 41 birds sampled at 14 dpi sustained virus infections in one or more organs for up to 13 days beyond the period of viremia and, in two cases, in birds with no detectable viremia (Table 9).

Reservoir Competence

We analyzed viremia data from mosquito-exposed birds to determine values for susceptibility, mean infectiousness, and duration of infectious viremia; from these, we calculated competence indices (Table 10). Species with high mean peak viremias and long duration of viremia generally also had high competence index values.

Discussion

Reservoir Competence

The principal goal of our experimental infection studies was to estimate reservoir competence for a variety of candidate reservoir species in the United States. We used a formula derived for evaluating vertebrate reservoir competence for Eastern equine encephalomyelitis virus (family: *Togaviridae*), a mosquito-borne alphavirus (12). The value derived for reservoir competence is an index that reflects the relative number of infectious mosquitoes that would be derived from feeding on these hosts. This value depends on the concentration of infectious virus particles in blood and the duration of an infectious level viremia. We have shown that WNV viremia profiles derived from mosquito-borne infection in birds vary greatly among the 25 species that we evaluated. Birds that sustained a viremic titer greater than $10^{5.0}$ PFU/mL were considered infectious for *C. pipiens* (13) and *C. quinquefasciatus* (14), two important enzootic vectors. These bird species were considered reservoir competent for WNV, whereas species that did not develop viremia of sufficient titer to infect these mosquitoes were considered incompetent. Some mosquito vectors may develop infections after imbibing lesser concentrations of virus. For example, the threshold WNV titer for infection of *C. univittatus* in South Africa was reported as $<10^{4.0}$ 50% suckling mice lethal doses ($SMLD_{50}$)/mL blood, and $<10^{4.6}$ $SMLD_{50}$/mL blood for infection of *C. perexiguus* in Egypt (15). Recent studies with WNV (New York 1999) indicate that some California populations of *C. tarsalis*, *C. pipiens*, and *C. erythrothorax* are susceptible to infection at titers $<10^{5.0}$ PFU/mL (16). Similarly, the threshold for SLEV infection of *C. tarsalis* may be as low

Table 8. Viral load, determined by Vero plaque assay, in organs harvested from fatal cases of West Nile virus infection in experimentally infected birds[a]

Species	ID no.	Sex	Mode of infection	Organ (log_{10} PFU/0.5 cm^3)										
				Br	Ki	He	Sp	Li	Lu	In	Es	Go	Sk	Ey
Ring-billed Gull	BDG	Male	M	2.7	2.8	1.0	3.3	3.0	3.2	<1.0	2.7	1.6	3.0	2.0
Ring-billed Gull	LG	Female	C	3.0	2.4	1.7	1.3	<1.0	<1.0	<1.0	2.3	3.0	2.4	3.0
American Crow	21	Male	C	7.2	7.6	5.4	7.9	7.5	8.5	7.6	7.8	6.8	4.4	7.7
American Crow	24	Female	M	6.0	8.2	7.7	7.0	7.3	7.1	8.4	7.4	7.0	4.6	6.6
American Crow	25	Female	M	5.9	7.2	5.7	6.7	6.8	6.7	10.6	6.2	7.9	3.8	6.9
American Crow	34	Female	C	8.3	8.2	7.8	7.9	7.7	8.3	9.0	8.2	8.1	5.3	7.9
American Crow	37	NR	C	7.2	8.2	7.7	7.8	7.4	8.5	10.0	7.3	NT	5.3	7.7
American Crow	41	Female	M	8.3	9.1	8.5	8.7	8.7	10.3	6.6	8.8	9.4	5.6	8.5
American Crow	42	Female	M	8.1	9.1	8.4	7.8	8.1	8.4	10.3	8.6	8.3	5.8	7.9
American Crow	529	Female	M	6.1	7.2	7.0	6.5	5.3	7.7	5.7	5.9	6.9	6.8	5.7
American Crow	543	Female	M	8.3	8.4	8.4	7.2	7.4	9.6	8.7	8.0	8.7	5.9	8.6
American Crow	562	NR	M	8.3	8.2	6.9	7.7	7.7	8.1	10.2	7.4	8.1	5.8	7.3
American Crow	574	Male	C	8.3	8.1	8.2	8.0	8.6	8.1	9.7	6.3	7.8	6.1	7.5
American Crow	805	Male	M	8.1	6.5	7.3	5.9	6.3	9.2	8.3	8.0	6.3	6.4	8.7
Fish Crow	005	Male	M	6.9	4.0	<2.0	3.3	3.5	4.0	4.5	8.5	4.4	3.0	6.2
Fish Crow	016	Male	M	2.7	3.2	6.6	1.0	1.0	4.6	5.2	5.1	3.6	4.0	4.8
Fish Crow	036	NR	M	3.0	3.1	6.6	3.3	1.5	5.3	3.0	6.5	3.3	4.9	3.1
Fish Crow	038	Male	M	4.1	3.9	4.7	1.3	1.0	4.4	2.0	5.4	4.5	2.8	5.0
Fish Crow	050	Female	M	6.9	8.1	8.5	8.2	7.8	7.6	8.6	7.7	8.0	5.5	6.9
Fish Crow	058	Male	M	4.5	5.1	6.9	2.4	1.6	5.8	6.0	5.1	2.7	5.0	5.3
Fish Crow	402	Male	U	4.7	3.6	3.8	3.7	1.5	3.0	2.8	7.4	1.8	3.7	5.8
Blue Jay	124	NR	M	7.4[b]	7.8	9.0	8.3	8.8	9.1	6.3	NT	7.1	NT	NT
Blue Jay	125	NR	M	7.3[b]	8.6	9.1	8.3	8.9	9.2	7.3	NT	7.3	NT	NT
Blue Jay	908	Male	C	8.2	9.0	8.8	8.6	8.5	9.0	8.0	8.7	9.1	6.9	7.9
Blue Jay	909	Male	C	2.7	6.1	6.6	5.5	4.3	5.8	3.4	4.5	5.0	5.9	7.1
Blue Jay	910	Female	M	8.2	9.0	9.0	8.1	8.4	9.1	7.9	8.9	9.1	6.0	8.0
Black-billed Magpie	LG	Male	M	4.7	6.6	6.6	3.9	5.4	6.5	4.8	6.7	5.9	5.5	6.3
Black-billed Magpie	RBLG	Male	M	5.9	5.5	5.0	4.0	4.8	4.4	2.7	5.9	4.8	4.1	4.6
Black-billed Magpie	NB	Male	C	5.8	6.2	7.2	5.9	6.4	5.7	5.1	5.9	6.5	4.8	5.5
Black-billed Magpie	RB	Male	C	5.0	7.7	6.2	4.5	6.8	6.3	5.2	5.8	4.7	5.9	5.3
Black-billed Magpie	RG	Female	M	6.4	5.4	6.7	4.7	1.9	6.1	1.0	6.6	4.5	5.9	4.7
Common Grackle	120	Male	M	3.5	>3.6	>3.6	>3.6	>3.6	>3.6	<1.0	5.0	<1.0	4.0	5.0
Common Grackle	123	Male	M	2.4	>3.3	NT	NT	NT	NT	<1.0	4.9	5.1	4.6	4.9
House Finch	0	Female	M	3.9	3.8	5.9	3.6	3.8	3.9	<1.0	5.5	3.8	4.1	6.2
House Finch	1	Male	M	4.9	3.0	6.1	3.5	2.7	6.0	<1.0	5.7	3.2	5.8	6.3

[a]B, brain; Ki, kidney; He, heart; Sp, spleen; Li, liver; Lu, lung; In, intestine; Es, esophagus; Go, gonad; Sk, skin; Ey, eye; M, mosquito-exposed; C, contact-exposed; U, undetermined mode of transmission; NR, not recorded; and NT, not tested.

[b]These Blue Jay brains were evaluated for viral load in both cerebellum and cortex. In both cases, cerebellum was negative.

as 10 PFU per bloodmeal (approximately $10^{3.3}$ PFU/mL blood) (17). Nonetheless, titers $<10^{5.0}$ PFU/mL probably result in few mosquito infections.

Our observation that passerine bird species were generally competent for WNV transmission is consistent with the role of these birds as hosts for other flaviviruses such as SLEV and Japanese encephalitis virus (18,19). Previous work with African strains of WNV also implicated passerine birds as the most competent. In Egypt, experimental infections of House Sparrows and Hooded Crows (*Corvus corone sardonius*), both passerine species, using a local strain of WNV (Ar-248), showed that these two species developed high titered viremia whereas three nonpasserine species (including a dove, an egret, and a falcon) were weakly competent (20). Adult chickens and pigeons were incompetent (21). Similarly, in South Africa, inoculation of 14 bird species with a local WNV strain showed two highly competent species, both passerine (Masked Weaver [*Ploceus velatus*] and Red Bishop [*Euplectes orix*], both closely related to House Sparrow) (22). Our finding that Anseriformes were weakly competent reservoir hosts for WNV is consistent with the findings of the South African study, which evaluated three species of ducks. Our study also coincided with both the Egyptian and South African studies in finding Rock Doves (pigeons) incompetent but other species of doves weakly competent. Reservoir competence index values derived from experimental infection studies must be combined with field-derived data to fully evaluate the importance of candidate reservoir hosts in a specific location. However, larger sample sizes should be studied to derive competence values with greater accuracy.

Illness and Death Associated with WNV infection

Our study confirms that some species of bird suffer high mortality rates from WNV infection. The mortality rates presented in this paper are preliminary because of small sample sizes, inadequate controls, and bias from the effects of captivity and handling. Nonetheless, birds sufficiently weakened by the infection to succumb in captivity are also likely to succumb in nature, where other stresses may contribute to death. Avian deaths were not reported in natural WNV infections until 1998

Table 9. Viral load, determined by Vero plaque assay in organs harvested from surviving birds 14 days after West Nile virus (WNV) infection by mosquito bite[a,b]

Species	ID no.	Sex	Organ (PFU/0.5 cm^3)											Days postviremia
			Br	Ki	He	Sp	Li	Lu	In	Es	Go	Sk	Ey	
American Kestrel[c]	F2	F	–[d]	20	–	20	–	–	–	–	NT	30	–	10
American Kestrel	F3	F	–	–	–	10	–	–	–	–	NT	–	–	11
Japanese Quail	902	U	10	10	–	–	–	–	–	–	–	–	–	8
Japanese Quail	904	U	–	–	–	10	–	–	–	–	10	–	–	14
Japanese Quail	907	U	–	–	20	20	–	–	–	–	–	–	–	14
Killdeer	CT	U	–	60	–	–	–	–	20	–	–	110	–	9
Killdeer	WT	U	–	–	–	550	–	–	–	–	–	$2x10^4$	–	10
Mourning Dove	LCW	U	–	20	–	–	–	–	–	–	–	–	–	10
Mourning Dove	RB	M	–	100	–	–	–	–	–	–	–	–	–	11
Budgerigar	13591	U	–	–	130	–	–	–	–	–	–	–	–	13
Blue Jay	911	U	20	–	–	–	–	–	–	–	–	–	360	9
Fish Crow	049	U	–	–	–	–	–	–	–	–	–	–	30	9
Red-winged Blackbird	711	M	–	–	10	–	10	–	–	–	–	–	–	11
Common Grackle	119	F	–	–	–	10	–	–	–	10	20	380	150	11
Common Grackle	122	M	–	–	–	–	–	–	–	–	–	–	10	10
House Sparrow	011	F	10	50	–	–	–	–	40	90	–	370	60	8
House Sparrow	012	M	–	–	–	120	–	590	10	–	–	10	–	10
House Sparrow	016	M	200	20	–	50	–	20	–	–	–	–	50	8

[a]For each bird, the number of days postviremia is indicated. Birds from which no virus was detected are not included. WNV was isolated from at least one organ sample from 18 birds, and at least one isolate was made from each of the 11 different organs. Liver had the fewest isolates with one; spleen had the most with eight. Titers were generally low. The highest titered specimen was a skin sample from a Killdeer. Twenty-three surviving birds had no WNV isolated from tissues at 14 days postinoculation, including three Northern Bobwhite, three Ring-necked Pheasants, three Monk Parakeets, two Budgerigars, one Great Horned Owl, one Mourning Dove, six European Starlings, two Common Grackles, and two Red-winged Blackbirds. In addition, five Rock Doves were sampled at 64 days postinoculation and were negative.

[b]M, male; F, female; U, undetermined gender or gender not recorded; Br, brain; Ki, kidney; He, heart; Sp, spleen; Li, liver; Lu, lung; In, intestine; Es, esophagus; Go, gonad; Sk, skin; Ey, eye; and NT, not tested.

[c]Kestrels were tested 15 days postinoculation.

[d]– indicates that no virus was isolated (threshold of detection 10 PFU/0.5 cm^3 tissue).

when domestic goslings in Israel were affected, as well as White Storks (*Ciconia ciconia*) (23). The 1998 goose strain is essentially identical to the New York 1999 strain that resulted in thousands of bird deaths beginning in 1999 in New York City (24).

The high mortality rate in corvids was presaged by the results of the Egyptian experimental infection study, in which all 13 infected Hooded Crows succumbed (20). However, the lack of observed crow deaths and the observation of high seroprevalence in natural crow populations (an indicator of survival of infection) led investigators in Egypt to speculate that the crow deaths in captivity were artifacts (21).

The observed high mortality rate in 8 of the 25 species tested in our study indicates that these 8 species (Table 4) may be useful in avian mortality surveillance. These species include all the corvids tested, as well as House Sparrow and Common Grackle, two abundant passerine bird species likely to be important reservoir hosts in some locations, and Ring-billed Gull. Deaths in experimentally infected passerines (House Sparrows and crows) have been reported previously (20). The Egyptian and the South African studies did not include Charadriiformes. However, in a Russian study of WNV infection in Black-tailed Gulls (*Larus crassirostris*), deaths were observed (25), as well as in naturally infected White-eyed Gulls (*L. leucophthalmus*) in Israel (23).

The lack of clinical signs and death in 17 species suggests that mortality rates in these birds are low. However, natural WNV-associated deaths have been reported from all 10 orders of birds included in this study (11). Even for birds that are generally resistant to fatal WNV infections, the virus may still be an important cause of death relative to the overall mortality rate of the population; this idea provides an explanation for why 7 (17%) of 41 dead pigeons tested positive for WNV in New York in 2000 (11) and yet none of 6 pigeons experimentally infected showed signs of illness. Whether WNV alone is capable of killing a pigeon is unknown; WNV infection may require underlying illness or immune suppression in a pigeon to result in death.

Oral Transmission

We have demonstrated that certain bird species may become infected by WNV (New York 1999) after ingesting it in infected dead animals and infected mosquitoes, both natural food items of some species. This phenomenon was previously observed in American Crows that ingested WNV-infected suckling mice (26) and in mammals on several occasions (27). Langevin et al. (28) were not able to infect chickens orally. We found that the viremia profiles generated by oral infection were essentially identical to those derived from mosquito-borne infection, although in some species, onset of viremia was delayed by approximately 1 day. The importance of oral WNV infection in birds is unknown but may contribute to the success of avian mortality surveillance compared with surveillance for infected mosquitoes (5,29) or other surveillance systems.

Table 10. West Nile virus reservoir competence index values derived for 25 species of birds

Common name	Susceptibility (*s*)	Mean infectiousness (*i*)	Mean duration (days) (*d*)	Reservoir competence index (C_i)[a]
Blue Jay	1.0	0.68	3.75	2.55
Common Grackle	1.0	0.68	3	2.04
House Finch	1.0	0.32	5.5	1.76
American Crow	1.0	0.50	3.25	1.62
House Sparrow	1.0	0.53	3	1.59
Ring-billed Gull	1.0	0.28	4.5	1.26
Black-billed Magpie	1.0	0.36	3	1.08
American Robin	1.0	0.36	3	1.08
Red-winged Blackbird	1.0	0.33	3	0.99
American Kestrel	1.0	0.31	3	0.93
Great Horned Owl	1.0	0.22	4	0.88
Killdeer	1.0	0.29	3	0.87
Fish Crow	1.0	0.26	2.8	0.73
Mallard	1.0	0.16	3	0.48
European Starling	1.0	0.12	1.8	0.22
Mourning Dove	1.0	0.11	1.7	0.19
Northern Flicker	1.0	0.06	1	0.06
Canada Goose	1.0	0.10	0.3	0.03
Rock Dove	1.0	0	0	0
American Coot	1.0	0	0	0
Japanese Quail	1.0	0	0	0
Northern Bobwhite	1.0	0	0	0
Ring-necked Pheasant	1.0	0	0	0
Monk Parakeet	1.0	0	0	0
Budgerigar	0.7	0	0	0

[a]$Ci = s*i*d$

Numerous dead crows may result from a single mosquito-borne transmission to a bird or mammal because of the carrion-feeding behavior of crows.

Contact Transmission

We have shown that certain bird species may become infected by WNV (New York 1999) after being in close contact with other infected birds, in the absence of mosquito-borne transmission. We observed this phenomenon in American Crows, Blue Jays, Black-billed Magpies, and Ring-billed Gulls. This type of direct transmission of WNV among birds was first reported in a cage mate of a chicken inoculated by needle (28). Subsequently, this transmission was observed in needle-inoculated domestic goslings (30) and American Crows held in a free-flight aviary with uninfected crows (26). The mode of this "cage mate transmission" is unknown. Viremia profiles in contact-exposed American Crows, Blue Jays, and Black-billed Magpies were indistinguishable from those of mosquito-exposed birds. Onset of viremia in contact-exposed crows and magpies occurred approximately 1 day after death of the mosquito-exposed cage mate, suggesting that exposure did not occur until death of the infected companion. Onset of viremia in contact-exposed Blue Jays, however, began before the death of the mosquito-exposed cage mates. Whether direct transmission of WNV between birds occurs in nature in the absence of mosquitoes is unknown. However, given the close cloacal and oral contact between birds that occurs within families during the breeding season and the sometimes high quantity of WNV in oral and cloacal fluids, this type of transmission likely occurs in nature.

Viral Shedding

In general, arboviruses are not thought to be shed by their hosts because of the requirement for arthropod vectors in the transmission cycle. However, Langevin et al. (28) detected low-level shedding in WNV-infected chickens by swabbing the cloaca and the oral cavity. We extended those observations to many other species of birds in this study. Viral shedding may be involved in the cage mate transmission that we observed in corvids and Ring-billed Gull. The prospect of shedding in naturally infected birds has other implications for both public health and potential spread of WNV within and between species. Does shedding in birds represent a health risk to humans? Can birds shed virus to the extent that other birds in close contact can become infected in the absence of mosquitoes? These questions require further study. Our observations of shedding in acutely infected birds led to the hypothesis that swabbing corvid carcasses could be useful for diagnosing WNV infection. This hypothesis was validated (31).

Viral Persistence and Viral Load in Organs

Several reports have suggested that WNV and other flaviviruses may persist in the organs of birds in such a way as to permit retransmission to vector mosquitoes after the period of initial viremia (32–34). The mode of retransmission, however, is unknown. To generate preliminary data on persistence of the New York 1999 strain of WNV in North American bird species, we monitored persistence of virus infection in organs of 41 surviving birds that were euthanized at 14 dpi. Almost half of these birds harbored infectious virus in organs. Although we isolated WNV in all 11 organs evaluated, we found no pattern of which organs are infected for which species, and viral titers were generally low. Organs most frequently infected were spleen, kidney, skin, and eye; the liver was the least likely to harbor infectious virus particles. These infections may have been remnants of the acute infection. However, some unexpected observations were made in these studies. For example, the organ with the highest density of infectious virus particles was a skin sample taken from a Killdeer. Skin samples were positive in six birds tested, including an American Kestrel, both Killdeer, a Common Grackle, and two House Sparrows (and high titers were consistently detected in skin samples collected from fatal infections). Persistent skin infections may be transmitted to mosquitoes that contact the skin while feeding even beyond the period of infectious-level viremia. Infected skin also suggests that ticks may become infected with WNV while feeding for several days within the skin. Interestingly, charadriiform birds are thought to serve as reservoir hosts in a soft tick-borne WNV transmission cycle in Eurasia (35). Another unexpected finding was infection of ovaries; persistent infections in these organs raise the possibility of transovarial transmission.

We also evaluated the viral load of organ samples from seven species of birds that died from WNV infection. Overall, our evaluation of viral loads in 11 organs of WNV-infected carcasses supports the current prioritization of brain or kidney for selective organ testing for WNV (36–38). Intestines had the highest WNV concentrations of the organs evaluated, yet several birds (the two gulls and two finches) had no detectable infectious WNV in their intestines. Although skin titers were lower, the universal positivity among the birds tested and the ease of specimen collection in the field warrant consideration of skin as a potential biologic specimen for collection from carcasses in the field.

Development of Neutralizing Antibodies

We expected all infected birds to generate a humoral immune response to WNV, with development of virus-neutralizing antibodies. Thus, the two Budgerigars that did not produce detectable antibodies (assayed at 14 dpi) were thought to have avoided infection. However, one of these parakeets harbored a persistent infection in heart tissue, indicating that infection did occur. This same bird had detectable viremia only at 1 dpi. With one species, Rock Doves, we followed the immune response through 9 weeks postinfection. All six Rock Doves generated a neutralizing immune response that persisted throughout the monitoring period. An early rise-fall-rise pattern in the neutralizing antibody response (Figure 3) is probably explained by the early, ephemeral contribution of immunoglobulin (Ig) M to virus neutralization, followed by a rise in neutralizing IgY. The Rock Dove (or domestic pigeon) is considered a candidate sentinel for monitoring WNV transmission in the United States (29). Our results indicate that Rock Doves have a strong immune response after a brief, low-level (noninfectious) viremia, both important criteria for candidate sentinels.

Conclusion

We have presented basic data on the course of WNV (New York 1999) infection in 25 species of birds, including viremia duration and magnitude, illness and death, persistent infection in organs, and viral shedding. We have also shown that some birds are susceptible to oral transmission and that some cage mates may become infected in the absence of mosquitoes, although the mode of this type of transmission remains unknown. An analysis of our data shows that passerine birds, charadriiform birds, and at least two species of raptors (American Kestrel and Great Horned Owl) are more competent than species evaluated from the following orders: Anseriformes, Columbiformes, Galliformes, Gruiformes, Piciformes and Psittaciformes. Indeed, many birds of the latter orders were found to be incompetent for transmission.

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Emergence of Ceftriaxone-Resistant *Salmonella* Isolates and Rapid Spread of Plasmid-Encoded CMY-2–Like Cephalosporinase, Taiwan

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Of 384 *Salmonella* isolates collected from 1997 to 2000 in a university hospital in Taiwan, six ceftriaxone-resistant isolates of *Salmonella enterica* serovar Typhimurium were found in two patients in 2000. The resistance determinants were on conjugative plasmids that encoded a CMY-2-like cephalosporinase. During the study period, the proportion of CMY-2–like enzyme producers among Escherichia coli increased rapidly from 0.2% in early 1999 to >4.0% in late 2000. *Klebsiella pneumoniae* isolates producing a CMY-2–like -lactamase did not emerge until 2000. The presence of bla_{CMY}-containing plasmids with an identical restriction pattern from *Salmonella*, *E. coli*, and *K. pneumoniae* isolates was found, which suggests interspecies spread and horizontal transfer of the resistance determinant. Various nosocomial and community-acquired infections were associated with the CMY-2-like enzyme producers. Our study suggests that the spread of plasmid-mediated CMY-2-like β-lactamases is an emerging threat to hospitalized patients and the public in Taiwan.

Because of increasing rates of antimicrobial resistance in salmonellae worldwide (1-4), extended-spectrum cephalosporins, especially ceftriaxone, are frequently used to treat invasive salmonellosis. Since the early 1990s, ceftriaxone-resistant salmonellae have been noted in many countries, including France, Argentina, Algeria, Tunisia, Turkey, Spain, Latvia, the United States, and Hungary (5-17), with resistance conferred by various class A extended-spectrum β-lactamases or class C cephalosporinases (18). These β-lactamases in salmonellae are usually encoded on transmissible plasmids (5-11,13-17), which could be acquired from other multidrug-resistant Enterobacteriaceae, such as *Klebsiella pneumoniae* or *Escherichia coli* (10,19).

Although the prevalence of multidrug-resistant salmonellae is a major public health concern, ceftriaxone-resistant salmonellae have not yet been reported in Taiwan (4,20,21). We conducted a retrospective survey of clinical *Salmonella* isolates collected over a 4-year period in a teaching hospital to investigate whether ceftriaxone-resistant salmonellae have emerged in Taiwan. Two ceftriaxone-resistant strains producing a CMY-2–like class C cephalosporinase were found. We also established a connection between the appearance of the resistant strains and the rapid spread of the bla_{CMY}-2–like gene in this area.

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Materials and Methods

Bacterial Isolates and Patients

From January 1997 to December 2000, a total of 384 Salmonella isolates from 324 patients were collected at the National Cheng Kung University Hospital, a tertiary-care referral center with 900 beds in southern Taiwan. According to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) for the disk diffusion method, we selected for further investigation the isolates that exhibited resistance or intermediate resistance to cefpodoxime, ceftazidime, aztreonam, cefotaxime, ceftriaxone, cefoperazone, or cefixime (22). For comparison, 5,520 E. coli isolates and 3,680 *K. pneumoniae* isolates collected during the same period were included. Of these isolates, 1,210 nonrepetitive *E. coli* isolates collected from January to September 1999 were investigated previously (23). The 1997 and 1998 isolates were randomly collected and the 1999 and 2000 isolates were consecutively collected. *Salmonella* isolates were serotyped according to the Kauffman and White scheme (24) by using somatic and flagellar antigens (Becton Dickinson Microbiology, Cockeysville, MD).

We reviewed the medical records of patients infected with or colonized by the organisms being studied. Patients who provided samples positive for the organisms collected from any body site but who had no related signs or symptoms of infections were considered colonized. Nosocomial acquisition of infections in the teaching hospital was defined according to the 1988 definitions from the Centers for Disease Control (25). Patients transferred from other hospitals or nursing homes with infections occurring <48 h after admission were considered to have acquired the infections at the other locations. Hospitalization histories for the previous locations were recorded for outpatients and for inpatients who were colonized by the studied organisms and who provided samples within 48 h after admission.

Susceptibility Testing

The susceptibilities of isolates to antimicrobial agents were determined by using the agar dilution or disk diffusion

method according to the NCCLS guidelines (22,26). The antimicrobial agents used for the agar dilution test included amoxicillin, clavulanic acid, ceftriaxone, cefoxitin, ceftazidime, cefotaxime, and imipenem. Sources of antimicrobial agents used in this study are described elsewhere (23). Breakpoints used for susceptibility meet NCCLS criteria; the ceftazidime, cefotaxime, and ceftriaxone breakpoint was 8 μg/mL (26).

Isoelectric Focusing

Crude β-lactamase extracts were prepared by using sonication (27) as described previously (23). We performed isoelectric focusing by the method of Matthew et al. (28) with an LKB Multiphor apparatus on prepared PAGplate gels (pH 3.5 to 9.5; Amersham Pharmacia Biotech, Hong Kong, China). Enzyme activities of β-lactamases were detected by overlaying the gel with 0.5 mM nitrocefin in 0.1 M phosphate buffer, pH 7.0. We used TEM-1, TEM-10, SHV-1, SHV-5, CMY-1, CTX-M-3, and CMY-2 β-lactamases as standards (18,23).

Conjugation Experiments and Plasmid Analysis

Conjugation experiments were performed by the liquid mating-out assay as described (29) with streptomycin-resistant *E. coli* C600 as the recipient (30). Transconjugants were selected on tryptic soy agar plates supplemented by 500 μg of streptomycin and 10 μg of ceftazidime per milliliter. Plasmids from transconjugants were extracted by using a rapid alkaline lysis procedure (31). We analyzed restriction fragment length polymorphism of transferred plasmids using agarose gel electrophoresis of plasmid DNA samples treated with the restriction endonuclease *Eco*RI (Roche Molecular Biochemicals, Mannheim, Germany). The restricted plasmid DNA samples were then transferred to a nylon membrane (Amersham Pharmacia Biotech) and subjected to Southern hybridization. The plasmid sizes of transconjugants were estimated by adding restriction fragments.

Molecular Techniques

Plasmid preparations from clinical isolates and their transconjugants were used as templates in polymerase chain reaction (PCR) assays. Genes related to bla_{TEM}, bla_{SHV}, bla_{CMY}-1, bla_{CMY-2}, and $bla_{CTX-M-3}$ were amplified with the oligonucleotide primers as described (23). Primers 5′-ATAAAATTCTTGAAGACGAAA-3′ and 5′-GACAGTTACCAATGCTTAATCA-3′, corresponding to nucleotides -5 to 15 and 1,074 to 1,053, respectively, of the bla_{TEM-1} structural gene (32), were used to amplify the entire sequences of bla_{TEM}-related genes. Primers AmpC-1C (5′-CTGCTGCTGACAGCCTCTTT-3′) and AmpC-1B (5′-TTTTCAAGAATGCGCCAGGC-3′) (23), which correspond to nucleotides 28-47 and 1,136-1,117, respectively, of the bla_{CMY-2} structural gene (33), were used to amplify an internal fragment of approximately 95% of bla_{CMY}-2-related genes. Both strands of the amplified products were sequenced on an ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA). Colony hybridization and Southern hybridization were performed as described (34,35) with DNA probes prepared from the PCR-generated amplicons. The probes were labeled with [α^{-32}P]dCTP (Amersham Pharmacia Biotech) by using the random priming technique with a commercial kit (GibcoBRL Life Technologies, Gaithersburg, MD).

The genetic relatedness of ceftriaxone-resistant *Salmonella* isolates was investigated by ribotyping by using the method described by Popovic et al. (36). The chromosomal DNA was extracted and digested overnight with 10 U of *Sph*I and *Pst*I or *Eco*RI (Roche Molecular Biochemicals) (36,37). A cDNA probe was prepared by reverse transcription of 16S plus 23S rRNA (Roche Molecular Biochemicals) and was labeled with [a^{-32}P]dCTP. DNA molecular marker II (Roche Molecular Biochemicals) and 1-kb molecular marker (Promega Corp., Madison, WI) were used as size standards.

Results

Emergence of *Salmonella* Isolates Producing CMY-2-Like Enzymes

Six of 384 *Salmonella* isolates displayed intermediate resistance to ceftriaxone by the disk diffusion method. The isolates were recovered from stool samples of two patients who had community-acquired enteric infections in August and November 2000, respectively, which were identified as *S. enterica* serovar Typhimurium. One isolate from each patient (isolates ST275/00 and ST595/00) was investigated further. Both isolates demonstrated reduced susceptibilities to ceftazidime (MIC 64 μg/mL), cefotaxime (MIC 16 μg/mL), ceftriaxone (MIC 32 μg/mL), cefoxitin (MIC 128 μg/mL), and amoxicillin-clavulanic acid (MIC 64 μg/mL) and were susceptible to imipenem (MIC 0.25 μg/mL). Both isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole by the disk diffusion method. Isolate ST275/00 was resistant to chloramphenicol, while isolate ST595/00 was susceptible. Isoelectric focusing showed that both isolates expressed two β-lactamases focusing at pIs 5.4 and 9.0, suggesting that they produced a TEM-1–like enzyme and an AmpC-like β-lactamase. Both isolates yielded an approximately 1.1-kb DNA fragment in PCR with primers for bla_{CMY-2}–like genes, and the amplified sequences obtained by nucleotide sequencing were identical to the homologous region of bla_{CMY-2}, which encodes a class C extended-spectrum cephalosporinase (33). A narrow-spectrum β-lactamase gene, bla_{TEM-1} (18) was also detected by PCR and nucleotide sequencing in both isolates. The two ceftriaxone-resistant *Salmonella* isolates had different ribotypes, suggesting that they are of different clones (Figure 1).

Prevalence of the bla_{CMY-2}–Like Gene in *E. coli* and *K. pneumoniae* Isolates

We found that 659 of 5,520 *E. coli* and 409 of 3,680 *K. pneumoniae* isolates showed resistance to at least one extended-spectrum cephalosporin by the disk diffusion test. These isolates were subjected to colony hybridization and PCR

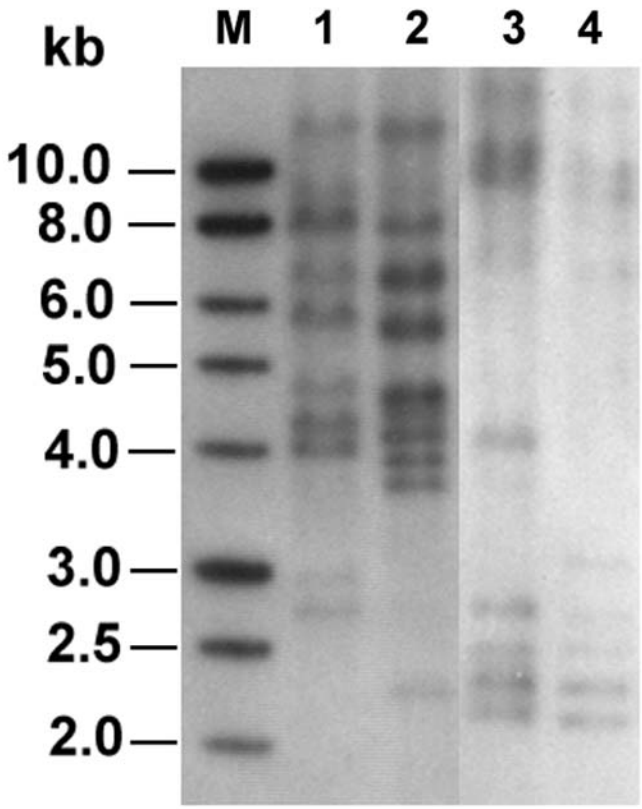

Figure 1. Ribotyping profiles of the two ceftriaxone-resistant Salmonella isolates generated by digestion of chromosomal DNA with SphI and PstI (lanes 1 and 2) or EcoRI (lanes 3 and 4). Lanes 1 and 3, isolate ST275/00; lanes 2 and 4, isolate ST595/00; lane M, 1-kb DNA ladder (Promega Corp., Madison, WI).

assays. Of these isolates, 97 *E. coli* isolates from 48 patients and 4 *K. pneumoniae* isolates from 2 patients gave a strong signal in colony hybridization with the bla_{CMY-2} probe and gave positive results in PCR with the primers for bla_{CMY-2}. Sequence analysis indicated that the sequences of all amplicons were identical to the homologous region of bla_{CMY-2}. The prevalence rates of CMY-2-like enzyme producers in *E. coli* increased from 0% in 1997 and 1998 to >4% in late 2000 (Figure 2). Among the patients with *E. coli* isolates, the incidence of patients with bla_{CMY}-positive isolates increased from 0.0% in 1997 and 1998 to 3.6% in late 2000 (Figure 2).

The sources of *E. coli* isolates harboring a bla_{CMY-2}–like gene and the infections associated with these isolates are summarized in the Table. The isolates and patients are distributed according to the likely locations of infection or colonization. Of the 97 bla_{CMY}-positive *E. coli* isolates, 8 isolates were likely acquired in the community by eight patients. Six of these patients had never been hospitalized, and two had been hospitalized 10 months or 2 years before isolation. *K. pneumoniae* isolates producing the CMY-2-like enzyme were associated with nosocomial bloodstream infections in two patients. Notably, *E. coli* (EC811/00) and *K. pneumoniae* (KP218/00) isolates were recovered from the blood sample of a single patient.

Conjugation Experiments and Plasmid Analysis

One isolate from each patient was subjected to conjugation experiments and plasmid analysis. The bla_{CMY}-positive plasmids were successfully transferred to *E. coli* C600 from 2 *Salmonella* isolates, 40 of 48 *E. coli* isolates, and 2 *K. pneumoniae* isolates. All *E. coli* transconjugants and their plasmid donors showed decreased susceptibilities to ceftazidime (MIC >32 µg/mL), cefotaxime (MIC >16 µg/mL), ceftriaxone (MIC >32 µg/mL), and cefoxitin (MIC >64 µg/mL). The sizes of the transferred plasmids ranged from approximately 65 kb to >100 kb.

Restricted by *Eco*RI, the plasmids from transconjugants of *E. coli* isolates showed 19 restriction patterns, designated TP1-TP19 (Figures 3A and 3C). Patterns TP17 (lane 17) and TP19 (lane 19), the most common patterns, were shown by 6 and 17 transferred plasmids, respectively. Of the 17 isolates with a TP19 plasmid, 3 were acquired from nursing homes and 5 from the community. Of the six isolates with a TP17 plasmid, two could have been acquired from the community. We considered that the remaining isolates with a TP17 or TP19 plasmid were acquired in the university hospital. Each of the remaining 17 patterns was shown by a single transferred plasmid. The TP19 pattern is the only restriction pattern displayed by transconjugants of all three studied bacterial species: *Salmonella* (lane 21), *K. pneumoniae* (lane 22), and *E. coli* (lane 24). Notably, *K. pneumoniae* isolate KP218/00 and *E. coli* isolate EC811/00, from the same patient, both had a TP19-type plasmid. The plasmids from transconjugants of *Salmonella* isolate ST275/00 (lane 20) and another *K. pneumoniae* isolate KP1905/00 (lane 21) showed two distinct restriction patterns. The presence of the bla_{CMY-2}-like gene on plasmids was confirmed by Southern hybridization with the bla_{CMY-2} probe (Figures 3B and 3D).

Discussion

Emergence of ceftriaxone-resistant *salmonellae* has become a great public health concern worldwide (5-17). In

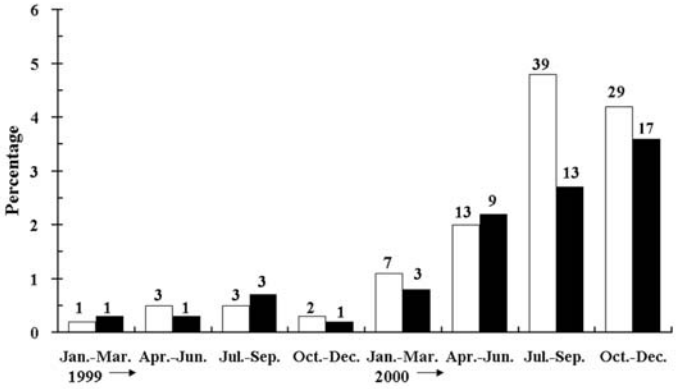

Figure 2. Prevalence rates of the bla_{CMY-2}–like gene among clinical isolates of *Escherichia coli* (□) and percentage of the new cases infected with or colonized by the *E. coli* isolates producing the CMY-2–like enzyme among patients with *E. coli* isolates (■), 1999 and 2000. Numbers over bars denote the numbers of isolates with a CMY-2–like ß-lactamase or the numbers of patients with these isolates.

Table. Likely sources of *Escherichia coli* isolates producing a CMY-2–like β-lactamase and types of infection associated with the isolates

Specimen or infection	No. (%) of isolates or patients[a]			Total
	NCKU[b]	Nursing home	Community	
Specimen				
Blood	12 (14.4)	2 (33.3)	0 (0)	14 (14.4)
Urine	23 (27.7)	1 (16.7)	6 (75.0)	30 (30.9)
Sputum	17 (20.5)	2 (33.3)	1 (12.5)	20 (20.6)
Wound	23 (27.7)	0 (0)	0 (0)	23 (23.7)
Body fluid	2 (2.4)	1 (16.7)	0 (0)	3 (3.1)
Miscellaneous	6 (7.2)	0 (0)	1 (12.5)	7 (7.2)
Total isolate no.	83 (100)	6 (100)	8 (100)	97 (100)
Infection				
Bacteremia	6 (16.7)	1 (25.0)	0 (0)	7 (14.6)
Urinary tract infection	8 (22.2)	0 (0)	3 (37.5)	11 (22.9)
Pneumonia	1 (2.8)	1 (25.0)	0 (0)	2 (4.2)
Wound infection	9 (25.0)	0 (0)	0 (0)	9 (18.8)
Colonization	12 (33.3)	2 (50.0)	5 (62.5)	19 (39.6)
Total patient no.	36 (100)	4 (100)	8 (100)	48 (100)

[a]Isolates and patients are distributed by the likely locations of infection or colonization.
[b]NCKU, National Cheng Kung University Hospital.

Taiwan, no ceftriaxone-resistant isolates were detected from several surveys, which included isolates collected from 1989 to 1998 (4,20,21). One of the surveys was conducted in National Cheng Kung University Hospital (4), and one survey conducted in 1998 included isolates from 22 hospitals (21). In the present study, *Salmonella* isolates resistant to extended-spectrum β-lactams were not detected until August 2000. Thus, the appearance of ceftriaxone-resistant *Salmonella* strains is likely a recent event.

Production of a CMY-2–like β-lactamase was responsible for resistance to extended-spectrum β-lactams in the *Salmonella* strains we isolated. The spread of bla_{CMY-2} in *salmonellae* recently was reported to be an emerging problem in the United States (14,15). In Taiwan, the bla_{CMY-2}–like gene was first detected in *E. coli* isolates collected in 1999 in the university hospital (23). The fact that the bla_{CMY-2}–like gene was found in few isolates from patients with community-acquired infections suggested that the genetic determinant had spread in the community environment (23). The appearance of the bla_{CMY-2}–like gene in the *Salmonella* isolates supports our previous speculation on the spread of the genetic determinant in the community environment. Moreover, the discovery of patients who might have acquired *E. coli* isolates with the CMY-2-like enzyme from nursing homes and in the community (Table) suggests widespread distribution of the bla_{CMY-2}–like gene in southern Taiwan.

Conjugation experiments and plasmid analysis demonstrated the prevalence of conjugative resistance plasmids TP17 and TP19 among *E. coli* isolates. These *E. coli* isolates were recovered from patients hospitalized in the teaching hospital, transferred from nursing homes, or without recent hospitalization histories (Figure 3). Furthermore, the TP19 plasmid was found in a *Salmonella* strain and a *K. pneumoniae* strain, which suggests the interspecies spread of the bla_{CMY-2}–like gene among different health-care settings and the community in Taiwan. The dissemination of the resistance determinant is probably partly because of horizontal transfer of endemic resistance plasmids.

All *K. pneumoniae* isolates producing the CMY-2-like enzyme were acquired by patients hospitalized in the university hospital. Producers of the CMY-2–like enzyme in *K. pneumoniae* were not found until 2000 and, even then, remained very rare (38). Isolate KP218/00 (lane 22, Figure 3C) and *E. coli* isolate EC811/00 (lane 24, Figure 3C) were obtained from the same patient, and both isolates had a TP19 plasmid, suggesting interspecies spread of a resistance plasmid. Thus, the acquisition of the bla_{CMY-2} gene by *K. pneumoniae* in this hospital was likely also a recent event, which occurred after spread of the resistance determinant.

Enterobacteriaceae with plasmid-encoded class C cephalosporinases are typically resistant to cephamycins, extended-spectrum cephalosporins, and monobactams (19). No standard methods exist to detect class C cephalosporinases (39). Failure to detect and report CMY-2–like enzyme producers, and a lack of infection control measures against such organisms might be partially responsible for the rapid spread of the bla_{CMY-2}–like gene in *E. coli* and the increased cases of infections caused by such organisms in our hospital (Table). With the discovery of *Salmonella* isolates producing the CMY-2-like enzyme in Taiwan, our study suggests that the cephalosporinase could become an emerging threat, not only to hospitalized patients, but also to public health. Constant and consistent surveillance is needed to prevent its further spread.

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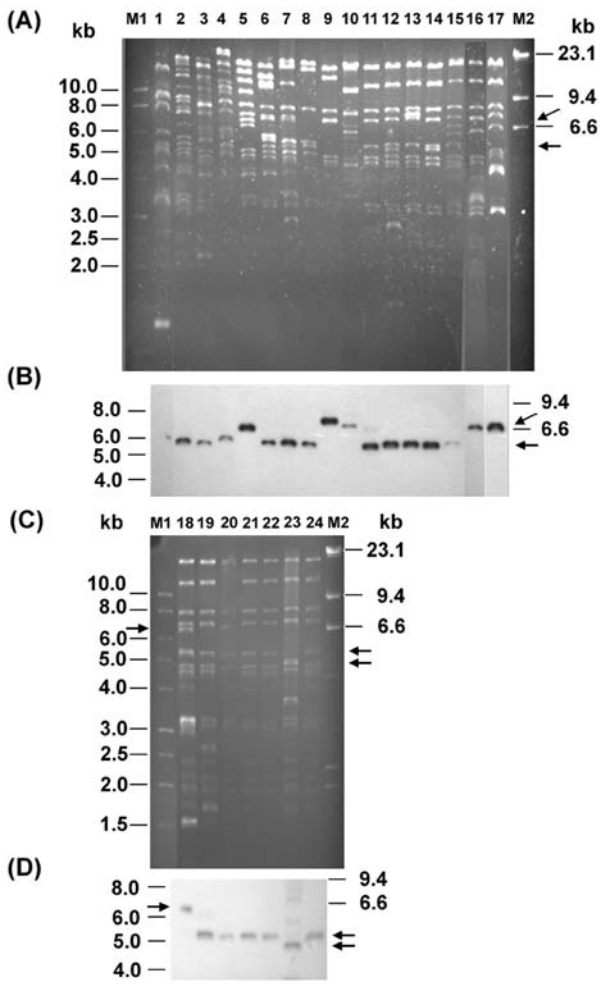

Figure 3. *Eco*RI restriction patterns of plasmids from Escherichia coli transconjugants of clinical isolates (A and C) with a CMY-2–like enzyme and bla_{CMY} Southern hybridization (B and D). Lanes 1–19, restriction profiles (TP1–TP19) of plasmids from 19 transconjugants of E. coli isolates; lanes 20–21, transconjugants of *Salmonella* isolates ST275/00 and ST595/00; lanes 22–23, transconjugants of *K. pneumoniae* isolates KP218/00 and KP1905/00; lane 24, transconjugant of *E. coli* isolate EC811/00; lanes M1 and M2, 1-kb molecular marker and molecular marker II, respectively. Arrows indicate the locations of restriction fragments that were hybridized with the bla_{CMY} probe.

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Bartonella henselae in *Ixodes ricinus* Ticks (Acari: Ixodida) Removed from Humans, Belluno Province, Italy

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The potential role of ticks as vectors of *Bartonella* species has recently been suggested. In this study, we investigated the presence of *Bartonella* species in 271 ticks removed from humans in Belluno Province, Italy. By using primers derived from the 60-kDa heat shock protein gene sequences, *Bartonella* DNA was amplified and sequenced from four *Ixodes ricinus* ticks (1.48%). To confirm this finding, we performed amplification and partial sequencing of the *pap*31 protein and the cell division protein *fts*Z encoding genes. This process allowed us to definitively identify *B. henselae* (genotype Houston-1) DNA in the four ticks. Detection of *B. henselae* in these ticks might represent a highly sensitive form of xenodiagnosis. *B. henselae* is the first human-infecting *Bartonella* identified from *Ixodes ricinus*, a common European tick and the vector of various tick-borne pathogens. The role of ticks in the transmission of bartonellosis should be further investigated.

Bartonella species are facultative intracellular bacteria associated with a number of emerging anthropozoonoses. They have been detected in or isolated from diverse vertebrate hosts, including humans (1–3), various intradomicillary mammals (4–7), and a wide range of wild animals (8,9), which serve as natural vertebrate hosts. Various hematophagous arthropods have been implicated in the ecoepidemiology of *Bartonella* species. *B. bacilliformis*, the etiologic agent of Carrión disease, is transmitted by the sand fly (*Lutzomyia verrucarum*) in the Andes Mountains in Peru, Columbia, and Ecuador (10). B. quintana, the agent of trench fever and bacillary angiomatosis, is found worldwide and is transmitted by the human body louse (*Pediculus humanus*) (11).

B. henselae is another cosmopolitan emerging human pathogen. This agent was first reported in 1990 in association with bacillary angiomatosis (12). The organism was later isolated from the blood of a febrile HIV-positive patient and subsequently described as a new species in 1992 (1). *B. henselae* is now recognized as the causative agent of cat-scratch disease (1), bacillary angiomatosis, peliosis hepatitis, oculoglandular syndrome, and endocarditis (13,14). *B. henselae* is associated with cats, which serve as its reservoir (13,15); the cat flea (*Ctenocephalides felis*) was demonstrated to be a vector (16). Other *Bartonella*-flea associations are apparent: for example, 61% of rat fleas (*Xenopsylla cheopis*) were found infected with bartonellae, including a known human pathogen, *B. elizabethae* (7).

Polymerase chain reaction (PCR) amplification and sequence analysis of various genes are now widely used to differentiate *Bartonella* species. The 16S/23S rRNA intergenic spacer region (17), the heat shock protein (*gro*EL) gene (18), the citrate synthase gene (*glt*A) (19), the riboflavin synthase a-chain gene (*rib*C) (20), the cell division protein (*fts*Z) (21), and the *pap*31 (22) gene sequences were used for detecting, identifying, and classifying the phylogenetic properties and subtyping of *Bartonella* isolates.

Ticks are vectors of more diverse microorganisms than any other arthropod vector (23). The sheep tick (*Ixodes ricinus*) is the most common hard tick species in western Europe and has been established as the vector of tick-borne encephalitis virus, *Babesia* sp., *Borrelia burgdorferi*, *Rickettsia helvetica*, and the agent of granulocytic ehrlichiosis, Anaplasma phagocytophila, (24). *I. ricinus* feeds on a large number of vertebrate hosts. The immature stages of *I. ricinus* are found mainly on small-size vertebrates and can readily feed on humans. Ticks have been suspected to transmit *Bartonella* (25). However, evidence of *Bartonella* infection in ticks has only recently been reported (26,27). Although these observations suggest the possibility of *Bartonella* transmission by ticks, more precise identification of these tick-infecting agents is required to establish their zoonotic potential.

Materials and Methods

Tick Collection and Identification

During 2000–2001, a total of 271 ticks were removed from asymptomatic persons who visited first aid departments in Belluno Province, Italy, for assistance with tick bites. Ticks were removed with tweezers by grasping their mouthpart and pulling straight out from the skin. The tick-bite site was disinfected, and individual ticks were placed in sterile tubes and kept frozen at –70°C for further study. The ticks were stored on ice during the identification procedure, which was done on the basis of their morphologic features by using standard taxonomic keys.

Tick DNA Extraction

All ticks were disinfected by immersion into a 70% ethanol solution for 5 min, rinsed with sterile water, and dried

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in a sterile filter paper. Ticks were then subjected to DNA extraction by using the QiaAmp tissue kit procedure (QIAGEN GmbH, Hilden, Germany). DNA was extracted from ticks according to the manufacturer's protocol. To serve as a negative control, DNA of lice from a laboratory colony that had been fed on an uninfected rabbit was extracted, along with tick DNA, to serve as control. DNA was eluted in a final volume of 200 µL and stored at 4°C until studied further.

PCR Screening of Ticks for the Presence of *Bartonella*

Tick DNA was screened by PCR amplification of the heat-shock protein–encoding gene (*gro*EL) sequences for the presence of *Bartonella*. Primers HSPF1d and BbHS1630.n were used as described (22) and are listed in the Table.

Subtyping of Detected *Bartonella* with *pap*31 and *ftsZ* Partial Sequences

Amplification

Primers used for amplification sequencing of each gene are listed in the Table. PCR reactions were performed in a Peltier model PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA). PCR was carried out in a total volume of 50 µL, consisting of 10 pmol of each primer, 0.5 U of ELONGase mix enzyme (GibcoBRL, Cergy Pontoise, France), 20 mM concentration of each deoxynucleoside phosphate, and 1.8 mM of MgCl2. Two negative controls were included in the reaction: DNA from uninfected lice, and the master mix with sterile water instead of the DNA template. DNA from a culture of *B. elizabethae* was used as the positive control. The following amplification program was used: a first denaturation step at 94°C for 4 min was followed by 44 cycles of denaturation at 94°C for 30 s, annealing at temperatures corresponding to each gene (53°C for *gro*EL and *pap*31 genes and 55°C for *fts*Z) for 30 s, and a hybridization step at 68°C for 1 min. The amplification reaction was terminated with a further extension step at 68°C for 10 min. PCR products were visualized under UV illumination after electrophoresis migration on a 1% gel agarose stained with ethidium bromide.

Sequencing

PCR products were purified by the QIAquick PCR purification kits (QIAGEN GmbH) as recommended by the manufacturer. Primers used for the sequencing of each gene are listed in the Table. PCR products were sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer, Inc., Coignières, France) according to the manufacturer's recommendations. Sequencing products were resolved in an Applied Biosystem automatic sequencer model 3100 (PerkinElmer).

Sequence Analysis

Nucleotide sequences were edited with the Autoassembler (version 1.4; Perkin Elmer) package. Multiple alignment with other *Bartonella* sp. sequences available from GenBank was carried out by using the Clustal W program (28).

Results

Of the 271 ticks collected from patients, 268 were *I. ricinus* (98.9%); the other specimens were one female *I. hexagonus* (0.4%), one female *Rhipicephalus sanguineus* (0.4%), and one female *I. ventalloi* (0.4%). Most of the ticks were nymphs (142; 52.3%), followed by females (115; 42.4%); larva (10; 3.6%), and males (1; 0.4%).

PCR Screening of Ticks for *Bartonella*

By using primers HSPF1d and BbHS1630.n, a single band of PCR product of approximately 1,490 bp was amplified and sequenced in four *I. ricinus* ticks (two females and two nymphs) (1.48%). No amplification product was yielded from

Table. Primers used for polymerase chain reaction or sequencing

Primer	*Bartonella* species	Primer sequence	Target gene	References
HSPF1d[a,b]	All	5′-GAACTNGAAGATAAGTTNGAA-3′	*gro*EL	22
BbHS1630.n[a,b]	All	5′-AATCCATTCCGCCCATTC-3′	*gro*EL	18
HSP1[b]	All	5′-GGAAAAAGTNGGCAATGAAG-3'	*gro*EL	22
HSP2[b]	All	5′-GCNGCTTCTTCACCNGCATT-3′	*gro*EL	22
HSPS1[b]	All	5′-AAGCNCCNGGNTTTGGTGA-3′	*gro*EL	22
HSPS2[b]	All	5′-TCACCAAANCCNGGNGCTT-3′	*gro*EL	22
HSPF2d[b]	All	5′-GAAAGANCGNGTNGATGAT-3'	*gro*EL	22
HSPR2d[b]	All	5′-GTNATNAGAAGNCTNGCAAT-3′	*gro*EL	22
PAPn1[a,b]	*B. henselae* and *B. quintana*	5′-TTCTAGGAGTTGAAACCGAT-3′	*pap*31	22
PAPn2[a,b]	*B. henselae* and *B. quintana*	5′-GAAACACCACCAGCAACATA-3′	*pap*31	22
PAPns2[a,b]	*B. henselae* and *B. quintana*	5′-GCACCAGACCATTTTTCCTT-3'	*pap*31	22
PAPns1[b]	*B. henselae* and *B. quintana*	5′-CAGAGAAGACGCAAAAACCT-3'	*pap*31	22
BaftsZF	*B. henselae*	5′-GCTAATCGTATTCGCGAAGAA-3′	*fts*Z	This study
BaftsZR	*B. henselae*	5′-GCTGGTATTTCCAAYTGATCT-3′	*fts*Z	This study
BhftsZ 1393.n	*B. henselae*	5′-GCGAACTACGGCTTACTTGC-3′	*fts*Z	21
Bh ftsZ 1247.p	*B. henselae*	5′-CGGTTGGAGAGCAGTTTCGTC-3′	*fts*Z	21

[a]Amplification primer.
[b]Sequencing primer.

the negative controls. We used the BLAST tool (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST/); the search of the 1,422-base sequenced fragment from all four ticks revealed a 100% homology with *B. henselae* Houston-1 (GenBank accession no. AF014829).

Subtyping of *Bartonella henselae*

Amplification of the *pap*31 and *fts*Z partial sequences yielded 257-bp and 885-bp, fragments, respectively. Sequences of these products had 100% identity with those of *B. henselae* Houston-1 (GenBank accession nos. AF001274 and AF061746, respectively).

Discussion

Recently, vector biologists and epidemiologists have suggested that ticks may have a role in *Bartonella* transmission (29). In 1996 Kruszewska et al. reported the preliminary finding of a *Bartonella* strain in *I. ricinus* ticks from a park in Walz, Poland (26). Unfortunately, the strain has not been further characterized. In a study conducted in the Netherlands, the 16S rRNA gene sequences of an unspecified *Bartonella* were amplified in >70% of *I. ricinus* ticks removed from roe deer (27). Such a high prevalence of *Bartonella* in ticks is surprising and may be because ticks were collected while they were feeding on bacteriemic hosts (30,31). According to Schouls et al., none of the *Bartonella* organisms detected was a known human pathogen (27). More recently, different *Bartonella* sp., including *B. quintana*, *B. henselae*, *Bartonella* strain cattle-1, *B. washoensis*, and *B. vinsonii* subsp. *berkhoffii*, have been detected in 19.2% of *I. pacificus* ticks collected in California by amplification and sequencing of a fragment of the *glt*A gene (32).

In this study, we report the detection of *B. henselae* in four *I. ricinus* ticks (1.4%) removed from persons in Italy. Because the primers used in the screening PCR generate rather large PCR fragments (1,490 bp), this prevalence could be expected to be greater (usually, the longer the PCR product, the lower the sensitivity). DNA from positive samples was further characterized by using the *gro*EL, the *pap*31, and the *fts*Z genes to establish their relationship with known *Bartonella* sp. and subsp. On the basis of the 16S rRNA genes and immunogenic characteristics, Drancourt et al. (33) suggested the presence of two variants of *B. henselae*. Ribosomal genes such as the 16S rRNA genes are, however, highly conserved within bacteria and can pose the risk of unspecific amplification. Protein-coding genes exhibit a higher degree of sequence variation and thus can be targeted as tools for differentiating strains of the same species. Although the two genogroups of *B. henselae*, Marseille and Houston-1, are closely related, and the respective pathogenicity spectrum of the two serotypes has not been established, the serotypes could be differentiated on the basis of sequences of the *gro*EL, C-terminal region of the *fts*z gene and the *pap*31 gene (21,22).

In northwestern Italy, about 89% of the ticks found to parasitize people were *I. ricinus* (34). In our study, four different species of ticks were recorded from humans, and *I. ricinus* was recorded most frequently (98.9%). All the active life stages of *I. ricinus* were represented.

Experimental studies and epidemiologic observations have suggested that ticks may play a role in the transmission of *Bartonella* sp. *Dermacentor andersoni* was proven to be a competent vector of *B. bacilliformis* in the experimental infection of nonhuman primates many years ago (35). *B. vinsonii* subsp. *berkhoffii* infection was correlated with heavy tick infestation of dogs (36). The cat flea (*Ctenocephalides felis*) is the main arthropod vector of *B. henselae* with cats serving as the main vertebrate reservoirs. Although finding *B. henselae* in ticks might suggest another possible reservoir, *I. ricinus*–like ticks have a very broad host range and are known to infest cats. In our study, none of the persons from whom the positive ticks were collected exhibited symptoms associated with *B. henselae* infection. However, serum specimens from these patients have not been tested. Because bacteremia levels in cat-scratch disease patients have never been consistently demonstrated (37), the tick was unlikely to have acquired *B. henselae* from feeding on patients with asymptomatic cat-scratch disease. Nevertheless, a number of wild animals, including the preferred hosts of both adults and immature stages of *I. ricinus* (38) have been found to be infected with *Bartonella* sp. (8,39). Questing ticks have been found infected with *Bartonella*, including *B. henselae* (32). Furthermore, ticks were suspected of being vectors of *B. henselae* in an epidemiologic study conducted by Lucey et al. in 1992 (25). These authors reported *B. henselae* bacteremia levels in patients who recalled a tick bite but had no history of contact with cats. Ticks were also reported as possible source of infection in some human cases of concurrent infection of the central nervous system by Borrelia burgdorferi and *Bartonella* henselae (40). The evidence that ticks may serve as *Bartonella* vectors appears to be rapidly accumulating.

In conclusion, we have confirmed that ticks feeding on humans were infected with the agent of cat-scratch disease, *B. henselae* (Houston-1). The source of infection of the ticks was not determined. No case of transmission to humans was observed. However, our findings suggest that the ticks were naturally infected. These results support the argument that ticks are involved in the transmission of *Bartonella* organisms and represent a potential source of infection for persons exposed to tick bites. Therefore, we encourage further investigation of ticks as vectors of human pathogenic *Bartonella* strains.

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New Lyssavirus Genotype from the Lesser Mouse-eared Bat (*Myotis blythi*), Kyrghyzstan

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The Aravan virus was isolated from a Lesser Mouse-eared Bat (*Myotis blythi*) in the Osh region of Kyrghyzstan, central Asia, in 1991. We determined the complete sequence of the nucleoprotein (N) gene and compared it with those of 26 representative lyssaviruses obtained from databases. The Aravan virus was distinguished from seven distinct genotypes on the basis of nucleotide and amino acid identity. Phylogenetic analysis based on both nucleotide and amino acid sequences showed that the Aravan virus was more closely related to genotypes 4, 5, and—to a lesser extent—6, which circulates among insectivorus bats in Europe and Africa. The Aravan virus does not belong to any of the seven known genotypes of lyssaviruses, namely, rabies, Lagos bat, Mokola, and Duvenhage viruses and European bat lyssavirus 1, European bat lyssavirus 2, and Australian bat lyssavirus. Based on these data, we propose a new genotype for the Lyssavirus genus.

The *Lyssavirus* genus includes seven genotypes: rabies virus (RABV, genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus 1 (EBLV-1, genotype 5), European bat lyssavirus 2 (EBLV-2, genotype 6), and Australian bat lyssavirus (ABLV, genotype 7) (1,2). Lagos bat virus was isolated from frugivorous bats (*Eidolon helvum*) in Nigeria in 1956 (3) and in 1974 from another bat (*Micropterus pusillus*) in the Central Africa Republic (4). Mokola virus was isolated from shrews (*Crocidura* sp.) and a child in Nigeria in 1968 (5,6), a girl in Nigeria in 1971 (7), and cats in Zimbabwe (8). Duvenhage virus was originally isolated from a human who died after being bitten by a bat in South Africa in 1970 (9) and from *Miniopterus* sp. bats in 1981 (10). EBLV-1 was isolated from bats (*Eptesicus serotinus*) in Germany in 1968 (11), in Poland in 1985 (12), in Denmark, Holland, and Spain in 1987, and in France in 1989 (13). Some isolates of EBLV-1 were obtained from bats in Ukraine and from one human case of bat origin in Russia in 1985 (14,15). EBLV-2 was isolated from a human in Finland in 1985 (16), and from bats in Holland, the Netherlands, Switzerland, and the U.K. EBLV-2 is mainly carried by bats of the *Myotis* genus (*Myotis dasycneme* and *M. daubentonii*) (17). ABLV was isolated from five species of flying fox bats, one species of an insectivorous bat, and two infected humans in 1996 (1,18,19).

Rabies viruses have been reported in Kazakhstan, central Asia (20). Terrestrial rabies viruses have been enzootic in all Central Asian countries and are mainly carried by dogs. Field rabies viruses have been isolated and characterized in Asia, specifically Pakistan, China, Indonesia, Thailand, the Philippines, Malaysia, India, and Sri Lanka (21–26). Isolation of lyssaviruses from bats has been reported only in India and Thailand; however, these viruses were reported as RABV (27,28). Recently, Arguin et al. detected neutralizing antibodies against ABLV in the serum of six bat species (*Mineopterus schreibersi*, *Taphozous melanopogan*, *Philetor brachypteus*, *Scotophilus kuhli*, *Pteropus hypomelanus*, and *Rousettus amplexicaudatus*) in the Philippines (29).

Aravan virus was originally isolated from the brain of a lesser mouse-eared bat (*Myotis blythi*) in Kyrghyzstan in 1991. The antigenic profile of the virus was analyzed by using two panels of antinucleocapsid (N) gene monoclonal antibodies developed at the Wistar Institute of Anatomy and Biology (USA) and the Central Veterinary Laboratory of Great Britain (Weybridge, U.K.) (30–32). These results demonstrated that the virus differed from rabies and serotypes 2 (Lagos bat virus), 3 (Mokola virus), 4 (Duvenhage virus), 5 (EBLV-1), and 6 (EBLV-2). Furthermore, 386 nucleotides (nt) of the N gene were determined from reverse transcription-polymerase chain reaction (RT-PCR) product. Phylogenetic analysis suggested that the Aravan virus did not belong to the rabies virus group (33). In the present study, we determined the entire coding region of the N protein of Aravan virus and evaluated the phylogenetic relationships with other members of the *Lyssavirus* genus.

Materials and Methods

Viruses

Aravan virus was isolated from the brain of one lesser mouse-eared bat (*Myotis blythi*) during a survey of 269 bats collected in the Osh region of Kyrghyzstan from 1988 to 1992 (30,32). A direct fluorescent antibody test was conducted. Aravan virus–infected mouse brains were impressed on glass slides, air-dried, and fixed with acetone. To detect the lyssavirus antigen, specimens were stained with fluorescein isothiocyanate (FITC)–labeled anti-rabies globulin (BBL,

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Cockeysville, MD) or FITC-labeled anti-rabies monoclonal globulin (Centocor Inc., Malvern, PA). FITC-labeled anti-nucleoprotein monoclonal antibodies (NC-MAbs, W502) cross- reactive to lyssaviruses were also used (19).

Amplification of Nucleoprotein cDNA and Direct Sequencing

Total RNA was extracted from virus-infected mouse brain emulsions with a commercial reagent (RNeasy Mini Kit, QIAGEN, Germany). cDNA was obtained with a T-Primed First-Strand kit (Amersharm Biosciences Corporation, Piscataway, NJ). PCR amplification and sequencing of the N gene were performed by using the sense primer AraN-S01 (5′-ATGTACCACCTCTACAATGG-3′, nt 55–74) and an antisense primer AraNC-1400 (5′-TCATGCTCAATTGTAAAAC-3′, nt 1456–1474). The cDNA template (2 μL) was amplified by using primers (AraN-S01 and AraNC-1400), according to the manufacturer's instruction (Super Taq Premix Kit, Sawady Technology, Tokyo, Japan). PCR reactions were incubated at 94°C for 2 min, subjected to 40 cycles of 94°C for 30 s, 48° for 20 s, and 68°C for 2 min, and a final extension at 68°C for 7 min in a DNA thermal cycler (GeneAmpPCR System 9700 Applied Biosystems, Perkin-Elmer Corporation, Japan) (24,25). PCR products were purified by using a commercial kit (QIAquick PCR Purification Kit, QIAGEN). The sequences of the purified DNA products were determined on an automated sequencer (ABI model 310, Applied Biosystems, Foster City, CA) by using a PRIMS Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic Analysis

The 1350-nt and the deduced 450 amino acid (aa) sequences of the N gene of the Aravan virus were aligned with 26 lyssaviruses by using Clustal W program (34). A phylogenetic tree was constructed with the computer software MEGA 2 (35). Pairwise evolutionary nucleotide distances, including both transitions and transversions, were estimated according to Kimura's two-parameter method. Phylogenetic trees were constructed by the neighbor-joining method with 1,000 replicates to generate bootstrap probabilities at each node (36).

Results and Discussion

Direct Fluorescent Antibody Assay

The three stains used in this study reacted against the Aravan virus infected mouse brain impressions. Fluorescence showed more scattered inclusions than those of the challenge virus standard in the acetone-fixed mouse brain smear (data not shown). The results confirmed that the Aravan virus is a lyssavirus.

Nucleotide and Deduced Amino Acid Sequence Identities among the Aravan Virus and Other Lyssaviruses

The 1350-nt and the deduced 450 aa sequences of the Aravan virus were compared with 26 representative lyssaviruses belonging to seven genotypes (Table 1). We selected 16 representative rabies variants from the eight diverse groups, including rabies variants from geographic areas of Asia near

Table 1. Lyssavirus isolates used in this study

Genotype[a]	Yr isolated	Virus (strain)	Country of isolation	Host	Accession no.
1 (Rabies)	?	CTN	China	?	AF367863
1 (Rabies)	1983	8738THA	Thailand	Human	U22653
1 (Rabies)	?	?	India	?	AF374721
1 (Rabies)	1996	SRL1032	Sri Lanka	Jackal	AB041964
1 (Rabies)	1992	9218TCH	Chad	Dog	U22644
1 (Rabies)	1988	9141RUS	Russia	Arctic fox	U22656
1 (Rabies)	?	9196FX	Canada	*Vulpes vulpes*	L20676
1 (Rabies)	1987	1500AFS	Rep.South Afr.	Yellow mongoose	U22628
1 (Rabies)	1985	9142EST	Estonia	Racoon dog	U22476
1 (Rabies)	1986	8681IRA	Iran	Dog	U22482
1 (Rabies)	1985	86118BRE	Brazil	Vampire bat	U22479
1 (Rabies)	1992	BBCAN	Canada	*Eptesicus fuscus*	AF351833
1 (Rabies)	1992	MYCAN	Canada	*Myotis lucifugus*	AF351839
1 (Rabies)	?	?	Chile	*Tadarida brasiliensis*	AF070450
1 (Rabies)	1988	Insectivorous bat	Chile	*Insectivorous bat*	AF351850
1 (Rabies)	1989	PA R89	USA	Raccoon	U27221
2 (Lagos bat)	1958	8619NGA	Nigeria	*Eidolon helvum*	U22842
3 (Mokola)	?	Y09762	?	?	Y09762
3 (Mokola)	1981	MOK	Zimbabwe	Cat	U22843
4 (Duvenhage)	1986	86132AS	Rep.South Africa	Human	U22848
5 (EBLV-1)	1985	8615POL	Poland	*Eptesicus serotinus*	U22844
5 (EBLV-1)	1989	8918FRA	France	*E. serotinus*	U22845
6 (EBLV-2)	1986	9007FIN	Finland	Human	U22846
6 (EBLV-2)	1986	9018HOL	Holand	*M. dasycneme*	U22847
7 (ABLV	1996	Ballina	Australia	*Pteropid alecto*	AF006497
7 (ABLV)	1996	Insectivorous isolate	Australia	Insectivorous bat	AF081020
?	1991	Aravan	Kyrghystan	*M. blythi*	AB094438

[a]EBLV-1, European bat lyssavirus 1; EBLV-2, European bat lyssavirus *2*; ABLV, Australian bat lyssavirus; MOK, strain name in Mokola virus

Kyrghyzstan and from bats and raccoons in North and South America (25,37). The nucleotide and amino acid sequence identities among all 27 lyssaviruses, including Aravan virus, were calculated. Then genotype 1 was represented by seven rabies viruses (SRL1032, 86118BRE, 1500AFS, 9218TCH, 8738THA, insectivorous bat/Chile, and PA R89), and genotypes 2, 3, 4, 5, 6, and 7 were represented by Lagos bat virus (8619NGA), Mokola virus (MOK/U22843), Duvenhage virus (86132AS), EBLV-1 (8918FRA), EBLV-2 (9007FIN), and ABLV (Balina/AF006497), respectively (Table 2). The nucleotide sequence identity of Aravan virus with the genotypes 4, 5, 6, and 7 was 77% to 78%; with genotype 1, 75% to 77%; and with genotypes 2 and 3, 72% to 74%. The most extensive nucleotide sequence differences among isolates of genotype 1 were between the raccoon isolate (PA R89) and the African and Asian isolates (82.8% to 82.9% identity). The Aravan virus demonstrated 92% aa sequence identity with genotypes 4, 5, and 7; 89% with genotype 6; and 81% to 85% with genotypes 2 and 3. The maximum variation of amino acid sequences within genotype 1 was exhibited between a vampire bat isolate from Brazil and an African isolate (93.1% to 93.3% identity). Genotype 4 (Duvenhage virus) was most closely related to genotype 5 (EBLV-1) with nucleotide and amino acid sequence identities of 79.8% and 93.3 %, respectively. ABLV (genotype 7) was closely related to SRL1032 (genotype 1, Sri Lankan rabies virus) with a 93.1% aa sequence identity. These values were almost same as maximum variation of genotype 1. Based on our present data, we determined that isolates sharing <79.8% nt and 93.1% to 93.3% aa sequence identities belonged to different genotypes. In several studies, thresholds of <80% nt and 92% or 93% aa sequence identities warranted the proposal of a new genotype (1,23,38). Hence, the nucleotide and amino acid percentage identity values demonstrated that Aravan virus should be regarded as a new lyssavirus genotype.

Phylogenetic Analysis

A phylogenetic tree of 27 lyssaviruses, including the Aravan virus, based on the 1350-nt sequence of the N gene was constructed by using the vesicular stomatitis Indiana virus (VSIV, tsW16B/U13898) as an outgroup (Figure, a). The lyssaviruses divided into two groups: one group consisted of genotypes 2 and 3, and the other consisted of genotypes 1, 4, 5, 6, 7, and the Aravan virus. The latter group was divided into six distinct clusters corresponding to genotypes 1, 7, 6, and 5 (high bootstrap values of 98%, 99%, 100%, and 100%, respectively), then Aravan virus and genotype 4. Moreover, the Aravan virus clustered with genotypes 4, 5, and 6 (low bootstrap value of 59%). Duvenhage virus (genotype 4) and EBLV-1 (genotype 5) formed the same cluster (high bootstrap value of 91%), and are therefore closely related. The Aravan virus occupied the phylogenetic position between genotype 6 and the cluster of genotypes 4 and 5. We also constructed a phylogenetic tree based on the deduced 450-aa sequences of the N gene (Figure,b). Similar to the nucleotide data, the amino acid sequences divided into two large groups and further subdivided into eight groups. One group consisted of genotypes 2 and 3 (bootstrap value of 89%), and the other group consisted of genotypes 1, 7, 6, 4, and 5, and the Aravan virus (high bootstrap value of 100%). The latter group had three distinct clusters corresponding to genotypes 1, 7, and 6 (high bootstrap values of 100%, 99%, and 100%, respectively), genotypes 4 and 5 (same cluster with a high bootstrap value of 98%), and the Aravan virus. The Aravan virus did not group with any other genotypes and is located at a position close to the cluster of genotypes 4 and 5 (bootstrap value of 66%).

These results, along with those in Table 2 and the Figure, suggest that the Aravan virus does not belong to any of the seven Lyssavirus genotypes (rabies, Lagos bat, Mokola, Duvenhage, EBLV-1, EBLV-2, and ABLV). Thus, we propose that the Aravan virus forms an independent cluster and is a new member of the Lyssavirus genus.

In this article, we have reported the first lyssavirus distinct from rabies virus originating on the Asian continent. The Aravan virus was more closely related to genotypes 4, 5, and, to a lesser extent, 6, which circulates among insectivorus bats in Europe and Africa. The lesser mouse-eared bat, from which

Table 2. Comparison of nucleotide and deduced amino acid sequences of Aravan virus with other 13 lyssaviruses

Amino acid sequence identity (%)	Nucleotide sequence identity (%)													
	Genotype 1 (rabies virus)								Genotype 2	Genotype 3	Genotype 4	Genotype 5	Genotype 6	Genotype 7
	Aravan	SRL1032	U22479BRE	U22628AFS	U22644CHAD	U22653THA	AF351850	U27221	LBU22842	MKU22843	86132AS	8918FRA	9007FIN	AF006497
Aravan	100.0	75.6	76.0	76.2	74.8	75.9	77.0	76.2	74.3	72.4	78.2	77.9	77.2	76.9
SRL1032	90.9	100.0	84.7	85.9	86.9	86.8	86.6	84.1	74.2	70.2	73.9	75.6	74.7	78.0
U22479BRE	88.2	95.3	100.0	83.3	**83.0**[a]	83.3	89.9	83.7	73.8	69.6	73.8	75.3	74.2	77.2
U22628AFS	89.3	96.7	**93.3**[b]	100.0	85.6	83.5	83.6	83.2	73.3	70.4	73.9	75.3	74.6	78.0
U22644CHAD	88.7	96.9	**93.1**[b]	94.4	100.0	86.0	84.0	**82.9**[a]	73.0	69.0	73.1	75.0	74.8	77.2
U22653THA	89.8	97.1	94.2	95.1	95.3	100.0	84.2	**82.8**[a]	73.4	69.7	74.4	75.9	74.7	77.3
AF351850	90.0	97.1	95.8	95.8	94.9	95.6	100.0	86.1	73.5	70.1	74.3	75.0	74.8	77.4
U27221	89.6	95.8	93.6	94.2	93.8	94.9	95.1	100.0	73.1	70.2	74.2	74.6	75.7	77.1
LBU22842	84.7	82.9	81.8	81.1	80.4	81.8	83.1	82.2	100.0	74.8	73.4	74.4	72.5	72.6
MKU22843	80.9	78.2	77.6	77.3	76.2	77.6	78.4	77.8	84.4	100.0	71.6	69.9	69.2	71.0
86132AS	91.8	88.9	86.9	87.6	87.1	87.6	88.7	87.8	85.8	80.7	100.0	**79.8**[c]	75.9	77.0
8918FRA	92.0	88.9	86.7	87.8	87.8	88.4	88.2	88.2	83.8	79.1	**93.3**[d]	100.0	78.0	76.9
9007FIN	88.9	88.0	86.2	87.1	86.9	88.0	87.3	87.3	79.1	76.2	86.2	88.0	100.0	77.2
AF006497	92.0	**93.1**[d]	91.1	91.6	91.1	91.8	91.8	91.3	82.0	79.8	90.0	89.8	87.8	100.0

[a]The values were shown as maximum variation of nucleotide sequence identities (%) within genotype 1.
[b]The values were shown as maximum variation of amino acid sequence identities (%) within genotype 1.
[c]Thresholds of nucleotide sequence identities % as different genotypes.
[d]Thresholds of amino acid sequence identities % as different genotypes.

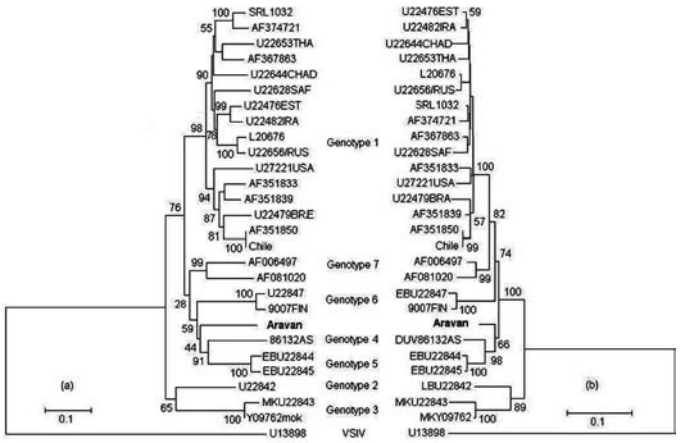

Figure. Rooted phylogenetic tree showing genetic relationships among Aravan virus and 26 lyssaviruses. Phylogenetic relationships were determined by comparing the 1350-nucleotide sequences of the nucleoprotein (N) gene (a) and the deduced 450-amino-acid sequences (b) by the neighbor-joining method (36). The sequences used were those of genotypes 1, 2, 3, 4, 5, 6, and 7 shown in Table 1 by using vesicular stomatitis Indiana virus (VSIV) as an outgroup (tsW16B/U13898).

the Aravan virus was isolated, is widely distributed in northern Africa, the Mediterranean, southern Europe, Crimea, Caucasus, Palestine, southwest Asia, and parts of central and eastern Asia. This information should be considered in the discussion of lyssavirus classification and evolution, as it suggests the possibility of a broader geographic distribution of the Aravan virus. We have no information about human rabies caused by bat exposure from central Asia, and rabies surveillance in this area is not known well. Based on this information and the virus' misdiagnoses as rabies, we consider that transmission of Aravan virus to humans is possible. Indeed, this finding stimulates interest in new genotypes of lyssaviruses and is important from the viewpoint of public health, necessitating further lyssavirus surveillance of bats on the Asian continent.

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Molecular Detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in Cat Fleas, France

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The prevalences of *Bartonella*, *Rickettsia*, and *Wolbachia* were investigated in 309 cat fleas from France by polymerase chain reaction (PCR) assay and sequencing with primers derived from the gltA gene for *Rickettsia*, the *its* and *pap*31 genes for *Bartonella*, and the 16S rRNA gene for *Anaplasmataceae*. Positive PCR results were confirmed by using the Lightcycler and specific primers for the *rOmpB* of *Rickettsia* and *glt*A of *Bartonella*. *R. felis* was detected in 25 fleas (8.1%), *W. pipientis*, an insect symbiont, in 55 (17.8%), and *Bartonella* in 81 (26.2%), including *B. henselae* (9/81; 11.1%), *B. clarridgeiae* (55/81; 67.9%), *B. quintana* (14/81; 17.3%), and *B. koehlerae* (3/81; 3.7%). This is the first report of the amplification of *B. quintana* from fleas and the first description of *B. koehlerae* in fleas from an area outside the United States. Cat fleas may be more important vectors of human diseases than previously reported.

Fleas can be found worldwide and are vectors of several important zoonoses, including plague caused by *Yersinia pestis* (1). The classic cycle of *Rickettsia* typhi, the agent of murine typhus, involves rats and the rat flea, *Xenopsylla cheopis*, the main vector (2). The disease is transmitted by flea bites or contact with flea feces. Recently, murine typhus has been shown to exist in some endemic foci where neither rats or their fleas are found. Subsequently, in the United States, *R. typhi* was found to be maintained in the cat flea, *Ctenocephalides felis*, collected from opossums (2). *R. felis* is the recently recognized agent of flea-borne spotted fever, which has been reported in various countries, including the United States, Mexico, Brazil, Germany, and France (3–6). *C. felis* is apparently the main vector of this new rickettsial disease, and *R. felis* has been found in this flea in several countries, including the United States (2), Brazil (7), Spain (8), and Ethiopia (6). A reservoir of flea-borne spotted fever in the United States may be the opossum. Of major importance to the epidemiology of the above rickettsioses is the maintenance of *R. typhi* or *R. felis* in their hosts by transovarial transmission (9) and the fact that neither organism is lethal for fleas.

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Bartonellae are gram-negative bacteria that cause various human diseases and have various arthropods, such as lice, ticks, and fleas, as vectors (10). Transmission to humans may also occur by scratches or bites from reservoir hosts, especially cats. Among the genus *Bartonella*, four species have been isolated from the blood of cats. Two of these species occur worldwide, *B. henselae*, the agent of cat-scratch disease, and *B. clarridgeiae*, which might be another agent of cat-scratch disease (11), and two have been reported only from the United States, *B. koehlerae* and *B. bovis* Bermond (*B. weissii*) (12–14). The main vector of *B. henselae* infections in cats is most likely the cat flea (2), whereas the vectors of *B. koehlerae* and *B. weissii* are unknown. Detection of *B. clarridgeiae* in cat fleas by polymerase chain reaction (PCR) amplification has indicated the possible role of fleas as vectors of the organism (15). Cats are the reservoirs of the bacteria, and the prevalence of cats with *B. henselae* and *B. clarridgeiae* bacteremia ranges from 4% to 70%, according to the geographic location (15).

We describe experiments in which we used PCR amplification and DNA sequencing to detect *Rickettsia*, *Bartonella*, and *Ehrlichia* species in *C. felis* collected from various sites in France.

Materials and Methods

Source and Identification of Cat Fleas

Cat fleas (*C. felis*), identified according to current taxonomic keys (16), were obtained from various departments by the Veterinary School of Toulouse, located throughout France. Cat fleas were sent to our laboratory in sealed, preservative-free, plastic tubes at room temperature. To prevent contamination problems, as positive controls we used DNA from *R. montanensis* (ATCC VR-611) and *Bartonella elizabethae* F9251 (ATCC 49927), which react with the primer pairs we used in PCR but give sequences distinct from the species we were investigating. For negative controls, we used sterile water and human body lice reared in our laboratory; both negative controls were tested after every seventh cat-flea sample in our PCR.

DNA Extraction

Fleas were immersed for 5 min in a solution of 70% ethanol/0.2% iodine, washed for 5 min in sterile distilled water

and crushed individually in sterile Eppendorf tubes with the tip of a sterile pipette. Their DNA was extracted by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. This kit was also used to extract DNA from the human body lice reared in our laboratory under standard conditions and used as negative controls.

Detection of *Bartonella* spp., *Rickettsia* spp., and Anaplasmataceae

DNA extracts were amplified in two different runs with different target genes to confirm the results. In the first run we tested all the cat fleas by using genus-specific primers (Table 1) derived from the intergenic spacer region (its gene), the *pap*31 gene for *Bartonella* (19,20), the citrate synthase–encoding gene for *Rickettsia* (17), and the 16S RNA gene for *Anaplasmataceae* (21,22). A total volume of 2.5 µL of the extracted DNA was amplified in a 25-µL reaction mixture containing 12.5 pmol of each primer, 200 µM of dATP, dCTP, dGTP, and dTTP, and 1 U of Elongase in 1X PCR buffer with 0.8 µL of 25 mM MgCl2 (Life Technologies, Cergy Pontoise, France). PCR was carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, MA) with an initial 3-min denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C (30 s), annealing at 50°C (30 s), and extension at 72°C (1 min). Amplification was completed by holding the reaction mixture at 68°C for 3 min to allow complete extension of the PCR products. PCR products were resolved by electrophoresis in 1% agarose gels, and when appropriately sized products were found, they were purified by using Qiagen columns (QIAquick Spin PCR purification kit; QIAGEN) before sequencing.

Any positive sample was tested again by using different primers and real-time PCR technology. For *Bartonella*, the forward primer of the *glt*A gene (Table 1) was used for all samples and species-specific primers targeting *B. quintana*, *B. henselae*, *B. clarridgeiae*, and *B. koehlerae* were designed as reverse primers. For *Rickettsia*, we used *rOmpB*-specific primers targeting *R. felis* (Table 1) (18). A real-time PCR assay was performed on DNA extracts in a Lightcycler instrument (Roche Biochemicals, Mannheim, Germany). The amplification program began with a denaturation step of 95°C for 120 sec, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 5 sec, and extension at 72°C for 10 s with fluorescence acquisition at 54°C in single mode. Melting curve analysis was done at 45°C to 90°C (temperature transition, 20°C/s) with stepwise fluorescence acquisition by real-time measurement of fluorescence directly in the clear glass capillary tubes. Sequence specific standard curves were generated by using 10-fold serial dilutions (105 to 106 copies) of standard bacterial concentration of *Bartonella*.

The positive PCR products of the two runs for *Bartonella*, *Rickettsia*, and *Wolbachia* were sequenced by using the d-rhodamine terminator cycle-sequencing ready reaction kit (PE Applied Biosystems, Les Ulis, France) according to the manufacturer's protocol. Sequences obtained were compared with those in the GenBank DNA database by using the program BLAST (version 2.0, National Center for Biotechnology Information; available from: URL: http://www.ncbi.nlm.nih.gov).

Results

Overall, 309 cat fleas from 92 cats from all areas of France (north, west, east, and south) were tested. Almost two thirds (60/92; 65%) of the cats lived both outdoors and indoors, 20% lived predominantly outdoors, and 15% lived exclusively indoors. Our negative controls consistently failed to yield detectable PCR products, whereas our positive controls always gave expected PCR products. We found a total of 89 fleas (28.8%) that were infected: 25 were positive for *R. felis* (25/309; 8.1%) as determined by citrate synthase–gene sequencing, and 81 were positive for *Bartonella* species (81/309; 26.2%) as determined either by its gene or *pap*31 gene sequencing (Table 2). The sequences of the DNA amplicons we obtained were identical to those of *R. felis* (Genbank accession no. U33922), *B. henselae* (Genbank accession no. AF369527), *B. clarridgeiae* (GenBank accession no. AF312497), *B.*

Table 1. Oligonucleotide primers used for polymerase chain reaction amplification and sequencing

Primer (reference)	Nucleotide sequence	Detected organism	References
CS-877 (*glt*A gene)	GGG GGC CTG CTC ACG GCG G	*Rickettsia* species	(17)
CS-1273 (*glt*A gene)	ATT GCA AAA AGT ACA GTG AAC A	*Rickettsia* species	(17)
BM59 (*rOmpB* gene)	CCG CAG GGT TGG TAA CTG C	*Rickettsia* species	(18)
B807 (*rOmpB* gene)	CCT TTT AGA TTA CCG CCT AA	*Rickettsia* species	(18)
URBarto1 (*its* gene)	CTT CGT TTC TCT TTC TTC A	*Bartonella* species	(19)
URBarto2 (*its* gene)	CTT CTC TTC ACA ATT TCA AT	*Bartonella* species	(19)
PAPn1 (*pap*31 gene)	TTC TAG GAG TTG AAA CCG AT	*Bartonella* species	(20)
PAPn2 (*pap*31 gene)	GAA ACA CCA CCA GCA ACA TA	*Bartonella* species	(20)
BartogltAForward (*glt*A gene)	TTC CGY CTT ATG GGT TTT GG	*Bartonella* species	This report
Bartokoehlerae (*glt*A gene)	AAC AAA ATA TTC ATC ATT CAG G	*B. koehlerae*	This report
Bartoclarridgeiae (*glt*A gene)	AAA GCA ATT TTT TCA AGT TCC	*B. clarridgeiae*	This report
Bartohenselae (*glt*A gene)	CAT TTC TGT TGG AAA TCC TAG	*B. henselae*	This report
Bartoquintana (*glt*A gene)	TTT TAA TGT AAT GCC AGA ATA A	*B. quintana*	This report
EHR16SD (16S rRNA gene)	GGT ACC YAC AGA AGA AGT CC	*Wolbachia*	(21)
EHR16SD (16S rRNA gene)	TAG CAC TCA TCG TTT ACA GC	*Wolbachia*	(21)

Table 2. Repartition of 89 *Bartonella*-positive fleas

	B. henselae	*B. clarridgeiae*	*B. quintana*	*B. koehlerae*	No coinfection	Total
Rickettsia felis	0	5	12	0	8	25
No coinfection	9	50	2	3	17	
No. of fleas positive for *Bartonella*	9	55	14	3		81

koehlerae (GenBank accession no. AF312490), or *B. quintana* (GenBank accession no. AF368391). The *Bartonella* species we identified were *B. henselae* (9/89; 11.1%), *B. clarridgeiae* (55/89; 67.9%), *B. quintana* (14/89; 17.3%), and *B. koehlerae* (3/89; 3.7%). Our results were confirmed by a second PCR with the Lightcycler and specific primers for the *glt*A gene for *Bartonella* and *rOmpB* for *Rickettsia*. Because the primers were species-specific, we were able to demonstrate that none of the fleas contained more than one *Bartonella* species. Seventeen fleas, however, contained *R. felis* and *B. quintana* (12 fleas) or *B. clarridgeiae* (5 fleas) (Table 2). Lastly, in 55 fleas (17.8%) our *Anaplasmataceae*-specific primers amplified DNA with a sequence identical to that of *Wolbachia pipientis* (Genbank accession no. U23709).

Discussion

Rickettsia and *Bartonella* infections occur worldwide and may cause serious diseases in people. Most of these pathogenic bacteria are transmitted to people by arthropod vectors such as ticks, fleas, and lice, which are also involved in the maintenance of the bacteria. The detection of these pathogenic bacteria in their vector arthropods can be used in epidemiologic studies and control strategies (2). We tested cat fleas from around France for the presence of *Rickettsia*, *Bartonella*, and *Ehrlichia* species. We found DNA of *R. felis* and various *Bartonella* species in these fleas by using PCR with primers for different specific genes and sequencing to confirm our results. All our negative controls gave no PCR products, and all fleas that tested positive were also positive with other PCRs with different target genes and different techniques.

We report for the first time the presence of *R. felis* in cat fleas from France. This bacteria has been detected previously in wild cat fleas from various countries, including the United States (2), Ethiopia (6), and, very recently, Spain (8), and Brazil (7). Since *C. felis* has a worldwide distribution and infestation with these fleas is very common, some have assumed that *R. felis* and flea-borne spotted fever should occur worldwide. We found that 8.1% of fleas from domestic cats were infected with *R. felis*, suggesting that clinical cases in humans may be prevalent in France and probably in Europe. In the United States, the infection rates of fleas, as determined by PCR amplification, have been reported to vary from 43% to 93% (2). Since bacteria are maintained transovarially, *R. felis* may be used as a marker to follow changes in the infection rates over time (2). In people, clinical cases of flea-borne spotted fever have been reported in the United States (Texas) (3), Mexico (4), France, and Brazil (6), and, very recently, in Germany (5). Preliminary serologic results indicate that flea-borne spotted fever might occur in France (6), and we have now shown that fleas in France are infected with *R. felis*. The disease is probably more prevalent than expected, even if the risk of transmission by fleas is unknown. Cross-reactions in serologic testing for *R. felis* are unpredictable in our experience, and thus serologic tests for *R. felis* should be performed in patients suffering from fever of unknown origin. Our findings of *R. felis* in French fleas indicate, then, that *R. felis* should be used, along with other spotted fever group rickettsiae, in serologic tests on patients suspected of having a spotted fever group rickettsiosis.

To date, *B. henselae* is the only recognized agent of cat-scratch disease; epidemiologic studies have implicated cats, which remain bacteremic for months to years, as the major reservoirs of *B. henselae* (23,24). Its DNA has been amplified from fleas found on bacteremic cats, and transmission of *B. henselae* to cats by *C. felis* has been demonstrated (23,25,26). Flea infestation was found to be more common in bacteremic cats than in nonbacteremic cats. The prevalence of *B. henselae* in fleas in our study was 3%, whereas *B. henselae* has been isolated from the blood of 4% to 70% of cats, depending on location, cat population, and flea infestation rate but not depending on the infection rate of fleas on the cats or the seropositivity of the cats (15,25).

Our study has confirmed that *B. clarridgeiae* may be detected in fleas, and we found a 17.8% prevalence in infected fleas, that is, 67.9% of all fleas positive for *Bartonella* by PCR. Until now, *B. henselae* was the most common species isolated from cats, and the prevalence of *B. clarridgeiae* has ranged from 16% to 30% (25). *B. clarridgeiae* is more prevalent in European cats than in American cats (25). The difference of recovery rate of *B. clarridgeiae* in these studies may be explained by the fact that the number of bacteria in the blood of cats infected with *B. clarridgeiae* is low (unpub. data) and because *B. clarridgeiae* grows more slowly in culture (27). Also, freezing of whole blood improves the recovery rate of *B. clarridgeiae* since bacteria are probably localized in erythrocytes (15).

We report, for the first time, the presence of *B. koehlerae* in cat fleas. This recently described species has only been reported in the blood of cats in the United States (12), and our findings support the idea that this bacteria might be transmitted by cat fleas, like other *Bartonella*, and that it has a worldwide distribution. The prevalence of this new *Bartonella* species in cats remains unknown and is probably underestimated because the bacteria are extremely fastidious and only grow on chocolate agar and not on heart infusion agar with rabbit blood, Columbia, or on sheep blood agar, which are currently widely used for the isolation of *Bartonella* (12).

We also report for the first time that cat fleas can contain *B. quintana*; this is surprising because, to date, the body louse

was the only known vector of this species, and people are the only known natural reservoirs. Previously, however, in two clinical reports of chronic adenopathy attributed to *B. quintana* infection, the only epidemiologic risk identified was the presence of infected cat fleas (28,29). The role of the cat flea as a potential vector of *B. quintana* and its related diseases needs to be clarified, and new investigations of patients in contact with cats and their fleas are indicated. Also, we have reported cases of endocarditis due to *B. quintana* for which no epidemiologic risks (alcoholism or homelessness) were found (30,31). These findings and the fact that *B. quintana* DNA sequences can be found in ticks (32) indicate that other arthropod vectors apart from lice may be involved in the epidemiology of *B. quintana* infections.

Coinfection of cats with *B. clarridgeiae* and *B. henselae* has been reported, as has infection with the two different genotypes of B. henselae, Marseille and Houston (25,33). Recent reports suggest that interspecific competition between closely related rickettsiae may control rickettsial establishment in arthropods (dual infections in arthropod vectors are rare and have not yet been observed in individual fleas) (34). Fleas, however, feed intermittently on different hosts and thus may acquire multiple bacterial strains and pass these to their progeny transovarially (2). Our finding of *R. felis* with either *B. clarridgeiae* or *B. quintana* in fleas suggests that dual infections may occur in humans infected by flea feces. We did not, however, find fleas infected with several species of *Bartonella* although cats may be bacteremic with two different species. We believe that the techniques we used should have been sensitive enough to detect such coinfections, and that competition between two *Bartonella* species may result in one species being eliminated and the flea becoming infected with the other *Bartonella* species. Further studies are indicated to confirm this possibility.

Finally, we report, for the first time, the presence of *W. pipientis* in fleas, which we found while seeking Ehrlichia using *Anaplasmataceae*-specific primers. This bacteria is known to be an endosymbiont of several arthropods, primarily insects, and it has a role in parthenogenesis (35). Any effects that it might have on fleas, however, are unknown.

In summary, our study has provided evidence that cat fleas are commonly infected by *R. felis* and *Bartonella* species in France. Further, we have shown for the first time that *B. quintana* may infect fleas. The description of *B. quintana*–related diseases in patients with histories of contact with fleas indicates the possibility that fleas may be vectors of the organism. Finally, we reported for the first time the presence of *B. koehlerae* outside the United States.

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European Echinococcosis Registry: Human Alveolar Echinococcosis, Europe, 1982–2000

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Surveillance for alveolar echinococcosis in central Europe was initiated in 1998. On a voluntary basis, 559 patients were reported to the registry. Most cases originated from rural communities in regions from eastern France to western Austria; single cases were reported far away from the disease-"endemic" zone throughout central Europe. Of 210 patients, 61.4% were involved in vocational or part-time farming, gardening, forestry, or hunting. Patients were diagnosed at a mean age of 52.5 years; 78% had symptoms. Alveolar echinococcosis primarily manifested as a liver disease. Of the 559 patients, 190 (34%) were already affected by spread of the parasitic larval tissue. Of 408 (73%) patients alive in 2000, 4.9% were cured. The increasing prevalence of *Echinococcus multilocularis* in foxes in rural and urban areas of central Europe and the occurrence of cases outside the alveolar echinococcosis–endemic regions suggest that this disease deserves increased attention.

Human alveolar echinococcosis, caused by the metacestode of the fox tapeworm *Echinococcus multilocularis*, is considered to be the most pathogenic zoonosis in temperate and arctic regions of the Northern Hemisphere. Transmission to humans occurs when eggs of the tapeworm, excreted by the final hosts (usually foxes), are accidentally ingested. The larva's primary target organ is the liver, where it proliferates slowly, but the larva also spreads into extrahepatic structures and even metastasizes to distant organs. In earlier untreated cohorts, the fatality rate exceeded 90% within 10 years (1). The introduction of benzimidazoles for alveolar echinococcosis treatment in 1976 has considerably improved the prognosis (2,3). Long-term follow-up of 117 patients showed that the 5-year actuarial survival rate increased to 88% with this improved management (4). As chemotherapy is parasitostatic only, long-term administration is mandatory for most patients (5,6). Radical surgical excision, the only curative treatment, is feasible in a few select cases (7).

In Europe, previous assessments of human cases did not cover all alveolar echinococcosis–endemic areas at comparable periods. In Switzerland, where laboratory-diagnosed alveolar echinococcosis was a reportable disease until 1997, the annual incidence ranged from 7.2 to 10.4 (0.10–0.18/100,000) and did not markedly vary during a 36-year period (8). In Austria, an average incidence of 2.5 cases per year corresponded to an incidence of 0.034/100,000 from 1985 to 1999 (9). These low numbers of human infections throughout a whole country failed to alarm public health authorities. However, two findings are beginning to attract more attention: 1) high annual incidence rates occurring regularly in particular regions, e.g., the Swiss Jura (0.74/100,000) (10); and 2) a presumed range extension of the parasite in its sylvatic life cycle.

In Europe, the Red Fox (*Vulpes vulpes*) is the most important final host for *E. multilocularis*. Reviews based on the data collected during the past decade have shown that the natural range of the parasite extends farther to the east and north in Europe than previously thought (11,12). Defined rural areas have been monitored regularly for many years, and increasing parasite prevalence rates in foxes have been recorded (13). Clusters of high endemicity (60% to 75%) have been found (14). Increasing fox populations have been reported from several European countries (13). Foxes migrating to urban areas are also causing concerns: *E. multilocularis* prevalence rates of 20% and 48% have been recorded in Stuttgart, Germany (11), and Zürich, Switzerland, respectively (15).

Knowledge of the parasite's range and prevalence in animal hosts has thus grown during recent years. However, comprehensive assessments of human alveolar echinococcosis covering the known risk areas across European countries have not been performed. To provide baseline data for future risk calculations and to establish a prospective case retrieval, the European Echinococcosis Registry (EurEchinoReg) created a network of reporting centers in 11 countries of western and central Europe and Turkey. This report provides the status of reporting, origin, and clinical and epidemiologic data of such patients reported to the registry up to the year 2000.

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[1]The European Echinococcosis Registry (EurEchinoReg) is a surveillance network for human alveolar echinococcosis coordinated by D.A. Vuitton (France) and P. Kern (Germany). Registration of human cases is organized by H. Auer (Austria), Y. Carlier (Belgium), L. Kolarova (Czech Republic), K. Bardonnet (France), P. Kern (Germany), P.S. Craig (Great Britain), I. Prousalidis (Greece), A. Siracusano (Italy), J. van der Giessen (Netherlands), Z. Pawlowski (Poland), E. Renner, R.W. Ammann (Switzerland), and N. Altintas (Turkey).

Methods

Case Retrieval

Case detection and data collection have been organized by each participating country according to the existing infrastructure of the national health systems and the availability of data sources. In the EurEchinoReg, experts from universities (research units and hospitals) and public health authorities cooperate in eight countries of the European Union (Austria, Belgium, France, Germany, Greece, Great Britain, Italy, and the Netherlands), and in Switzerland, Poland, the Czech Republic, and Turkey. Patient data are stripped of identifiers and sent to two subregistries (the University of Franche-Comté, Besançon, France and the University of Ulm, Ulm, Germany), where they are controlled and approved for electronic recording.

Case Definition and Period of Inclusion

Diagnosis of alveolar echinococcosis is confirmed by 1) positive histopathology, if available and/or 2) typical liver lesion morphology identified by imaging techniques (ultrasound scan, computed tomography, and magnetic resonance imaging) with or without the detection of serum antibodies (serology). Positive serologic results without suggestive imaging findings or positive histopathology does not qualify for a case definition.

The period of inclusion began in 1982, when benzimidazoles, ultrasound, and other imaging techniques (which facilitated diagnosis, treatment, and follow-up) were introduced. The registry includes all confirmed new cases from January 1982 to December 2000, as well as cases with a diagnosis from earlier periods, provided the patients were alive in 1982 and their diagnosis was confirmed with the appropriate techniques in 1982 or later.

Case Report Form and Completeness of Registration

Patients were asked to allow their nominal registration at their national center, in conformity with the national legislation for data privacy to avoid double registration and to facilitate follow-up. Two questionnaires are used: an epidemiologic part to be answered by the patient and a clinical part to be completed by the reporting physician. In addition to demographic baseline data, we gathered information on year of diagnosis, disease manifestation at the time of the diagnosis, co-existing conditions; diagnostic and therapeutic measures, year of death, presumed cause of death, places of residence, and occupation in agriculture, forestry, and gardening.

Data files from the study groups in Austria, France, Germany, and Switzerland were the basis for the European patient registry; additional case files were collected by active case finding and with the help of physicians from hospitals and private practices. Completeness of registration can be assumed: 1) in France, since access to patient files is facilitated by a centralized distribution of albendazole by a few university hospitals; 2) in Austria, since laboratory diagnosis is made in a single institution; and 3) in Switzerland, where alveolar echinococcosis was a reportable disease until 1997; case reports are thus complete from the 1970s until 1997. Underreporting is likely in Germany, where reporting relies entirely on the cooperation of family physicians and clinicians. In Belgium, Greece, and Poland, alveolar echinococcosis seems to be newly emerging, and cases are discussed in the medical community; the cases reported to the registry should reflect the true prevalence in these countries.

Data Analysis

The combined data sets for all European patients are kept in an Access database (Microsoft Corp., Redmond, WA). Descriptive analyses were made with SAS software V8 (SAS Institute, Inc., Cary, NC). The regional distribution of alveolar echinococcosis cases was mapped with the software package RegioGraph 5.1 (GfK MACON AG, Waghäusel, Germany).

Results

Epidemiology

The total number of verified alveolar echinococcosis cases reported to the registry was 559; 42.0% were diagnosed in France, 23.6% in Germany, and 21.1% in Switzerland (Table 1). Fifteen patients acquired the infection outside their reporting country, 7 of these cases originated from one of the neighboring countries, 8 were of non-European origin.

During the reporting period, the number of new cases varied from year to year. From 1981 to 2000, a peak incidence of 36 was noted in 1988; aside from this 1 year, reports ranged from 15 to 27 patients. A total of 258 patients were male (46.2%) and 301 female (53.8%) (gender ratio 1:1.2). The median age at first diagnosis was 56 (mean 52.5, range 5–86 years) and was almost equal in men and women (Figure 1). The

Table 1. Number of patients with alveolar echinococcosis, Europe

Yr of first diagnosis	Reporting country									Total[a]
	Austria	Belgium	France	Germany	Great Britain	Netherlands	Switzerland	Poland	Greece	
Until 1980	8	0	23	30	0	0	43	0	0	104
1981–1985	12	0	60	11	0	0	29	0	0	112
1986–1990	11	0	80	17	0	0	17	2	0	127
1991–1995	13	0	40	26	0	0	16	6	0	101
1996–2000	10	3	32	48	1	1	13	6	1	115
Total no. of patients[b]	54 (1)	3	235	132(6)	1 (1)	1 (1)	118 (6)	14	1	559

[a]Status of notification to the European Echinococcosis Registry as of August 2001.

[b]Includes 15 non-autochthonous cases (in parentheses); 7 of them originated from neighboring countries, 3 from Turkey, 3 from the Newly Independent States, 1 from Kazakhstan, and 1 from Afghanistan.

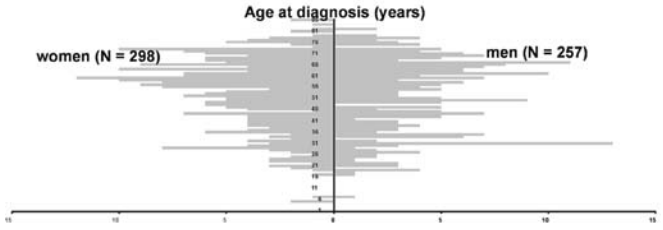

Figure 1. Patients with alveolar echinococcosis reported to the European Registry. Age at first diagnosis by gender (N=555, year of birth missing for 4 patients).

proportion of patients <20 years old was 2.1% (12/559); 88 (15.7%) were >69 years of age. For four patients (0.7%), the year of birth was missing. Three of the four children in this case series, ages 5 and 7 when diagnosed, had severe organ damage; two were immunocompromised.

Information on potential risk factors was available for 210 (37.6%) patients from Austria, Germany, Greece, and France (Table 2), including 97 men and 113 women. Of these, 21.9% were farmers. In addition, of all the patients engaged in other professions (including housewives and students), 46.2% regularly farmed, gardened, or performed related activities as a pastime. Of all pensioners and unemployed patients, 62.2% also gardened, farmed, or the like. Most patients (70.5%) owned or formerly kept dogs and cats. Among these pet owners, 105 persons also actively farmed or gardened. Only 15 patients (7.1%) did not farm, garden, or own pets.

Geographic Distribution

Figure 2 gives the residence at the time of diagnosis or at the time of the last medical report for 532 alveolar echinococcosis patients; cases were autochthonous from the countries represented on the map. The patient from Greece lived in Macedonia. Data were unavailable for 18 patients.

Most residences were clustered in defined regions: central France, French Jura and Savoy, Swiss Jura and northeastern Switzerland, southern Germany, and western Austria. Single cases were identified in Belgium, the northern regions of France, Germany, and Poland, and northeastern Austria. For the period 1980–1999, a total of 201 cases were reported from Turkey; all originated from the Asian part of the country, mostly from eastern Anatolia. However, the aggregated data (reviewed by Altintas et al. [16]) could not be combined with the detailed datasets from western and central Europe. No autochthonous cases were reported from the Netherlands, the Czech Republic, the Slovak Republic, Italy, or the U.K.

Clinical Data

Table 3 lists the main diagnostic procedures, conducted within a time span of 6 months after initial examination, which led to the diagnosis of alveolar echinococcosis. A total of 53.5% of diagnoses were definitely confirmed by positive histopathology; 38.5% were ascertained by imaging techniques combined with serology, or imaging alone, when obtaining tissue specimens for analysis was not possible. Information on diagnostic procedures was missing for 7.7% of the patients.

In 397 (71.0%) of the 559 cases, the diagnosis was made after the patients reported symptoms; 66 (11.8%) cases were disclosed by chance in the course of a general medical checkup or an examination related to other diseases; and 18 (3.2%) cases were found during studies that screened for alveolar echinococcosis. Data on these circumstances were not available for 78 (14%) of 559 patients.

The primary infection site was the liver for almost all patients, and primary extrahepatic lesions without any involvement of the liver were diagnosed in 13 patients (Table 4). At first diagnosis, the liver was the only affected organ for 351 (62.8%) of 559 patients. Damage to the liver included single or multiple lesions in one or more segments of one or both liver lobes, the hilus region, the intrahepatic portal vein, hepatic vein, or bile duct. Approximately one third (34%) of the patients (190/559) were already affected by a spread of the larval tissue either in continuum into neighboring organs, by the formation of distant metastases, or both. Specific details of organ damage were available for 178 of 190 patients. The organs most frequently affected by continuous growth were the diaphragm (59 patients), kidneys or adrenal glands (26 patients), and lungs and pleura (15 patients). Metastases occurred mainly in the lungs (39 patients), brain (17 patients), and spleen (10 patients).

At the time of reporting, 267 (47.8%) of the 559 patients had undergone surgery and received benzimidazoles; 200 (35.8%) were treated with these drugs alone, and 48 (8.6%) by surgery alone. A total of 29 patients (5.2%) underwent liver transplantation. Twenty-two patients (3.9%) had not received any treatment during the time between diagnosis and notification; another 13 (2.3%) had apparently had inadequate treatment. For 9 patients (1.6%) the chosen treatment options were not specified.

Table 2. Possible exposure risks assessed for 210 patients with alveolar echinococcosis

		Activity in agriculture, gardening, forestry, hunting			Ownership of dogs, cats, or both		
Occupation	N (%)	Yes	No	Missing	Yes	No	Missing
Farmers	46 (21.9)	46	0	0	39	2	5
Nonfarmers[a]	119 (56.7)	55	56	8	80	13	26
Occupation not specified, including unemployed and pensioners	45 (21.4)	28	13	4	29	6	10
Total	210 (100.0)	129	69	12	148	21	41

[a]For example, tailors, hairdressers, cooks, nurses, drivers, teachers, students, and housewives.

Figure 2. Regional distribution of autochthonous alveolar echinococcosis in Europe, from 532 diagnoses ascertained from 1982 to 2000. Dots represent place of residence (at time of diagnosis or last medical record) of 1–5 patients. In Austria, Belgium, Germany, and Poland, administrative units for locating patients are the municipality; in France and Switzerland, dots are placed at random in larger units ("Arrondissement" for France, "Kanton" for Switzerland). Source: European Echinococcosis Registry, Ulm, Besançon, 2001. Used with permission.

By December 2000, 73.0% of the patients were alive, 21.3% had died, and 5.7% were lost to follow-up (Table 5). Of the patients still alive, disease activity was assessed at their last clinical examination as follows: cured (20 patients, 4.9%); stable or regressive (226 patients, 55.4%); or progression, sequelae, or complications caused by larval growth or occurring after intervention (43 patients, 10.5%). An assessment was not provided for 119 patients (29.2%); many of them had been diagnosed recently, and treatment had just begun. Death was definitely associated with alveolar echinococcosis in 13 (10.9%) of the 119 cases; in 15 cases (12.6%) death was probably related to this disease. In 20 patients (16.8%), death was definitely independent of the diagnosis of alveolar echinococcosis. No assessment was available for 71 patients (59.7%).

Discussion

In 1998, the EurEchinoReg network initiated the assessment of human alveolar echinococcosis across European borders. The reasons for promoting concerted efforts to survey a disease thought to be rare in Europe were as follows: 1) the disease is one of the most aggressive chronic liver diseases, 2) comprehensive assessments of human cases covering all known risk areas were not available, 3) the routes of transmission to humans are still hypothetical, and 4) the range of the parasite in its life cycle seems to have extended, posing threats in previously unaffected areas.

The assessment included patients from former clinical studies and cases identified by active case finding. Nine European countries reported on 559 patients; cases were autochthonous from seven of these countries. The median numbers per year did not vary during two decades (24 in 1980s; 22 in 1990s). Underreporting from previous years was responsible for a seemingly increasing incidence in Germany; underreporting since 1998 explains a decline in Switzerland. High numbers in France in the mid-1980s could be an effect of mass screenings performed at that time in alveolar echinococcosis–endemic areas, which may have raised awareness of the disease. In the past, the number of verified and published cases from Europe (Austria, France, Germany, and Switzerland) amounted to 844 cases or 10.6 cases per year (published between 1900 and 1980) (17). The patient numbers from our report reflect what is probably an optimal detection rate owing to improved technology. Thus, low but constant incidence is characteristic of the occurrence of human alveolar echinococcosis in Europe today.

In this parasitic infection, a long incubation period seems to precede diagnosis. Albeit difficult to prove, the initial asymptomatic period is assumed to last 5–15 years (1). (This conclusion is derived from the small proportion of patients <20 years old at diagnosis [2.1% in this report]). Determining the time and place of infection is difficult. Assuming that in humans, who are unsuitable hosts for *E. multilocularis*, repeated or long-term exposure is required before an infection becomes established, these conditions are more likely to be met by outdoor activities close to the place of residence than by travels to alveolar echinococcosis–endemic areas. We therefore assume that for most cases, the place of residence is most likely the area of infection. A complete documentation of all the places where the patients had lived during their lives was available for approximately 30%. Mobility of this patient subgroup was low, in conjunction with long-term farming.

The distribution of alveolar echinococcosis in Europe shows a core area with a high density of cases and border areas with clusters of a few patients or single cases. The core area covers large parts of the classic alveolar echinococcosis–

Table 3. Diagnostic procedures to ascertain the diagnosis of alveolar echinococcosis[a]

Histopathology[b]	Imaging[c]	Serology[d]	No. of patients (%)
+	+	+	176 (31.5)
+	+	–	48 (8.6)
+	–	+	19 (3.4)
+	–	–	56 (10.0)
Subtotal			299 (53.5)
-	+	+	192 (34.3)
-	+	-	25 (4.5)
Subtotal			217 (38.8)
Data not available			43 (7.7)
Total			559 (100.0)

[a]All documented techniques, applied during 6 months after initial examination. +, positive result in the respective tests/examinations; –, negative result, tests/examinations not done, or data not available.

[b]Examination of liver tissue samples carried out on material removed by surgery, diagnostic laparoscopy, or, in rare instances, by fine needle biopsy.

[c]Comprised one or several examinations, i.e., ultrasound, computed tomography (CT), or magnetic resonance imagry (MRI) of the abdomen. In some cases, X-ray, CT, or MRI were available on brain, chest, or other organs.

[d]Included screening methods using different crude antigen preparations in indirect hemagglutination or enzyme-linked immunosorbent assays (ELISA). In addition, purified and recombinant antigen preparations such as Em2+, Em10 or Em18 were used in ELISA, Western blot, or both.

Table 4. Location of the primary lesions at first diagnosis in alveolar echinococcosis

Primary infection site	No. of patients
Liver	541 (96.8%)
Spleen, peritoneum, lung, vertebra, brain, kidneys, heart	13 (2.3%)
Data not available	5 (0.9%)
Total	559

endemic regions in Austria, France, Germany, and Switzerland, including those where the index cases from each of these countries have been identified since 1855 (17). In these areas, recent screening studies have detected not only a small number of manifest diseases but also self-cured infections (aborted lesions, first described by Rausch et al. [18]), and seropositivity rates of up to 2% (14,19,20). Fifteen persons with aborted hepatic lesions (lesions with characteristic calcification) and positive serologic results were reported to the registry but were excluded from this analysis, since a definite diagnosis based on histopathologic or molecular findings had not been provided. Together with a persistent *E. multilocularis* seroprevalence, such reports point to a manifest infection pressure in the core area.

In the core area, a consistently high prevalence of *E. multilocularis* in foxes has been reported, e.g., >50% in southwestern Germany (11), 44% in western Bavaria (21), 65% in eastern France (13), and 35% in western Austria (9). In the border area with less frequent and more dispersed human cases, fewer investigations have been undertaken to establish parasite prevalence, and the figures determined rely on low numbers of examined foxes. The prevalence was generally low, e.g., 13% in northern Germany (11), 8% to 21% in eastern Bavaria (21), and 10% in eastern Austria (9). In Belgium, the first three patients with autochthonous infections lived in areas with low parasite prevalence (final report to the European Commission, Directorate General V (EurEchinoReg, unpub. data, 1999). No prevalence data are available for northern France. In Poland, parasite prevalence was initially investigated in 1994. All registered patients live in the northeastern districts with the highest prevalence (20% and 36%) (22). In Greece, sporadic cases had been reported previously (23).

Whether low parasite prevalence exerts an infection pressure relevant for transmission to humans remains questionable. Recent investigations have shown that foci of high prevalence can persist, even for long periods, in regions where the overall infection rates in foxes are low (e.g., foci of 25% in areas with 5% in eastern Germany) (24). Similar foci may exist in other regions but are undetected to date. Thus, human infection can probably occur in regions with low overall parasite prevalence, and we regard case reports from areas remote from the core area as strong hints of new areas at risk. Therefore, threshold findings at diagnosis should not be rated as incompatible with the disease when the patient lived in an area where human alveolar echinococcosis was unknown before. According to Eckert et al. (12), all regions with a proven occurrence of *E. multilocularis* in Red Foxes indicate a "potential risk area," irrespective of the magnitude of prevalence rates. This view is the basis for the current concept of a continuous distribution of the parasite in Europe from central France to Poland. Future studies should, therefore, address the redefinition of risk areas for alveolar echinococcosis and the population at risk.

Transmission of the parasite to humans occurs rarely, and individual risk factors for human disease are not well understood. In Europe, only one case-control study has been published; this study included 21 patients and 84 controls from Austria (25). A high association of the disease was found with cat ownership and hunting, but because of the low case number the study was of limited power. Farming did not seem to have an impact on infection risk. In China, a population-based study showed that farming was the most important risk factor (26). In Alaska, dog ownership was found to be associated with the disease (19 patients, 38 controls) (27). None of these studies found an association of the disease with a history of picking and eating wild berries and mushrooms or raw produce from unfenced gardens. Also, neither fox hunters in China (26) nor trappers in South Dakota, United States, are affected by the disease (28). The records of 210 patients from the European registry data show that 21.9% were farmers; another 39.5% were engaged in farming, gardening, hunting, or working in forestry as a pastime; 70.5% of all patients kept dogs or cats. These data point to a high frequency of putative exposure, but the lack of a comparison group does not allow an evaluation of the risk potential of these activities. These activities may be characteristic of most people in rural communities in Europe. For Europe, the questions of how risk behavior can be defined and how exposure can best be prevented are, therefore, still unanswered.

Within the last 20 years, major improvements have been made in the diagnosis and treatment of alveolar echinococcosis. Definite diagnosis by histopathology was available in 53.3% of this case series; the remaining cases were ascertained by imaging with or without specific serology. At diagnosis, 34% of the patients were already affected by advanced larval growth; when the parasitic tissue does not affect important organs or vessels, it may go unnoticed for prolonged periods. This fact may also explain a diagnosis late in a patient's life. In Europe, the mean age at diagnosis was slightly higher than in non-Caucasian populations, i.e., in Hokkaido, Japan (48.7 years) (29), or in China (35.7 years) (30).

Immunodeficiencies, e.g. HIV infection (31) or immunosuppressive therapy after liver transplantation (32), may possibly accelerate the manifestation of alveolar echinococcosis.

Table 5. Vital status of patients with alveolar echinococcosis, as of December 2000

Yr of first diagnosis	No. of patients			Interval between diagnosis and death (yrs)
	Alive	Lost to follow-up	Deceased	
Until 1980	63	4	37	4–29
1981–1985	73	11	28	<1–14
1986–1990	84	9	34	<1–10
1991–1995	85	6	10	1–4
1996–2000	103	2	10	<1–1
Total	408	32	119	

Chemotherapy with benzimidazoles was the only treatment for one third of the registered patients; complete cure after surgery and adjuvant chemotherapy was achieved in only 4.9%. For most patients, stability can be achieved with long-term chemotherapy, with or without surgery or interventional radiology, but the disorder remains chronic.

This report is the first collection of data on human alveolar echinococcosis in Europe. Our study confirms that an infection with this parasite is still dangerous. A low annual incidence persists in the previously known foci. However, case reports from regions remote from the core area indicate that the disease is spreading. We therefore recommend that the occurrence of this potentially reemerging zoonosis should be continuously monitored in western and central Europe.

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Tularemia on Martha's Vineyard: Seroprevalence and Occupational Risk

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We conducted a serosurvey of landscapers to determine if they were at increased risk for exposure to *Francisella tularensis* and to determine risk factors for infection. In Martha's Vineyard, Massachusetts, landscapers (n=132) were tested for anti–*F. tularensis* antibody and completed a questionnaire. For comparison, serum samples from three groups of nonlandscaper Martha's Vineyard residents (n=103, 99, and 108) were tested. Twelve landscapers (9.1%) were seropositive, compared with one person total from the comparison groups (prevalence ratio 9.0; 95% confidence interval 1.2 to 68.1; p=0.02). Of landscapers who used a power blower, 15% were seropositive, compared to 2% who did not use a power blower (prevalence ratio 9.2; 95% confidence interval 1.2 to 69.0; p=0.02). Seropositive landscapers worked more hours per week mowing and weed-whacking and mowed more lawns per week than their seronegative counterparts. Health-care workers in tularemia-endemic areas should consider tularemia as a diagnosis for landscapers with a febrile illness.

Tularemia is a potentially severe zoonosis caused by *Francisella tularensis*, a small, pleomorphic, gram-negative bacterium. The bacterium can be transmitted by an arthropod bite, ingestion, inhalation, or direct contact with infected tissues. The clinical signs and symptoms of tularemia depend, in part, on the route of inoculation. The ulceroglandular form, in which an ulcer develops at the site of inoculation and is accompanied by regional lymphadenopathy, is the most common. The less common but more severe primary pneumonic form develops after inhalation of the bacteria; pneumonic tularemia can be difficult to diagnose because the respiratory signs and symptoms may be minimal or absent and, when present, are often nonspecific. The typhoidal form of tularemia has no localizing signs and is, therefore, also often difficult to diagnose. Tularemia can also occur in glandular, oculoglandular, and oropharyngeal forms. An average of 124 cases of tularemia was reported annually in the United States from 1990 to 2000 (1).

Tularemia is endemic on Martha's Vineyard, an island off the coast of Cape Cod, Massachusetts. The only two reported outbreaks of pneumonic tularemia in the United States occurred on Martha's Vineyard in 1978 and 2000 (2,3). During the outbreak in the summer of 2000, 15 patients with tularemia were identified; 11 had pneumonic tularemia. A case-control study demonstrated that lawn mowing or brush-cutting were risk factors for pneumonic tularemia (adjusted odds ratio 6.7; 95% confidence interval [CI] 1.1 to 39.9). Five patients were professional landscapers, and patients with pneumonic tularemia were approximately 32 times more likely to have worked as a landscaper than controls were (3).

Tularemia transmission on Martha's Vineyard continued in the summer of 2001; one case of ulceroglandular and three cases of primary pneumonic tularemia were identified. Of the patients with pneumonic tularemia, two were professional landscapers, and one was a farmer who had mowed fields 4–6 hours a day the week before illness. We conducted a serosurvey to determine the prevalence of antibodies to *F. tularensis* among landscapers and three comparison groups and to evaluate potential risk factors for exposure to *F. tularensis* among landscapers.

Methods

In July 2001, landscapers on Martha's Vineyard were offered free testing for anti-*F. tularensis* antibody during an all-day event publicized at a local small-engine repair shop and through community-wide advertisements. After providing informed consent, participating landscapers gave serum samples and completed a risk factor questionnaire about their professional activities, contact with animals and arthropods, and past medical history. A professional landscaper was defined as anyone who reported their occupation as landscaper, tree worker, property manager, caretaker, professional gardener, or land or lot clearer.

For comparison, serum samples were obtained from three control groups. Group 1 (n=103) comprised nonlandscaper patients at two local physicians' offices who were having blood drawn for other reasons (n=56), nonlandscaper members of various Martha's Vineyard civic organizations (n=27), and persons who participated in our serosurvey but did not meet the definition of landscaper (n=20); all participants gave informed consent. Groups 2 and 3 comprised individual serum samples from anonymous, healthy Martha's Vineyard residents who had blood drawn for other reasons (n=99 in July and n=108 in October). All serum samples were tested at the Centers for Disease Control and Prevention (CDC) for anti–*F. tularensis* antibodies with a microagglutination assay (4); titers of at least 1:128 were considered positive.

The seroprevalence of antibody to *F. tularensis* in landscapers was compared to the seroprevalence in each of the three control groups. Seropositive landscapers were compared to seronegative landscapers to determine risk factors for

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seropositivity. Statistical analyses were performed in Epi Info 2000 (CDC, Atlanta, Georgia) and SAS version 8 (SAS Institute, Inc., Cary, NC). For univariate analyses, prevalence ratios were determined for dichotomous variables; the Mann-Whitney U test was performed to compare the median values of the continuous variables. Multivariable logistic regression was used to determine significant associations with seropositivity while controlling for variables that were significant on univariate analyses. Forward, backward, and stepwise selection procedures were used to obtain a parsimonious model with variables that were significant on univariate analysis.

A CDC ethics review coordinator reviewed the study plan and determined that the survey represented a public health response that did not require additional ethics review. The Harvard School of Public Health Institutional Review Board also approved our serosurvey as part of a broader study of zoonotic diseases in North Atlantic communities.

Results

One hundred thirty-two landscapers requested serologic testing and completed risk factor questionnaires. These landscapers included 117 persons who described their occupation as landscaper on the questionnaire and 15 persons who listed their occupations as tree worker, property manager, caretaker, professional gardeners, or land or lot clearer. Compared to persons in the control groups, the landscapers were younger (median 37 years of age, compared with median 58, 49, and 50 years for groups 1, 2, and 3, respectively) and more likely to be male (79%, compared with 60%, 49%, and 41%, respectively, in the control groups). Of the 56 persons in control group 1 who were enrolled at local physicians' offices, 27 (48%) went to their physician for a complete physical exam, 23 (41%) had an office visit, and 6 had no recorded reason for the office visit. None of these 56 patients reported a febrile illness.

Twelve (9.1%) of the 132 landscapers were seropositive for anti–*F. tularensis* antibodies (titer range 1:256–1:2048), compared to one person total from the three control groups (titer 1:128). The seropositive control sample was from a healthy Martha's Vineyard resident who had blood drawn in July (group 2). Compared to control group 2 (99 residents who had blood drawn in July), Martha's Vineyard landscapers were nine times more likely to be seropositive (95% CI 1.2 to 68.1; p=0.02) (Table 1). All 12 seropositive landscapers described their occupation as landscaper on the questionnaire, and they reported working as landscapers for 2–52 years (median 11 years). Two seropositive landscapers reported having been diagnosed with tularemia by a physician (one in 1985 and one in 1986); two others reported having had an undiagnosed febrile illness in 2000 or 2001. (We did not ask about febrile illnesses before 2000 because of concern about recall bias.)

Of the 12 seropositive landscapers, 11 were male; seropositive and seronegative groups had no significant difference in the proportion of males. The median age of seropositive landscapers was 35 years of age (range 18–66 years), compared with a median of 38 years of age (range 12–75 years) in seronegative landscapers (p=0.83). Of landscapers who used a power blower, 15% (11/72) were seropositive, compared to 2% (1/60) of landscapers who did not use this device (prevalence ratio 9.2; 95% CI 1.2 to 69.0; p=0.02) (Table 2). Of 132 landscapers, 116 (88%) mowed lawns, and 106 (80%) used a weedwhacker. Seropositive landscapers worked more hours per week mowing (median 29.5 vs. 15 hours; p=0.03) and weedwhacking (median 10 vs. 3 hours; p=0.01) and mowed more lawns per week (median 25 vs. 3 lawns; p=0.0003) than their seronegative counterparts (Table 3). Seropositive and seronegative landscapers reported similar frequencies of exposure to arthropods or sick or dead mammals (Table 2). A multivariable logistic regression model was constructed by using all variables significant on univariate analysis (power blower use, number of lawns mowed, hours mowed per week, and hours weedwhacked per week). No single variable was significantly associated with seropositivity after adjustment for the effects of all other variables. When forward, backward, and stepwise selection procedures with a 0.05 significance requirement for inclusion in the model were used, the final model contained only the number of lawns mowed per week (OR=1.04; 95% CI 1.01 to 1.07; p=0.004).

Forty-one percent of landscapers reported that they wore a mask either sometimes or always while performing landscaping activities in 2001, compared with 23% in 2000 (p=0.005). However, few landscapers reported always wearing a mask in either year (3% in 2000 and 6% in 2001). Ninety-two percent of seropositive landscapers reported never wearing a mask in 2000, and 58% reported never wearing a mask in 2001; these proportions were not significantly different from seronegative landscapers. When mask-wearing was dichotomized into wearing a mask always versus sometimes or never, no significant differences between seropositive and seronegative landscapers occurred in either year.

Discussion

In 2001, after 2 years of increased tularemia transmission on Martha's Vineyard, 9.1% of 132 tested landscapers on the island were seropositive for anti–*F. tularensis* antibody, com-

Table 1. Relative seropositivity of Martha's Vineyard landscapers compared with three control groups, Martha's Vineyard, 2001[a]

Population	Seropositive landscapers/total no. (%)	Seropositive controls/total no. (%)	Seroprevalence ratio (95% CI)	Yates corrected p value
1) Landscapers vs. physicians' office patients and members of civic organizations	12/132 (9.1)	0/103 (0)	Undef (undef, undef)	0.004
2) Landscapers vs. residents (July)	12/132 (9.1)	1/99 (1)	9.0 (1.2 to 68.1)	0.02
3) Landscapers vs. residents (October)	12/132 (9.1)	0/108 (0)	Undef (undef, undef)	0.004

[a]CI, confidence interval; undef, undefined.

Table 2. Risk factors among landscapers (dichotomous variables), Martha's Vineyard, 2001

Potential risk factor	Seropositive among exposed no. (%)	Seropositive among unexposed no. (%)	Prevalence ratio (95% CI)	Yates corrected p value
Mow or brush-cut during summer	12/116 (10.3)	0/16 (0)	Undef (undef, undef)	0.38
Recall mowing or brush-cutting over dead animal	4/30 (13.3)	8/79 (10.1)	1.3 (0.4 to 4.1)	0.90
Use power blower during summer	11/72 (15.3)	1/60 (1.7)	9.2 (1.2 to 69.0)	0.02
Ticks crawling on body	10/112 (8.9)	2/18 (11.1)	0.8 (0.2 to 3.4)	0.89
Ticks attached to skin	4/73 (5.5)	8/59 (13.6)	0.4 (0.1 to 1.3)	0.19
Seen sick or dead rabbits in past year	6/60 (10)	6/71 (8.5)	1.2 (0.4 to 3.5)	0.99

[a]CI, confidence interval; undef, undefined.

pared with <1% of nonlandscaper residents in each of three comparison groups. The seroprevalence observed in landscapers is comparable to that described in groups traditionally considered at risk for tularemia; for example, 2.4% to 17.5% of Native Americans and trappers in North America have been reported to have detectable antibody to *F. tularensis* (5–12). In Europe, where only the milder type B *F. tularensis* is found, seroprevalence estimates of 9.7% and 19.7% have been reported among populations affected by outbreaks (13). Estimates of tularemia seroprevalence in the general populations of North America and Sweden have been reported to range from 0% to 1.8% (9,13–15). While agglutination tests were also used in these earlier reports to determine antibody levels, different reagents and techniques may have been employed. In addition, the cutoff for a positive result was generally set much lower (often >1:8 or >1:20) than that used by CDC, with a potential loss in test specificity and exaggerated reported seroprevalence.

Historically, disproportionate numbers of tularemia cases have been reported among laboratory workers, farmers, veterinarians, sheep workers, hunters or trappers, and cooks or meat handlers (16). Our results indicate that landscapers on Martha's Vineyard have increased exposure to *F. tularensis*. Of the eight sporadic case-patients identified in the tularemia outbreak on the island in 1978, two were gardeners (2). Sporadic tularemia cases in landscapers or persons participating in landscaping activities in Colorado and South Carolina suggest that this increased exposure is not unique to Martha's Vineyard (17,18). Health-care workers in tularemia-endemic areas should consider a diagnosis of tularemia in landscapers who have fever or pneumonia.

Arguably, landscapers are more likely to be exposed to *F. tularensis* because they spend most of their time outdoors and are thus more likely to encounter infected ticks and animals. The case-control study conducted in the summer of 2000 showed an association between pneumonic tularemia and mowing or brush-cutting activities, but case-patients and controls did not differ in their exposure to ticks and animals (3). In 2001, seropositive landscapers were more likely to have used a power blower, spent more hours mowing and weed-whacking, and mowed more lawns than seronegative landscapers, but the groups did not differ in frequencies of exposure to arthropods or sick or dead mammals. Mowing or brush-cutting was not significantly associated with seropositivity when analyzed as a dichotomous variable, which may be caused by a lack of ability to detect a significant difference because only 16 of 132 landscapers did not mow lawns. The number of lawns mowed was the factor most robustly associated with seropositivity in our study, but the small number of seropositive landscapers limits the ability to detect other significant differences. The association between seropositivity and increased participation in potential aerosol-generating activities in the absence of an association with arthropod or animal exposure further supports the hypothesis that *F. tularensis* persists in the environment and is aerosolized and inhaled during mowing activities. Lawn mowing has previously been implicated in an outbreak of *Chlamydia psittaci* (19), and infection with *Legionella* spp. has been attributed to aerosolization of the organism in potting soil during gardening activities (20).

The clinical manifestations and severity of illness after infection with *F. tularensis* depend on the portal of entry, infectious dose, virulence of the organism, and immune status of the infected person; despite the organism's high infectivity, asymptomatic infection with *F. tularensis* is known to occur. Eight of the seropositive landscapers did not report a previous diagnosis of tularemia or recall an undiagnosed febrile illness in 2000 or 2001. Because of concerns about recall bias, we did not ask about an undiagnosed febrile illness before 2000; thus, some of these eight landscapers might have had such an illness, which resolved without intervention or was treated empirically with an agent effective against many tick-borne infections. Some or all of them may also have had an asymptomatic infection. Many of the seropositive Native Americans and trappers previously surveyed in North America also did not recall clinical illness (5,6), yet at least some of them were likely infected with

Table 3. Risk factors among landscapers (continuous variables), Martha's Vineyard, 2001

Exposure	n	Mean	Median	Mann-Whitney p value
Average hrs mowing/wk				
Seropositive	12	35	29.5	
Seronegative	118	21	15	0.03
Average hrs weed-whacking/wk				
Seropositive	11	26	10	
Seronegative	114	9	3	0.01
Average no. lawns mowed/wk				
Seropositive	11	31	25	
Seronegative	112	12	3	0.0003

the more virulent type A *F. tularensis*. To date, only type A *F. tularensis* has been isolated from Martha's Vineyard specimens (2,3), including one isolate from a patient who died of pneumonic tularemia in 2000, and one isolate recovered from a dead rabbit in 2001.

Efforts on Martha's Vineyard to prevent tularemia should focus on landscapers who participate in aerosol-generating activities, as well as other persons who mow many lawns. Preventive efforts should include educating landscapers to survey their work areas for carcasses or excreta, and if encountered, to avoid or properly dispose of them. Equipment should be maintained in good working order; for example, the protective skirting and collection bags found on mowers should be kept intact. Landscapers not already using respiratory protection might consider doing so when generating aerosols. Following increased awareness of tularemia in 2000, landscapers did increase their use of respiratory protection; however, the effectiveness of masks in preventing occupational exposure to *F. tularensis* has not been evaluated. Since none of the seropositive landscapers reported always wearing a mask in either 2000 or 2001, we cannot draw any conclusions about the potential protective effect of masks from our data. Seronegative landscapers wore masks at the same low frequency, and exposure of the seropositive landscapers might have occurred in the past, before they became aware of the potential benefits of mask-wearing. Landscapers should be made aware of the risk for tularemia and should seek prompt medical attention if a febrile illness develops after they participate in aerosol-generating activities. We have shown that landscapers are at increased risk for infection with *F. tularensis*; however, some patients in the 2000 outbreak had mowed only their own lawns. The recommendations for landscapers apply to all persons who mow lawns.

Several possible limitations to this study exist. Both our landscaper and control populations were enrolled through convenience sampling and may or may not be representative of all landscapers on Martha's Vineyard or the general population of Martha's Vineyard residents. Samples from persons in control groups 2 and 3 were anonymous; therefore, we have no information on the occupations of those persons. Persons in these groups may be landscapers and could even be in our landscaper series. If any of those persons were landscapers, nondifferential misclassification occurred and would bias our results to the null, so the actual prevalence ratios could be stronger than what we observed. Landscapers on the island include both permanent and seasonal residents and determining the size of the total landscaper population is not possible; therefore, knowing what proportion of landscapers participated in our study is also not possible. The small number of seropositive landscapers limited statistical power for risk factor analyses and multivariable analysis, and our cross-sectional study design did not permit us to assess temporal relationships between exposure to potential risk factors and seropositivity.

Professional landscapers on Martha's Vineyard are a newly identified occupational group with increased exposure to *F. tularensis*. Landscapers appear to be at least nine times more likely to have measurable anti–*F. tularensis* antibodies than nonlandscapers, and seropositive landscapers mow more lawns per week than seronegative landscapers. Health-care workers in tularemia-endemic areas need to be aware of this occupational risk when evaluating landscapers with a febrile illness. Landscapers in tularemia-endemic area should be aware of the potential risk of acquiring infection and should seek prompt medical attention if a febrile illness develops after landscaping activities.

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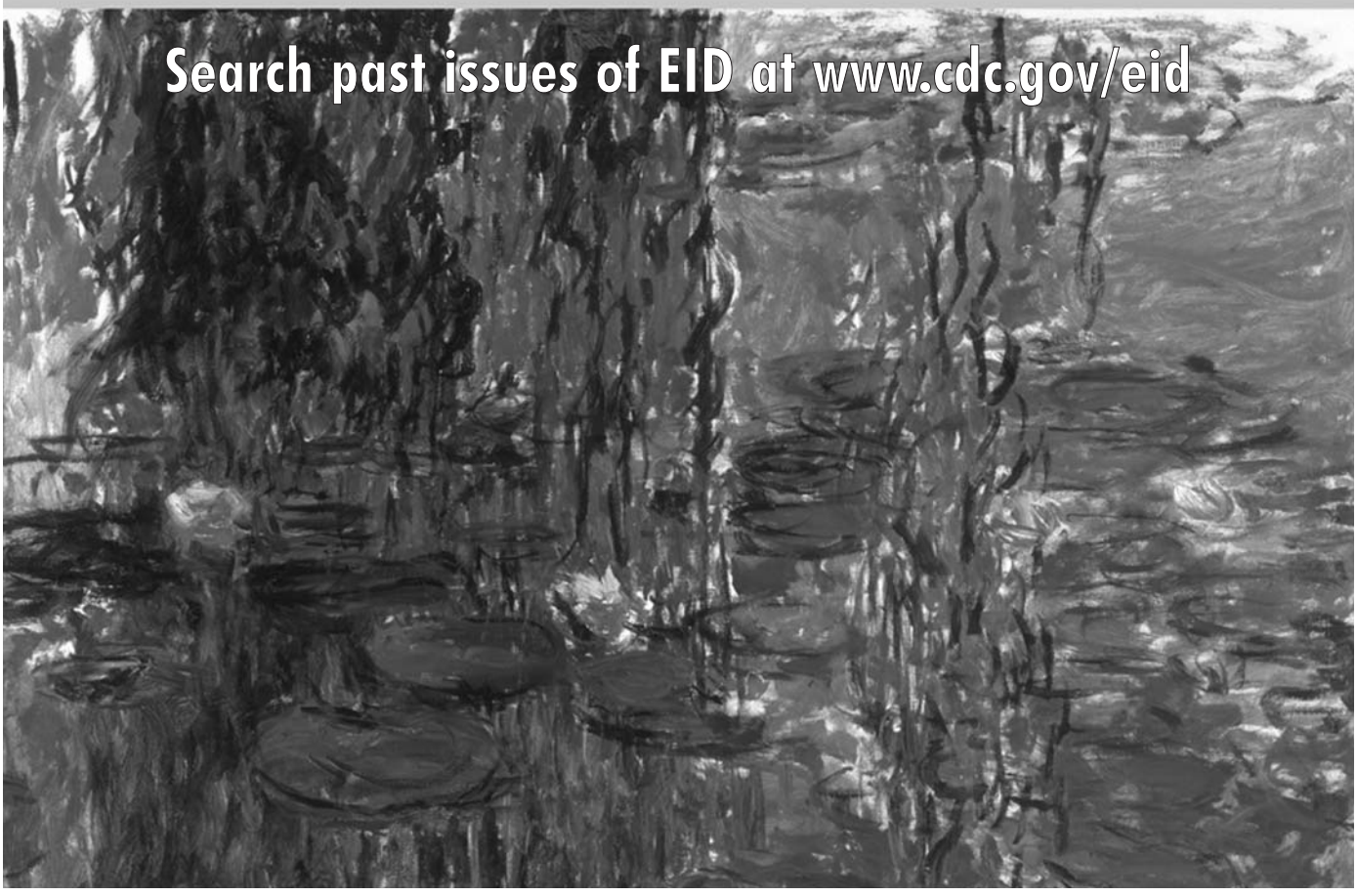

Epidemiology of Meningococcal Disease, New York City, 1989–2000

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Study of the epidemiologic trends in meningococcal disease is important in understanding infection dynamics and developing timely and appropriate public health interventions. We studied surveillance data from the New York City Department of Health and Mental Hygiene, which showed that during 1989–2000 a decrease occurred in both the proportion of patients with serogroup B infection (from 28% to 13% of reported cases; p<0.01) and the rate of serogroup B infection (from 0.25/100,000 to 0.08/100,000; p<0.01). We also noted an increased proportion (from 3% to 39%; p<0.01) and rate of serogroup Y infection (from 0.02/100,000 to 0.23/100,000; p<0.01). Median patient age increased (from 15 to 30 years; p<0.01). The case-fatality rate for the period was 17%. As more effective meningococcal vaccines become available, recommendations for their use in nonepidemic settings should consider current epidemiologic trends, particularly changes in age and serogroup distribution of meningococcal infections.

Meningococcal disease is a broad term used to describe the different clinical syndromes resulting from *Neisseria meningitidis* infection. Its two major clinical illnesses, meningitis and meningococcemia (i.e., sepsis caused by meningococcal infection), occur more often as sporadic cases, but occasional outbreaks are an important cause of illness and death worldwide.

In the United States, a substantial proportion of cases of meningitis and sepsis are caused by *N. meningitidis* (1). The incidence rate of meningococcal disease in the United States is estimated to be 0.7–1.4/100,000 population, and the case-fatality rate (CFR) is approximately 10% (2,3). Both the incidence rate and CFR have been relatively constant, with no major changes observed in the past decade (2).

Serogroups B and C are the most common strains found in the United States; however, increased rates of infection from serogroup Y were observed in the 1990s (2,3). Changes in the age distribution of those infected have also been noted, and the conventional concept that meningococcal disease predominately affects infants and young children should be revised because the median age of meningococcal disease case-patients has increased (2).

We describe the epidemiology of meningococcal disease in New York City from January 1989 to December 2000 with an emphasis on the trends of serogroup incidence, age, and fatality rates. The hypotheses tested were: Consistent with the trends in the epidemiology of meningococcal disease in the United States, the incidence of serogroup Y infection in New York City is increasing, the median age of patients is increasing, and the CFR is comparable to national figures.

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Methods

The study included New York City residents who met the case definition for confirmed or probable meningococcal disease, as defined by the Centers for Disease Control and Prevention and the Council of State and Territorial Epidemiologists. Inclusion in the study as a confirmed case required a clinically compatible course with the isolation of *N. meningitidis* from a sterile site (e.g., blood, cerebrospinal fluid, joint fluid, or pleural fluid); inclusion as a probable case required a positive antigen test from cerebrospinal fluid or clinically described purpura fulminans (4). The period of study was January 1989–December 2000.

We obtained the meningococcal disease cases from the New York City Department of Health and Mental Hygiene (referred to hereafter as NYC Department of Health) surveillance database of reportable diseases. Meningococcal disease is a national reportable disease; in New York City, all cases are required by health code to be reported to the NYC Department of Health. Physician reports, investigation forms, and laboratory reports were reviewed for all the meningococcal disease patients included in the NYC Department of Health database. All cases with evidence of the study criteria were included. Data on meningococcal disease used in this study were collected through routine passive surveillance, and serogroup identification was performed by the NYC Department of Health Public Health Laboratory. Antibiotic resistance profiles and pulsed-field gel electrophoresis results were available only for a subset of isolates after 1999 and are not included in this report. Archival data and population estimates (before 1989) were obtained from New York City Vital Statistics Annual Summary reports.

The database contained information on each cast-patient's age, sex, race, ethnicity, borough of residence, and death. Information on race and ethnicity was incomplete and therefore was not analyzed. When death information was missing, patient identifiers were submitted to the New York City Vital Records and Registry for a death certificate search, which was accomplished by searching by name and International Classification of Diseases (ICD) code. Using name search, staff

in the New York City Vital Records and Registry department used visual inspection to search the New York City death certificates, looking for the name of each patient with an unknown cause of death in the 1-month period after the date of onset of the disease. We also conducted a search using the ICD codes that correspond to meningococcal disease (ICD-9 036.0–036.9 and ICD-10 A39.0–A39.9); the search identified all death certificates from 1989 to 2001 that included these codes. We used the information found through this second search method if the death certificate referred to a patient already in the database with an unknown outcome. We did not include death certificates with meningococcal disease ICD codes that referred to patients not previously included in the database (i.e., they had not been reported to the NYC Department of Health as having meningococcal disease) because of the lack of data to confirm the diagnosis. Patients whose names did not appear in the death certificate search file were considered survivors in the CFR calculation. This study was based on electronic data and surveillance records; we ensured confidentiality by excluding all identifying information from the active analysis database.

Statistical Methods

Incidence rates were calculated by using 1990 and 2000 population files from the U.S. Census Bureau. We used the Pearson chi-square test or Fisher exact test to assess the statistical significance of categorical variables and the Kruskal-Wallis test to assess continuous variables.

Time trend analysis was performed to detect an association between time (e.g., year or year group) and response variables (e.g., serogroup and outcome). We used the Spearman correlation test and chi-square test for linear trends to assess statistical significance. Logistic regression models were built to provide coefficients for significant trends.

Independence can be assumed from the data because most cases were sporadic throughout the study period; we considered the vast majority of cases to be unrelated. In addition, no patient had more than one episode of the disease during the study period, and the analyses were performed with the patients grouped into 3-year intervals to minimize any existing correlation between sequential years (5). The SPSS (SPSS Inc., Chicago, IL) statistical software package and Epi Info 2000 (Centers for Disease Control and Prevention, Atlanta, GA) software were used to perform the statistical calculations.

Results

Among New York City residents, 615 cases of meningococcal disease were reported to the NYC Department of Health from January 1989 to December 2000, with an average annual incidence of 0.67/100,000; of cases reported, 582 cases (95%) were confirmed and 33 cases (5%) were included as probable. Meningococcemia occurred in 54% of the cases, meningitis in 44%, and pneumonia, septic arthritis or other sterile site infections in 2%. All cases were considered to be sporadic except for two case-patients in 1997 who were contacts of a primary case-patient in a juvenile detention center resident and one culture-negative case-patient in 2000 who was linked to a subsequent confirmed case-patient by household contact.

For the period 1989–2000, the meningococcal disease rate decreased by 33%, compared to the period 1953–1988, and declined by 90%, compared to the period 1905–1952 (Table 1). During the period under study, a 69% reduction occurred at the beginning of the 1990s, with the rates dropping from 1.19 per 100,000 population in 1989 to 0.37 per 100,000 population in 1992 (chi square for trend = 9.1; $p<0.01$). Since then, rates have increased slightly and remained relatively constant (Table 1). When children <1 year of age are excluded, the declining trend in incidence is no longer statistically significant (chi square for trend = 2.4; $p<0.12$).

When stratified by borough of patient residence, the average incidence rates were highest in the Bronx (0.88/100,000) and Manhattan (0.81/100,000) and lowest in Brooklyn (0.65/100,000), Staten Island (0.65/100,000), and Queens (0.55/100,000). However, the differences between boroughs were not statistically significant (chi square = 1.4; df = 4; $p = 0.23$). Rates by United Hospital Fund neighborhoods ranged from 0.23 to 1.08 per 100,000. The highest rates occurred in two northern Manhattan and one central Bronx neighborhoods; the lowest rates were all in Queens.

The highest average annual incidence rate was observed among patients <1 year of age (8.49/100,000), with substantially lower rates observed for older age groups (Table 2). A statistically significant declining trend for the age groups of <1 years of age (chi square for trend = 21.5; $p<0.01$) and 1–4 years of age (chi square for trend =14.3; $p<0.01$) was seen over the four 3-year groups. No other decrease or increase in age-specific incidence trends was statistically significant. The proportion of

Table 1. Rates of meningococcal disease, New York City, 1905–2000

Yr group or yr	Cases[a]	Annual rate/100,000
1905–1916	7,038	12.3
1917–1928	3,715	5.44
1929–1940	3,844	4.29
1941–1952	4,505	4.75
1953–1964	1,007	1.08
1965–1976	707	0.75
1977–1988	986	1.16
1989	87	1.19
1990	79	1.08
1991	30	0.41
1992	27	0.37
1993	40	0.55
1994	42	0.57
1995	54	0.73
1996	59	0.80
1997	54	0.73
1998	35	0.47
1999	59	0.79
2000	50	0.62
1989–2000	615	0.67

[a]Before 1945, meningococcal disease was classified as cerebrospinal fever or epidemic spinal meningitis.

Table 2. Meningococcal incidence rates and case-fatality rates by age group and year group, New York City, 1989–2000

Age group (yr)	1989–1991	1992–1994	1995–1997	1998–2000	1989–2000	Case-fatality rate (%)
<1	15.9	8.15	7.25	4.23	8.49	13.0
1–4	2.83	1.10	1.24	0.85	1.50	13.0
5–14	0.77	0.37	0.61	0.40	0.53	7.8
15–24	0.99	0.76	0.45	0.72	0.77	10.6
25–44	0.34	0.21	0.49	0.47	0.38	17.1
45–64	0.47	0.26	0.61	0.55	0.48	24.4
≥65	0.77	0.45	0.75	0.60	0.64	32.9
All ages	0.89	0.50	0.69	0.60	0.67	16.9

[a]Rates are per 100,000.

cases occurring in young children (<5 years of age) decreased from 39% in 1989–1991 to 17% in 1998–2000.

The overall median age of the patients with meningococcal disease was 22 years; stratification by year group showed that median age has increased from 15 years of age in 1989–1991 to 30 years of age in 1998–2000 (Kruskal-Wallis test; chi square = 20.0; df =3; p<0.01). To assess the effect of changes in serogroup on the median age, serogroups B, C, Y, and unknown were sequentially excluded from the computation of median age. Only the removal of serogroup Y resulted in a loss of statistical significance of the trend in median age (Kruskal-Wallis test; chi square = 7.6; df=3; p=0.06).

Overall incidence rates were higher for males (0.73/100,000) than females (0.61/100,000; relative risk [RR] = 1.19; 95% confidence interval [CI] 1.02 to 1.40). However, CFR was higher (20.1% vs. 13.9%; RR=1.45; 95% CI 1.02 to 2.07) for females. No statistically significant differences were found in gender-specific incidence rate by age category.

Serogroup was determined for 423 (72%) of 582 culture-positive cases. From 1989 to 2000, serogroups B, Y, and C were the most commonly identified serogroups (32% [n=137], 28% [n=119], and 27% [n=112], respectively) of the cases for which a serogroup was known. Serogroup W135 constituted 7% (n=28); nongroupable, 3% (n=13); A, 2% (n=9); and other serogroups, 1% (n=5) of the isolates. The median age of the case-patients differed by serogroup, with the highest median age for nongroupable (48 years of age), followed by other (43 years of age), Y (37 years of age), A (34 years of age), W (27 years of age), C (23 years of age), and B (11 years of age).

Incidence rates for serogroup B infections declined in all age groups with the largest decline in the <1-year and 1–4-year age groups in 1989–2000. Serogroup Y incidence rates increased twofold to tenfold in all age groups except 1–4 years during the period (Table 3).

Over the 12-year interval, the proportion of cases caused by strains included in the quadrivalent vaccine available in the United States (A, C, Y, and W135) increased from 28% to 65% of reported cases (Kruskal-Wallis test; chi square = 57.4; df=3; p< 0.01). This increase is due in part to the decline in incidence of serogroup B infections and the decline in the number of cases for which a serogroup could not be determined (Figure 1).

To assess whether changes in serogroup B and Y incidence were independent from the changes observed in age, we performed logistic regression analyses. The likelihood of serogroup Y infection compared with all other serogroups increased by a factor of 2.47 (99% CI 1.84 to 3.33) for each successive year group while controlling for age. The likelihood of serogroup B infections compared with all other serogroups decreased by a factor of 0.77 (99% CI 0.61 to 0.91) for each successive year group.

Information about patient outcome was initially available for 478 (77.7%) of the cases. After the vital records search, one additional death was identified for a patient with missing outcome. The overall CFR during 1989–2000 was 16.9% (104 deaths, 615 cases). The CFR varied during the study period, being lowest in the interval 1992–1994 (14%; 15/109) and highest in 1989–1991 when 20% (39/196) of the case-patients died; however, the difference between year groups was not statistically significant. When we analyzed CFR for each year separately, we found a surprisingly high CFR of 27% (16/59 cases) in 1999.

Table 3. Annual incidence rates of *Neisseria meningitidis*, serogroups B and Y, New York City, 1989–2000[a]

Age group (yr)	1989–1991				1992–1994				1995–1997				1998–2000			
	B		Y		B		Y		B		Y		B		Y	
	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate
<1	15	5.8	2	0.78	11	4.3	1	0.39	9	2.7	3	0.91	4	1.2	5	1.5
1–4	13	1.0	0	0	5	0.39	2	0.16	4	0.31	2	0.15	2	0.15	1	0.08
5–14	3	0.11	1	0.04	4	0.15	0	0	3	0.09	3	0.09	1	0.03	9	0.27
15–24	9	0.29	1	0.03	5	0.16	4	0.13	2	0.06	3	0.09	3	0.09	7	0.21
24–44	6	0.08	1	0.01	4	0.05	1	0.01	8	0.10	15	0.19	3	0.04	7	0.09
45–64	5	0.12	0	0	1	0.02	2	0.04	2	0.04	11	0.22	4	0.08	17	0.33
≥65	3	0.10	0	0	4	0.14	3	0.10	2	0.07	8	0.28	2	0.07	10	0.36
All ages	54	0.25	5	0.02	34	0.15	13	0.06	30	0.12	45	0.19	19	0.08	56	0.23

[a]Rates are per 100,000 and use 1990 and 2000 census figures.

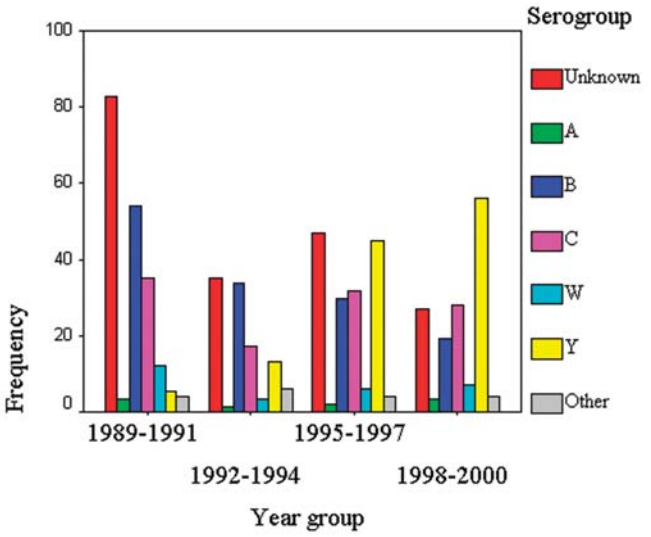

Figure 1. Distribution of meningococcal serogroups by year group, New York City, 1989–2000.

CFR increased linearly with age after 5 years of age and was lowest for those 5–14 years of age (8%) and highest for >65 years of age (33%) (Table 2). Figure 2 shows CFR by age category and year group. The CFR also differed by serogroup and was the highest for serogroup A (44.4%; 4/9), compared to that observed among serogroups C (22.3%; 25/112), Y (18.5%; 22/119), W (17.9%; 5/28), and B (12.4%; 17/137). However, the high CFR for serogroup A should be interpreted cautiously because of the low number of cases in the study period. No statistically significant difference of CFR between serogroups was noted (Bonferoni adjustment for multiple comparisons, p>0.002).

Discussion

Our study has shown that a significant decrease in meningococcal disease incidence rates occurred at the beginning of the 1990s in New York City, and low incidence rates were observed throughout the rest of the decade. The decline in incidence rates are unlikely to have occurred because of changes in surveillance; no modifications in the diagnostic criteria for meningococcal disease were made, and only passive surveillance was conducted throughout the entire study period.

Compared to national surveillance data, the overall incidence rate in New York City during the period was 34% lower (0.67 vs. 1.02/100,000) (6). Age-specific rates in children <5 years of age have declined both nationally and in New York City, although the magnitude of the decline in the city has been greater. Nationally, the rate for patients <1 year of age declined from 13.5/100,000 in 1989 to 6.79/100,000 in 2000. For children 1–4 years of age, the rate declined from 4.18/100,000 to 2.04/100,000 over the same period (6). Much of the decline occurred during the years 1998–2000. For New York City, the rates in the <1-year age group declined from 18.6/100,000 in 1989 to 2.72/100,000 in 2000. For the 1–4 year age group, the rate declined from 4.95/100,000 in 1989 to 0.93/100,000 in 2000. A similar trend for New York State (excluding New York City) has occurred, with overall meningococcal rates dropping 44% (from 1.28/100,000 in 1989 to 0.72/100,000 in 2000) and the rate <5 years declining 85% (from 8.85/100,000 to 1.29/100,000, unpub. data, New York State Department of Health, Division of Epidemiology).

The median age of New York City case-patients was higher than that observed in epidemiologic reviews for the United States and the New England region (2,6). The increase in the median age of cases from 1989 to 2000 observed in New York City is predominately due to a decrease in the incidence rates among young children (1–4 years of age) and infants (<1 year of age), along with slight increases in rates among adults (25–64 years of age). This finding of higher median age may be a result of the greater decline in meningococcal disease seen in children <5 years of age in New York City compared to the rest of the United States.

In accordance with trends observed in other areas of the United States (2,3), a significant increase in the incidence of serogroup Y infection occurred in New York City. The median age of patients with serogroup Y infections in New York City (30 years) was comparable to that of Connecticut patients (29 years) but greater than that seen in Illinois patients (16 years) when comparison data from 1989–1996 were used (3). Serogroup C was responsible for an increasing number of sporadic cases and outbreaks in the United States (7) and Canada (8) in the late 1980s and early 1990s. In New York City, the single cluster involving three people in 1997 was caused by serogroup C infection. The incidence of serogroup C infection did not change substantially in New York City throughout the 12 years of the study; serotype C infection accounted for 18.2% of the total number of cases (range per year group 15.6–19.4).

Serogroup W-135, important worldwide because of the cases associated with returning pilgrims from Saudi Arabia, accounted for 4.6% of all cases in New York City from 1989 to

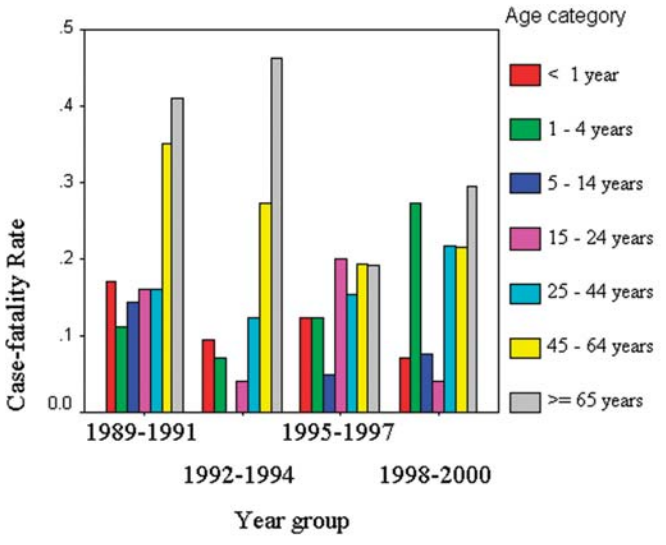

Figure 2. Meningococcal case-fatality rate by age category and year group, New York City, 1989–2000.

2000. This association with the pilgrimage to Mecca accounted for the three cases in New York City caused by serogroup W-135 reported from January to April in 2000: One patient was a returning pilgrim, another was a household contact of a returning pilgrim, and the third patient reported having interacted with returning pilgrims or their families (9).

The incidence rate of serogroup B declined threefold during the period of study, and the infection has nearly disappeared in children <5 years of age in New York City (one case in 1999–2001). The number of serogroup B meningococcal cases in New York State (excluding New York City) has also declined, from 10 in 1997 to 3 in 2000 (unpub. data, New York State Department of Health, Division of Epidemiology). Data from New Jersey indicate that the number of meningococcal infections in children <5 years of age has similarly declined, from 16 in 1995 to 3 in 2000, although the number of serogroup B cases has remained relatively constant (unpub. data, New Jersey Department of Health and Human Services). In Oregon, where an increase in serogroup B meningococcal disease occurred in the last decade (10), no similar decline in serogroup B has been seen in children <5 years of age (Frederick Hoesly, pers. comm.). An interesting discovery is the coincident increase in the use of *Haemophilus influenzae* conjugate vaccine containing serogroup B meningococcal outer membrane protein. Private provider vaccine orders received by the Vaccine for Children program for NYC indicate that the proportion of *H. influenzae* vaccine containing serogroup B meningococcal outer membrane protein has risen steadily from 0% in 1994 to 52% in 2000 (unpub. data, Department of Health, Immunization Program). Comparison Vaccine for Children data for the public sector are incomplete. Studies conducted by the manufacturer found that immunity to serogroup B meningococcus was induced by the serogroup B meningococcal outer membrane protein vaccine in a primate animal model (11) and in children during phase III vaccine trials (Alan Shaw, pers. comm.). Further epidemiologic and immunologic research are needed to explore the protective immunity and potential use of this vaccine for meningococcal serogroup B disease.

A significant change occurred in the prevalence of vaccine-preventable strains during the period. During the years 1989–1991, only 29.7% (43/145) of the meningococcal infections that occurred in patients >2 years of age were caused by vaccine-preventable strains. The proportion of vaccine-preventable strains increased steadily in each 3-year interval reaching 66.4% (85/128) in 1998–2000 (chi square for trend; $p<0.01$). Currently, the quadrivalent meningococcal polysaccharide vaccine, the only licensed and approved vaccine in the United States, provides good efficacy against serogroups A, C, W-135, and Y infections in older children and adults. The vaccine is not routinely recommended for the general population because of its short duration of protection, poor efficacy in children <2 years of age, and the low incidence of meningococcal infections in the United States (1,12). To overcome the problems of immunity in young children, conjugate vaccines have been recently developed and might dramatically improve the prevention of meningococcal disease because of their greater efficacy among infants and longer duration of immunity (13). The conjugate vaccine that is currently licensed in the United Kingdom only protects against serogroup C infection; its addition to the routine childhood vaccine schedule in New York City would have limited impact based on current serogroup incidence (13). Meningococcal serogroup C accounted for 12% (3/25) of infections in children <5 years of age and 2% (3/144) of all meningococcal infections in 1998–2000. During the entire 12-year period, only one serogroup A meningococcal infection occurred in a child <5 years of age. To make an impact on rates of meningococcal disease in New York City through routine childhood vaccination, a vaccine is needed that produces good, long-lasting immunity in young children to serogroups B, C, W-135, and Y.

In contrast with the overall lower incidence rates, the CFR for 1989–1998 was 16.6% for New York City, compared to 9.3% for the rest of the United States (6). Possible explanations for this finding include differential reporting of severe cases, presence of virulent clones in the population, and timely access of medical care. Additionally, not all public health jurisdictions include probable cases in their surveillance reports to the Centers for Disease Control and Prevention, raising the possibility that the national number of deaths is low because of underreporting of culture-negative fatal cases. A small proportion of cases (5%) in the New York City surveillance database met the definition for probable cases, suggesting that such cases may be underreported; however, no statistically significant difference existed in deaths by case status (confirmed CFR = 16.7%; probable CFR = 21.1%; chi square =0.46; $p=0.50$). This proportion of probable cases in New York City is comparable to that found in a review of meningococcal disease in New England for 1993–1998, where 4% of the cases met the probable case definition and the CFR was 10% (14). The proportion of probable cases and CFR for meningococcal disease in New Jersey in 1990–2000 were 10.5% and 11.6%, respectively (unpub. data, New Jersey Department of Health and Senior Services, Infectious and Zoonotic Diseases Program). An assessment of meningococcal surveillance in New York State (excluding New York City) found delays but relatively complete reporting (15). The exceptionally high death rate in 1999 prompted a closer examination of these data. Median age for the year was 35, higher than median age for any other year cohort and significantly higher than median age for all other years (median age excluding 1999 = 21; $p=0.013$). The proportion of group Y disease was 41%, which also differed significantly from the years excluding 1999 (chi square = 19.0, $p<0.01$). Further epidemiologic investigation, including molecular typing, is necessary to explain the excess meningococcal deaths in New York City.

Limitations

A limitation of surveillance-based studies is the bias introduced by underreporting. However, because of the severity of

the disease and the need for intravenous antibiotic treatment, most meningococcal disease case-patients are hospitalized, and the local health department is usually notified in order to track down close contacts and ensure that they receive antibiotic prophylaxis. A study assessing the completeness of the New York State surveillance system for meningococcal disease by using hospital discharge data as the basis for comparison showed that 93% of estimated cases in hospitalized patients in 1991 were properly reported (15). Hospital practices, such as antibiotic administration before acquisition of cultures, might render samples from case-patients culture-negative; however, no evidence suggests that a change in such practice has occurred in the study interval.

Because our inclusion criteria required a positive bacterial culture, positive cerebrospinal fluid antigen test, or purpura fulminansis, cases that were culture-negative where antigen testing was not available or nonmeningitis cases without purpura fulminans might have been missed. The use of these inclusion criteria was important to ensure the validity of the study and comparability to other jurisdictions but could have slightly inflated the CFR if fatal cases tend to be reported more often to the health departments.

Another limitation was the large proportion of missing information for outcome (22.1%) that may have underestimated the CFR. We minimized this problem by performing death certificate searches using multiple search criteria.

Approximately three quarters of the New York City isolates during the study period were identified by serogroup; this proportion was similar to that observed in other surveillance-based studies conducted in the United States (2,3,9). Assuming that the lack of serogroup information for a proportion of the cases was not related to problems in identifying any specific serogroup (i.e., independent from serogroup), bias was unlikely to have been responsible for the observed trends in serogroup.

The time-trend analysis performed in this study assessed the presence of epidemiologic trends during the 1990s but not the factors responsible for them. Therefore, our study was important in identifying trends, but further studies need to be conducted to test specific hypotheses about the factors responsible for them.

National surveillance data used for comparison of rates and CFR were based on the same case definitions as used in our study; however, not all jurisdictions follow these definitions. For example, New York State Department of Health excludes probable cases, and this variation in surveillance methodology may affect the national CFR used for comparison.

Conclusions

In New York City, during the period from 1989 to 2000, the overall incidence rates of meningococcal disease decreased. This reduction was more evident in the younger age groups, and therefore the median age of patients with meningococcal disease increased. Independent of the changes in the age distribution, the proportion of cases caused by serogroup Y increased and those caused by serogroup B decreased. The CFR did not change significantly throughout the study period and is higher than national figures. The incidence of serogroup B infections has dramatically declined. Evidence suggests that this decline may be the unintended result of *H. influenzae* type b vaccine use that incorporates the meningococcus serogroup B outer membrane protein. The implications of this finding require further research because currently no available vaccine or satisfactory method exists for controlling outbreaks from serogroup B.

Understanding trends in meningococcal disease epidemiology is important in redefining appropriate measures of control and prevention. The identification of groups at high risk and the distribution of prevailing meningococcal serogroups will be critical in future decisions and recommendations regarding the nonepidemic use of meningococcal vaccine.

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Amplification of the Sylvatic Cycle of Dengue Virus Type 2, Senegal, 1999–2000: Entomologic Findings and Epidemiologic Considerations

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After 8 years of silence, dengue virus serotype 2 (DENV-2) reemerged in southeastern Senegal in 1999. Sixty-four DENV-2 strains were isolated in 1999 and 9 strains in 2000 from mosquitoes captured in the forest gallery and surrounding villages. Isolates were obtained from previously described vectors, *Aedes furcifer*, *Ae. taylori*, *Ae. luteocephalus*, and—for the first time in Senegal—from *Ae. aegypti* and *Ae. vittatus*. A retrospective analysis of sylvatic DENV-2 outbreaks in Senegal during the last 28 years of entomologic investigations shows that amplifications are periodic, with intervening, silent intervals of 5–8 years. No correlation was found between sylvatic DENV-2 emergence and rainfall amount. For sylvatic DENV-2 vectors, rainfall seems to particularly affect virus amplification that occurs at the end of the rainy season, from October to November. Data obtained from investigation of preimaginal (i.e., nonadult) mosquitoes suggest a secondary transmission cycle involving mosquitoes other than those identified previously as vectors.

Dengue is a viral disease transmitted by mosquitoes and caused by four viral serotypes (dengue virus serotypes 1–4 [DENV 1–4]) belonging to the genus *Flavivirus* of the *Flaviviridae* family. In terms of illness and death, dengue is the most important viral disease transmitted to humans by mosquitoes (1). This virus is distributed nearly worldwide and represents a serious public health problem in Southeast Asia, the Caribbean, the Pacific Islands, and Latin America. While in Asia and the Americas human-to-human transmission by mosquitoes is the current form of virus circulation, in West Africa sylvatic circulation is predominant. In Africa, the existence of dengue dates back to 1956,when a retrospective serosurvey confirmed that a dengue epidemic occurred in Durban, South Africa, in 1926–1927 (2). Additional evidence was obtained in the 1960s, when DENV-1 and DENV-2 were isolated for the first time from human samples in Nigeria (3).

In Senegal, evidence of dengue virus circulation was obtained when DENV-2 was isolated for the first time in 1970 from human blood (4). After this isolation, an entomologic surveillance program was undertaken in Senegal. By that time, in light of the yellow fever virus transmission cycle established by Haddow (5), much interest was being shown in sylvatic cycles of arboviruses in general. The main objective of this program was to identify the vectors and describe the sylvatic cycle of dengue virus transmission. Results obtained from these studies identified several DENV-2 epizootics through periodic amplification of the sylvatic cycle in Kedougou, Senegal (6–8). Although DENV 1–4 were isolated incidentally in Senegal from humans, only DENV-2 was shown to be circulating regularly in mosquito, human, and monkey populations with a sylvatic focus in Kedougou (9,10).

After 8 years of silence, DENV-2 reemerged in 1999. In this paper we analyze data obtained during this 1999–2000 epizootic in light of climatic changes and vector ecology. We also discuss new insights in terms of the maintenance and emergence mechanisms of DENV-2.

Materials and Methods

Study Sites and Calendar of Investigations

Our study area was located in the southeastern part of Senegal (12°11'W, 12°33'N) in Kedougou, a department (the first-level administrative subdivision of the region) named after the town Kedougou, which is surrounded by an area of savannah and forest. In this paper the expression Kedougou area refers to the Savannah and forest galleries area, where most of our study took place. The population is essentially rural. The Kedougou area, which belongs to the Sudan-Guinean climate, is located at 1,200–1,300 mm isohyets; the area is part of the rainiest Senegalese region. The rainy season generally lasts from May/June to October/November, with maximum rainfall generally recorded in August or September. We conducted entomologic investigations in the Kedougou area in June, October, and November 1999. This schedule for site visits was based on our previous experience in the field and had two objectives: 1) to investigate vertical transmission of arboviruses with the emergence of nulliparous adult mosquitoes at the beginning of a rainy season after an arbovirus amplification, and 2) to isolate the maximum amount of virus at the end of the rainy season, the period of maximum arbovirus amplification. After results of virus isolation from mosquitoes showed evi-

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dence of DENV-2 circulation in 1999, we conducted intensive mosquito collections during the rainy season in June, August, September, October, and November 2000.

Mosquito Sampling

Because many arboviruses of medical and veterinary interest circulate in the study area, we used a variety of sampling methods to collect a wide range of mosquito species. Mosquitoes were then sampled by using human landing catches, CDC light traps with or without CO2, and animal bait traps. However, for collection of dengue vectors, we captured mosquitoes exclusively by human landing collections, using persons vaccinated against yellow fever virus and taking malaria chemoprophylaxis. These captures occurred between 5:30 p.m. and 8:30 p.m. in the forest gallery (located 10 km from Kedougou) and the villages of Ngari, Silling, Bandafassi, and Kénioto (Figure 1). The ecologic characteristics of the area have been described (7–11). Twenty-four human volunteers participated each evening, including 18 persons in the forest gallery and 6 in one village. In the forest gallery, mosquito catches were performed at the ground level and in the canopy. Five platforms 6–9 m high served as capture sites in the canopy. Captured mosquitoes were frozen and then sorted on a chill table by using identification keys established by Edwards (12), Ferrara et al. (13), Huang (14), and Jupp (15). Mosquitoes were sorted into monospecific pools and frozen in liquid nitrogen for virus isolation attempts.

To verify virus maintenance in the field by vertical transmission (after evidence of DENV-2 circulation was obtained in 1999), wild mosquito breeding sites were investigated. This investigation was undertaken during the dry season (outside of the adult mosquito's activity season), in February 2000, when all sylvatic mosquito breeding sites were dry. Tree holes were scraped with spoons and knives to collect *Aedes* eggs, known to be resistant to desiccation. Samples from these tree holes were stored in plastic boxes and flooded in the laboratory for egg hatching. Larvae were reared to the adult stage, then frozen, identified, and pooled by species for virus isolation attempts.

Virus Isolations

Virus isolations were performed on AP61 (*Aedes pseudoscutellaris*) mosquito cells lines, as described by Digoutte et al. (16). Virus was identified by using immunofluorescence with a specific immune ascitic fluid and confirmed by complement fixation or neutralization tests.

Monkey Serum Sampling

After initial results of virus isolation from mosquitoes showed evidence of DENV-2 circulation, serologic investigations on monkeys were undertaken January 31–February 6, 2000. Blood samples were collected from each wild monkey captured with flexible nets in the forest gallery. Briefly, animals were immobilized with ketamine HCl (Merial, Lyon, France), 10–15 mg/kg of body weight, injected intramuscularly. Blood was collected by femoral venipuncture into a heparinized vacuum tube, and gender was determined by inspection. Dental casts, morphologic measurements, and features relating to reproductive status (i.e., nipple length, scrotal pigmentation) were used to assign age classes of the animals. Each was tagged and released in the wild. In the field laboratory, heparinized blood was centrifuged for 15 minutes at 2,000 rpm, and the plasma was removed and stored in liquid nitrogen until testing. Serum samples were tested for DENV-2 immunoglobulin M (IgM)/IgG antibodies by using the enzyme-linked immunosorbent assay, as described (17).

Figure I. Map of the Kedougou area, Senegal, showing geographic position of villages and forest gallery where dengue virus serotype 2 vectors were collected.

Rainfall Analysis

Rainfall fluctuations from 1972 to 1999 were retrospectively analyzed with respect to 1961–1990 mean rainfall (the normal level, as defined by the World Meteorological Organization) and correlated to DENV-2 emergence in the same period. Anomalies were calculated by subtracting the recorded seasonal rainfall during May to October in each year from the seasonal rainfall mean (or normal) from 1961 to 1990. Rainfall data were provided by Agence pour la Sécurité de la Navigation Aérienne, Dakar, Senegal. Only rainy season months were taken into account in the analysis.

Data Analysis

Several entomologic indices were estimated: 1) the true infection rate (estimated number of dengue virus–positive mosquitoes per 100 mosquitoes tested) by using the methods of Chiang and Reeves (18) and Walter and others (19); 2) and the entomologic inoculation rate (number of infected mosquito bites per human per evening). Rates obtained were compared by using the chi-square test with $p < 0.05$ considered significant.

Results

A total of 24,747 mosquitoes belonging to six genera and 55 species were collected by using all sampling methods in 1999. Table 1 lists the number of pools and infection rates of

Table 1. Mosquitoes collected and dengue virus serotype 2 infection rates of potential vectors, Kedougou, 1999

Species	June		October				November				Total			
	No. specimens captured	(No. positive/ total pools)	No. specimens captured	(No. positive/ total pools)	True infection rate[a]		No. specimens captured	(No. positive/ total pools)	True infection rate[a]		No. specimens captured	(No. positive/ total pools)	True infection rate[a]	
Aedes furcifer male	2	(0/1)	–	(0/0)	–		27	(0/6)	–	–	29	(0/7)	-	-
Ae. furcifer female	1,132	(0/36)	1,998	(10/56)	1.72	[0.34]	1,398	(25/41)	2.84	[0.58]	4,528	(35/133)	0.91	[0.15]
Ae. taylori male	21	(0/7)	2	(0/1)	–	–	32	(0/5)	–	–	55	(0/13)	-	-
Ae. taylori female	358	(0/12)	122	(1/5)	0.92	[0.92]	543	(10/19)	2.63	[0.85]	1,023	(11/36)	1.28	[0.39]
Ae. luteocephalus female	1,064	(0/35)	682	(8/22)	1.45	[0.52]	392	(8/13)	3.06	[1.17]	2,138	(16/70)	0.84	[0.21]
Ae. aegypti female	54	(0/8)	6	(0/2)	–	–	15	(2/4)	17	[11.57]	75	(2/14)	2.74	[1.97]
Other mosquitoes[b]	5,549	(0/197)	3,366	(0/165)	–	–	7,984	(0/276)	–	–	24,747	(0/638)	-	-

[a]TIR, true infection rate (estimated number of positive mosquitoes per 100 mosquitoes tested according to Chiang and Reeves [18] and Walter et al. [19]) [standard error]; –, no data obtained.

[b]Other mosquitoes: *Anopheles coustani, An. ziemanni, An. brohieri, An. brunnipes, An. domicola, An. flavicosta, An. funestus, An. gambiae s.l., An. hancoki, An. maculipalpis, An. nili, An. pharoensis, An. pretoriensis, An. rufipes, An. squamosus, Ae. argenteopunctatus, Ae. centropunctatus, Ae. dalzieli, Ae. fowleri, Ae. hirsutus, Ae. minutus, Ae. ochraceus, Ae. vexans, Ae. vittatus, Ae; mcintoshi, Ae. cozi, Ae. metallicus, Ae. neoafricanus, Ae. unilineatus, Culex annulioris, Cx. antennatus, Cx. bitaeniorhynchus, Cx. decens, Cx. duttoni, Cx. ethiopicus, Cx. neavei, Cx. perfuscus, Cx. poicilipes, Cx. tritaeniorhynchus, Cx. cinereus, Cx. nebulosus, Cx. tigripes, Mansonia africana, Ma. uniformis, Mimomyia mediolineata, Mi. mimomyiaformis, Mi. plumosa, Uranotaenia fusca, Ur. mashonaensis, Ur. balfouri,* and *Ur. mayeri.*

mosquito species infected with DENV-2. Sixty-four DENV-2 strains were isolated and distributed as follows: *Ae. furcifer* (35 strains), *Ae. taylori* (11 strains), *Ae. luteocephalus* (16 strains), and *Ae. aegypti* (2 strains) captured in October and November. From mosquitoes collected in June, no DENV-2 strain was isolated.

The highest mean infection rates were obtained from *Ae. aegypti* (2.74%), followed by *Ae. taylori* (1.28%). However, mean infection rates of species did not differ significantly (p=0.34). Infection rates showed temporal and spatial variations. Most virus strains were isolated from mosquitoes captured in November. Infection rates observed during that month were often higher than those obtained in October, but only *Ae. furcifer* showed a significant difference between these 2 months (p<0.001). Table 2 shows the spatial distribution of biting, infection, and inoculation rates for mosquito species associated with DENV-2. In the forest gallery, *Ae. furcifer*, *Ae. taylori*, and *Ae. luteocephalus* were very aggressive, whereas *Ae. aegypti* displayed weak biting activity. The highest biting rate was obtained from *Ae. furcifer* (average 4.16 bites per person per hour). The lowest biting rate was obtained from *Ae. aegypti* (0.08 bite per person per hour). The maximum rate for *Ae. furcifer* was obtained in October (5.87 bites per person per hour). For *Ae. luteocephalus* and *Ae. taylori*, the maximum rate occurred in June (3.28 bites per person per hour) and November (1.64 bite per person per hour), respectively. In the villages, only *Ae. furcifer* had significant activity, with a maximum of 8.75 bites per person per hour in Ngari. When collections from Silling, Bandafassi, and Ngari were compared, the highest biting rates of the species regularly collected in the villages were obtained in October.

Our results (Table 2) show that DENV-2 circulated in the forest gallery and all the sampled villages. Indeed, among the 64 DENV-2 strains isolated, 58 were isolated from mosquitoes caught in the forest gallery and 6 from those caught in the villages: Ngari (2 strains), Silling (1 strain), Bandafassi (2 strains), and Kenioto (1 strain). In the forest gallery, except for *Ae. furcifer*, which exhibited significantly higher infection rates in November than in October (p<0.001), infection rates for all species were comparable in October and November. In the Kedougou area, the highest infection rate was obtained from *Ae. aegypti* (17%). However, the highest inoculation rate was obtained from *Ae. furcifer*, which was estimated to be responsible for at least two infected bites per person per week in

Table 2. Temporal and spatial distribution of vector activity and infection in Kedougou, 1999

Species	Nos. and rates[a]	Mosquitos captured in forest gallery			Ngari			Silling			Bandafassi			Kenioto
		June	Oct	Nov	June	Oct	Nov	June	Oct	Nov	June	Oct	Nov	Nov
Aedes furcifer male	n	1	–	8	–	1	7	1	–	–	–	–	3	2
Ae. furcifer female	n	1,053	1,586	1,204	2	315	110	37	33	23	21	66	50	18
	BR	3.25	5.87	3.65	0.16	8.75	3.66	2.05	2.5	1.27	1.75	5.5	4.17	1
	TIR	–	0.51	2.99	–	–	2.85	–	3.13	–	–	16.12	–	5.55
	EIR	–	0.08	0.32	–	–	0.31	–	0.23	–	–	2.67	–	0.65
Ae. luteocephalus female	n	1,063	682	390	–		2	1	–		–		–	-
	BR	3.28	2.52	1.18	–	–	0.07	0.05	–		–	–	–	-
	TIR	-	1.45	3.09	–	–	–	–		–	–	–	–	-
	EIR	–	0.11	0.11	–	–			–	–	–		–	-
Ae. aegypti female	n	45	5	15	5	1	–	4	–	–	1	–	–	-
	BR	0.13	0.45	0.04	0.42	0.03	–	0.22			0.08	–	–	-
	TIR	–	–	17	–	–	–	–	–	–	–	–	–	-
	EIR	–	–	0.02	–	–	–	–	–	–	–	–	–	-
Ae. taylori male	n	21	2	32	–	–	–	–	–	–	–	–	–	-
Ae. taylori female	n	358	122	543	–	–	–	–	–	–	–	–	–	-
	BR	1.1	0.45	1.64	–	–	–	–	–		–		–	-
	TIR	-	0.92	2.63	–	–	–	–	–	–	–	–	–	-
	EIR	-	0.01	0.13	–	–	–	–	–	–	–	–	–	-

[a]n, number of specimens captured; BR, biting rate (number of mosquitoes captured per human per hour); Oct, October; Nov, November; TIR, true infection rate (estimated number of positive mosquitoes per 100 mosquitoes tested according to Chiang and Reeves [18] and Walter et al. [19]); EIR, entomologic inoculation rate (number of infected mosquito bites per human per evening); –, no data obtained.

Table 3. Mosquito adults emerging from tree hole samples

Mosquito species	No. of specimens	No. of pools	DENV-2[a] isolations
Aedes (Diceromyia) taylori	75	2	0
Ae. (Diceromyia) furcifer	1	1	0
Ae. (Aedimorphus) dalzieli	24	2	0
Ae. (Albuginosus) stokesi	27	1	0
Ae. (Finlaya) longipalpis	16	2	0
Ae. (Stegomyia) aegypti	1,305	26	0
Ae. (Stegomyia) luteocephalus	331	15	0
Ae. (Stegomyia) bromeliae	510	16	0
Ae. (Stegomyia) unilineatus	134	3	0
Total	2,423	68	0

[a]DENV-2, dengue virus serotype 2.

November. *Ae. furcifer* was also the only mosquito infected with DENV-2 in the villages. The highest entomologic inoculation rate was obtained in Bandafassi, where a person might receive at least two infectious bites each evening.

A total of 2,423 adult mosquitoes belonging to the genus *Aedes* and the subgenera *Albuginosus*, *Stegomyia*, *Aedimorphus*, *Diceromyia*, and *Finlaya* emerged from the tree hole samples collected during the dry season in 2000. *Ae. aegypti*, *Ae. bomeliae*, and *Ae. luteocephalus* were the most common species. Out of 68 pools, no DENV-2 was isolated (Table 3).

During the 2000 rainy season, out of 31,521 mosquitoes collected in the same locations, nine DENV-2 strains were isolated, all associated with circulation of yellow fever virus. Isolations were obtained from female mosquitoes including *Ae. furcifer* (one strain), *Ae. taylori* (two strains), *Ae. luteocephalus* (two strains), *Ae. aegypti* (one strain), Ae. vittatus (two strains); isolations were obtained from males of *Ae. furcifer* (two strains) captured in the forest gallery. Isolations were obtained in August (three strains), October (five strains), and November (one strain).

DENV-2 IgG was detected in nonhuman primate blood samples, but no evidence of recent infection (IgM antibody) was obtained. Of a total of 17 African green monkeys (*Chlorocebus sabaeus*) collected, 8 juveniles (<4 years of age) and 9 adults were captured in a forest gallery near Ngari. In all samples, serologic test results were negative for DENV-2 IgM antibody. A seroprevalence of 58% for DENV-2 IgG antibodies was detected; no significant difference (p=0.09) was detected according to age (77% in adult and 37% in juvenile monkeys).

The years 1999–2000 were characterized by a rainfall surplus compared to the 1960–1999 seasonal mean rainfall. Retrospective analysis showed no clear relationship between dengue emergence and rainfall anomalies. DENV-2 amplifications were detected during periods of heavy rainfall as well as during periods of low precipitation (Figure 2).

Discussion

After 8 years of silence, DENV-2 reemerged in a sylvatic cycle in the Kedougou area in 1999. With new isolations, the number of DENV-2 strains isolated from mosquitoes in Senegal reached 350. Except for *Ae. aegypti* and *Ae. vittatus*, which were associated with DENV-2 for the first time in Senegal, all strains were obtained from vectors previously described for the DENV-2 sylvatic cycle in West Africa and belonging to the subgenus Diceromyia (*Ae. furcifer* and *Ae. taylori*) and Stegomyia (*Ae. luteocephalus*) (20–22). Population density of vector species and their spatial dynamics permitted us to assess a potential role in the transmission cycle for each infected mosquito species. *Ae. taylori* and *Ae. luteocephalus*, because of their scarcity in villages, may have a role limited to the forest gallery. However, *Ae. furcifer* may contribute to both sylvatic transmission and virus dissemination from the forest zone to human habitats since this species is the only infected one abundant in the domestic environment. *Ae. aegypti*, the principal DENV-2 epidemic vector worldwide, while very scarce in our catches, was more abundant in the forest gallery than in the villages. *Ae. aegypti*'s low rate of biting humans may reflect the zoophilic tendency of this species, which probably exists in the area only in its sylvatic form, *Ae. aegypti* formosus (11), which uses tree holes as breeding sites. (By contrast, the domestic form, *Ae. aegypti aegypti*, preferentially colonizes artificial water containers.) However, the low entomologic inoculation rates of *Ae. aegypti* in sylvatic areas suggests its limited role in sylvatic amplification cycles of DENV-2 despite its high infection rates. Although *Ae. vittatus* was infected with DENV-2 in our study, its role in dengue transmission has never been demonstrated. Further studies are needed to determine its involvement in the sylvatic cycle.

Sylvatic amplifications of DENV-2 have previously been observed in Senegal in 1974, 1980–1982, and 1989–1990 (6–8), defining periods of occurrence with silent intervals of 5–8 years (23). This periodicity of occurrence shows the similarity between DENV-2 and yellow fever virus, which share the same vectors, vertebrate hosts, and ecologic niche (8). Moreover, this pattern of occurrence shows that DENV-2 is maintained in the Kedougou area by a still-unknown mechanism, although two have been hypothesized: vertical transmission of the virus or its maintenance through a secondary cycle. Vertical transmission is the most probable hypothesis since it has been supported by laboratory and field evidence for *Ae. aegypti* (24,25) and *Ae. taylori* (6). The isolation from male *Ae. furcifer* during this investigation is the first field evidence of vertical transmission of DENV-2 by this species in Kedougou and reinforces this hypothesis. Our investigation of tree holes

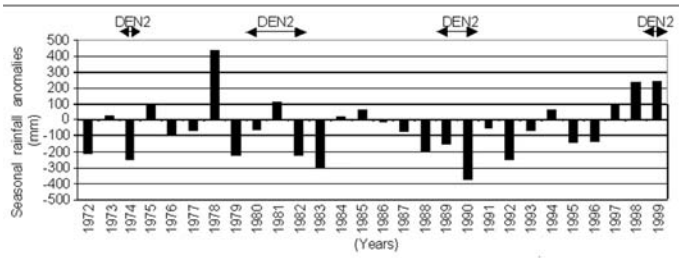

Figure 2. Seasonal rainfall anomalies in Kedougou, Senegal, 1972–1999. Anomalies were calculated by subtracting the recorded seasonal rainfall during May to October in each year from the seasonal rainfall mean (normal), 1961–1990.

also supports the hypothesis of a secondary transmission cycle involving mosquito species other than those identified as vectors. These investigations showed high representation of species considered scarce or absent, which was the case for *Ae. longipalpis* belonging to the *Finlaya* subgenus, a forest/enzootic vector of dengue in Asia; *Ae. bromeliae*, one of the major yellow fever virus vectors in East Africa; and *Ae. stockesi* (1,14,26). The role of these species may have been overlooked because of biased sampling methods for mosquito captures. Further research about the existence of a secondary cycle is needed.

In DENV-2 sylvatic circulation, vertebrate hosts may serve either as amplifiers or reservoirs. Unfortunately, data obtained in this study from monkey serum samples were not conclusive about their role during amplification because of IgG cross-reactions between flaviviruses (27). Thus, the role of monkeys in sylvatic cycle of DENV-2 remains unresolved. Extensive investigations are needed to assess the role of vertebrate hosts in the amplification and maintenance of DENV-2 in natural conditions through herd immunity, turnover of susceptible vertebrates hosts, or persistent infection (28,29).

Analysis of rainfall amount and distribution did not show any correlation with sylvatic DENV-2 emergence unlike the situation with other arboviruses such as Rift Valley fever (30,31). Rainfall has an impact on two epidemiologic parameters important in arbovirus transmission: vector density, which controls the transmission level, and adult mosquito longevity, which makes transmission possible and durable. For the sylvatic DENV-2 vectors in the Kedougou area, rainfall seems to interfere, particularly with the virus amplification period (generally October–November), coinciding with the end of the rainy season. Indeed, although vector population densities are already high at the beginning of the rainy season, the virus emergence and the maximum amplification period occur only at the end of the rainy season. Probably the greater longevity of the female vectors at the end of the rainy season, attributable to low intensity of precipitation, allows them to achieve a complete extrinsic incubation of DENV-2.

DENV-2 isolations from *Ae. furcifer* captured in peridomestic habitats demonstrate that the virus circulates in all villages where mosquito catches were undertaken. However, no DENV-2 clinical case was recorded in the region. This finding suggests that DENV-2 is confined to the forest or, if human-mosquito-human transmission occurs, the level is low and leads to nonsymptomatic cases. Similar situations were observed during the 1981 and 1990 DENV-2 epizootics, when only a few sporadic human cases were recorded (32,33). The same situation occurred in Burkina Faso and Côte d'Ivoire (21,22,34), where sylvatic transmission of DENV-2 was observed without an epidemic. Only one epidemic, which occurred in Burkina Faso in 1982, has been reported in West Africa; that epidemic was suspected of being caused by the introduction of a virus strain from the Seychelles Islands (35). Therefore, the risk of a DENV-2 epidemic in Senegal is very low if only the sylvatic cycle is taken into account. However, a risk exists since importation of an epidemic strain in an urban area cannot be excluded, as witnessed by the epidemic in Burkina Faso in 1982.

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Rabies in Sri Lanka: Splendid Isolation

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Rabies virus exists in dogs on Sri Lanka as a single, minimally divergent lineage only distantly related to other rabies virus lineages in Asia. Stable, geographically isolated virus populations are susceptible to local extinction. A fully implemented rabies-control campaign could make Sri Lanka the first Asian country in >30 years to become free of rabies virus.

Rabies, an encephalomyelitis caused by infection with rabies virus or other lyssaviruses, is responsible for 40,000–50,000 human deaths each year in Asian countries (1). Human rabies is preventable, but the high cost of antirabies biologics limits their use. Because the source of almost all human rabies infections in Asia is a bite by a domestic dog, effective dog rabies control programs not only serve to reduce human deaths but also can reduce the overall costs associated with rabies prevention.

Sri Lanka is an island with a land area of approximately 62,000 km^2 situated in the Indian Ocean, 35 km from the southern end of the Indian Peninsula. More than 95% of approximately 100 human rabies deaths each year in Sri Lanka are the result of bites by unvaccinated dogs, and U.S.$1.5 million is spent each year for antirabies biologics (2). A national dog rabies elimination program begun 20 years ago has yet to reduce the number of bite exposures requiring rabies treatment or the number of human rabies deaths. Low vaccination coverage in the resident dog population and ineffective management of stray animals are the most likely reasons for the program's lack of success, but the abundant wild fauna in Sri Lanka could provide unrecognized reservoirs for rabies virus. Serologic data also suggest that lyssaviruses other than rabies virus may be present in bats in Asia (3). Additionally, Sri Lanka's close proximity to India might allow frequent introduction of rabies virus–infected animals. Our research expands an earlier study of rabies in Sri Lanka (4) by using a panel of monoclonal antibodies (MAbs) to survey Sri Lankan rabies samples for lyssaviruses other than rabies, by using genetic typing to identify viral lineages that might signify a wildlife reservoir for rabies virus, and by using phylogenetic analysis to obtain estimates of the frequency of introductions of rabies virus from neighboring Asian countries that might jeopardize a dog rabies–control program in Sri Lanka.

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The Study

Most samples submitted for rabies testing in Sri Lanka originate from the area around the capital, Colombo. Our study included 44 samples: 36 from cases chosen randomly from the approximately 180 animals diagnosed with rabies at the Medical Research Institute, Colombo, from January to May 2001; 2 from elephants whose infections in 1998 and 1999 occurred in an area of Colombo with large active bat colonies; and 5 from human case-patients (1999–2001). A dog rabies virus collected in 1986 from Colombo served as a reference sample for mutational change in Sri Lankan rabies virus over time. Sri Lankan samples were submitted to virus typing by both antigenic and genetic methods.

The antigenic profile of 31 Sri Lankan samples, displaying 3+ to 4+ antigen distribution, was determined by indirect immunofluorescence methods with a panel of 20 nucleoprotein-specific MAbs obtained from the Centers for Disease Control and Prevention and the Wistar Institute and compared with published reaction patterns for these MAbs (5). Only two antibodies were useful. C4, which is not known to react with any lyssavirus other than rabies virus, reacted with all samples from Sri Lanka, indicating the absence of any other lyssavirus among these samples. A negative reaction with MAb CR54 distinguished rabies virus from Sri Lanka from that from the Philippines. The reaction pattern for Sri Lankan virus was identical to that reported for rabies virus from Indonesia and Thailand.

A phylogenetic analysis included the 44 Sri Lankan samples and nucleotide sequences for rabies virus and other lyssaviruses obtained from GenBank and from a sequence repository at CDC (Table, Figure 1). Data analyzed included representatives of available sequence for mainland and island Asian countries and sequence data for samples representing a similarly broad geographic distribution of mainland and island countries of East Africa and the Arabian Peninsula. With the use of standard methods (6), RNA was extracted from the Sri Lankan rabies virus samples, reverse transcribed, and amplified by polymerase chain reaction. Genetic typing was based on nucleotide sequence differences in complementary DNA (cDNA) for the nucleoprotein gene as aligned with sequence for the Pasteur vaccine strain of rabies virus (7).

The 44 samples from Sri Lanka shared approximately 99% homology over a 320-nucleotide region of the carboxy terminus of the N gene (bp 1157–1476). Only 10 nucleotide substitutions (all synonymous) were found when the entire protein encoding sequence of the N gene (1350 bp) was compared for isolates from 1986 and 2001 (1986 dog case no. 1294, GenBank accession no. AY138549; 2001 cow case no. 5657, GenBank accession no. AY138550), and the two samples were identical over an additional 56 bp of nontranslated sequence between the stop codon and polyadenylation signal (bp 1421–1476).

No evidence was found for a wildlife reservoir for rabies independent of rabies in domestic dogs. Mutations at nucleotide position 1231 and 1408 separated the virus samples into two clusters (Figure 1), but the clusters corresponded

Table. Virus samples from countries other than Sri Lanka included in phylogenetic analysis

Sample ID	Sample history and GenBank accession numbers
Australian bat lysssavirus	Australian bat lyssavirus from insectivorous bat *(Saccolaimus flaviventris);* 1996. Lyssavirus most closely related to rabies virus. Used as outgroup to root phylogenetic analysis. AF081020.
Duvenhage virus	Duvenhage virus from human bitten by bat, South Africa, 1970. Bat lyssavirus used as outgroup to root phylogenetic analysis. U22848.
Nepal	U.S. citizen bitten by dog in Nepal, 1996. AY138578.
Pakistan	Dog, 1990. AY138565.
India	Resident of U. K., bitten by dog in India, circa 1988. AY138566.
Kenya	U.S. Peace Corps volunteer bitten by dog in Kenya, 1983. AY138567.
Tanzania	Goat, 1992, Zanzibar, Tanzania. AY138568.
Saudi Arabia	Arabian-American Oil Company employee infected in Saudi Arabia, circa 1981, AY138569; fox, 1987. U22481.
Oman	Red fox, 1990. U22480.
Iran	Dog, 1986. U22482.
Israel	Dog, 1993. AY138570.
Madagascar	Human, 1980. AY138571.
Pasteur rabies virus	Cow bitten by dog, Paris, 1882, multiple laboratory passages. M13215.
Namibia	Jackal, 1992. U22649.
Algeria	Dog, 1982. U22643.
Ethiopia	Hyena, 1987. U22637.
India-Madras	Unpublished, 2001. AF374721.
Sri Lanka, reference dog sample, 1986	Dog case sample collected by Dr. Alex Wandeler, Colombo, 1986. AY138549.
Sri Lanka, human cases	Five human case-patients with diagnosed rabies, 1999–2001. Cluster A samples originated from Kandy (n=2) and Ratnapura. Cluster B samples originated from Colombo and Karapitiya. Two cluster A human case samples with nucleotide substitutions not found in consensus sequence were submitted to GenBank. AY138554 and AY138558.
Sri Lanka, dog cases	24 dogs with diagnosed rabies, January–May 2001. Cluster A samples originated from Ragama, Polgasowita, Katunayake, Puttalam, Meerigama, Panadura, Dewulapitiya. Cluster B samples originated from Ragama, Polgasowita, Mirihana, Dehiwela, Moratuwa, Chillaw, Katana, Mount Lavinia, Balapitiya, Wattala (n=2), Payagala (n=2), Colombo (n=3), Nugegoda. Dog samples with nucleotide substitutions not found in consensus sequence for cluster A (AY138553, AY138555, AY138557, AY138559) and cluster B (AY138551, AY138552) were submitted to GenBank.
Sri Lanka, goat cases	Two goats with diagnosed rabies, January–May 2001. Cluster A sample originated from Seeduwa. AY138561. Cluster B sample originated from Kandy. AY138556.
Sri Lanka, mongoose cases	Two mongooses (cluster B) with diagnosed rabies, January–May 2001, Panadura and Moratuwa. Sample with nucleotide substitutions not found in consensus sequence for cluster B was submitted to GenBank. AY138562.
Sri Lanka, cow cases	Four cows (cluster B) with diagnosed rabies, January–May 2001, Kandy, Kaduwela, Horana, Polgasowita. Samples with nucleotide substitutions not found in consensus sequence for cluster were submitted to GenBank. AY138550 and AY138560.
Sri Lanka, elephant cases	Two elephants (cluster B) with diagnosed rabies in 1998 and 1999, in an area of Colombo with large active bat colonies. Sample with nucleotide substitutions not found in consensus sequence for cluster B was submitted to GenBank. AY138564.
Sri Lanka, cat cases	Four cats (cluster B) with diagnosed rabies, January–May 2001, Kelaniya, Nittambuwa, Horana, Kaduwela. Sample with nucleotide substitutions not found in consensus sequence for cluster B was submitted to GenBank. AY138563.
Philippines	Resident of California, 1987, bitten by dog in the Philippines, AY138575; resident of California, 1972, bitten by dog in the Philippines. AY138576.
Viet Nam	Vietnamese refugee in Sidney, Australia, 1991. AY138579.
Thailand	Human, 1983, AY138572; dog, 1995, U22653.
Laos	Laotian immigrant, Texas, 1984. AY138577.
Indonesia	Human, 1989, Jakarta, Java; AY138573. dog, 1989, Java; AY138574.

roughly to sample collection sites (Figure 2), and domestic dog samples were found in both clusters. Because the analysis included only two mongooses, we cannot exclude mongooses as a cryptic reservoir for rabies. Nevertheless, rabies cases in mongooses and other wild animals are only infrequently identified in Sri Lanka and occur in areas where dog rabies cases are common. Arai et al. (4) found similar results for wildlife samples in their study. Given that >95% of human rabies cases report a dog bite as the source of infection, expanding rabies-control programs to include wild animals in Sri Lanka without more specific evidence of a reservoir status for these animals would not be helpful.

In contrast to the limited sequence diversity among samples from Sri Lanka, extensive sequence divergence was found when samples from Sri Lanka were compared with samples from other Asian countries, and genetic change was much more evident in the nontranslated sequence (17.2% to 31.2% divergence) as compared with the protein-encoding sequence (11.8% to 21.5% divergence). Identity for nontranslated sequence in some intercountry comparisons was as low as 50% (e.g., 50% to 58% identity over the nontranslated sequence for comparison of viruses from Indonesia and India). Additionally, samples from Vietnam, Indonesia, Laos, the Philippines, and Thailand contained an extra nucleotide in the nontranslated

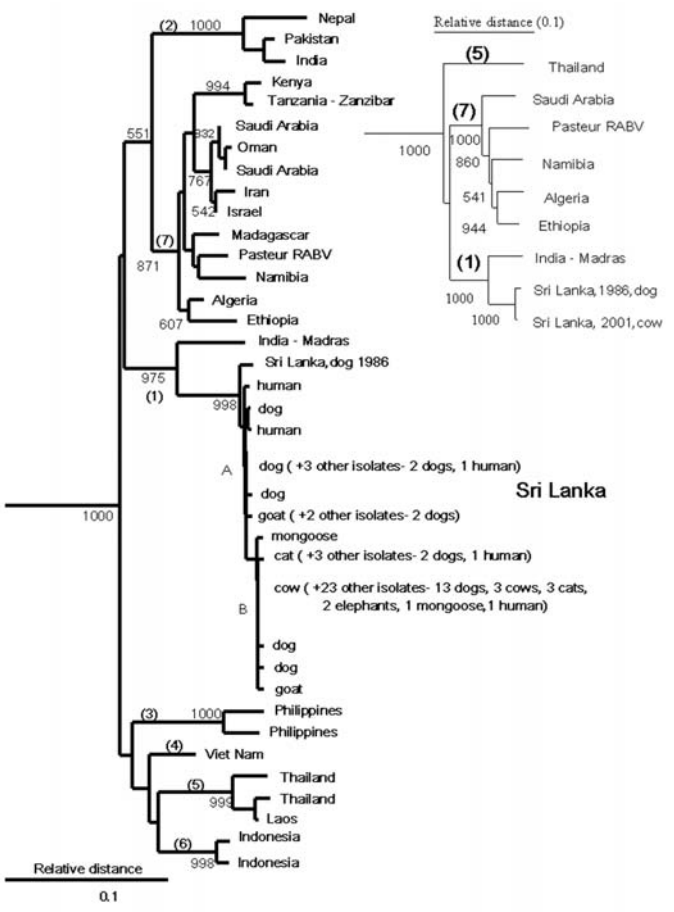

Figure 1. Neighbor-joining tree for 264 base pairs (bp) of nucleoprotein gene sequence (bp 1157–1420) for 44 rabies virus samples from Sri Lanka compared with samples from other areas of Asia and Africa with uncontrolled dog rabies. Samples were aligned with Pasteur rabies virus (GenBank accession no. M13215) by using the PileUp program of the Wisconsin Package version 10 (Genetic Computer Group, 1999, Madison, WI). The phylogenetic analyses were perfomed by using the DNADIST (Kimura two-parameter method), Neighbor (Neighbor-joining method), SEQBOOT, and CONSENSE programs of the PHYLIP package, version 3.5 (available from: URL: http://evolution.genetics.washington.edu/phylip.html). The program TREEVIEW was used to obtain the graphic output (available from: URL: http://taxonomy.zoology gla.ac.uk/rod/treeview.html). Australian bat lyssavirus (GenBank AF081020) and Duvenhage virus (GenBank U22848) were used as outgroups (not shown). Bootstrap values >50% in 1,000 resamplings of the data are shown at nodes corresponding to the different lineages and sample clusters. The nodes signifying highest order clustering of rabies virus lineages (supported by bootstrap values >70%) are numbered 1–7. The two clusters of Sri Lankan samples, designated by mutations at nucleotides 1231 and 1408, are indicated as A and B. Inset shows a similar tree achieved by analysis of complete nucleoprotein gene sequence (1414 bp). All nonidentical Sri Lankan samples were submitted to GenBank (AY138549–AY138564). The Table lists sample collection information.

region as compared with the Pasteur vaccine strain of rabies virus. The exact position of this extra nucleotide could not be determined because it varied in relation to different sample groups, and the single extra nucleotide likely represents multiple insertion and deletion events.

Molecular aspects of the epidemiology of rabies within Sri Lanka and between Sri Lanka and other countries were determined by constructing phylogenetic trees (Figure 1). Because the possibility of multiple insertions and deletions prevented an exact alignment of the nontranslated sequence, phylogenetic analysis was conducted with only the protein-encoding sequence.

The phylogenetic analysis showed that all samples positive for rabies in diagnostic tests from Sri Lanka were rabies virus. Samples from Sri Lanka clustered with the Pasteur vaccine strain of rabies virus and other samples identified as genotype 1 lyssavirus (8) and were monophyletic with respect to other lyssavirus genotypes (not shown). Rabies virus samples from Asia were segregated into six clades on the basis of the geographic origin of the samples. Despite the exclusion of the highly variable nontranslated sequence from the analysis, no evidence was found for a common ancestry for all six Asian lineages (Figure 1). Three lineages were composed of samples from a single country (Philippines, Vietnam, and Indonesia). The remaining three lineages comprised samples from geographically proximate countries (Sri Lanka and the sample from Madras, India; Thailand and Laos; and Pakistan, India, and Nepal). Similar findings were achieved with a more limited sample set encompassing the entire nucleoprotein gene (inset in Figure 1).

In contrast to the genetic diversity of Asian rabies virus samples, samples of African 1 rabies virus (8) collected from the Arabian Peninsula and mainland and island countries of East Africa shared much higher sequence identity (approximately 93%) and could be resolved as a single lineage sharing a common ancestry with European vaccine strains of the virus dating from the 19th century (Pasteur rabies virus). Molecular clock data suggest that this virus emerged 300–500 years ago (9) and support historical data for rabies virus dissemination during early European exploration and colonization of Africa (8–10). The genetic divergence between samples from Sri Lanka and the most closely related Asian sample (Madras, India; >6.3% difference) suggests a similarly distant common ancestor for these two rabies virus populations.

Conclusions

As depicted by the antigenic and genetic analysis of samples in this report and the earlier work by Arai et al. (4), rabies in Sri Lanka is associated with a single lineage of rabies virus, which shares only a distant common ancestry with rabies viruses from any other Asian country. Genetic diversity between rabies virus lineages from mainland or island Asian countries was much greater than among samples collected over a similar geographic expanse in Africa. Differences between Asia and Africa in the partitioning and degree of spread of rabies virus in host populations might be explained by agricultural practices that evolve in areas of high rainfall versus the agricultural practices necessary in areas where rain is seasonal and scarce. For example, nomadic herding, a common agricultural practice in Africa, would spread an introduced rabies virus over great dis-

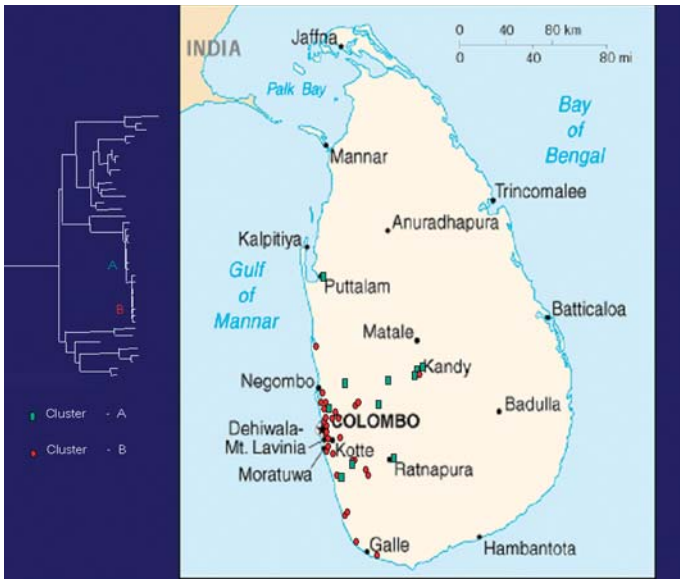

Figure 2. Map of Sri Lanka showing location of rabies samples included in this study. Green, cluster A; red, cluster B.

tances, whereas the primarily small-scale farming operations in Sri Lanka and other Asian countries, maintained locally for generations, could restrict virus spread. These observations suggest that restrictions to domestic animal movement can be exploited in rabies-control efforts in Asia.

Rabies viruses in Sri Lanka and India may be more closely related than described in this and previous studies (4). Rabies virus more closely related to Indian viruses may be present in the northern and eastern parts of Sri Lanka, where civil unrest prevented sample collection. The Sri Lankan variant of the virus may still be actively transmitted by animals in India; only limited virus typing has been conducted on Indian rabies samples. However, although considerable genetic diversity was found in the four available sequences for rabies virus from the Indian subcontinent, only one Indian lineage shared a common ancestry with Sri Lankan samples. The degree of divergence between this Indian sample and the Sri Lankan samples and the lack of heterogeneity in Sri Lankan samples collected over a 16-year period suggest that the introduction of the ancestral virus occurred only once and in the distant past.

While the data reported here and in the work by Arai et al. (4) represent only a fraction of all rabies cases that occur each year in Sri Lanka, the island is small and heavily populated. If other rabies virus lineages are present, or if introductions from India are more frequent than our data indicate, future virus typing should detect them easily.

Antigenic and genetic typing of samples collected for rabies testing in Sri Lanka found no lyssavirus other than rabies virus, no evidence for cycles of rabies virus transmission in wild species independent of endemic rabies virus in domestic dogs, no evidence of a recent introduction of the virus to Sri Lanka through the translocation of animals from other areas of Asia, and (surprisingly, given the close proximity to mainland Asia) no evidence for outside introduction of the virus to Sri Lanka in the recent past. Historically, rabies of this type has been susceptible to control with a fully implemented vaccination campaign and an animal-control effort that addresses local cultural and religious attitudes toward management of stray animals. The economic impact of rabies on Sri Lanka argues for national and international cooperation in a strong rabies-control program for this island nation. The prospects are good that such an effort could eliminate rabies virus from the island and create the first rabies virus–free country of the millennium.

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Human Metapneumovirus in Severe Respiratory Syncytial Virus Bronchiolitis

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Reverse transcription-polymerase chain reaction was used to detect segments of the M (matrix), N (nucleoprotein), and F (fusion) genes of human metapneumovirus in bronchoalveolar fluid from 30 infants with severe respiratory syncytial virus bronchiolitis. Seventy percent of them were coinfected with metapneumovirus. Such coinfection might be a factor influencing the severity of bronchiolitis.

Bronchiolitis is an important cause of illness and death in infants. An estimated 25 in every 1,000 children will require hospitalization with bronchiolitis; for 1% to 2% of these children, infection will be severe enough to require ventilatory support (1). The etiologic agents of bronchiolitis include adenovirus, influenza and parainfluenza viruses, and *Bordetella pertussis*. However, most infections are due to respiratory syncytial virus (RSV) (2). In June 2001, Osterhaus and colleagues reported the discovery of a "new" human respiratory virus (*Pneumovirinae* subfamily, *Paramyxoviridae* family) (3). RSV is in the genus *Pneumovirus*, and the new virus, although related to RSV, is more closely related to avian pneumovirus serotype C (previously known as turkey rhinotracheitis virus) (4), the only other member of the Metapneumovirus genus. The new virus, however, does not infect chickens or turkeys (3), and they are unlikely to be a zoonotic source. Thus, the new virus was termed human metapneumovirus (hMPV). Of 28 isolates, all isolated in the winter, 13 came from infants <12 months of age who had disease patterns resembling those seen in RSV infections. Subsequently, human metapneumovirus has been detected in children in Australia (5) and Canada (6).

The Study

As part of a larger study examining the immunopathogenesis of severe bronchiolitis, nonbronchoscopic bronchoalveolar lavage samples were collected from 30 infants ventilated with RSV bronchiolitis (determined by antigen detection in nasopharyngeal aspirates) in 2000 and 2001. The sampling was performed according to recent European Respiratory Society guidelines (7). Briefly, a suction catheter was passed down the endotracheal tube beyond the bifurcation of the bronchi. Two 1-mL/kg aliquots of sterile normal saline were instilled separately down the suction catheter. Lavage fluid was recovered with constant suction pressure into a mucous trap. Samples were frozen at –80°C until analyzed. Control samples were obtained in winter from children within the same age range who were being intubated and ventilated for noninfection-related conditions. The local research ethics committee approved the study, and informed consent was obtained from parents.

Detection of the human metapneumovirus genome was performed by reverse transcription-polymerase chain amplification (RT-PCR) of the matrix (M), fusion (F), and nucleoprotein (N) genes. RNA was extracted from the specimens by the guanidine/silica method (8), and 15 µL was added to a 50-µL RT reaction with a polyT primer and 1.25 U AMV-RT (Gibco, Basingstoke, U.K.) to produce cDNA. Each PCR reaction contained 10 µL of cDNA with 2.5 U Amplitaq Gold (Applied Biosystems, Warrington, U.K.), 2.0 mM Mg Cl2, 300 µM deoxynucleoside triphosphate, 0.4 µM of each primer, and the buffer supplied. For amplification of the M gene, primers hMPV-MF1 (AAG TGA ATG CAT CAG CCC AAG) and hMPV-MR1 (CAC AGA CTG TGA GTT TGT CAA A), which amplified the region between nucleotides 212 and 331, were used to give an amplicon of 120 bp. PCR cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 10 min in a Perkin-Elmer (Warrington, Cheshire, U.K.) 2400 model thermal cycler. Positive samples were confirmed by PCR amplification for the F or N gene products, or by amplification of a longer product of the M gene. The longer M product used primer hMPV-MR1 and a new primer, hMPV-MF2 (ATG GAG TCC TAT CTA GTA GAC A), amplifying the region between nucleotides 1 and 331 of the M gene, yielded a 331-bp product. For the F PCR, primers hMPV-FF1 (GTG AGC TGT TCC ATT GGC AG) and hMPV-FR1 (CCC TCA ACT TTG CTT AGC TGA TA) amplified the region between nucleotides 1162 and 1295 of the F protein to give a 134-bp PCR product. PCR cycling conditions were as for the M protein but with an annealing temperature of 62°C. The N PCR used primers hMPV-NF1 (GTA TTA CAG AAG TTT GTT CAT TGA G) and hMPV-NR1 (GAG AAC AAC ACT TGC AAA GTT GG), which amplified the region between nucleotides 710 and 1034 of the N protein to give a 325-bp product. PCR cycling conditions were as for the F protein. In each case, the primers were designed by aligning the M, N, and F gene sequences of the original Dutch isolates (93.1, 93.2, 93.3, 94.1, 94.2, 99.1, 99.2, and 00.1) (3) to define regions that were held in common. The primers did not amplify sequences from either human RSV or avian pneumovirus.

Selected PCR products were cloned into a TA cloning vector (pGEM-T, Promega, Southampton, Hampshire, U.K.), and the sequence was determined to confirm the identity of the virus detected by the PCR reaction. Sequences from the M, N,

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and F proteins of one virus (LIV08) have been deposited in EMBL, with the accession numbers AJ439505, AJ439504, and AJ439503, respectively.

The phylogenetic relationship between isolates was examined. Human metapneumovirus sequences were aligned with the CLUSTALX multiple alignment program (EMBL, Heidelberg, Germany), and analyses were carried out by using the Phylogeny Inference Package (PHYLIP version 3.5c, (J. Felenstein and the University of Washington). DNA sequences were analyzed by using DNADIST followed by neighbor joining; 100 replica samplings were analyzed. Detection of RSV by RT-PCR and N gene typing was as described (9).

M gene and at least one other of N, F, or long M amplicons of human metapneumovirus were detected in bronchoscopic bronchoalveolar lavage fluids from 21 (70%) of 30 infants ventilated for RSV bronchiolitis (Table). Four of the M-gene amplicons were cloned and sequenced. Amplicons were cut from agarose gels, eluted, and purified by using a gel purification kit (QIAGEN, West Sussex, U.K.). The amplicons were cloned into pGEM-T (Promega), and sequencing was conducted with MI3 primers by Lark Technologies (Essex, U.K.). In each case, the amplicons were very similar (1 base different) to each other and closely related to those reported for human metapneumovirus (82% to 97%) (3). The Figure shows a neighbor-joining tree that uses a 102-bp (nucleotide 208–309) region of the M gene, demonstrating the relationship of a Liverpool (LIV08) strain to those from Holland. RSV was detectable by RT-PCR in only 24 (80%) of 30 nonbronchoscopic bronchoalveolar lavage samples, despite RSV antigens being detectable from all nasopharyngeal aspirates at the onset of illness. Of these 24, 23 could be assigned an N genotype (18 NP-4, 4 NP-2, 1 NP-3). Human metapneumovirus coinfection was detectable with each of the genotypes, although only one (25%) of the four infants infected with NP-2 was coinfected with human metapneumovirus, compared with 13 (76%) of 17 of those with NP-4. Human metapneumovirus was detectable in three infants from whom no RSV amplicon could be obtained. Neither RSV nor human metapneumovirus amplicons were detectable in nonbronchoscopic bronchoalveolar lavage fluid from 10 control patients ventilated for reasons unrelated to bronchiolitis. Most infants have serologic evidence of infection with RSV by 2 years of age (10), but other than host factors such as underlying cardiac, respiratory, or genetic factors and prematurity, why severe disease develops in some infants and

Table. Characteristics of infants with severe bronchiolitis[a]

Pt. no.	Age (weeks)	Weight (kg)	Predisposing factors	RSV	hMPV	Days of illness before BAL
1	3	3.5	None	NP-4	+	8
2[b]	48	6.6	Premature, BPD	–	+	6
3	18	7.5	None	NP-4	+	7
4	35	6.0	Cardiac, genetic	–	+	7
5	11	2.8	Premature	NP-4	+	6
6	21	5.5	Premature	NP-4	+	4
7	3	2.3	Premature	–	–	7
8	5	2.5	Premature	NP-4	+	3
9	18	5.1	Premature, cardiac	NP-2	–	6
10	11	5.7	None	NP-4	+	10
11	21	7.5	None	NP-2	+	9
12	9	2.0	Premature	NP-4	–	3
13[b]	16	4.4	Premature, cardiac	NP-2	–	3
14	7	2.6	Premature	NP-3	+	5
15	15	2.8	Premature	NP-4	+	5
16	4	4.0	None	NP-4	+	2
17	7	4.3	None	NP-4	+	5
18	12	3.2	Premature, cardiac	NP-4	–	6
19	12	5.4	None	–	+	13
20	5	3.2	None	NP-4	+	7
21	5	2.8	Premature	NP-2	–	5
22	9	4.7	Cardiac	NP-4	–	8
23[b]	22	5.7	Genetic	NP-4	+	4
24	7	3.4	Premature	+	+	11
25	19	4.8	Premature, BPD	NP-4	–	6
26	13	3.4	Premature	NP-4	+	6
27	8	2.5	Premature	NP-4	+	6
28	29	3.5	Premature, BPD	–	+	6
29	3	4.1	None	NP-4	–	5
30	5	4.1	None	–	+	5

[a]Abbreviations used: Pt., patient; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; BPD: bronchopulmonary dysplasia; BAL, bronchoalveolar lavage; NP, nucleoprotein genotype.

[b]Died.

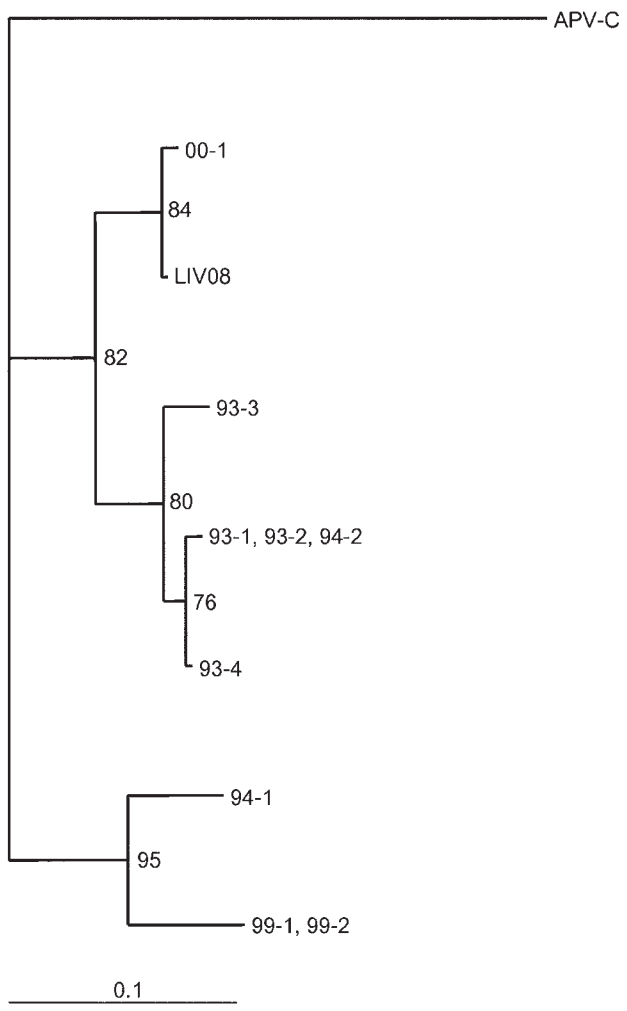

Figure. The phylogenetic relationship of human metapneumovirus from Liverpool (LIV08) to those from Holland and to avian pneumovirus. The divergence bar is shown at the bottom of the figure.

mild disease in others is unclear. For example, although 87% of children have antibodies to RSV by age 18 months (11), most children have only upper respiratory tract symptoms such as rhinitis, cough, and coryza (12). The age of the patient is also important because lower respiratory tract signs develop in up to 40% of infants <1 year, and 0.5% to 2% of all infants need to be hospitalized (12). Coinfection with RSV and other pathogens has been described previously. In studies in Northern Ireland (13) and India (14), coinfection occurred in 1.1% (all with rhinovirus) and 15% (with influenza and parainfluenza viruses) of patients with RSV acute lower respiratory tract infection, respectively. However, apart from in viral infections that might alter host immunity (15), acute respiratory tract infections involving RSV alone were not significantly different from those caused by RSV and another virus (16). RSV and human metapneumovirus might have similar seasonal patterns (3), so coinfection is possible. In the present study, we detected coinfection with human metapneumovirus in 70% of infants with RSV bronchiolitis sufficiently severe to require admission to the pediatric intensive-care unit for ventilatory support.

Sequence analysis of a portion of the M gene from four of the detected human metapneumoviruses indicated that they were closely related both to each other and to the Dutch isolates. This high rate of coinfection raises the possibility that coinfection with RSV and human metapneumovirus might be another determinant of RSV disease severity. Indeed, of 10 studied infants with severe RSV bronchiolitis and no other risk factors for severe disease, 9 (90%) were coinfected with human metapneumovirus. Confirmation of a role for coinfection will require longitudinal studies over several bronchiolitis seasons, comparing disease severity with frequency of coinfection across the full spectrum of RSV disease. Finally, this high RSV–human metapneumovirus coinfection rate in severe disease means that previous studies into RSV subtype/genotype, disease severity, and cytokine and chemokine expression in RSV disease may need to be reevaluated.

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Dr. Greensill is a postdoctoral fellow working on characterization of Kaposi's sarcoma–like herpesviruses in Old World monkeys. Her doctoral degree focused on investigating the pathogenesis and molecular epidemiology of respiratory syncytial virus.

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Persistence of Virus-Reactive Serum Immunoglobulin M Antibody in Confirmed West Nile Virus Encephalitis Cases

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Twenty-nine laboratory-confirmed West Nile virus (WNV) encephalitis patients were bled serially so that WNV-reactive immunoglobulin (Ig) M activity could be determined. Of those patients bled, 7 (60%) of 12 had anti-WNV IgM at approximately 500 days after onset. Clinicians should be cautious when interpreting serologic results from early season WNV IgM-positive patients.

In late summer and early fall of 1999, human West Nile virus (WNV) infections were recognized for the first time in the Western Hemisphere (1–6). Since its original introduction into the New York City (NYC) area, WNV virus has caused disease in humans, horses, and a wide variety of birds and other vertebrates, spreading into the eastern two thirds of the United States and also into Canada and the Caribbean Basin (7,8). The apparent ability of WNV virus to be disseminated by infected birds and to persist from year to year indicates that it will continue to be a public health problem for the foreseeable future.

The detection of human cases of WNV encephalitis early in the transmission season is a valuable tool to identify human risk and seasonal virus activity. Diagnosis of WNV encephalitis is made by using an immunoglobulin (Ig) M antibody capture enzyme-linked immunosorbent assay (MAC-ELISA), which demonstrates virus-reactive IgM in serum or cerebrospinal fluid (CSF) from a person with a clinically compatible illness (6,9–16). However, if WNV-reactive IgM is long lasting (e.g., from one transmission season to the next), an early season positive IgM test could result from either a recent or past infection.

At least four previous studies, three with Japanese encephalitis virus (JEV) (13,17,18) and one with WNV (19), included evaluations of flavivirus-reactive IgM persistence. Only one of the JEV studies evaluated the IgM activity in case-patients >6 months after illness (17). Three of 41 JEV case-patients demonstrated JEV-reactive IgM in serum >10 months (300, 330, and 350 days) after onset. In the latter study of human WNV infections, 50% of patients were still IgM positive after 2 months (19). We report the results of a longitudinal study that followed laboratory-confirmed human WNV encephalitis case-patients for up to 18 months to determine the longevity of their serum WNV-reactive IgM.

The Study

Of the 55 surviving laboratory-confirmed case-patients diagnosed with WNV encephalitis in the United States in 1999 (6), 29 agreed to participate in follow-up studies to assess their recovery from disease and their WNV-reactive antibody levels. Serum specimens were obtained and analyzed for the presence of anti-WNV IgM and IgG antibodies by using MAC-ELISA and indirect IgG ELISA as described (16,20). All cases were originally laboratory confirmed by presence of WNV-reactive IgM in acute cerebral spinal fluid specimens, identification of WNV-reactive IgM in serum samples in the presence of WNV-specific neutralizing antibodies, a >4-fold increase in WNV-reactive neutralizing antibodies in serial serum specimens, or a combination of these antibody activities.

For this analysis, all serum specimens from these case-patients were considered independent samples, and multiple specimens obtained at different times during the acute phase of illness of the same patient were included in the temporal analysis (Figure). MAC-ELISA results for acute-phase serum specimens of the 33 remaining 1999 case-patients who were not followed longitudinally were also included (Figure). When case-patients became serologically negative for WNV-reactive IgM, they were not subsequently resampled. Since the timing of the sequential bleeds depended on the availability of the patients, not all patients were sampled at all follow-up time intervals. Results from the ELISA testing were expressed as a positive-to-negative (P/N) ratio of observed A450nm (MAC-ELISA) or A405nm (IgG ELISA) as described (16,20). In these tests, P/N ratios >3.0 were considered positive, and P/N ratios >2.0 and <3.0 were considered equivocal, requiring additional laborato-

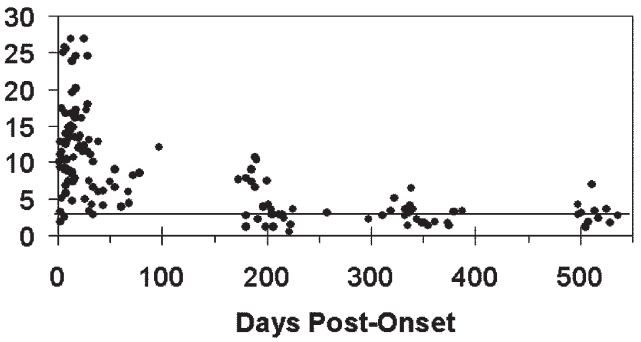

Figure. Scatterplot of anti–West Nile virus immunoglobulin M positive-to-negative (P/N) values of individual serum specimens over time. Dotted line represents P/N=3.0 cut-off.

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ry testing. The MAC-ELISA and IgG ELISA were run in triplicate for each specimen.

Scatter plot analysis of the IgM activity of all tested specimens showed a typical WNV-reactive IgM response (Figure), similar to that seen with human infections with that virus (19). The IgM activity peaked within the first 20 days after onset. Of the 29 case-patients followed for long-term antibody activity, 22 had serum specimens obtained approximately 200 days after onset, 21 had serum specimens obtained 200–400 days after onset, and 12 had serum specimens obtained >500 days after onset (Table 1). Of the 22 specimens obtained approximately 200 days after onset, 14 (64%) were WNV-reactive IgM-positive (P/N values >3.0; range 3.0–10.8; average 6.3). An additional four specimens had equivocal results (P/N values from 2.0 to 2.99), which would require additional laboratory testing. In total, 18 (82%) of 22 specimens had either positive or equivocal results at approximately 200 days after onset.

Of the 21 serum specimens obtained approximately 300–400 days after onset, 9 (43%) were WNV-reactive IgM-positive (P/N values >3.0; range 3.1–6.5; average 4.0). An additional four specimens had equivocal results (P/N values of 2.2, 2.2, 2.7, and 2.7), which would require additional laboratory testing. In total, 13 (62%) of 21 specimens had either positive or equivocal results at 300–400 days after onset.

Of the 12 serum specimens obtained approximately >500 days after onset, 5 (42%) were WNV-reactive IgM-positive (P/N values≥3.0; range 3.1–6.9; average 4.6). An additional two specimens had equivocal results (P/N value 2.8), which would require additional laboratory testing. In total, 7 (58%) of 12 specimens had either positive or equivocal results at approximately 500 days after onset, with the latest positive specimen having been drawn 525 days (17.5 months) after onset (P/N = 3.6).

As expected, most of the 29 case-patients had positive WNV-reactive IgG results (P/N≥3.0; range 3.2–8.8; average 6.3) in their last tested serum specimen. The one patient whose P/N was <3.0 had a P/N of 2.8, which means that all specimens would probably be considered WNV antibody-positive or at least require additional laboratory testing. Although we did not follow longitudinally the WNV-reactive IgM activity in the CSF of these case-patients, the latest WNV-positive CSF specimen ever submitted to Centers for Disease Control and Prevention for diagnostic testing from a laboratory-confirmed WNV human infection was obtained at 47 days after onset (data not shown).

The percent of patients with detectable IgM antibodies at 9 months differed by age (Table 2), with 56% of those≥65 years of age being positive compared to 44% of those <65 years; the difference was not statistically significant. No difference existed in the percentage positive at 1 year by sex or initial clinical syndrome, with 18% of encephalitis patients being positive, compared with 0% of meningitis patients. Patients with acute IgM P/N ratios above the median appeared to be more likely to remain positive over time than those with acute P/N ratios below the median.

Table 1. IgM and IgG P/N values of WNV virus patient serum samples by days after onset[a]

Case no.	Sample	Days after onset	WNV IgM	WNV IgG
	S1	12	14.3	2.0
	S2	197	3.9	7.8
	S3	340	3.5	8.2
1	S4	498	2.8	nd[a]
	S1	97	12.0	5.3
2	S2	378	3.2	nd
	S1	72	8.2	5.0
	S2	186	7.3	7.0
3	S3	388	1.0	nd
	S1	27	17.3	3.4
4	S2	311	2.7	6.4
	S1	31	11.0	5.5
	S2	215	2.8	7.9
5	S3	374	1.3	nd
	S1	39	5.9	2.8
	S2	173	7.6	3.9
	S3	332	3.5	4.3
6	S4	502	3.1	nd
	S1	29	11.5	4.8
	S2	189	6.7	6.3
	S3	332	2.7	6.2
7	S4	536	2.8	nd
	S1	3	3.3	6
	S2	15	10.7	5.0
	S3	30	nd	5.3
	S4	43	4.1	5.4
	S5	204	3.5	6.2
	S6	343	2.2	7.0
8	S7	508	1.8	nd
	S1	21	13.6	4.4
9	S2	199	1.7	3.2
	S1	55	9.0	3.7
10	S2	189	10.8	6.0
	S1	17	24.6	5.2
	S2	255	3.6	4.3
	S3	336	3.1	7.5
11	S4	512	6.9	nd
	S1	11	13.4	5.3
	S2	13	15.0	5.6
	S3	24	11.4	5.8
12	S4	533	1.8	nd
	S1	8	16.7	3.4
13	S2	202	4.2	nd
	S1	16	7.8	2.4
	S2	30	3.5	5.6
14	S3	349	1.7	7.0
	S1	17	17.2	5.6
	S2	192	2.2	6.3
15	S3	335	1.4	6.8
	S1	7	14.0	2.0
	S2	29	24.5	3.7
16	S3	338	6.5	6.6

Table 1 (continued). IgM and IgG P/N values of WNV virus patient serum samples by days after onset[a]

Case no.	Sample	Days after onset	WNV IgM	WNV IgG
	S1	79	8.5	5.7
	S2	200	7.5	4.5
	S3	337	4.1	4.6
17	S4	525	3.6	nd
	S1	50	7.3	4.8
	S2	55	6.6	3.3
	S3	68	4.4	nd
	S4	207	2.9	7.3
18	S5	505	1.3	nd
	S1	4	10.5	4.0
	S2	14	7.3	4.1
19	S3	355	1.3	nd
	S1	29	18.0	3.1
	S2	257	3	8.8
20	S3	373	1.7	nd
	S1	1	10.1	3.6
	S2	7	9.2	3.5
21	S3	180	1.2	2.8
	S1	6	25.7	2.4
	S2	185	9.1	6.9
	S3	319	3.4	8.2
22	S4	497	4.2	Nd
	S1	25	12.3	5.5
	S2	204	3.0	7.2
23	S3	529	1.7	nd
	S1	4	11.4	3.2
	S2	8	9.1	3.5
	S3	14	19.6	2.7
	S4	212	2.9	6.3
24	S5	361	1.9	nd
	S1	9	9.1	4.8
	S2	34	6.7	5.2
25	S3	223	1.5	5.0
	S1	10	14.8	2.3
	S2	22	16.1	3.7
	S3	191	10.4	3.2
	S4	387	3.4	nd
26	S5	514	5.2	nd
	S1	3	11.1	3.7
	S2	180	7.8	5.8
	S3	322	5.1	8.3
27	S4	518	2.4	nd
	S1	26	4.9	6.0
	S2	32	4.3	5.6
28	S3	298	2.2	nd
	S1	217	2.4	6.7
29	S2	351	1.8	8.1

[a] Ig, immunoglobulin; P/N, positive-to-negative; WNV, West Nile virus; nd, not done.

Conclusions

Identification of a recent human infection with WNV is a important event that usually triggers public health alerts, mosquito control measures, and media attention. Because laboratory tests for the presence of WNV-reactive IgM are used to identify such infections, they should be conducted properly, and the test results must be interpreted accurately. Proper interpretation criteria include considering clinical context (encephalitis or meningitis), previous travel history, flaviviral vaccination history, and evidence of previous and current WNV activity in the region. Cumulatively during 1999–2000, onset dates for human WNV disease cases in the United States ranged from early July to early December, roughly indicating a 5-month transmission season and a 7-month nontransmission season (7,8). Based on the serologic results presented here, approximately 50% of persons with an acute WNV infection of the central nervous system would be expected to have persistent IgM antibody for >8 months. The presence of WNV-reactive IgM in serum alone, therefore, is not necessarily diagnostic of an acute WNV infection. These IgM results with human WNV infections concur with the previously published results of human JEV infections and suggest that the temporal characteristics of the human antibody response to related neurotropic flaviviruses (e.g., WNV, JEV, St. Louis encephalitis virus, and Murray Valley encephalitis virus) are similar (17).

Given the low incidence of indigenously acquired neurotropic flavivirus infections in the United States, however, this similarity would seem to be more of a theoretical concern than a practical one (i.e., the chance of a person in the United States acquiring WNV encephalitis or meningitis during a given transmission season, maintaining a significant level of virus-specific IgM activity over the ensuing 8–12 months, and then again developing a viral encephalitis, meningitis, or being re-exposed to WNV during the subsequent transmission season is highly unlikely). Therefore, when evaluating a patient with acute viral encephalitis acquired in the United States, a positive serum test for IgM antibody to WNV would be expected to have a high predictive value, particularly during July to December, and especially when additional evidence exists of current epizootic or epidemic WNV activity in the area.

Nevertheless, especially in areas where WNV is known to have circulated previously or has an extended transmission season (e.g., Florida), suspected cases of acute WNV disease of the central nervous system should be confirmed by the demonstration of WNV-reactive IgM in CSF, the development of WNV-specific IgG antibody in convalescent-phase serum (ideally, by demonstrating a fourfold change in neutralizing antibody titer between the acute and convalescent phases), or both. We have determined empirically that the cross-reactivity of the WNV-reactive IgM appears to be less than that of Saint Louis encephalitis virus-reactive human IgM; therefore, conducting concurrent tests with the other indigenous neurotropic flaviviruses, which now coexist with WNV in some parts of the United States (21), is also important.

The observed apparent-to-inapparent WNV infection ratio in the United States is approximately 1:140, which indicates that a large group of persons with subclinical WNV infections exist (22). While the serologic results presented here are for patients with neuroinvasive WNV disease and may not be generalizable to patients with clinically mild or inapparent WNV infection, the possibility exists that many mild (and probably

Table 2. Percentage of persons with IgM-positive serology by months after onset

	No. WNV IgM positive/total (%)				
	Acute (n=27)	3 months (n=15)	6 months (n=23)	9 months (n=23)	12 months (n=18)
Age					
<65 yrs	9/9 (100)	6/6 (100)	6/9 (67)	4/8 (50)	0/5 (0)
+65 yrs	18/18 (100)	9/9 (100)	9/14 (64)	5/15 (33)	2/13 (15)
Sex					
Male	13/13 (100)	8/8 (100)	8/11 (73)	3/10 (30)	1/9 (11)
Female	14/14 (100)	7/7 (100)	7/12 (58)	6/13 (46)	1/9 (11)
Clinical syndrome					
Encephalitis	16/16 (100)	8/8 (100)	8/13 (62)	5/13 (39)	2/11 (18)
Meningitis	11/11 (100)	7/7 (100)	7/10 (70)	4/10 (40)	0/7 (0)
Baseline IgM[b]					
<Median P/N	14/14 (100)	6/6 (100)	6/11 (55)	3/12 (25)	0/10 (0)
≥median P/N	13/13 (100)	9/9 (100)	9/12 (75)	6/11 (55)	2/8 (25)

[a]WNV, West Nile virus; Ig, immunoglobulin; P/N, positive-to-negative; P/N ratio ≥ 3.0.
[b]Median P/N = 11.4.

undiagnosed) infections occur and further emphasizes the need for careful laboratory assessment before a diagnosis of acute WNV infection.

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Isolation of *Escherichia coli* O157:H7 from Intact Colon Fecal Samples of Swine[1]

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Escherichia coli O157:H7 was recovered from colon fecal samples of pigs. Polymerase chain reaction confirmed two genotypes: isolates harboring the *eaeA*, stx_1, and stx_2 genes and isolates harboring the *eaeA*, stx_1, and hly_{933} genes. We demonstrate that swine in the United States can harbor potentially pathogenic *E. coli* O157:H7.

During the past two decades, disease caused by *Escherichia coli* O157:H7 has been increasing (1). Currently, the Centers for Disease Control and Prevention estimates that *E. coli* O157:H7 causes an average of 500 outbreaks that affect >73,000 persons and result in >61 deaths each year in the United States (2). The epidemiology of *E. coli* O157:H7 has become an important research topic as manure harboring *E. coli* O157:H7 is dispersed, and soil, food, and water are cross-contaminated with feces containing *E. coli* O157:H7 (1,3). Although cattle feces are the most important source of *E. coli* O157:H7, the need to evaluate the presence of *E. coli* O157:H7 in the feces of other animal species has been recognized (1). The presence of *E. coli* O157:H7 in swine feces has been reported in Japan (4), Norway (5), and Chile (6); however, to date, *E. coli* O157:H7 has not been reported in swine in the United States.

The Study

Colon samples were collected at a cooperating swine slaughter facility from 305 swine carcasses during evisceration. Two to three inches of distal colon that contained feces at the first point proximal to the rectum was resected and maintained on ice for approximately 2 hours before processing (Figure). Ten grams of feces from each colon was transferred to filter-lined sterile plastic bags. One hundred milliliters of brilliant green bile broth (Difco Laboratories, Detroit, MI), prewarmed to 37°C, was added to each filter stomacher bag containing feces and incubated at 37°C for 6 h with shaking (150 rpm) (7). After enrichment, 1.0-mL aliquots were processed by using Dynabeads anti–*E. coli* O157 (Dynal Biotech, Oslo, Norway), according to manufacturer's instructions with modification. Bead/sample suspensions were incubated at room temperature for 30 min with continuous mixing on a Bellco roller drum (Bellco Glass, Inc., Vineland, NJ) before plating onto sorbitol MacConkey (SMAC; Difco Laboratories), cefixime/tellurite (CT; cefixime-tellurite supplement, Dynal Biotech)-SMAC agars, and rainbow agar O157 (Biolog, Inc., Hayward, CA). Black colonies from rainbow agar O157 and sorbitol-negative colonies from CT-SMAC and SMAC agars were tested for the absence of β-glucuronidase and the ability to ferment lactose by using *E. coli* broth containing 4-methylumbelliferyl-β-D-glucuronide (MUG) (EC medium with MUG; Difco Laboratories) and MacConkey broth (Difco Laboratories), respectively. Lactose-positive/MUG-negative isolates were serotyped by using the RIM *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS). Up to 10 *E. coli* O157 latex agglutination–positive isolates per colon fecal sample were tested for the presence of the rfb_{O157} gene by using polymerase chain reaction (PCR) (8). Isolates positive for the rfb_{O157} gene were further characterized for the presence of genes encoding the H7 flagellar protein ($fliC_{H7}$), Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), intimin protein (*eaeA*), and hemolysin (hly_{933}) (9). We conducted further analysis using antimicrobial resistance patterns, pulsed-field gel electrophoresis (PFGE), and ribotyping on all *E. coli* O157 PCR–positive isolates containing $fliC_{H7}$, stx_1, stx_2,

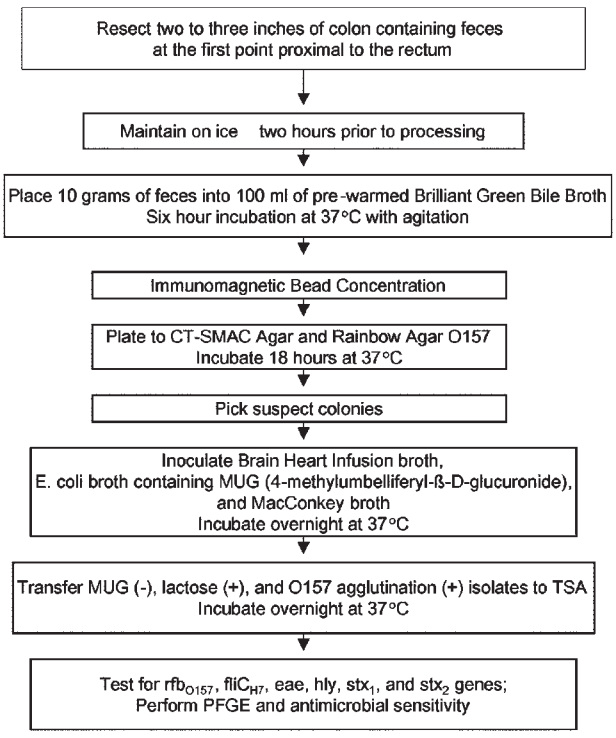

Figure. Procedure for isolating *Escherichia coli* O157 from swine colon fecal samples.

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eae_A, or hly_{933}. However, for tabulation purposes, each sample ultimately contributed one isolate. When $fliC_{H7}$, stx_1, stx_2, *eaeA*, or hly_{933} was not detected in PCR-confirmed *E. coli* O157 isolates, further analysis was performed on only one *E. coli* O157 isolate per colon sample.

E. coli O157 isolates were tested for susceptibility to 17 antimicrobial agents (amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole) as described (10) by using a custom-made semiautomated broth microdilution assay (Sensititre, Trek Diagnostics, Westlake, OH). Imipenem was used at concentrations of 0.25–8.0 µg with the following breakpoints: sensitive (<4) and resistant (16).

For PFGE, DNA was digested with 50 U *Xba*I (Invitrogen Corp., Carlsbad, CA) for 4 h at 37°C. PFGE was performed by using a CHEF Mapper XA system (Bio-Rad, Hercules, CA) at 14°C with pulses ramping from 2.16 s to 63.8 s over 18 h. PFGE patterns were evaluated visually, and isolates were assigned to the same pulsotype when exhibiting a difference of <3 bands from the index isolate. Ribotyping of the *E. coli* O157 isolates was done by using a RiboPrinter (Qualicon, Inc., Wilmington, DE) as described in the user's manual. Restriction digests were performed on *E. coli* O157 isolates by using the *Eco*RI enzyme (Qualicon, Inc.).

A total of 305 colon samples were randomly collected on 8 different days over a 6-month period as follows: collection day 1 (February 16, 2001), n=5; collection day 2 (March 8, 2001), n=20; collection day 3 (March 22, 2001), n=40; collection day 4 (April 20, 2001), n=40; collection day 5 (May 4, 2001), n=50; collection day 6 (May 16, 2001), n=50; collection day 7 (June 20, 2001), n=50; and collection day 8 (July 10, 2001), n=50 (Table). Eighteen (5.9%) of the 305 colon samples had isolates positive for rfb_{O157}. Isolates from 6 of these 18 colon samples also contained $fliC_{H7}$. Two gene combinations based on the presence or absence of stx_1, stx_2, *eae*, and hly_{933} were detected in these *E. coli* O157:H7 PCR-confirmed isolates. The stx_1, *eaeA*, and hly_{933} virulence pattern was detected in two isolates (isolates 1 and 2) from two of the five colon samples collected on February 16, 2001, and the stx_1, stx_2, and *eaeA* virulence pattern was detected in 22 isolates (isolates 6–27) from 4 of the 50 colon samples collected on May 4, 2001. None of the *E. coli* O157:H7 isolates recovered contained all four of the virulence genes (stx_1, stx_2, *eaeA*, and hly_{933}). None of the *E. coli* O157:non-H7 isolates (isolates 3–5, 28–36) in the present study contained stx_1, stx_2, *eaeA*, or *hly* genes. Non–Shiga toxin–producing *E. coli* O157:non-H7 isolates have been previously isolated from the feces of pigs (11,12). For slaughterhouse visits on March 8, March 22, June 20, and July 10, 2001, *E. coli* O157 and *E. coli* O157:H7 were not recovered from any of the colons sampled.

All *E. coli* O157:H7 isolates recovered in this study were sensitive to the antimicrobial agents tested, with the exception of one isolate (isolate 15) that was resistant to streptomycin. This isolate was recovered from a colon from which a pan-sen-

Table. Characterization of *Escherichia coli* O157:H7 and non-H7 isolates recovered from 305 swine fecal colon samples[a,b]

Collection date	Swine *E. coli* O157 isolate no.	*E. coli* O157 positive colon samples/total colon samples collected	Colon ref. no.	No. of isolates recovered from sample	PCR characteristics	*E. coli* O157 ribotyping	PFGE type	Antimicrobial resistance pattern[b]
Feb. 16, 2001	1	2/5	1	1	rfb_{O157}, $fliC_{H7}$, stx_1, *eae*, hly_{933}	H7	1	Pan-sensitive
	2		2	1	rfb_{O157}, $fliC_{H7}$, stx_1, *eae*, hly_{933}	H7	1	Pan-sensitive
Mar. 8, 2001	No isolates	0/20						
Mar. 22, 2001	No isolates	0/40						
Apr. 20, 2001	3	3/40	3	1	rfb_{O157}	Non-H7	3	kan, strept, sulfa, tet
	4		4	1	rfb_{O157}	Non-H7	3	kan, strept, sulfa, tet
	5		5	1	rfb_{O157}	Non-H7	3	kan, sulfa, tet
May 4, 2001	6–11	4/50	6	6	rfb_{O157}, $fliC_{H7}$, stx_1, stx_2, *eae*	H7	2	Pan-sensitive
	12–16		7	5	rfb_{O157}, $fliC_{H7}$, stx_1, stx_2, *eae*	H7	2	Pan-sensitive, except isolate no. 15 resistant to strept
	17–25		8	9	rfb_{O157}, $fliC_{H7}$, stx_1, stx_2, *eae*	H7	2	Pan-sensitive
	26,27		9	2	rfb_{O157}, $fliC_{H7}$, stx_1, stx_2, *eae*	H7	2	Pan-sensitive
May 16, 2001	28	9/50	10	1	rfb_{O157}	Non-H7	4	Pan-sensitive
	29		11	1	rfb_{O157}	Non- H7	4	tet
	30		12	1	rfb_{O157}	Non-H7	4	tet
	31		13	1	rfb_{O157}	Non-H7	4	strept, tet
	32		14	1	rfb_{O157}	Non- H7	4	tet
	33		15	1	rfb_{O157}	Non-H7	4	tet
	34		16	1	rfb_{O157}	Non- H7	4	tet
	35		17	1	rfb_{O157}	Non- H7	4	tet
	36		18	1	rfb_{O157}	Non-H7	4	tet
June 20, 2001	No isolates	0/50						
July 10, 2001	No isolates	0/50						
	Total=36	18/305	18					

[a] Each isolate listed in the following table represents an isolate from an individual colon sample.

[b] Feb., February; Mar., March; Apr., April; ref., reference; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; kan, kanamycin; strept, streptomycin; sulfa, sulfamethoxazole; tet, tetracycline.

sitive *E. coli* O157:H7 was also recovered. The antimicrobial sensitivity pattern of the *E. coli* O157:non-H7 isolates was more varied than that of the *E. coli* O157:H7 isolates with five different susceptibility patterns noted. Only one of the *E. coli* O157:non-H7 isolates was pan-sensitive. These data are similar to previous reports in which antimicrobial resistance among *E. coli* O157 non–Shiga toxin–producing isolates was higher than that of Shiga toxin–producing *E. coli* O157 isolates (11).

As previously shown, ribotyping did not discriminate among isolates within the *E. coli* O157:H7 serotype (13). Additionally, the *E. coli* O157:non-H7 isolates were indistinguishable from one another. Four PFGE profiles were noted. The *E. coli* O157:H7 isolates obtained from colon 1 and colon 2 on February 16, 2001, exhibited the PFGE type 1 pattern, whereas the *E. coli* O157:H7 isolates obtained from four colons on May 4, 2001 exhibited the PFGE type 2 pattern. The *E. coli* O157:non-H7 isolates obtained on April 20, 2001, and May 16, 2001, exhibited PFGE patterns 3 and 4, respectively.

Conclusions

Results from this study demonstrate that pigs in the United States can harbor *E. coli* O157:H7. The recovery rate of *E. coli* O157:H7 from colon fecal samples of pigs reported in this study was 2.0% (6/305). Previous attempts to isolate *E. coli* O157:H7 from swine feces in the United States have been unsuccessful (12,14). Use of more appropriate methods for sampling, processing, and culturing swine feces may have accounted for the ability to recover and isolate *E. coli* O157:H7 from swine feces in our study. For example, samples were obtained from the colon, transported on ice, and processed within 2 h of collection. The absence of antibiotics in our enrichment step may have also facilitated the recovery of *E. coli* O157:H7 from swine feces. Furthermore, although direct comparisons cannot be made between cattle studies, the recovery rate of Shiga toxin–producing *E. coli* O157 from cattle feces has improved over the past 10 years. This is most likely due to more conducive sampling procedures, culture practices, and detection methods than an increase in true carriers. The detection of *E. coli* O157 in swine feces has previously been based on the isolation techniques used for the recovery of *E. coli* O157 from cattle feces. The difficulty in detecting *E. coli* O157 from swine feces may in part be attributable to differences in the physiologic environment between swine and cattle feces. More appropriate isolation techniques may still be discovered for detecting *E. coli* O157 in swine.

Although our recovery rates of *E. coli* O157:H7 from swine are similar to recovery rates in Japan (4), we recovered a genotype in addition to the stx_1, stx_1, and *eaeA* genotype: the stx_1, *eaeA*, and hly_{933} genotype. In Norway, the recovery rate (0.1%) of *E. coli* O157:H7 from pig feces was much lower (5). Isolates recovered from Norway possessed the stx_2 and *eaeA* genes only; however, the presence of the hly_{933} gene was not determined (5).

The ability to produce one or more Shiga toxins is an important virulence characteristic of *E. coli* O157:H7 (1). However, production of Shiga toxins alone may not be sufficient for *E. coli* O157:H7 to be pathogenic (1). Other virulence factors such as the intimin protein (involved in the attachment of the *E. coli* O157 to enterocytes), the presence of a plasmid-encoded hemolysin, or both, are important in the pathophysiology of hemorrhagic disease (1). *E. coli* O157:H7 isolates recovered in this study possessed either two virulence factors, *eaeA* and hly_{933}, in addition to stx_1 or one virulence factor, eae*A*, in addition to stx_1 and stx_2. These isolates can potentially cause disease and should be considered pathogenic to humans. Since human *E. coli* O157:H7 clinical isolates contain the stx_1, stx_2, *eaeA*, and hly_{933} genes, the human pathogenicity of *E. coli* O157:H7 isolates from pigs that lack the *hly* gene requires further study.

The clonal nature of the isolates that were isolated on a particular day suggests transmission of *E. coli* O157 between pigs. Unfortunately, we did not have access to information concerning the source of the pigs from which the samples were collected, the number of pigs slaughtered from a given farm, or the holding facilities and grouping of the pigs before slaughter. Therefore, we do not know whether *E. coli* O157 transmission between pigs occurred on the farm, in transit, or while the pigs were in a holding pen at the slaughterhouse.

This study did not permit inferences of *E. coli* O157:H7 isolation rates with respect to the season, nor can inferences of *E. coli* O157:H7 isolation rates be made with respect to swine or herd prevalence. The relatively low recovery rate of *E. coli* O157:H7 from swine feces compared to cattle feces warrants further study to determine the significance and prevalence of *E. coli* O157:H7 in swine and if different enrichment and isolation methods would have an impact on the recovery of *E. coli* O157:H7 from swine feces. In addition, future studies should be conducted to determine the occurrence of *E. coli* O157 on swine hides, in swine mouths, and in swine stomachs.

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Dr. Feder is a research microbiologist with the U.S. Department of Agriculture. Her research interests include diagnostic microbiology, molecular epidemiology, and assay development of zoonotic pathogens, primarily *Escherichia coli* O157:H7 and *Salmonella*.

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Echinococcus multilocularis: An Emerging Pathogen in Hungary and Central Eastern Europe?

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Echinococcus multilocularis, the causative agent of human alveolar echinococcosis, is reported for the first time in Red Foxes (*Vulpes vulpes*) in Hungary. This parasite may be spreading eastward because the population of foxes has increased as a consequence of human interventions, and this spread may result in the emergence of alveolar echinococcosis in Central Eastern Europe.

Echinococcus multilocularis is the causative agent of alveolar echinococcosis in humans. The life cycle of this tapeworm is indirect and sylvatic; eggs shed by the definitive host, mainly the Red Fox (*Vulpes vulpes*) in Europe, develop to the metacestode stage in arvicolid rodents, which serve as intermediate hosts. In accidental cases, humans as aberrant intermediate hosts may also acquire *E. multilocularis* infection by egg ingestion. Although a rare disease in humans, alveolar echinococcosis is of considerable public health importance because it can be lethal in up to 100% of untreated patients (1). Treatment is still difficult, and therapy may cost $300,000 per patient (1).

The parasite has an extensive geographic distribution in the Northern Hemisphere, including parts of North America (Alaska, Canada, and some of the lower contiguous states of the United States), Asia (some of the newly independent states of the former Soviet Union, China, and Japan), and some European countries. Until the end of the 1980s, parasite-endemic areas in Europe were known to exist only in France, Switzerland, Germany, and Austria (2). In the 1990s and early 2000s, the infection rate of foxes increased drastically in some areas of France and Germany; several new endemic foci were detected in Switzerland, Germany, and Austria; and the parasite was reported from the surrounding countries, including The Netherlands, Belgium, Luxembourg, Poland, the Czech Republic, the Slovak Republic, and Italy (1,3,4). Here we report *E. multilocularis* infection from Red Foxes in the northern areas of Hungary and give a possible explanation for the spreading of the parasite from the west to the east.

Carcasses of Red Foxes sent to the Central Veterinary Institute, Budapest, from January to July 2002, in connection with the rabies immunization and control program, were included in this study. Carcasses were transported and stored in individual plastic bags at 4°C. The approximate delay between death and necropsy was 2 days. We examined the intestinal tracts by the sedimentation and counting technique as described (5). The whole sediment was examined in petri dishes under stereomicroscope at a magnification of X66, and worms were ascertained, counted, and subsequently washed and stored in 70% ethanol until DNA purification. Of 100 foxes (18 subadults and 82 adults) examined during the screening of the parasitologic status of the foxes in 15 counties in Hungary, 5 adults shot in April and May 2002 were found to be infected with 2, 3, 5, 6, and 254 mature worms of *Echinococcus*, respectively (Figure 1).

All five foxes were shot in two northern Hungarian counties, Nógrád and Borsod-Abaúj-Zemplén, in the Nógrád Basin (Drégelypalánk, 48°02' North, 19°04' East, and Pusztaberki, 47°58' North, 19°11' East), in the Cserhát Mountains (Salgótarján, 48°03' North, 19°47' East and 48°01' North, 19°45' East) and in the Borsod Basin (Kelemér, 48°19' North, 20°27' East), near the Hungarian-Slovak border. The five places are in the Northern Mountain Range and at a distance of 60–120 km from the nearest known *E. multilocularis*–endemic region, the Muránska Planina Mountains (48°44' North, 20°02' East) in Slovakia (6). These territories are 200–400 m above sea level and are mainly forested, nonagricultural, or extensive agricultural areas. Based on the most important morphometric parameters of *Echinococcus* adult stages (length of the worm: 1.3–2.5 mm; number of proglottids: 3–5; length of terminal proglottids: 0.5–1.1 mm; terminal proglottids in percentage of total worm length: 26–44; position of genital pore: anterior to middle; form of uterus: sacklike without lateral sacculations), the parasites were identified as *E. multilocularis* (7). Although the overall prevalence of *E. multilocularis* seems to be low in Hungary, in the two *E. multilocularis*–endemic counties, the

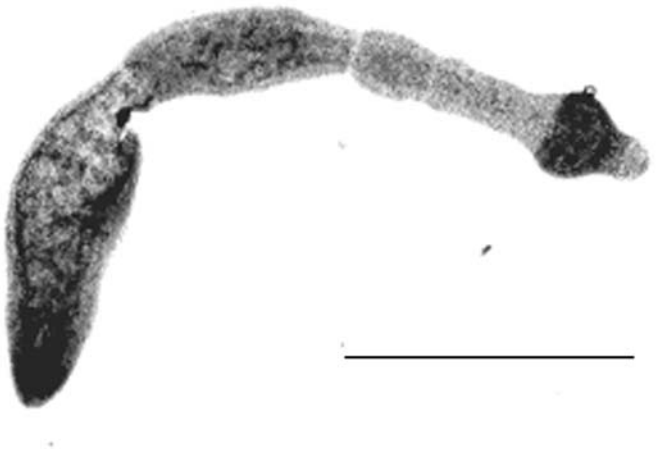

Figure 1. *Echinococcus multilocularis* isolated from a fox in Hungary. Scale bar: 0.5 mm.

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prevalence was considerably higher (5 of 17 foxes were infected). These prevalence data are similar to those observed in the surrounding countries, Austria and The Slovak Republic (8,9).

The taxonomic status of the isolates was also confirmed by a diagnostic polymerase chain reaction (PCR) assay. The five isolates were treated separately. Two different genes coding for U1 snRNA and the mitochondrial 12S rRNA genes have been used in diagnostic PCR for detecting *E. multilocularis* DNA (10–12); however, the species specificity of the PCR amplifying a fragment of the U1 snRNA gene could not be confirmed in a study (12). Thus, the nested PCR method described by Dinkel et al. (11) was used in our study. To exclude the possibility of contamination with specific DNA, a negative control was included and underwent the entire procedure starting with DNA extraction. DNA was purified as described (13). PCR reactions were performed by using a GeneAmp 2400 PCR system (Perkin Elmer, Foster City, CA). The conditions used for PCR were identical to those described (12). PCR products were detected on ethidium bromide–stained 1.5% agarose gels by visualizing them with UV light. PCR products of the expected size (373 bp and 250 bp) were amplified in all cases (Figure 2), confirming the results of the morphologic comparisons, i.e., *E. multilocularis* was responsible for all infections.

According to some authors (1,2), researchers cannot confirm whether *E. multilocularis* is spreading from historically known *E. multilocularis*–endemic foci (eastern France, southern Germany, northern Switzerland, and western Austria) to new regions, or whether the Central European *E. multilocularis*–endemic area is connected with the *E. multilocularis*–endemic area in Asia, and the tiny worms previously escaped the attention of parasitologists. Our findings may suggest that the parasite's range has recently expanded, rather than the first identification of formerly unknown *E. multilocularis*–endemic areas. The parasite was not identified previously in either Red Foxes or wild rodents in Hungary, despite the extensive studies conducted by Murai, Mészáros, Gubányi, and other parasitologists of the Natural History Museum, Budapest. Moreover, human cases have never been reported in Hungary. The photograph and the description of macroscopic lesions (two fist-sized, undulating cysts) in the only presumed report of alveolar echinococcosis written by two surgeons (14) clearly indicate that the case was indeed cystic echinococcosis.

The appearance of *E. multilocularis* in Hungary might be explained by changes in the size of the Red Fox population in Central and Central Eastern Europe. From the 1970s, a continuous increase in the size of the Red Fox population was observed in Switzerland and Germany, probably as a consequence of the initiation of the antirabies vaccination programs (2). The larger population led to a continuous migration of young foxes from territories with high population density toward those with lower density, i.e., partly eastward. This migration might have resulted in the appearance of foxes infected with *E. multilocularis* and the establishment of small disease-endemic foci in Poland and the Czech Republic. After

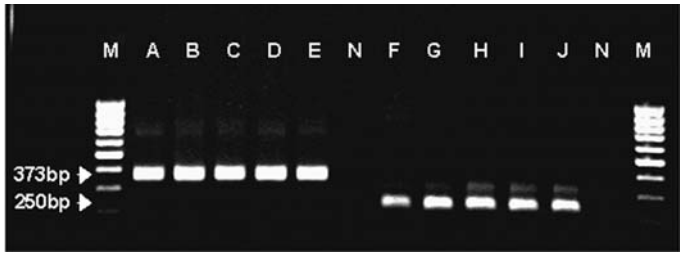

Figure 2. Nested polymerase chain reaction amplification of mitochondrial 12S rRNA gene from five Hungarian *Echinococcus multilocularis* isolates. Lanes A-E: amplification with outer primers; lanes F-J: amplification with inner primers; N, appropriate negative controls; M, molecular weight marker (100 bp).

the political changes of 1990, considerable changes in land use were observed in the former communist countries because of the disintegration of large state farms. The probable consequences of these changes, the decrease of annual hunting index resulting from a decrease in the price of fox fur, and the initiation of antirabies vaccination of foxes in Central Eastern European countries (Poland, the Czech Republic, the Slovak Republic, and Hungary), caused a corresponding increase in the fox population size (8,15), and probably the coincidental increase of *E. multilocularis* population and prevalence, and the expansion of *E. multilocularis*–endemic regions. A similar positive correlation between the population size of foxes and the prevalence of the parasite was also observed in Switzerland and Germany (2).

In the historically known *E. multilocularis*–endemic region, almost 400 patients are currently under continuous therapy, and the annual incidence of human alveolar echinococcosis has not varied markedly in the past few decades (1,2). In contrast with the stable epidemiologic situation in that region, the first 16 sufficiently documented and undoubtedly confirmed autochthonous human infections have been reported in Central Eastern European countries only from the late 1990s (2,9). Based on Central European annual incidence data (approximately 0.1–0.3/100,000 population) (2) and the similar overall prevalence of infection in foxes in Central and Central Eastern European countries (2,9), hundreds of cases would have been expected in the past few decades. The tiny worms may have escaped the attention of Central Eastern European parasitologists earlier. However, failing to recognize the characteristic and extensive lesions in humans in the past is unlikely.

Data from The Netherlands, Italy, Hokkaido Island and the surrounding islands of Japan, and North America provide clear evidence for the spreading and emergence of *E. multilocularis* infection (2,4,12). In the past, *E. multilocularis* has spread from the tundra zone of Northern Canada to the central regions of the continental United States (7) and from a small focus to the entire Hokkaido Island (2). Based on the above data, a similar spreading and emergence are likely being observed in Central Eastern European countries. As a result of their increasing population, foxes are inhabiting urban areas in several European countries, including Hungary (1,15). The appearance of foxes in a synanthropic environment may result in the infection of

domesticated dogs and cats and may increase the risk for human infections in *E. multilocularis*–endemic areas. Thus, knowing that *E. multilocularis* is likely to continue to spread, one can predict that human alveolar echinococcosis will become an emerging infectious disease in Central Eastern European countries in a few years as has already occurred in some other European countries, Hokkaido Island of Japan, Canada, and the United States (2,4,12,16).

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Early and Definitive Diagnosis of Toxic Shock Syndrome by Detection of Marked Expansion of T-Cell-Receptor Vβ2-Positive T Cells

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We describe two cases of early toxic shock syndrome, caused by the superantigen produced from methicillin-resistant *Staphylococcus aureus* and diagnosed on the basis of an expansion of T-cell-receptor Vβ2-positive T cells. One case-patient showed atypical symptoms. Our results indicate that diagnostic systems incorporating laboratory techniques are essential for rapid, definitive diagnosis of toxic shock syndrome.

Toxic shock syndrome (TSS) is a severe illness caused by methicillin-resistant *Staphylococcus aureus* (MRSA) infection, usually during menstruation but also in the postpartum period. *S. aureus* produces several superantigenic exotoxins, including TSS toxin-1 (TSST-1), which activate a vast number of T cells in a T-cell-receptor Vβ-selective manner (1,2). Cytokines produced by T cells and activated by TSST-1 are thought to cause the abnormal changes of TSS (1,2). Polymerase chain reaction (PCR) analysis of peripheral blood T cells from adults with TSS has shown a protracted expansion of TSST-1–reactive Vβ2-positive T cells persisting for 4–5 weeks (3). TSS in neonates, referred to as neonatal TSS-like exanthematous disease, has been shown by flow cytometric analysis to involve an expansion of T-cell–receptor Vβ2-positive T cells (4,5).

Because many cases do not satisfy the strict diagnostic criteria for TSS proposed by the Centers for Disease Control and Prevention (6), revised clinical diagnostic criteria for TSS, including probable cases, have been proposed (Table) (7). In Japan, several clinicians have described a TSS-like clinical entity that could not be diagnosed as TSS even according to the revised criteria.

We report two cases of TSS with puerperal infection that could be diagnosed at the early stage of the clinical course by detecting a marked expansion of T-cell–receptor Vβ2-positive T cells, as measured by flow cytometric analysis. The symptoms of one patient were too complex to permit diagnosis according to the clinical criteria without evaluation of the TSST-1-reactive T cells. We discuss the role of T-cell analysis in peripheral blood mononuclear cells in the diagnosis of TSS.

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Case Reports

Case 1

A 29-year-old Japanese woman underwent a cesarean section at a private clinic after premature membrane rupture. On postpartum day 3, shock with hypotension (67/37 mmHg) developed. No rash occurred during this period. She was transferred to the Maternal and Perinatal Center, Tokyo Women's Medical University Hospital.

On admission, the patient was awake and alert, but her face was pale. Her body temperature was 37°C, blood pressure was 104/80 mmHg, heart rate was 140 bpm, and respiratory rate was 28 times/min. A pelvic examination showed a brownish discharge from the cervix. The uterus was approximately 10 x 10 cm in diameter, with no tenderness. Her systolic blood pressure subsequently decreased to 80 mmHg, respiratory rate increased to 44 times/min, and body temperature rose to 39°C. Mild hypoxemia (pO_2 = 65 mmHg while breathing room air) became apparent, and the cardiothoracic rate shown on a chest x-ray film had increased to 54%. To treat shock, dopamine and fresh frozen plasma were administered with antithrombin III and antibiotic therapy (initially, pentocillin 2 g/day + panipenem/betamipron 1 g/day + amikacin 100 mg/day, and subsequently, panipenem/betamipron 1 g/day + vancomycin 1 g/day). The results of laboratory tests led to a suspected diagnosis of septic shock with disseminated intravascular coagulopathy (Table). Therefore, ulinastatin and gabexate mesilate, which possess both antifibrinolytic and anticoagulative effects, were administered (8,9). Because the patient's general condition did not improve, she was admitted to the intensive care unit.

On day 3 after admission to the intensive care unit, an abscess was observed around the surgical wound. Bacteriologic tests showed that the vaginal discharge was positive for MRSA, and a preliminary diagnosis of TSS was made. Peripheral blood mononuclear cells were stained with antibodies to CD3, CD4, CD8, and T-cell-receptor–Vβ2 elements and examined for the percentage of Vβ2-positive T cells by a flow cytometer as described (4,5). Five hours after staining, a marked expansion of Vβ2-positive–T cells, unrelated to the CD4:CD8 ratio, was confirmed (Figure), indicating the definitive diagnosis of TSS in the early clinical course. Rash and desquamation, important clinical symptoms of TSS, did not develop. On day 7 after admission, the patient was discharged from the intensive care unit and entered the general ward. On day 17, intrapelvic abscess was incised and drained. Subsequently, her general condition improved. The percentage of Vβ2-positive–T cells decreased gradually over the course of 5 weeks (Figure). She has no long-term sequelae. MRSA isolated from this patient was later confirmed to be positive for TSST-1.

Table. Laboratory data on admission of case-patients

Data	Case-patient 1 day 2 postpartum	Case-patient 2 day 7 postpartum
Laboratory findings (normal range)		
Leukocytes (μL) (5,000–8,500)	2,800	17,500
Platelets (x104/μL) (13–40)	12.2	19.8
C-reactive protein (mg/dL) (0–0.4)	46.4	22
Total protein (g/dL) (6.5–8.2)	3.8	5.9
Albumin (g/dL (3.8–5.1)	1.6	3
Aspartate aminotransferase (IU/L) (0–35)	30	51
Alanine aminotransferase (IU/L) (0–35)	10	53
Lactic dehydrogenase (IU/L) (200–450)	712	698
Blood urea nitrogen (mg/dL) (5–12)	29.5	12.4
Creatinine (mg/dL) (<0.8)	2.58	0.72
Uric acid (mg/dL) (1.2–4.5)	9.2	2.5
Sodium (mEq/L) (136–145)	135	133
Potassium (mEq/L) (3.5–5)	4.6	3.3
Chloride (mEq/L) (98–108)	104	99
Creatine kinase (IU/L) (10–70)	244	
Prothrombin time (sec) (12–14)	11.7	13.7
Activated partial thrombosplastin time (sec) (24–36)	36.7	43.2
Fibrinogen (mg/dL) (400–650)	674	668
Antithrombin-III (%) (70–120)	60	82
Fibrinogen degeneration product (μg/mL) (<10)	8.8	11.7
D-dimer (μg/mL) (<0.2)	5.22	3.93
Thrombin/antithrombin complex (ng/mL) (<3.0)	40	20.4
Criteria for definite TSS (all criteria must be present)	No	Yes
≥38.9°C	Yes	Yes
Rash with desquamation		Yes
Hypotension <90 mmHg	Yes	Yes
Clinical or laboratory abnormalities (≥3 organs)		
Gastrointestinal		
Hepatic	Yes	Yes
Muscular		
Mucous membrane		Yes
Renal	Yes	Yes
Cardiovascular		
CNS		
Criteria for probable TSS	No	Yes
≥3 criteria and desquamation or ≥5 criteria without desquamation		
≥38.9°C	Yes	Yes
Rash		Yes
Hypotension	Yes	Yes
Myalgia		
Vomiting and/or diarrhea		
Mucous membrane inflammation		Yes
Clinical or laboratory abnormalities ≥2 organs		
Gastrointestinal		
Hepatic	Yes	Yes
Muscular		
Mucous membrane		Yes
Renal	Yes	Yes
Cardiovascular		
Central nervous system		

Case 2

A previously healthy 33-year-old Japanese woman had a fever (38.4°C) 1 week after an uncomplicated spontaneous vaginal delivery without episiotomy at a private clinic. She was transferred to Tokyo Women's Medical University Hospital. On admission, the patient was awake and alert. Her body temperature was 38.8°C, blood pressure was 80/52 mmHg, heart rate was 127 bpm, and respiratory rate was 28 times/min.

A diffuse erythematous rash was present on the chest. It spread to the face and extremities and resolved after 8 days. A pelvic examination disclosed a brownish discharge from the cervix. The uterus was approximately 6 cm x 8 cm in diameter, with no tenderness. The laboratory test results and clinical symptoms suggested a diagnosis of TSS (Table). To treat the hypotension, dopamine and fresh frozen plasma were administered. The patient also received antithrombin III, ulinastatin, and gabexate mesilate as well as antibiotics (initially, imipenem/cilastatin 2 g/day + amikacin 200 mg/day, and subsequently, vancomycin 2 g/day).

One day after admission, bacteriologic tests showed that the vaginal discharge and breast milk were positive for MRSA. On day 5 after admission, peripheral blood mononuclear cells were examined for expansion of Vβ2 T cells. A marked expansion of Vβ2 T cells was confirmed (Figure), indicating the definitive diagnosis of TSS. The Vβ2-positive T cells gradually diminished to normal levels. Desquamation of the extremities occurred on day 11 after admission. From day 3 after admission, the patient's general condition improved gradually, and she was discharged on day 14. She had no long-term sequelae. Isolates of MRSA isolated were later found to be TSST-1–positive.

Discussion

Puerperal infection is a major cause of maternal death. Postpartum nonmenstrual TSS has received attention as a potential cause of puerperal infection (10–12). Knowing the incidence of MRSA infections in medical institutions would be helpful. For example, Fujino et al. reported that 246 MRSA isolates were obtained from 74 inpatients in December 2000 in a Tokyo hospital with 27 wards and 925 beds (13). During the past 4 years, no TSS cases have occurred in our department of obstetrics and gynecology. The two patients in this report were transferred from a private clinic. Although the incidence of TSS is rare in our department, we are concerned that the incidence is not rare in small private clinics. Our two cases may provide important clues to the actual incidence of TSS in women with puerperal infection.

In both of our patients, the diagnosis of TSS was confirmed during acute illness on the basis of expansion of TSS-1-reactive Vβ2 T cells in peripheral blood mononuclear cells. Diagnosis solely on the basis of clinical symptoms was not possible in case 1 because of the absence of skin rash and desquamation, cardinal symptoms of TSS, and the presence of signs of severe multiple organ failure. In case 2, diagnosis of TSS was straightforward because of typical clinical symptoms. The

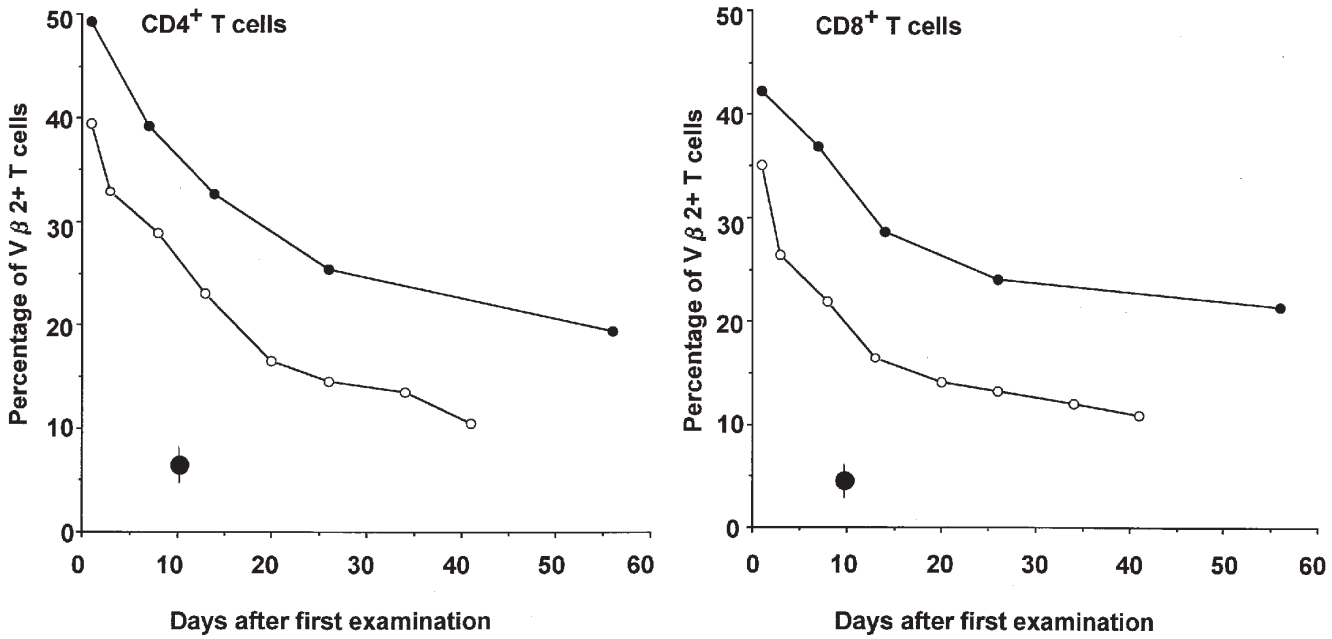

Figure. Results of T-cell–receptor Vβ2 positive T cells in two women with toxic shock syndrome. The percentages of Vβ2+ CD4+ cells (left panel) and Vβ2+ CD8+ T cells (right panel) in peripheral blood mononuclear cells were determined after admission. Patient 1 (open circles) and patient 2 (closed circles) refer to the same cases as in the text and Table ; ● represents mean±standard deviation in healthy adults (four men and three women).

severe multiple organ failure in case 1 may have suppressed the development of skin reactions. Our report strongly suggests that some TSS cases that cannot be correctly diagnosed because of a complicated clinical picture. Our results indicate that diagnostic systems incorporating laboratory techniques are essential for the rapid, definitive diagnosis of TSS.

Our experience suggests the necessity for better estimates of the incidence of postpartum staphylococcal infections, TSS associated with MRSA, and TSS that does not satisfy generally accepted diagnostic criteria. Several clinical trials in fields other than obstetrical infections with MRSA are now underway in Japan to address these issues.

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Removing Deer Mice from Buildings and the Risk for Human Exposure to Sin Nombre Virus

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Trapping and removing deer mice from ranch buildings resulted in an increased number of mice, including Sin Nombre virus antibody–positive mice, entering ranch buildings. Mouse removal without mouse proofing will not reduce and may even increase human exposure to Sin Nombre hantavirus.

Sin Nombre virus (SNV), carried by the deer mouse (*Peromyscus maniculatus*) is the etiologic agent of hantavirus pulmonary syndrome (1). Most cases of this syndrome occur after exposure to deer mice in peridomestic settings (2); the prevalence of antibody to SNV may be higher in peridomestic populations than in sylvan populations (3). In addition, some rodent species move readily between sylvan and peridomestic settings (3). Rodent removal combined with mouse proofing of human dwellings eliminates rodent-human contact in treated structures (4); however, removal without mouse proofing may not be effective (4) because removal may induce mouse immigration into the area (5). Additionally, if mice are trapped alive and released outdoors even at some distance, they will often return (6). Our study consisted of two experiments designed to determine the efficacy of reducing human exposure to rodents by removing deer mice from outbuildings that were not mouse proofed.

The Study

To determine how removal from outbuildings affects abundance of mice in structures, two removal experiments were conducted in Montana, where deer mice are commonly found in buildings. For both experiments, we followed the handling protocols as described (7), except that we did not anesthetize the mice. We collected data as described (8).

In experiment 1, we trapped live deer mice in 16 ranchyard outbuildings (peridomestic area), as well as nearby sylvan habitats, for 3 nights each week from mid-June to mid-August, 1999. The peridomestic area, about 1 ha in size, contained buildings and corrals. We trapped mice only in buildings in the peridomestic area. Four of the sites were designated "removal buildings," and all animals captured from these buildings were euthanized. Captured mice from the remaining 12 "control buildings" were marked and released. We set a total of 100 traps in buildings; the number of traps per building was determined by building size (16–40 m^2 with an average of one trap/4 m^2). During all trapping periods, the number of traps set was always more than the number of animals captured in every building. In the sylvan area (1.1 ha), we placed 100 traps in four parallel rows. Sets of two rows were placed on either side of the ranch yard; traps were located 20–100 m from the nearest building. We marked and released animals for 7 days during study week 1, then we removed them for weeks 2–8.

In experiment 2, we examined the effect of deer mouse removal on SNV-antibody prevalence in buildings. The site for this experiment was approximately 6 km from the site of experiment 1. On the experiment 2 site, we had conducted extensive work from November 1996 to April 1999; the site included three buildings as previously described (3). Two buildings were designated removal buildings and the third a control building. For 11 weeks in fall 1999 and 5 weeks in spring 2001, we collected blood from all removed and control animals (control animals at first capture only). In fall 1999, we trapped and removed mice daily from removal buildings during week 1, for 5 days during week 2, and for 3 days weekly during weeks 3–11. In spring 2001, animals were removed or marked and released (control building) for 3 days each week for 5 weeks.

In experiment 1, we captured a total of 133 deer mice in the sylvan (38 mice) and peridomestic (95 mice) areas (Table). We removed 52 deer mice from the four removal buildings. Immigrant mice quickly replaced resident deer mice removed from these buildings. This replacement resulted in a higher average number of deer mice captured in the four buildings from which we removed animals (13.8 individual mice/building; 95% confidence interval [CI] 7.6 to 20.0) than in buildings from which no mice were removed (5.8 mice/building; 95% CI 3.6 to 8.0). Of the deer mice previously captured in the sylvan area (20–100 m away), 7.9% immigrated into the removal buildings, and 16.8% moved from building to building (Table).

In experiment 2, a total of 54 deer mice were captured from all three buildings. Thirty deer mice were taken from the two removal buildings in 1999 and six in 2001. In the 1999 sample, more deer mice were captured in each of the two removal buildings than had been captured in the same buildings during either of the previous two fall seasons (3). The number of deer mice that occupied the control building was similar to the number reported for the previous two fall seasons. Although the spring 2001 sample was too small for statistical analysis, five deer mice were captured in one removal building and two in the control building. One deer mouse was captured from the other removal building (Figure). Notably, the two deer mice captured in the control building continued to occupy the build-

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Table. Deer mice removed from buildings and sources of immigrating deer mice on a ranch, southwestern Montana, 1999

Capture location					Source of post removal immigration	
Sylvan	Buildings	Total	Removed	Moved[a]	Sylvan to building	Building to building
38	95	133	52	19	7.9% (n=3)[b]	16.8% (n=6)[b]
					5.7%[c]	30.1%[c]

[a]No. of deer mice that moved from previous capture areas (source) to removal site.
[b]Percentage of marked source population.
[c]Percentage of total deer mice removed.

ings for all trapping periods. During the initial removal (fall 1999), none of 15 captured deer mice had detectable antibody to SNV. However, subsequent to removal, three immigrant mice were found to be antibody-positive when first captured, while deer mice occupying the control building remained antibody-negative (Figure). At various times during previous years of sampling (3) in these buildings, the control building for this study contained antibody-positive mice, as did the removal buildings. During the preceding two falls, no antibody-positive deer mice had been captured in the removal building in which antibody-positive mice were captured in the experiment.

Conclusions

Our data show that removing deer mice did not reduce their population numbers in any building. Outbuildings are normal habitats for deer mice in Montana, and all of the buildings in experiment 2 had resident deer mice for 3 years before this study (3). In sylvan habitats when resident deer mice are removed, immigrant mice quickly replace them (9) and often travel long distances to do so (10). Entire sylvan populations can be replaced in 2 weeks (5).

Under certain circumstances, removal could substantially reduce the number of mice. The number of dispersing deer mice is linearly related to the density of the source population, and the rate of dispersal is correlated with the rate of increase in the source population (11). Extremely large fluctuations have been documented in Montana deer mouse populations (8). These fluctuations affect dispersal rates and entrance into buildings.

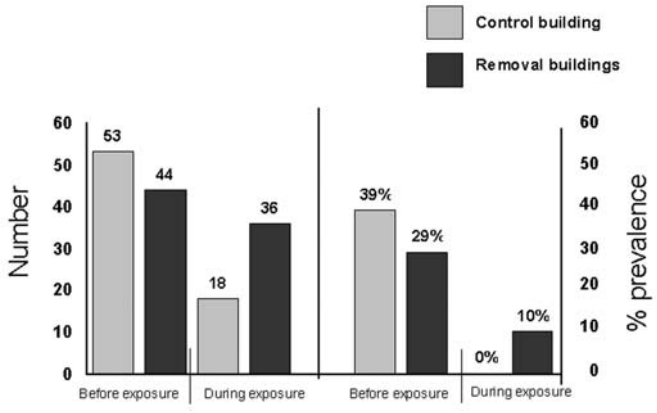

Figure. Number and Sin Nombre virus–antibody prevalence in deer mice found in removal and control buildings, Montana. Data combined from previous study (left side, [3]) and from experiments conducted in fall 1999 and spring 2001 (right side).

Outbuildings, though originally colonized from sylvan populations of deer mice, also act as sources (Table). The total peridomestic area occupied by buildings in experiment 1 (approximately 1 ha) is only slightly larger than the home ranges of some deer mice (10). Removing animals from 4 of the 16 buildings may have rearranged territories within a small area without creating the large vacant habitats reported in previous removal studies (9,5,11). Removal of deer mice from all buildings within the ranch yard might have resulted in migration from surrounding sylvan habitats larger than those identified in this study.

Removing animals from outbuildings also creates a constant turnover in a building's deer mouse population; thus, more deer mice would be captured in a building over time than if mice had not been removed. This constant turnover increases the probability that an antibody-positive mouse will enter the building. The entrance of antibody-positive mice into the removal building in experiment 2 is consistent with this concept. Removing animals from some but not all buildings initiates movement of mice from other buildings. Such removal rearranges local territories and may alter the proportion of SNV-infected deer mice, which in turn, may alter the probability of human exposure to SNV.

In summary, our study showed that removal of deer mice from non–rodent-proofed ranch buildings did not reduce rodent infestation of these buildings. An increase in the number of deer mice occurred in most buildings from which mice had been removed. In three instances, SNV antibody-negative mice in the buildings were replaced after their removal by antibody-positive mice. These results suggest that rural homeowners who trap deer mice in homes or outbuildings without first attempting to seal the structures against renewed infestation are not decreasing their risk of exposure to SNV. Detailed procedures for rodent proofing have been described (12), as well as procedures for safe trapping and handling of captured mice (7).

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The National Capitol Region's Emergency Department Syndromic Surveillance System: Do Chief Complaint and Discharge Diagnosis Yield Different Results?

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We compared syndromic categorization of chief complaint and discharge diagnosis for 3,919 emergency department visits to two hospitals in the U.S. National Capitol Region. Agreement between chief complaint and discharge diagnosis was good overall (kappa=0.639), but neurologic and sepsis syndromes had markedly lower agreement than other syndromes (kappa statistics 0.085 and 0.105, respectively).

Syndromic surveillance systems monitor disease trends by grouping cases into syndromes rather than specific diagnoses. U.S. state and local health departments are developing and implementing such systems in hopes of reducing the impact of bioterrorism attacks through earlier detection and action than is possible with traditional diagnosis-based surveillance. The rationale for this approach is that the organisms identified by the Centers for Disease Control and Prevention (CDC) as high priority potential bioterrorism agents cause diseases that are rare, often misdiagnosed initially (1,2), and can have overlapping clinical presentations (3). Syndromic surveillance systems may also have secondary benefits, including better disease monitoring after an attack and more rapid detection of naturally occurring outbreaks.

Deciding which data sources to use for syndrome assignment is an important consideration for health departments implementing syndromic surveillance. Several systems use emergency department (ED) chief complaint, discharge diagnosis, or both. We hypothesized that systematic differences between chief complaint and discharge diagnosis might affect syndrome assignment and that characterizing such differences would outline the potential strengths and weaknesses of using each type of data.

The National Capitol Region's ED syndromic surveillance system, a cooperative effort between Maryland, the District of Columbia, and Virginia, uses chief complaint for syndromic assignment, except for a few hospitals that only provide discharge diagnoses. Differences between these data types are

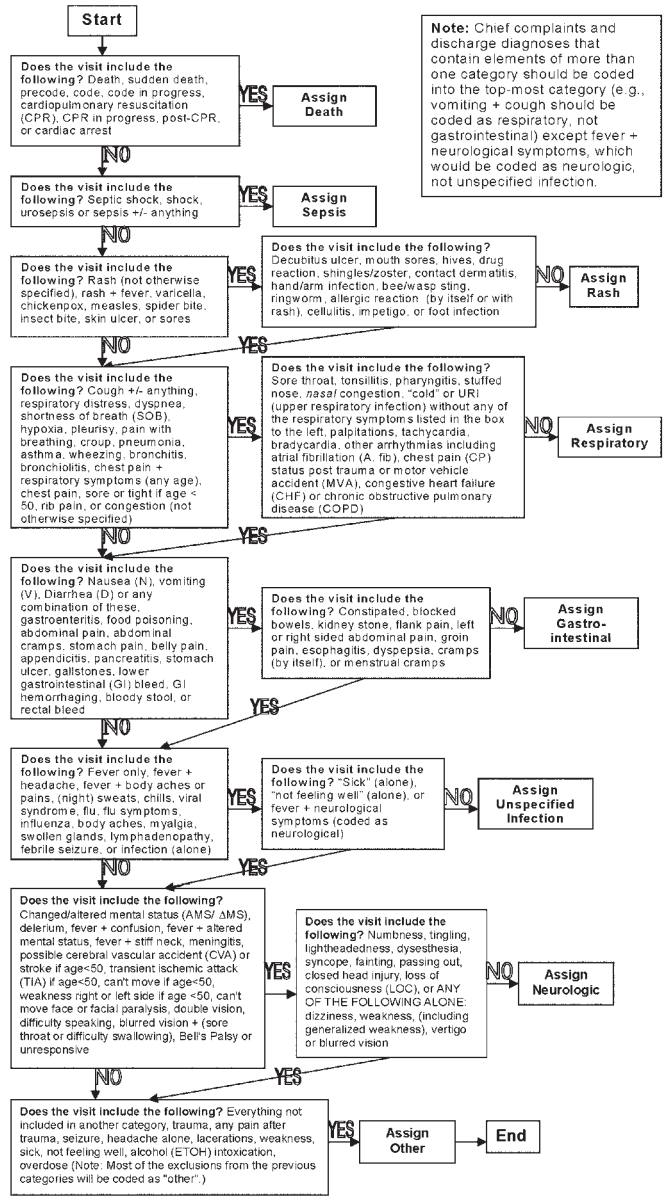

Figure. National Capitol Region's emergency department algorithm for syndrome assignment, United States. A larger version of this figure may be seen online at http://www.cdc.gov/ncidod/EID/vol9no3/02-0363-G.htm.

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functionally important for this system because not all participating hospitals report every day and the comparison statistic is based on each syndrome's proportion of all ED visits. Therefore, differences exist in the proportion of each data type contributing to the comparison statistic from day to day. This study was not intended to evaluate the utility of the specific syndrome categorization matrix used by the National Capitol Region system, which is still undergoing refinement.

The Study

The National Capitol Region's ED syndromic surveillance system has been operating continuously since September 11, 2001. Each day, the preceding day's ED logs from up to 25 hospitals are faxed to participating health departments. Depending on the hospital's routine, these logs provide chief complaint, discharge diagnosis, or both, for ED visits initiated the preceding day. Using a syndrome assignment matrix (Figure) modified from one developed by the Centers for Disease Control and Prevention (T.A. Treadwell, M.K. Glynn, and J. Duchin, unpub. data), all ED visits are coded into one of eight mutually exclusive syndromes: "death," "sepsis," "rash," "respiratory" illness, "gastrointestinal" illness, "unspecified infection," "neurologic" illness, and "other." The unspecified infection category was designed to capture infectious illnesses that would be categorized into other system-specific syndromes if additional information were available. The neurologic syndrome was intended to identify meningitis and botulism cases. The "other" category includes visits not consistent with any of the seven specified syndromes. Syndrome assignment is hierarchical, following the order listed above, from death to other, and is based on chief complaint or, if chief complaint is not available, discharge diagnosis.

For this study, we used ED logs from two participating hospitals (hospitals 1 and 2) that routinely provide both chief complaint and discharge diagnosis data. Approximately 24,000 and 60,000 ED visits, respectively, occur at these suburban general community hospitals annually. On these logs, both data types are free text because none of the participating EDs assign International Classification of Disease (ICD) codes to visits within 24 hours of ED discharge. In hospital 1, a certified nurse assistant transcribes chief complaints to the log, shortening the chief complaint to a few words from the several sentence version recorded by the triage nurse. The nurse assistant also transcribes the treating clinician's discharge diagnosis from the medical record to the ED log. At hospital 2, a certified nurse assistant takes and summarizes the patient's chief complaint in the log. Later, a clinician records the discharge diagnosis using a computerized pick list. These procedures are generally similar in other participating hospitals.

A single trained individual (E.M.B.) reviewed a total of twenty-eight 24-hour ED logs for 14 days in December 2001. Each visit was assigned a syndrome solely on the basis of chief complaint (i.e., the visit's discharge diagnosis was not viewed) and then rechecked. All logs were then reviewed again to assign each visit a syndrome by using only discharge diagnosis and then rechecked. In all, 4,040 visits were reviewed. One hundred twenty-one visits (3%) were excluded because of missing or illegible chief complaints (n=9), missing or illegible discharge diagnoses (n=100), or both (n=12).

For the 3,919 visits included in the comparison analysis, we calculated overall and syndrome-specific counts, frequencies, and kappa statistics using Stata 7 software (Stata Corp., College Station, TX). All analyses were repeated by hospital. Binary variables for each syndrome for discharge diagnosis and chief complaint were used to calculate kappa by syndrome. Kappa was chosen as the comparison statistic since neither chief complaint nor ED discharge diagnosis accurately provides the patient's true diagnosis on a consistent basis. Also, kappa corrects for the agreement expected by chance, improving the comparability of the agreement between syndromes of differing prevalence (4).

Overall agreement between chief complaint and discharge diagnosis for the 3,919 ED visits compared was good (kappa=0.639, Table 1) (4). Respiratory and gastrointestinal syndromes had the highest agreement (kappa statistics 0.684 and 0.677, respectively). The kappa statistic for unspecified infection was in the midrange (0.419). Poor agreement was found for sepsis and neurologic syndromes (kappa statistics 0.105 and 0.085, respectively).

Table 2 shows counts of concordant and discordant visits. Sepsis had only one concordant visit, which was the only visit coded as sepsis by chief complaint. Another 17 visits were

Table 1. Relative frequencies of clinical syndromes and kappa statistics for emergency department syndromic-coding results comparing chief complaint vs. discharge diagnosis, National Capitol Region, December 2001[a]

Syndrome	Chief complaint, %	Discharge diagnosis, %	Kappa[a]	Standard error
Death	0.23	0.26	0.6307	0.0160
Sepsis	0.03	0.46	0.1048	0.0071
Rash	1.38	0.79	0.5841	0.0154
Respiratory	13.37	10.61	0.6839	0.0158
Gastrointestinal	13.24	9.26	0.6768	0.0157
Unspecified infection	3.85	2.68	0.4191	0.0157
Neurologic	0.82	0.33	0.0846	0.0145
Other	67.08	75.61	0.6548	0.0156
Overall	–[b]	–[b]	0.6385	0.0104

[a]A total of 3,919 emergency department visits from two regional hospitals were used for all analyses.

[b]Frequencies not applicable to calculation of overall kappa as two categorical variables with eight values, one for each syndrome, were used for this analysis.

Table 2. Emergency department visits by syndromic-coding results, by chief complaint and discharge diagnosis, at two U.S. National Capitol Region hospitals, December 2001[a,b]

	Syndrome by discharge diagnosis								
Syndrome by chief complaint	Death	Sepsis	Rash	Resp	GI	UI	Neur	Other	Total
Death	**6**	1	0	0	0	0	0	2	9
Sepsis	0	**1**	0	0	0	0	0	0	1
Rash	0	0	**25**	2	0	2	0	25	54
Respiratory	2	1	0	**339**	6	18	0	158	524
Gastrointestinal	0	1	0	18	**314**	15	1	170	519
Unspecified infection	0	5	0	20	5	**56**	1	64	151
Neurological	0	2	0	1	0	0	**2**	27	32
Other	2	7	6	36	38	14	9	**2,517**	2,629
Total	10	18	31	416	363	105	13	2,963	3,919

[a]Resp, respiratory; GI, gastrointestinal; UI, unspecified infection; Neur, neurologic.
[b]Areas in bold indicate counts of visits with concordant results for both data types.

coded as sepsis by discharge diagnosis only. Seven (41%) were coded as "other" by chief complaint, and the remaining 10 chief complaints (59%) were distributed throughout the syndromes.

Neurologic syndrome had 2 concordant visits and 41 discordant visits. Among the 30 discordant visits coded neurologic for chief complaint but not for discharge diagnosis, the most common chief complaint was altered or decreased mental status and level of consciousness (21/30; 70%). Discharge diagnoses for these 30 visits included syncope; sepsis, and other infections; cerebral vascular events or asymmetric weakness in a person >50 years of age; hypoglycemia; and cancer. Eleven discordant visits were coded neurologic for discharge diagnosis only. Nine of these had chief complaints coded "other," including three patients with Bell's palsy with chief complaints of facial numbness and three patients with psychiatric chief complaints but discharge diagnoses of change in mental status.

Ninety-five cases were coded as unspecified infection by chief complaint but not by discharge diagnosis. Chief complaints were predominantly "flu" or fever alone or with other nonspecific symptoms. Corresponding discharge diagnoses generally specified the organ system affected and included respiratory infections (bronchiolitis, pneumonia, and bronchitis), gastroenteritis, sepsis, and infections coded as "other" (otitis media, pharyngitis, urinary tract infections, sinusitis, and upper respiratory infection). For the 51 visits coded as unspecified infection for discharge diagnosis but not for chief complaint, the discharge diagnoses were predominately nonspecific terms such as "febrile illness" or "viral illness/syndrome" with syndrome-specific complaints such as cough, vomiting, diarrhea, and rash coded as respiratory, gastrointestinal, or rash.

For three visits, the chief complaints suggested ongoing cardiopulmonary resuscitation and were coded as deaths; however, because all three patients were resuscitated, all had discharge diagnoses other than death. In addition, two visits with chief complaints of respiratory illness and two visits coded as "other" had discharge diagnoses coded as deaths because these four patients subsequently died in the ED.

Hospitals 1 and 2 recorded 971 visits (25%) and 2,948 visits (75%), respectively. Kappa statistics by hospital were similar overall (0.5899 and 0.6504 for hospitals 1 and 2, respectively) and by syndrome, except for rash (0.2822 and 0.6430, respectively) and unspecified infection (0.1786 and 0.4648, respectively).

Conclusions

Public health officials implementing ED syndromic surveillance systems must decide what data types to use when assigning visits to syndrome categories. Overall we found good agreement between ED chief complaint and discharge diagnosis, but substantial variability existed by syndrome. Sepsis, neurologic, and unspecified infection syndromes were found to have lower agreement than death, rash, respiratory, and gastrointestinal syndromes. These results suggest that several important differences exist between chief complaint and discharge diagnosis.

We found poor agreement between chief complaint and discharge diagnosis for sepsis syndrome. Our matrix terms for sepsis syndrome are sepsis, septic shock, shock, and urosepsis. Sepsis and shock are clinical terms rarely seen as a patient's chief complaint, even when the ED staff translate patients' complaints into medical terminology, making this life-threatening clinical entity difficult to track by using chief complaint only.

For neurologic syndrome, which was designed to capture botulism and meningitis cases, we also observed poor agreement between the two data types. Which data source best serves the aims of syndromic surveillance remains unclear, as no botulism or meningitis cases were diagnosed during the study period. Many key components of a meningitis diagnosis are available after a relatively brief ED evaluation, such as classic physical exam findings and spinal fluid analysis results, suggesting that discharge diagnosis may provide a better positive predictive value than chief complaint. Coding initial ED visits of patients with culture-confirmed cases retrospectively, by using chief complaint and discharge diagnosis, would test this hypothesis.

Unspecified infection syndrome is intended to identify nonspecific infectious conditions not captured elsewhere. As expected, we found a low agreement here, since patients with fever alone or other nonspecific chief complaints are often

given a specific diagnosis after clinical evaluation. In some situations, organ-specific discharge diagnoses reasonably rule out the possibility of illness caused CDC's high priority bioterrorism agents. In other situations, these diagnoses may be less informative if they place febrile patients into diagnoses infrequently associated with fever, such as upper respiratory infections, without ruling out serious rare disease.

One limitation of the kappa statistic is its dependence on the prevalence of the condition being detected (4). However, the gross differences seen here cannot be accounted for by underlying prevalence. For example, similar prevalences were found for death, sepsis, rash, and neurologic syndromes (range 0.23 to 1.38), but death and rash had substantially higher kappa values than sepsis and neurologic syndromes (0.6307 and 0.5841 vs. 0.1048 and 0.0846, respectively). Another limitation of this analysis is the inability because of sample size to examine interhospital differences in detail. However, kappa statistics were similar for both hospitals overall and differed only by syndrome for unspecified infection and rash. A larger dataset of visits from several hospitals using automated coding would be a better setting for investigating such interhospital variation.

Further work is needed to assess the ability of our syndrome-coding matrix to appropriately classify infections; this matrix continues to undergo refinement. However, our results illustrate important systemic differences between chief complaints and discharge diagnoses. Overall, chief complaint seems to best capture illnesses for which nonspecific symptoms like fever are the most important features. Discharge diagnosis appears better at tracking illnesses that can be identified after brief ED clinical evaluation and testing, such as sepsis and possibly meningitis. Since we are interested in monitoring both types of illness, we recommend coding both data types, if resources allow, or carefully defining system objectives if only one data type can be used. Additionally, linking supplemental clinical information, such as laboratory or radiographic data, to these data sources may substantially improve the predictive value of syndromic surveillance system results overall.

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Dr. Begier completed this work as preventive medicine resident at the Johns Hopkins Bloomberg School of Public Health during a practicum year rotation at the Maryland Department of Health and Mental Hygiene. Her research interests include traditional and alternative approaches to disease surveillance.

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Asymptomatic Visceral Leishmaniasis, Northern Israel

Irit Adini,* Moshe Ephros,† Jacopo Chen,* and Charles L. Jaffe*

Asymptomatic human visceral leishmaniasis was identified in Israel by using an enzyme-linked immunosorbent assay. Positive serum samples were more prevalent in visceral leishmaniasis–endemic (2.97%) compared to nonendemic (1.01%) regions (p=0.021). Parasite exposure was higher than expected, despite the small number of clinical cases, suggesting factors other than infection per se influence clinical outcome.

Human visceral leishmaniasis (HVL), caused by the *Leishmania donovani* complex, is lethal if not promptly diagnosed and treated (1). Yet in the Mediterranean and Middle East, only a small percentage of infections progress to clinical disease (2,3). HVL in this region is primarily a disease of young children; however, its epidemiology is changing. In southern Europe, 50% of new cases are in adults coinfected with HIV (4).

HVL is endemic in northern Israel; 68 cases, mostly from Arab villages, were documented from 1960 to 1989 (5). Since 1993, a drastic reduction in the disease was observed, despite the identification of nine active sites of canine visceral leishmaniasis (VL) in this region (G. Baneth and C. L. Jaffe, unpub. data). Recent studies have identified an emerging focus of human and canine disease near major population centers in central Israel and the Palestinian Authority (6,7). In this study, HVL seroprevalence in northern Israel was compared with that in a region free of clinical disease.

The Study

Prevalence of asymptomatic disease was examined by random cluster sampling of serum samples from 12 sites (494 serum samples) in a region presumed to be non-HVL-endemic (i.e., HVL has not been reported) and 11 sites (2,086 serum samples) in the HVL-endemic northern region, where the disease was previously reported in 8 of 11 sites (Figure). Coded samples were analyzed blindly by enzyme-linked immunosorbent assay on *L. donovani* antigen for anti-leishmanial antibodies (5). Each plate was read when the positive control serum (1/1,000 dilution) absorbance $_{\lambda 405\ nm}$ = 1.0-1.2. Most serum samples were from women ages 18-45. The mean ages for patients in the non-disease-endemic and HVL-endemic regions were 36 and 30 years, respectively (range <1 to >75). No symptomatic HVL was diagnosed during the study.

The mean absorbance for serum samples from HVL-endemic areas (0.0956; 95% confidence interval [CI] 0.0926 to 0.0986) was significantly higher (p=0.0001; unpaired t test with Welch's correction) than that for serum samples from non-disease-endemic areas (0.0832; 95% CI 0.0779 to 0.0885). The percentage of positive samples (mean of the non-HVL-endemic population + 3 standard deviations) was significantly higher (p=0.021, chi-square test) for the endemic, 2.97% (n=62) than for the non-HVL-endemic region, 1.01% (n=5). Age was directly standardized by examining the percent positive serum samples in children ≤14 years of age, who are most likely to have clinical HVL, and older study participants (>14 years). The standardized prevalence ratio of positive serum samples in the HVL-endemic to non-HVL-endemic regions was 2.90. Western blotting was used to confirm asymptomatic VL (8). Strong reactions were observed to 14- and/or 18-kDa antigens with all positive samples; weak or no bands were observed with negative samples.

The percentage of positive serum samples for each site is shown (Figure). In the non-HVL-endemic region, most serum samples collected (64.6%) came from Atlit (Jewish, n=146) and Isfiya (Arab, n=173). The remaining serum samples (n=175) originated from 10 sites, with a maximum of 39 samples per site. Only 1.1% of these samples (2/175) were positive, a value similar to that found for the larger towns of Atlit and Isfiya (p=1.0, Fisher exact test). Essentially no difference in the percentage of positive serum samples (p=1.0, Fisher exact test) was found between non-leishmaniasis-endemic Arab (Isfiya, 1.2%) and Jewish (Atlit, 0.7%) towns, even though residents of Isfiya are more likely to visit villages where the disease occurs.

Large numbers of samples (>140/site) were collected from 9 of 11 HLV-endemic sites. The percentage of positive samples from Arab areas (3.1%) was not significantly higher (p=0.46, chi-square test) than for samples from Jewish areas (1.8%), even though most HVL case-patients in this region are from Arab villages. However, the percentage of positive serum specimens in 7 of 11 HVL-endemic sites was three- to six-fold the average percentage found in the non-HVL-endemic region. The number and percentage of positive HVL-endemic serum samples during the second year were almost twice (3.9%; p=0.038, unpaired t test) the figures observed for the first year (2.2%). The opposite trend was found in the non-HVL-endemic region, where the number and percentage of positive samples decreased from 1.9% to 0.3% (p=0.044) in the first and second year, respectively. Nine of the 11 HLV-endemic sites showed increases in the percentage of positive serum samples during the second year.

During the 1960s, 45 cases of VL were diagnosed in western Galilee; more than half were from eight sites, all Arab villages, included in this study (5). Since then, the incidence of HVL in northern Israel has decreased: only three cases have been diagnosed in the last 7 years. This finding is not due to the absence of parasite transmission, since canine VL remains

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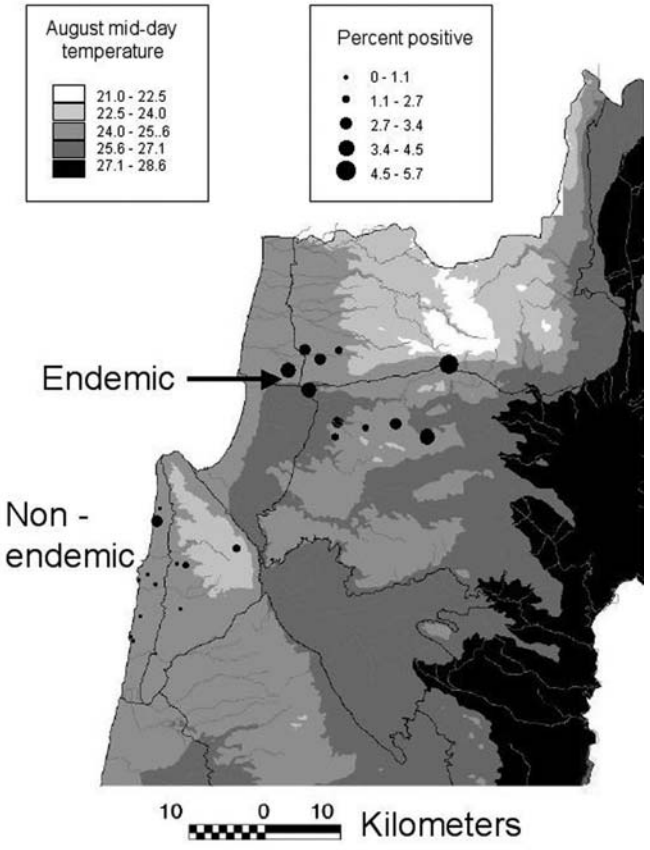

Figure. Percentage of positive serum samples and average mid-day temperatures, visceral leishmaniasis study sites, northern Israel

common (G. Baneth and C. L. Jaffe, unpub. data). While HVL is decreasing in northern Israel, the disease has emerged in central Israel, where numerous dogs and seven persons have been diagnosed with VL since the index HVL case in 1994 (6). Villages in both regions are rural, and stray dogs and jackals are frequently observed. The conditions contributing to the changes in disease epidemiology in Israel are unknown but are likely multifactorial. Studies in Brazil on risk factors associated with asymptomatic infection concluded that infection is associated with age (≥2 years), location of dwellings, and presence of relatives with acute VL. Malnutrition was not associated with infection (9). A higher standard of living and health was also postulated to be involved in decreased VL incidence over the past 10 years (10). During the last decade, improvements in the standard of living, health care, and infrastructure of Arab villages in Israel have taken place. Though data are not available for most study sites, the percentage of seropositive persons in Yirka, for example, decreased from 10% in 1989 (5) to 1.5% in the current study (1994-1996), suggesting that exposure to parasites in Yirka has decreased during the last decade.

The percentage of seropositive people is significantly greater in the HVL-endemic northern than the non-HVL-endemic coastal and Carmel Mountain regions. The presence of positive samples in the non-HVL-endemic region suggests that parasite transmission occurs, even though HVL has not been reported. Since people from this region frequently travel to northern Israel, we cannot exclude the possibility that they were infected there. Although the incidence of the disease is lower in Israel than in other HVL-endemic regions, our results suggest that cumulative exposure to the parasite and asymptomatic infection in Israel are more frequent than otherwise predicted, based on the yearly incidence of VL. These data will be important in understanding the spread of human and canine disease into new regions, transmission by blood transfusion, and the potential risk for VL/HIV coinfection.

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Mycobacterium celatum Pulmonary Infection in the Immunocompetent: Case Report and Review

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Mycobacterium celatum has been shown to cause disease in immunocompromised patients. We report a case of serious pulmonary infection caused by *M. celatum* in an apparently immunocompetent patient and review the characteristics of two other reported cases. Clinical and radiologic symptoms and signs included cough, malaise, and weight loss associated with cavitary lesions and pulmonary infiltrates. Although *M. celatum* is easy to detect in clinical specimens by liquid and solid media, it may be misidentified as a member of the *M. tuberculosis* complex or as *M. xenopi*. *M. celatum* pulmonary infection appears to respond to antimycobacterial chemotherapy, particularly with clarithromycin.

Mycobacterium celatum was first described in 1993 as a new species whose mycolic acid pattern closely resembled that of *M. xenopi* (xenopi-like) but was biochemically indistinguishable from *M. avium* complex (MAC) (1). By sequencing studies of the 16S rRNA gene, now known to exist in two different copies within *M. celatum* genome (2), three different types could be differentiated (1,3). Cross-reactivity with the Accuprobe assay (Gen-Probe Inc., San Diego, CA) for the *M. tuberculosis* complex (MTB) was observed by probing types 1 and 3 but not type 2 (3,4). DNA sequencing showed that *M. celatum* type 1 differs by one nucleotide from the probe used in the Accuprobe assay, type 2 differs by four nucleotides (5), and the type 3 DNA sequence for the probe region is unknown.

Although *M. celatum* has been reported to cause infection mainly in AIDS patients (6–10), recently a growing amount of clinical evidence indicates that the infection can lead to serious disease in immunocompetent subjects as well (11–13). In this case, the principles for diagnosis of diseases caused by nontuberculous mycobacteria, recently updated by the American Thoracic Society (14), need to be properly followed. We report a well-documented case of a pulmonary disease with this mycobacterium in an apparently immunocompetent woman and review the characteristics of two similar published cases.

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The Study

A 63-year-old, HIV-negative woman was referred in June 2000 by a general practitioner to the outpatient pulmonary service because she had exhibited a fever and night sweats for 3 months. The patient's medical history included a mild hypertensive cardiovascular disease and a pulmonary tuberculosis episode when she was 16 years old. Her father and brother had pulmonary tuberculosis. She lived in an urban area and had no history of alcoholism, smoking, or use of steroids or immunosuppressive drugs. No other pulmonary diseases that could potentially lead to bronchiectasis and further mycobacterial colonization were reported. Physical examination revealed no pathologic findings except a strong positive reaction (24 mm of induration) with tuberculin skin test (5 U of purified protein derivative–standard [PPD-S]). Results of clinical laboratory tests were unremarkable, apart from an elevated erythrocyte sedimentation rate (77 mm/h). A computed tomographic (CT) scan of the chest showed multifocal bronchiectases in the middle right lobe, multiple small nodules in the lower right lobe, and a cavitary lesion in the upper right lobe, which appeared to have thin walls and was surrounded by a scant or absent inflammatory reaction. Bronchoscopy showed no inflammatory mucosal changes on the right side. Smears of bronchial washing were negative for acid-fast bacilli (AFB), and smears of two sputum specimens were positive, while all specimens gave negative results when tested by a ligase chain reaction commercial assay specific for MTB. A reactivation of tuberculosis was assumed, and chemotherapy with isoniazid, rifampin, and ethambutol was begun. Cultures of bronchial washing and sputum specimens produced a slow-growing Mycobacterium, which was identified as *M. celatum* in July 2000. The isolate was not considered clinically important, and chemotherapy was unchanged.

In December 2000, although the general condition of the patient had slightly improved after 6 months of treatment, a control CT scan showed a worsening (enlargement) of the nodular lesions. Smears of two sputum specimens and one bronchial washing specimen were positive for AFB, whereas all specimens were negative when tested by the MTB ligase chain reaction assay. All of these specimens yielded *M. celatum*. On the basis of susceptibility data obtained from the first *M. celatum* isolate, chemotherapy was changed to a schedule that included isoniazid, ethambutol, and clarithromycin, which lasted until November 2001. This regimen proved to be well tolerated by the patient. The bacteriologic results (specimens from two additional bronchial washings were negative for AFB by smear and culture) and clinical response were good. In December 2001, chest x-ray examinations and a CT scan showed a considerable reduction of nodular lesions as well as improvement of lung cavitation (Figure). The patient was considered cured after taking medication for approximately 18 months.

Clinical, microbiologic, and radiologic data as well, as information on response to treatment and follow-up, were obtained from the patient's records. All material was carefully

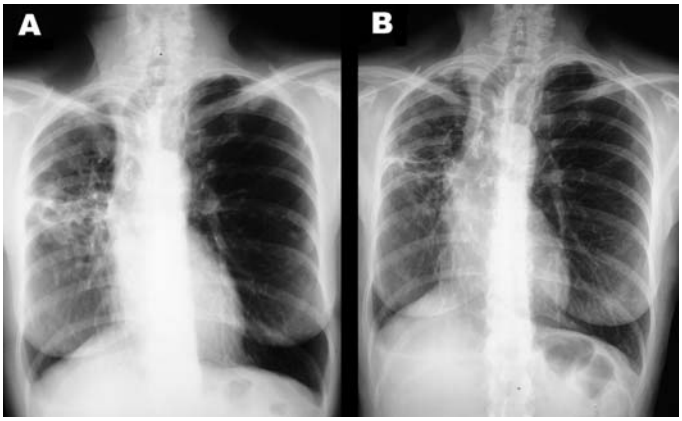

Figure. Chest x-ray showing nodular lesions and lung cavitation before (A) and after (B) treatment.

reviewed to evaluate the clinical significance of the findings according to the criteria proposed by the American Thoracic Society (14).

We examined sputum and bronchoaspirate specimens for acid-fast organisms. They were stained with routine Ziehl-Neelsen stain and cultured by standard procedures (15) by using a combination of radiometric Bactec system (Becton Dickinson Biosciences, Sparks, MD) and Löwenstein-Jensen medium. Amplification for MTB was performed by the LCx-MTB assay (Abbott, Diagnostics Division, Chicago, IL). Conventional identification and growth tests were performed according to standard methods (15). We tested recovered strains with the Accuprobe system using specific probes for MAC and MTB. Assays were run from liquid culture setting the unbound probe hydrolysis incubation time at 5 and 10 min and temperature at 60° ± 1°C (16). The amount of chemiluminescence emitted was estimated with a Leader 50 luminometer (Gen-Probe Inc., San Diego, CA, USA) and quantified as relative light units (RLUs). Definitive identification was achieved by mycolic acids high-performance liquid chromatography (HPLC) analysis (17). Drug susceptibility pattern was determined in 12B liquid medium (Becton Dickinson Biosciences) by using the radiometric macrodilution method developed for MAC (18).

Six isolates were recovered in a 6-month period. *M. celatum* was isolated from different specimens: two isolates were from bronchial washing and four from sputum. Acid-fast smears were positive in five of the six specimens. Cultures on Löwenstein-Jensen medium yielded multiple colonies, ranging from 100–150 CFU to a confluent growth. No additional microorganisms were isolated during the admission and follow-up period. Amplification tests performed on all respiratory specimens were repeatedly negative for MTB on smear samples positive and negative for *M. celatum*. *M. celatum* strains were isolated on both currently used media, radiometric liquid medium (Bactec 12B) and conventional Löwenstein-Jensen solid medium. We used an extended panel of biochemical and cultural tests for conventional identification. On the basis of these test results, the most likely identification, estimated by a program for the computerized identification of mycobacteria (19), appeared to be *M. avium*-intracellulare (Table). All strains isolated from this patient produced a weak yellow pigment in the dark, which grew at 45°C and were negative for arylsulfatase activity when tested after 3 days by the method of Kubica and Rigdon (20). The Bactec NAP test (Becton Dickinson Biosciences) did not demonstrate, as expected, any inhibition by para-nitro-α-acetilamino-β-hydroxypropiophenone (NAP). The hybridization test performed with Accuprobe specific for MAC gave negative results. Weak positive or slightly below the cutoff (30,000 RLUs) results were observed with Accuprobe MTB (mean RLU value 51,170; range 28,219–111,365). When selection reagent (unbound probe hydrolysis) incubation time was set at 10 min, *M. celatum* strains clearly showed negative results (range 5,721–9,574; mean RLU value 7,210). A reference strain (*M. tuberculosis* ATCC 25177), run in the hybridization assay as a positive control, gave RLUs of 580,321, about 20 times the cut-off value. Finally, the strains were sent to the Mycobacteria Reference Centre in Florence, Italy (Dr. E. Tortoli), and studied by HPLC analysis of mycolic acids. They showed the same profile that allowed all of our strains to be attributed to *M. celatum*.

MICs (μg/mL) determined for the first isolate were the following: streptomycin, 2.0; isoniazid, 0.5; rifampin, >64.0; ethambutol, 2.5; ciprofloxacin, 1.0; ofloxacin, 1.0; clarithromycin, 1.0; ethionamide, 2.5; and rifabutin, 0.5. Drug susceptibility testing was completed after 6 days.

We searched the English-language literature from 1993 to 2001 for all previously reported cases of pulmonary infections with *M. celatum* in immunocompetent patients. The search yielded two published reports (12,13). Descriptions of the clinical

Table. Biochemical analysis of the described isolate (*Mycobacterium celatum*) compared to those of *M. avium* complex and *M. xenopi*

	Results[a] for:		
Characteristics	Our isolate	*M. avium* complex	*M. xenopi*
Niacin	–	–	–
Nitrate reduction	–	–	–
Thermostable catalase	+	+	+
Tween 80 hydrolysis (10 days)	–	–	–
Tellurite reduction	+	+	V
Arylsulfatase (3 days)	–	–	+
Urease	–	–	–
Catalase (over 45 mm of foam)	–	–	–
Photochromogenicity	–	–	–
Scotochromogenicity	+	–	+
Growth at 25°C	+	+	–
Growth at 37°C	+	+	+
Growth at 45°C	+	–	+
MacConkey w/o CV	–	–	–
Tolerance to NaCl (5%)	–	–	–
Tolerance to TCH (5 mg/mL)	+	+	+
Growth rate	Slow	Slow	Slow
Colonial morphologic features	Smooth	Smooth	Smooth

[a]– negative; +, positive; v, variable; TCH, thiophene-2-carboxylic acid hydrazide.

signs, radiologic findings, and treatment were not always available, however.

Clinical and radiographic features of *M. celatum* pulmonary infection resemble those of tuberculosis and other nontuberculous mycobacterial infections. Cough and weight loss are the main symptoms, while pulmonary infiltrates and extensive cavitations have been described in all published reports. Patients did not suffer from any underlying diseases when pulmonary infection with *M. celatum* was diagnosed and had a strongly positive tuberculin skin test, suggesting immunocompetence. Published data and our findings indicate that *M. celatum* caused pulmonary disease, and not merely colonization, in the affected patients. Indeed, all of these cases met the American Thoracic Society criteria for the diagnosis of nontuberculous mycobacterial disease. The patients displayed radiographic evidence of pulmonary disease that could not be attributed to other causes and that was associated in two of three cases with the repeated isolation of the same mycobacterial strain. By contrast, clinical presentation in immunocompromised patients differs greatly, being characterized by interstitial pulmonary infiltrates or by clinical and micobiologic evidence of disseminated disease (7).

M. celatum strains were isolated on currently used liquid media in all reported cases (radiometric Bactec 12B and MB/BacT [Organon Teknika B.V., Boxtel, the Netherlands]), while samples from one of the published case-patients did not grow on conventional Löwenstein-Jensen solid medium, and growth has not been reported for samples from the second case-patient. Data from an extended panel of biochemical and cultural tests for conventional identification have not been reported; however, the appearance of a scotochromogenic pale yellow pigment with growth at 45°C and a negative 3-day arylsulfatase test are in full agreement with our present and previous findings (7). All strains showed a partial hybridization with Accuprobe MTB, which disappeared after 10 min of incubation with selection reagent. From a practical standpoint, setting the selection time at 10 min is advisable when the Accuprobe identification is performed on a mycobacterial culture grown in a liquid medium. In this situation, the characteristics of mycobacterial colonies cannot be observed and false-positive reactions may lead to misidentification. However, if the Accuprobe assay is carried out on a mycobacterial culture grown on a solid medium, a 5-min selection time is recommended. In this case, the appearance of mycobacterial colonies and their characteristics of growth may help to evaluate Accuprobe results. Thus, a weak positive reaction when testing nontuberculous mycobacteria with the *M. tuberculosis* complex Accuprobe strongly suggests that the organism is likely to be *M. celatum*.

In one case (13), a misleading positive reaction occurred when a clinical sample containing *M. celatum* was tested for MTB by the Amplified *M. tuberculosis* direct test (AMTD, Gen-Probe, Inc.). The test amplifies 16S rRNA of Mycobacterium species by transcription-mediated amplification (21). The resulting amplicons are then detected by a hybridization protection assay by using a probe that targets the same genomic region as the probe of the Accuprobe kit (22). In our case, when we tested clinical samples by a different amplification system, no false-positive results potentially leading to a delay of appropriate treatment were reported. Patients (including our case-patient) were given different treatment regimens, with three or four antibiotics, mainly ethambutol, rifabutin, clarithromycin, and ciprofloxacin. Clinical improvement, as defined by resolution of symptoms and improved radiographic findings (infiltrates and cavitary lesions), was obtained within 6 months after initiation of therapy in two of three patients. One patient who received antimycobacterial therapy died 10 weeks after admission from complications apparently related to the *M. celatum* infection (12). Data from necropsy were not reported. Both cured patients showed a marked improvement when clarithromycin was added the chemotherapy regimen; the patient who later died felt better for 3 weeks when his chemotherapy was changed to a new schedule that included clarithromycin. Despite evidence of clinical and radiologic improvement, one patient was still sputum-positive 1 year after the initiation of therapy (13). In-vitro susceptibility data (when available) seemed to correlate with patients' clinical improvement as the MICs of tested drugs were below the serum concentrations achievable during therapy.

Our findings show that *M. celatum* is an infrequent cause of potentially treatable pulmonary disease in immunocompetent subjects. Clinical picture and repeated isolation from respiratory specimens enabled us to consider *M. celatum* strains as clinically relevant in all the patients reported. Treatment with a three- or four-drug combination, including clarithromycin and ethambutol, should result in considerable reduction in the illness associated with this disease. Although at present only 16S rDNA sequencing or mycolic acid HPLC analysis can confirm *M. celatum* identification, the most practical way to get a preliminary identification relies on a positive DNA hybridization signal for MTB at 5 min but negative hybridization at 10 min with the Accuprobe test. Finally, although no major immunologic disorder could be detected in these patients, the host's defense failure associated with *M. celatum* infection suggests a possible "hidden immunodeficiency" rather than a true immunocompetence. Recent data on the immunology of nontuberculous mycobacterial infections (23) seem to support this hypothesis.

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Emerging Human Infectious Diseases: Anthroponoses, Zoonoses, and Sapronoses

To the Editor: The source of infection has always been regarded as an utmost factor in epidemiology. Human communicable diseases can be classified according to the source of infection as anthroponoses (when the source is an infectious human; interhuman transfer is typical), zoonoses (the source is an infectious animal; interhuman transfer is uncommon), and sapronoses (the source is an abiotic substrate, nonliving environment; interhuman transfer is exceptional). The source of infection is often the reservoir or, in ecologic terms, the habitat where the etiologic agent of the disease normally thrives, grows, and replicates. A characteristic feature of most zoonoses and sapronoses is that once transmitted to humans, the epidemic chain is usually aborted, but the clinical course might be sometimes quite severe, even fatal. An ecologic rule specifies that an obligatory parasite should not kill its host to benefit from the adapted long-term symbiosis, whereas an occasionally attacked alien host, such as a human, might be subjected to a severe disease or even killed rapidly by the parasite because no evolutionary adaptation to that host exists (1). In this letter, only microbial infections are discussed; metazoan invasion and infestations have been omitted.

Anthroponoses (Greek "anthrópos" = man, "nosos" = disease) are diseases transmissible from human to human. Examples include rubella, smallpox, diphtheria, gonorrhea, ringworm (*Trichophyton rubrum*), and trichomoniasis.

Zoonoses (Greek "zoon" = animal) are diseases transmissible from living animals to humans (2). These diseases were formerly called anthropozoonoses, and the diseases transmissible from humans to animals were called zooanthroponoses. Unfortunately, many scientists used these terms in the reverse sense or indiscriminately, and an expert committee decided to abandon these two terms and recommended "zoonoses" as "diseases and infections which are naturally transmitted between vertebrate animals and man" (3). A limited number of zoonotic agents can cause extensive outbreaks; many zoonoses, however, attract the public's attention because of the high death rate associated with the infections. In addition, zoonoses are sometimes contagious for hospital personnel (e.g., hemorrhagic fevers). Zoonotic diseases can be classified according to the ecosystem in which they circulate. The classification is either synanthropic zoonoses, with an urban (domestic) cycle in which the source of infection are domestic and synanthropic animals (e.g., urban rabies, cat scratch disease, and zoonotic ringworm) or exoanthropic zoonoses, with a sylvatic (feral and wild) cycle in natural foci (4) outside human habitats (e.g., arboviroses, wildlife rabies, Lyme disease, and tularemia). However, some zoonoses can circulate in both urban and natural cycles (e.g., yellow fever and Chagas disease). A number of zoonotic agents are arthropod-borne (5); others are transmitted by direct contact, alimentary (foodborne and waterborne), or aerogenic (airborne) routes; and some are rodent-borne.

Sapronoses (Greek "sapros" = decaying; "sapron" means in ecology a decaying organic substrate) are human diseases transmissible from abiotic environment (soil, water, decaying plants, or animal corpses, excreta, and other substrata). The ability of the agent to grow saprophytically and replicate in these substrata (i.e., not only to survive or contaminate them secondarily) are the most important characteristics of a sapronotic microbe. Sapronotic agents thus carry on two diverse ways of life: saprophytic (in an abiotic substrate at ambient temperature) and parasitic (pathogenic, at the temperature of a homeotherm vertebrate host). Typical sapronoses are visceral mycoses caused by dimorphic fungi (e.g., coccidioidomycosis and histoplasmosis), "monomorphic" fungi (e.g., aspergillosis and cryptococcosis), certain superficial mycoses (*Microsporum gypseum*), some bacterial diseases (e.g., legionellosis), and protozoan (e.g., primary amebic meningoencephalitis). Intracellular parasites of animals (viruses, rickettsiae, and chlamydiae) cannot be sapronotic agents. The term "sapronosis" was introduced in epidemiology as a useful concept (6–8). For these diseases the expert committee applied the term "sapro-zoonoses," defined as "having both a vertebrate host and a nonanimal developmental site or reservoir (organic matter, soil, and plants)" (3,9). However, the term sapronoses is more appropriate because animals are not the source of infection for humans. While anthroponoses and zoonoses are usually the domains for professional activities of human and veterinary microbiologists, respectively, sapronoses may be the domain for environmental microbiologists. The underdiagnosis rate for sapronoses is probably higher than that for anthroponoses and zoonoses, and an increase should be expected in both incidence and number of sapronoses. Legionellosis, Pontiac fever, nontuberculous mycobacterioses, and primary amebic meningoencephalitis are a few sapronoses that have emerged in the past decade. In addition, the number of opportunistic infections in immunosuppressed patients has grown markedly; many of these diseases and some nosocomial infections are, in fact, also sapronoses.

As with any classification, grouping human diseases in epidemiologic categories according to the source of infection has certain pitfalls. Some arthropod-borne diseases (urban yellow fever, dengue, epidemic typhus, tickborne relapsing fever, epidemic relapsing fever, and malaria) might be regarded as anthroponoses rather than zoonoses because the donor of the infectious blood for the vector is an infected human and not a vertebrate animal. However, the human infection is caused by an (invertebrate) animal in which the agent replicates, and the term zoonoses is preferred. HIV is of simian origin with a sylvatic cycling among wild primates and accidental infection of humans who hunted or ate them; the human disease (AIDS)

might thus have been regarded as a zoonosis in the very first phase but later has spread in the human population as a typical anthroponosis and caused the present pandemic. Similarly, pandemic strains of influenza developed through an antigenic shift from avian influenza A viruses. For some etiologic agents or their genotypes, both animals and humans are concurrent reservoirs (hepatitis virus E, Norwalk-like calicivirus, enteropathogenic *Escherichia coli*, *Pneumocystis*, *Cryptosporidium, Giardia*, and *Cyclospora*); these diseases might conditionally be called anthropozoonoses. Other difficulties can occur with classifying diseases caused by sporulating bacteria (*Clostridium* and *Bacillus*): Their infective spores survive in the soil or in other substrata for very long periods, though they are usually produced after a vegetative growth in the abiotic environment, which can include animal carcasses. These diseases should therefore be called sapronoses. For some other etiologic agents, both animals and abiotic environment can be the reservoir (*Listeria*, *Erysipelothrix*, *Yersinia pseudotuberculosis, Burkholderia pseudomallei*, and *Rhodococcus equi*), and the diseases might be, in fact, called saprozoonosis (not sensu 9) in that their source can be either an animal or an abiotic substrate.

For a concise list of anthropo-, zoo-, and sapronoses, see the online appendix available from: URL: http://www.cdc.gov/ncidod/EID/vol9no3/02-0208-app.htm.

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Multidrug-Resistant *Shigella dysenteriae* Type 1: Forerunners of a New Epidemic Strain in Eastern India?

To the Editor: Multidrug-resistant *Shigella dysenteriae* type 1 caused an extensive epidemic of shigellosis in eastern India in 1984 (1). These strains were, however, sensitive to nalidixic acid, and clinicians found excellent results by using it to treat bacillary dysentery cases. Subsequently, in 1988 in Tripura, an eastern Indian state, a similar outbreak of shigellosis occurred in which the isolated strains of *S. dysenteriae* type 1 were even resistant to nalidixic acid (2). Since then, few cases of shigellosis have occurred in this region, and *S. dysenteriae* type 1 strains are scarcely encountered (3). In other regions of the world, especially in Southeast Asia, low-level resistance to fluoroquinolones in S*higella* spp. has been observed for some time (4,5).

After a lapse of almost 14 years, clusters of patients with acute bacillary dysentery were seen at the subdivisional hospital, Diamond Harbour, in eastern India. No cases of dysentery had been reported during the comparable period in previous years. A total of 1,124 case-patients were admitted from March through June 2002. The startling feature of these infections was their unresponsiveness to even the newer fluoroquinolones such as norfloxacin and ciprofloxacin, the drugs often used to treat shigellosis. Clinicians tried various antibiotics, mostly in combinations, without benefit. Clinicians also randomly used anti-amoebic drugs without success.

An investigating team collected nine fresh fecal samples from dysentery patients admitted to this hospital; 4 (44%) yielded *S. dysenteriae* type 1 on culture. For isolation of *Shigella* spp., stool samples were inoculated into MacConkey agar and Hektoen Enteric agar (Difco, Detroit, MI), and the characteristic colonies were identified by standard biochemical methods (6). Subsequently, serogroups and serotypes were determined by visual inspection of slide agglutination tests with commercial antisera (Denka Seiken, Tokyo). Antimicrobial susceptibility testing was performed by an agar diffusion disk method, as recommended by the National Committee for Clinical Laboratory Standards (7). Results showed that the organisms were resistant to all commonly used antibiotics, including the fluoroquinolones (norfloxacin and ciprofloxacin) but were sensitive to ofloxacin. On our advice, the clinicians used ofloxacin with good results.

A similar outbreak of *S. dysenteriae* type 1 occurred in the northern part of West Bengal in eastern India among tea garden laborers from April 2002 to May 2002; 1,728 persons were affected (attack rate of 25.6%). Sixteen persons died. The isolated *S. dysenteriae* type 1 strains were found intermediately sensi-

tive to fluroquinolones with an MIC of 2 µg/mL (K. Sarkar, S. Ghosh, S.K. Niyogi, S.K. Bhattacharya, pers. commun.).

This drug-resistant Shiga bacillus is highly likely to spread further and will certainly pose a major therapeutic challenge unless adequate preventive measures are immediately instituted to contain its spread. Appropriate awareness programs for the community and reorientation training for physicians and other health personnel would be helpful to prevent further transmission of these resistant organisms. Alternative drugs to treat drug-resistant cases and an effective vaccine are also needed.

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Correction, Vol. 8, No. 12

In the article "Induction of Inflammation by West Nile virus Capsid through the Caspase-9 Apoptotic Pathway" by Joo-Sung Yang, et al., errors occurred in the Figure 5 caption on page 1384: delta symbols inadvertantly dropped or were replaced by the letter "d." The corrected caption appears below and online http://www.cdc.gov/ncidod/EID/vol8no12/02-0224-G5.htm.

Figure 5. Apoptosis determining domain in WNV-Cp gene. a, Schematic diagram of pcWNV-Cp-DJY, pcWNV-CpWT, and pcWNV-CpΔ3'constructs. b, Immunoprecipitation of in vitro translated protein from pcWNV-Cp-DJY (Cp-DJY), pcWNV-CpWT (CpWT), and pcWNV-Cp-3'(CpΔ3') plasmids. As a negative control, pcDNA3.1 in vitro translated supernatants were analyzed. c , Colorimetric caspase-3 activity assay using pcWNV-Cp-DJY (Cp-DJY), pcWNV-CpWT (CpWT), or pcWNV-Cp 3'(CpΔ3') plasmid-transfected cells. d, The cell lysates were assayed for caspase-9-like activity, and the pcDNA3.1-transfected cell lysate was used as the negative control. e, Inhibition of WNV-Cp-induced apoptosis by a dominant negative (DN) caspase-9 plasmid (DN Casp9) was assayed with equal amount of cell lysates from co-transfection of pcWNV-Cp-DJY (Cp-DJY) or pcWNV-CpWT (CpWT); an expression level of pro-caspase-9 cleavage products (35-37 kDa) was compared to 3'-terminal deletion mutant, pcWNV-CpΔ3'(CpΔ3') and pcDNA3.1 by Western blot analysis with anti-human caspase-9 mAb.

Correction Vol. 8, No. 12

In the article, "Vector Competence of California Mosquitoes for West Nile virus" by Laura B. Goddard et al., errors occurred in the table on page 1387. The corrected table appears below and online at http://www.cdc.gov/ncidod/eid/vol8no12/02-0536.htm.

We regret any confusion these errors may have caused.

Table 2. Infection and transmission rates for California mosquito species orally infected with $10^{7.1\pm0.1}$ PFU/mL of West Nile virus (WNV)

Species	Source by county	Day when transmission was attempted	No. tested	Infection rate[a]	Transmission rate[b]
Culex tarsalis	Yolo	7	30	87	60
		14	1	100	100
	Kern	7	15	93	40
		14	35	74	60
	Riverside	7	49	94	10
		14	55	85	62
Cx. pipiens quinquefasciatus	Kern	7	50	86	4
		14	50	58	52
	Riverside	7	60	8	0
		14	60	13	2
	Orange	14	58	28	19
Cx. p. pipiens	Shasta	7	45	80	9
		14	50	66	36
Cx. stigmatosoma	San Bernardino	7	17	100	0
		14	31	100	71
Cx. erythrothorax	Orange	7	15	67	0
		14	48	77	19
	Riverside	7	15	100	33
		14	25	100	64
Ochlerotatus dorsalis	San Luis Obispo	7	30	50	13
		14	29	41	34
Oc. melanimon	Kern	7	50	46	18
		14	60	48	20
Oc. sierrensis	Lake	7	40	5	3
		14	50	14	6
Aedes vexans	Riverside	14	22	32	23
Culiseta inornata	Kern	14	28	75	21

[a]Percent of mosquito bodies positive for WNV.

[b]Percent of transmission attempts positive for WNV.

About the Cover

Edvard Munch (1863-1944)
Self-Portrait After the Spanish Flu (1919-20)
Oil on canvas, 59 cm x 73 cm Munch Museum, Oslo, Norway

"Illness, insanity, and death…kept watch over my cradle and accompanied me all my life," noted innovative Norwegian artist Edvard Munch. Deeply affected by the untimely death of his mother (when he was 5) and his 15-year-old sister (when he was 14), he devoted his early artistic efforts to painting their predicament and the ravages of tuberculosis, "…the wan face in profile against the pillow, the despairing mother at the bedside, the muted light, the tousled hair, the useless glass of water" (1). His own fragile physical and emotional state dominated the way he viewed and executed his art. In his middle years, incapacitated by depression, he spent time in a sanatorium in Denmark, and even though he recovered, his work never regained its initial expressiveness (2).

Munch studied in Oslo and traveled extensively to Italy, Germany, and France, where he took in the influences of his contemporaries (particularly Toulouse-Lautrec, van Gogh, and Gauguin) who were turning the angst of modern civilization into symbolism and stark expressionism (3). Preoccupation with decadence and evil pervaded the artistic and literary climate of the day. Darkness and horror inspired deeply personal, highly expressive art in a variety of styles, all of which fit under the umbrella of symbolism, as long as they embodied its peculiarly gloomy state of mind (2). The movement's emphasis on inner vision rather than observation of nature captured Munch's haunted imagination and engaged his moody genius.

Inspired by the work of Henrik Ibsen, Munch studied psychoanalysis and created art that unraveled the mysteries of the psyche. His canvases are filled with agonizing uncertainty and excruciating loneliness, anticipating Ingmar Bergman's theater and cinematic work (3). His personal neuroses and physical ailments permeate the cultural anxiety expressed in his work. Even as he painted the existential drama of his own life, Munch did so without graphic depictions of monsters or apparitions. Rather, he provoked emotional response through unnatural color, internal rhythm, and undulating lines, as in The Scream, one of the most reproduced and universally acclaimed paintings in the history of art. Munch's most ambitious (unfinished) work, The Frieze of Life, comprised a sequence of connected panels intended to expose the illusory nature of optimism and bring to public view the painter's innermost feelings about life—from birth to death (3).

Pestilence, which traumatized Munch's early years in the form of tuberculosis, continued to rule his life and fuel his journey through the vagaries of the human condition. In Self-Portrait after the Spanish Flu on this month's cover of Emerging Infectious Diseases, the tormented painter appears judge and victim of this pandemic killer. The terse yet unsteady demeanor, the puffy discolored glare, the quivering lines of fever and chills, only highlight the despair and isolation of the "grippe" patient, the oppression, the weakness, the malaise, the lack of air, the stupor, the hopelessness.

Munch's preoccupation with suffering in this self-portrait is fully understood by those who study the Spanish flu pandemic. Erupting during the final stages of World War I, this global disaster reinforced the era's nihilism and apocalyptic visions of despair. "I had a little bird/Its name was Enza/I opened the window/And in-flew-enza," morbidly sang the children as they skipped rope (4). Specimens from the remains of flu victims buried in permafrost provide some clues about the 1918–1919 strain. Highly contagious and unusually virulent, the deadly flu, circled the globe, taking its toll among the youngest and healthiest. Medicine was then only beginning to understand infectious diseases and to take modest steps towards diagnostics and therapy.

Infectious disease medicine has come a long way, yet Munch's specter of the flu is alarmingly current. Surveillance of circulating viruses is increasing and flu vaccination has entered the mainstream, but epidemics are still frequent and strains arising from antigenic shift keep the next flu pandemic just around the corner.

Polyxeni Potter

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Upcoming Infectious Disease Conferences

March 4-7, 2003
11th International Congress on Infectious Diseases
Cancun, Mexico
E-mail: info@isid.org/programs

March 9-12, 2003
The American Society for Microbiology (ASM) Meeting
Future Directions for Biodefense Research: Development of Countermeasures
Baltimore Marriott Waterfront, Baltimore, Maryland
Abstract Deadline: January 30, 2003
Phone: 202-942-9248
Fax: 202-942-9340
Email: meetingsinfo@asmusa.org
www.asmbiodefense.org

March 9-12, 2003
American Society for Microbiology Meeting
Future Directions for Biodefense Research: Development of Countermeasures
Baltimore, MD
Phone: 202-942-9248
E-mail: meetingsinfo@asmusa.org

March 17-20, 2003
27th National Immunization Conference
Chicago, IL
Contact: Suzanne Johson DeLeon
(404) 639-8225
E-mail: msj1@cdc.gov
Website: http://www.cdc.gov/nip/nic

March 31-April 4, 2003
EIS Conference
Atlanta, GA
Contact: Erica Lowe
Phone: 404-639-4000
Email: ELowe@cdc.gov
Website: http://www.cdc.gov/eis/conference/conference.htm

April 2-5, 2003
37th Annual Meeting of the European Society for Clinical Investigation
The Pathophysiology of Diseases: From Bench to Bedside
Verona, Italy
Contact: Giovanni Ricevuti, M.D.
phone: 39-0382-502499
E-mail: g.ricevuti@smatteo.pv.it
Website: www.esci.eu.com

EMERGING INFECTIOUS DISEASES

A Peer Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.3, March, 2003

Upcoming Issue

For a complete list of articles included in the April issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm

Look in the April issue for the following topics:

Bacterial Resistance to Penicillin G by Decreased Affinity of Penicillin Binding Proteins: A Mathematical Model

The Elderly and Waterborne Cryptosporidium Infection: Gastroenteritis Hospitalizations before and during the 1993 Milwaukee Outbreak

Antimicrobial-Drug Prescriptions in Ambulatory-Care Settings, United States, 1992-2000

Dengue Fever in Travelers to the Tropics, 1998 and 1999

Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes

Seed Disinfection and Alfalfa Sprout-Associated Salmonellosis

Early-Season Avian Deaths from West Nile Virus as Warnings of Human Infection

Class I Integrons and SXT Elements in El Tor Strains Isolated before and after 1992 Vibrio cholerae O139 Outbreak, Calcutta, India

Fear of Bioterrorism and Implications for Public Health Preparedness

Converging Issues in Veterinary and Public Health

Cicero and Burkholderia cepacia: What's in a Name?

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and 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, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at http://www.cdc.gov/eid.

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 e-mail 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. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

Manuscript Types

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) 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) and a brief biographical sketch of first author—both authors if only two. 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) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should 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) and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 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 two); and a brief biographical sketch of first author—both authors if only two. 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 should not include 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. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

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.)

Conference Summaries. (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.