EMERGING INFECTIOUS DISEASES

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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Vol.10, No.12, December 2004

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends pages 2065–2274

EMERGING INFECTIOUS DISEASES

EDITOR-IN-CHIEF
D. Peter Drotman

www.cdc.gov/eid

Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-371-5329, fax 404-371-5449, email eideditor@cdc.gov.

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On the Cover

Edward Hicks (1780–1849).
Peaceable Kingdom (c. 1833)

Oil on canvas
(44.5 cm x 60.2 cm)
Worcester Art Museum

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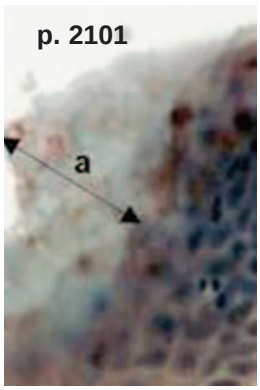

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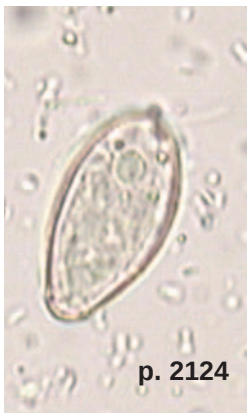

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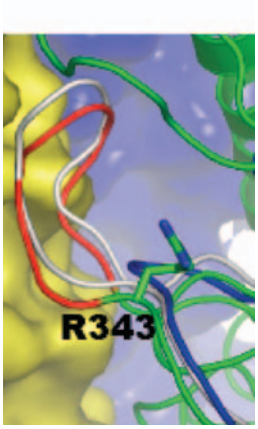

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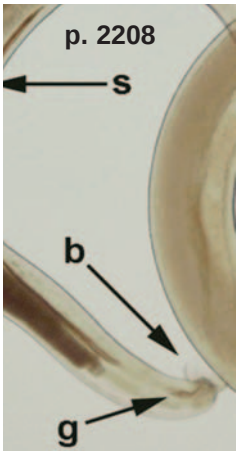

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Historical, New, and Reemerging Links between Human and Animal Health

Nina Marano* and Marguerite Pappaioanou*

A wide spectrum of microbes and infectious diseases have been transmitted from domesticated and wild animals to humans for thousands of years (1). In the last 5 years, infectious diseases such as West Nile virus infection and monkeypox have appeared in North America, and severe acute respiratory syndrome and avian influenza have emerged on a global scale. We learn from each new event, and we hope that we will be sufficiently prepared to prevent, or to detect and effectively respond to, the next event. These diseases, which disregard national borders, include new infections caused by changes or evolution of existing organisms (e.g., recent report of rabies virus transmission through organ transplantation) (2), known infections expanding to new geographic locations (e.g., emergence of West Nile virus in North America beginning with the United States in 1999), previously unrecognized infections appearing in areas undergoing ecologic transformation (e.g., Nipah virus in humans and swine in Malaysia) (3), new infections reemerging as a result of antimicrobial resistance developing in existing agents (e.g., emergence of infections caused by multidrug-resistant strains of *Salmonella* Newport) (4), or breakdowns in public health measures (e.g., *Mycobacterium bovis* tuberculosis [5]) (6).

The World Health Organization has defined zoonoses as those diseases and infections naturally transmitted between nonhuman vertebrate animals and humans (7), and emerging zoonotic disease as a "zoonosis that is newly recognized or newly evolved or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range" (8). Strikingly, 75% of emerging infectious diseases have been identified as zoonotic in origin (9).

All of the following factors have been identified as risk factors for the emergence of zoonotic diseases: international travel; global trade; increasing interactions among humans, wildlife, and exotic and domesticated food and companion animals; human behavior; rapid microbial adaptation; changing climates and ecosystems; and changing livestock management methods (10). Gaining a better understanding of zoonotic disease emergence, prevention, and control requires quality basic and applied research, which results from extensive interaction and collaboration among professionals from multiple disciplines. These disciplines should include ecology; entomology; occupational medicine; pathology; animal and human behavioral science; epidemiology; biostatistics; economics; clinical veterinary and human medicine; human and veterinary public health; environmental health; and regulatory, wildlife, and agricultural sciences.

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Emerging Infectious Diseases was established to promote the recognition of new and reemerging infectious diseases around the world and to improve the understanding of factors involved in disease emergence, prevention, and elimination. It is appropriate, therefore, that an entire issue of this journal be devoted to the topic of emerging and reemerging zoonotic diseases. This issue features articles from multiple countries that encompass a wide range of diseases and disease agents, including tularemia, Nipah virus, prion diseases, West Nile virus, cryptosporidiosis, hantavirus, bartonellosis, salmonellosis, parastrongylus, and lyssavirus. Multiple species are involved in transmission (e.g., wildlife, companion animals, fish, and amphibians) and a myriad of human behavioral risk factors (e.g., pet ownership, contact between pets and wildlife, direct contact with farm animals or wildlife) for these diseases.

The artwork featured on the cover of this December issue emphasizes the theme of humans living in harmony with animals. We hope this theme issue promotes greater awareness among our readers of the strong link between human and animal health and underscores the importance of establishing new partnerships between human and animal health, agricultural, natural resource, environmental, and other sectors to truly achieve a "Peaceable Kingdom."

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Wildlife as Source of Zoonotic Infections

Hilde Kruse,* Anne-Mette Kirkemo,* and Kjell Handeland*

Zoonoses with a wildlife reservoir represent a major public health problem, affecting all continents. Hundreds of pathogens and many different transmission modes are involved, and many factors influence the epidemiology of the various zoonoses. The importance and recognition of wildlife as a reservoir of zoonoses are increasing. Cost-effective prevention and control of these zoonoses necessitate an interdisciplinary and holistic approach and international cooperation. Surveillance, laboratory capability, research, training and education, and communication are key elements.

Throughout history, wildlife has been an important source of infectious diseases transmissible to humans. Today, zoonoses with a wildlife reservoir constitute a major public health problem, affecting all continents. The importance of such zoonoses is increasingly recognized, and the need for more attention in this area is being addressed.

Wildlife is normally defined as free-roaming animals (mammals, birds, fish, reptiles, and amphibians), whereas a zoonosis is an infectious disease transmittable between animals and humans. The total number of zoonoses is unknown, but according to Taylor et al. (1), who in 2001 catalogued 1,415 known human pathogens, 62% were of zoonotic origin. With time, more and more human pathogens are found to be of animal origin. Moreover, most emerging infectious diseases in humans are zoonoses. Wild animals seem to be involved in the epidemiology of most zoonoses and serve as major reservoirs for transmission of zoonotic agents to domestic animals and humans.

Zoonoses with a wildlife reservoir are typically caused by various bacteria, viruses, and parasites, whereas fungi are of negligible importance. Regarding prion diseases, chronic wasting disease occurs among deer in North America. This prion disease is thus far not known to be zoonotic. However, hunters and consumers are advised to take precautions (2,3).

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Historical Aspects

Zoonoses have affected human health throughout times, and wildlife has always played a role. For example, bubonic plague, a bacterial disease for which rats and fleas play a central role in transmission, has caused substantial illness and death around the world since ancient times (4). A possible epidemic of bubonic plague was described in the Old Testament, in the First Book of Samuel. The so-called Black Death emerged in the 14th century and caused vast losses throughout Asia, Africa, and Europe. The epidemic, which originated in the Far East, killed approximately one third of Europe's population. However, bubonic plague still occurs in Asia, Africa, and the Americas, and the World Health Organization annually reports 1,000–3,000 cases. In the western United States, acquisition of plague in humans is linked to companion animals infested with *Yersinia pestis*–carrying fleas in areas of endemic sylvatic disease (5).

Rabies was described in Mesopotamia, in hunting dogs, as early as 2,300 BC. Recognizable descriptions of rabies can also be traced back to early Chinese, Egyptian, Greek, and Roman records (6). In Europe in the medieval age, rabies occurred in both domestic animals and wildlife. Rabid foxes, wolves, badgers, and bears have been described in the literature as well as in figurative art.

Ancient accounts and modern hypotheses suggest that Alexander the Great, who died in Babylon in 323 BC, died of encephalitis caused by West Nile virus (7), a virus that has a wild bird reservoir. Marr and Calisher reported that as Alexander entered Babylon, a flock of ravens exhibiting unusual behavior died at his feet (7). In 1999, West Nile virus was introduced into the United States, where it caused the ongoing epizootic in birds with a spillover of infections to humans and equines.

Transmission Modes

Zoonoses with a wildlife reservoir represent a large spectrum of transmission modes. Several zoonotic agents can be directly transmitted from wildlife to humans, e.g., *Francisella tularensis*, the causative agent of tularemia, can be transmitted by skin contact with an infested,

diseased, or dead hare or rodent. By contrast, rabies virus is transmitted by bite (saliva) from a rabid animal. Hantaviruses are spread from rodents to humans by aerosols in dust from rodent excreta. Zoonotic agents can also be spread from wildlife to humans indirectly by contaminated food and water, for example *Salmonella* spp. and *Leptospira* spp.

Many zoonoses with a wildlife origin are spread through insect vectors. For example, mosquitoes are well-known vectors of several wildlife zoonoses, such as Rift Valley fever, equine encephalitis, and Japanese encephalitis. *Y. pestis* can be spread by fleas, *Bacillus anthracis* spores by flies, and *Leishmania* by sand-flies, whereas ticks are essential in the spread of *Borrelia burgdorferi* and *Ehrlichia/Anaplasma*.

A good example of a zoonotic agent with many different transmission modes is *F. tularensis*. Rodents and hares constitute the main sources of infections, and hunters are at particular risk of acquiring the disease. The transmission mode also affects the clinical manifestation in humans. The agent can be transmitted by direct contact through the handling of an infected carcass and through tick or mosquito bites, which cause initial skin symptoms such as ulcers. Infection may also occur after eating insufficiently cooked meat from an infected animal or contaminated drinking water, causing symptoms from the digestive tract, and by inhalation of contaminated dust, causing a pneumonialike illness.

Salmonella spp. can also be spread from wildlife to humans in different ways. Reptile-associated salmonellosis is a well-described phenomenon, especially among children. The increasing popularity of keeping reptiles and other exotic animals as pets presents a public health problem, as such animals are commonly carriers of *Salmonella* and thereby can infect humans directly or indirectly. In Norway, special types of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) occur endemically in hedgehogs and wild passerine birds, causing sporadic cases and small outbreaks in humans. In 1987, a nationwide outbreak of *S.* Typhimurium infections was traced to chocolate bars that had been contaminated by wild birds in the factory. In 1999, a waterborne outbreak of *S.* Typhimurium infections was linked to a dead seagull that had contaminated a reservoir water source from which the water was used untreated (8–10).

B. anthracis, the etiologic agent of anthrax, primarily a disease of herbivores, can also be transmitted from wildlife to humans by various modes. The spores formed by the bacteria are very resistant and have been found to remain dormant and viable in nature for >100 years (11). Anthrax is spread by food and water contamination or by the spread of spores by flies, vultures, and other scavengers. Humans can be infected by eating meat from infected carcasses or drinking contaminated water, through the skin by contact with infected material or by insect bites, and through the lungs by inhaling spores. Although livestock anthrax is declining in many parts of the world, the disease remains enzootic in many national parks, for example, in southern Africa and North America. Anthrax in wildlife represents a persistent risk for surrounding livestock and public health (12).

Factors Influencing the Epidemiology of Zoonoses with a Wildlife Reservoir

The ecologic changes influencing the epidemiology of zoonoses with a wildlife reservoir can be of natural or anthropogenic origin. These include, but are not limited to, human population expansion and encroachment, reforestation and other habitat changes, pollution, and climatic changes.

The spirochete *Borrelia burgdorferi*, which causes Lyme borreliosis, has its main reservoir among small rodents and deer and uses various *Ixodes* species as vectors (13). Lyme borreliosis was first recognized in Lyme, Connecticut, in 1975, and since then, an increasing number of cases have been reported in North America, Europe, and Asia. The increasing incidence of Lyme borreliosis in the northeastern United States in recent years can be explained by reforestation that has favored transmission of the disease through increased populations of white-tailed deer and deer mice and abundance of the tick vector, *Ixodes scapularis*.

Wild rodents also constitute a reservoir of hantaviruses (14). The viruses are shed in urine, droppings, and saliva, and humans are mainly infected aerogenically by inhaling aerosols containing the virus. Precipitation, habitat structure, and food availability are critical environmental factors that affect rodent population dynamics as well as viral transmission between animals and subsequently the incidence of human infection. The deer mouse is a reservoir host for Sin Nombre hantavirus, which causes hantavirus pulmonary syndrome in the southwestern United States. Because of climatic changes with increased rainfall in recent years, host abundance, and thereby spread of the pathogen, has increased, with subsequent transmission to humans.

The movement of pathogens, vectors, and animal hosts is another factor influencing the epidemiology of zoonoses with a wildlife reservoir. Such movement can, for example, occur through human travel and trade, by natural movement of wild animals including migratory birds, and by anthropogenic movement of animals. For instance, infectious agents harbored within insects, animals, or humans can travel halfway around the globe in <24 hours in airplanes. Thus, infectious agents can be transported to the farthest land in less time than it takes most diseases to

incubate. The appearance of West Nile virus infection in New York in 1999, and the subsequent spread within the United States, is an example of introduction and establishment of a pathogen that apparently originated in the Middle East (15).

Movement of infected wild and domestic animals is an important factor in the appearance of rabies in new locations. Rabies virus, which is widely distributed and affects various animals, especially canids, was introduced into North America by infected dogs in the early 18th century, with subsequent spillover to a variety of wild terrestrial mammals. Rabies became established in raccoons in the mid-Atlantic states in the late 1970s when raccoons were translocated from the southeastern United States, where rabies was endemic in this species (16). Finland experienced an outbreak of rabies linked to raccoon dogs in 1988. The raccoon dog had spread to Finland after this species was released in western Russia for fur trade. Rabies most probably arrived in Finland by wolves migrating from Russia during wintertime along the ice-packed coast (17). In the Arctic, the ice links the continents together. The movement of the arctic fox from the archipelago of Spitzbergen to Novaja Zemlja in Siberia and from Canada to Greenland has been described, indicating another way that rabies can be spread to new areas (18,19).

Bovine tuberculosis caused by *Mycobacterium bovis* is another zoonosis in which both natural and anthropogenic movement of animals has influenced the epidemiology. This zoonosis is emerging in wildlife in many parts of the world, and wildlife can represent a source of infection for domestic animals and humans. Bovine tuberculosis was probably introduced into Africa with imported cattle during the colonial era and thereafter spread to and became endemic in wildlife (20). In Ireland and Great Britain, badgers maintain the infection, whereas the brushtail possum constitutes a main wildlife reservoir in New Zealand. In parts of Michigan, bovine tuberculosis is endemic among white-tailed deer, whereas in Europe, both wild boars and various deer species can be a reservoir of the pathogen. The natural movement of these reservoir animals increases the spread of the disease to domestic animals and thereby its public health impact (21).

The epidemiology of multilocular echinococcosis, caused by the small tapeworm *Echinococcus multilocularis*, has also been influenced by the translocation of animals. The main hosts are canids, especially foxes; the intermediate hosts are small rodents. Humans can become accidental intermediate hosts, by ingesting eggs. Multilocular echinococcosis occurs in large parts of the Northern Hemisphere. In 1999, *E. multilocularis* was detected for the first time in Norway, in the archipelago of Spitzbergen (10,22). The parasite most probably spread from Russia, by natural movement of the main host, the Arctic fox. Establishment of the parasite was possible because the intermediate host, the sibling vole, had previously been translocated to Spitzbergen, most likely through imported animal feed (23). In Copenhagen, Denmark, in 2000, *E. multilocularis* was detected in a traffic-killed red fox. The theory is that the fox had traveled by train from central Europe, where the disease is endemic (H.C. Wegener, pers. comm.).

Figure 1. Foxes may be a reservoir of zoonotic agents such as rabies virus and the parasite *Echinococcus multilocularis*.

During the summer of 2003, an outbreak of monkeypox occurred in the United States with 37 confirmed human cases (24). Monkeypox is a rare zoonosis caused by a poxvirus that typically occurs in Africa. It was first found in monkeys in 1958 and later on in other animals, especially rodents. The African squirrel is probably the natural host. Transmission to humans occurs by contact with infected animals or body fluids. The cases in the United States, the first outside Africa, were associated with contact with infected prairie dogs. The outbreak was epidemiologically linked to imported African rodents from Ghana. Most likely, infected imported rodents have transmitted the virus to prairie dogs in United States. This transmission illustrates how non-native animal species can create serious public health problems when they introduce a disease to native animal and human populations. Thus, the transportation, sale, or distribution of animals, or the release of animals into the environment, can represent a risk for spread of zoonoses.

Microbial changes or adaptation also influence the epidemiology of zoonoses with a wildlife reservoir. These changes include mutations, such as genetic drift in viruses; activation and silencing of genes; genetic recombinations, such as genetic shift in viruses; and conjugation, transformation, and transduction in bacteria. Natural selection and evolution also play a role. Transmission of adaptive or genetically changed microorganisms from wildlife to humans, either directly or indirectly through domestic animals, may occur in many ways. In this respect an interna-

Figure 2. The pathologic role of marine *Brucella* spp. in animals, such as pinnipedes, remains unclear, as does their zoonotic potential.

tional wildlife trade, often illegal, in which wild animals end up in live-animal markets, restaurants, and farms, is important because such practices increase the proximity between wildlife, domestic animals, and humans (25).

Severe acute respiratory syndrome (SARS) is a current example of likely microbial adaptation. This viral respiratory illness, caused by SARS-associated coronavirus, is believed to have emerged in Guangdong, China, in November 2002. SARS was first reported in Asia in February 2003, and over the next few months, the illness spread to a global epidemic before it was contained. According to the World Health Organization, 8,098 cases, including 774 fatalities, have occurred. The virus has an unknown reservoir, but wildlife is a likely source of infection. Natural infection has been demonstrated in palm civet cats in markets and also in raccoon dogs, rats, and other animals indigenous to the area where SARS likely originated (26).

Genetic changes typically influence the epidemiology of influenza A. Natural infections with influenza A viruses have been reported in a variety of animal species, including birds, humans, pigs, horses, and sea mammals, and its main reservoir seems to be wild waterfowl, especially ducks. Influenza A virus has two main surface antigens; hemagglutinin with 15 subtypes and neuraminidase with 9 subtypes. All these subtypes, in most combinations, have been isolated from birds, whereas few combinations have been found in mammals. In the 20th century, the sudden emergence of antigenically different strains transmissible in humans, termed antigenic shift, has occurred on four occasions, each time resulting in a pandemic. "New" pandemic strains most certainly emerged after reassortment of genes of viruses of avian and human origin in a permissive host (27). The H5N1 strain of a highly pathogenic avian influenza that caused a severe outbreak in poultry in Southeast Asia in 2004 (28) demonstrated its capacity to infect humans; 39 cases, 28 of them fatal, were officially reported (29). For the human population as a whole, the main danger appears to be simultaneous infection with an avian and a human influenza virus. Reassortment could then occur either in humans or in pigs with the potential emergence of a virus fully capable of spread among humans but with antigenic characteristics for which the human population was immunologically naïve.

Enhanced recognition can also result in an apparent change in the epidemiology of a zoonosis, for example, the recognition of an agent that has been present for a long time but was previously undetected because of lack of diagnostic tools. Improved methods for molecular characterization have helped describe a larger repertoire of zoonotic agents.

Recognition and emergence of human tickborne ehrlichiosis are recent and continuing events, beginning with human monocytic ehrlichiosis and human granulocytic ehrlichiosis, reported first in the United States in 1987 and 1994, respectively. The causative agents, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, are intracellular bacteria that are maintained in zoonotic cycles involving persistently infected deer and rodents (30).

From 1994 to 2004, three zoonotic paramyxoviruses with a wildlife reservoir have emerged. The Hendra, Menangle, and Nipah viruses all have a fruit bat reservoir (31). Humans are infected by close contact with infected pigs or horses. Hendra virus infection was described in Australia in 1994, where it caused acute, fatal respiratory disease in horses and humans. Menangle virus was also described in Australia, in 1996, where it caused reproductive disorders in pigs and an influenzalike disease in humans. Nipah virus was detected in 1998, in Malaysia, when it caused severe disease with respiratory and neurologic symptoms among pigs and encephalitis with a 40% death rate in humans in close contact with pigs.

Since 1994, when the isolation of *Brucella* spp. from marine mammals was reported for the first time, such infections have been detected in a wide range of marine mammal species and populations. The pathologic role of marine *Brucella* spp. in animals remains unclear, as does their zoonotic potential. In 2003, two human cases of community-acquired granulomatous central nervous system infections caused by marine *Brucella* spp. were reported (32).

Human behavior and demographic factors can also influence the epidemiology of zoonoses with a wildlife reservoir. Hiking, camping, and hunting are activities that may represent risk factors for acquiring certain zoonoses with a wildlife reservoir, e.g., tickborne zoonoses and tularemia. Eating habits can also play a role. For example, eating meat from exotic animals such as bear increases the risk of acquiring trichinellosis (33). AIDS represents a

disease in which demographic factors and human behavior have contributed to its development into a global public health problem. The origin of HIV, the virus causing AIDS, is still a matter of controversy, but HIV likely spread to humans from nonhuman primates in West Africa (34).

Prevention and Control

Although prevention and control strategies for the various zoonoses associated with wildlife share many common aspects, specific strategies are also needed to address the etiology and epidemiology of the disease, characteristics of the pathogen involved, ecologic factors, and the population at risk. As wildlife is an essential component in the epidemiology of many, if not most, zoonoses, wildlife should be taken into account in the risk analysis framework. Consequently, cost-effective prevention and control of zoonoses in humans, including risk communication, necessitate an interdisciplinary and holistic approach that acknowledges the importance of wildlife as a reservoir.

To increase the capability of recognizing zoonoses with a wildlife reservoir, better national surveillance systems for humans and animals are needed, as well as better international integration and sharing of information from such systems. Which diseases should be reportable also needs to be evaluated on a continuous basis. Improved reporting systems and screening programs for human infections, including the application of syndromic surveillance, are warranted to detect new and emerging zoonoses. Efficient surveillance is dependent upon a laboratory system that is capable of identifying and characterizing the pathogens in question. More research is needed to better understand the epidemiology and pathogenesis of various zoonoses, to improve diagnostic methods, and to develop cost-effective vaccines and drugs. Training and education are prerequisites to enable the personnel involved at the various stages, from field to laboratory personnel, to detect zoonoses, both new and old.

Information and communication are key components in any prevention and control strategy. Public education and behavioral change are also important factors for successful intervention. Implementing restrictions on anthropogenic animal movement is another important preventive measure. For vector-borne zoonoses, vector control should be an integral part of any intervention strategy.

Interdisciplinary and international collaboration is necessary for the rapid identification and effective management of outbreaks of zoonoses. The pivotal role of international organizations such as World Health Organization and Office International des Epizooties is becoming clearer, exemplified by the 2004 avian influenza outbreak in Southeast Asia. Containing zoonoses with a wildlife reservoir relies on efficient national, regional, and international cross-sectional networks that can improve data sharing and thereby alertness and the timely and effective response to disease outbreaks.

Dr. Kruse is the head of the Norwegian Zoonoses Centre and a deputy director at the National Veterinary Institute, Norway. Her research interests include the epidemiology of zoonotic diseases and antimicrobial resistance.

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Potential Mammalian Filovirus Reservoirs

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Ebola and Marburg viruses are maintained in unknown reservoir species; spillover into human populations results in occasional human cases or epidemics. We attempted to narrow the list of possibilities regarding the identity of those reservoir species. We made a series of explicit assumptions about the reservoir: it is a mammal; it supports persistent, largely asymptomatic filovirus infections; its range subsumes that of its associated filovirus; it has coevolved with the virus; it is of small body size; and it is not a species that is commensal with humans. Under these assumptions, we developed priority lists of mammal clades that coincide distributionally with filovirus outbreak distributions and compared these lists with those mammal taxa that have been tested for filovirus infection in previous epidemiologic studies. Studying the remainder of these taxa may be a fruitful avenue for pursuing the identity of natural reservoirs of filoviruses.

The virus family *Filoviridae* has been known since 1967, when Marburg virus caused an outbreak of hemorrhagic disease associated with exposure to primates imported into Germany; Marburg and Ebola viruses were subsequently the cause of isolated cases or epidemics of hemorrhagic fever in humans or nonhuman primates across Africa (1–3) and in parts of southeast Asia (4), and in outbreaks among nonhuman primates in North America and Europe that resulted from importation of infected primates (5). Despite numerous epidemiologic analyses of the disease (6–8), laboratory tests of effects of infection on potential hosts (9), and searches for natural virus infections among animals in localities where outbreaks have occurred (10–12), the source of these viruses in nature has remained obscure.

This article is the second step in an effort to marshal a new set of tools and approaches, designed to increase the likelihood of detecting the natural reservoirs of filoviruses. We define the reservoir that we are seeking as a set of populations or species of animal or plant that sustains the pool of virus from which infections in primates have sprung. An earlier article described the large-scale ecology and geographic distribution (Figure 1) of filovirus disease occurrences (13). Here, we attempt to identify the clade that constitutes the reservoir hosts of filoviruses that have caused disease in humans and in nonhuman primates. We use a series of biologic inferences regarding host-parasite interactions and make explicit assumptions to arrive at a much-reduced list of potential reservoir taxa. This approach aims to identify taxa that, under explicit assumptions, have a higher probability of constituting the reservoirs of these viruses. These lists can be used to focus future sampling and testing of potential reservoir taxa.

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Rationale

The challenge of identifying the reservoirs of Ebola and Marburg viruses is complex. As previous authors (7,14) have emphasized, such a search is difficult because of the unpredictable nature of virus population dynamics in wild hosts. The challenge is also made larger because of the staggering biodiversity of tropical Africa: the array of potential hosts is immense. Subject to assumptions made explicit to the extent possible, the following rationale is an attempt to provide a scientific basis for narrowing the list of possibilities. Although any element of this list of assumptions could prove to be incorrect, the assumptions are explicit, making it possible to consider their effects.

Mammalian Reservoir

The reservoir for filoviruses has been variously hypothesized to be a mammal, some other vertebrate, an arthropod, or even a plant (7,14). Recent evidence of a relationship between filoviruses and avian retroviruses (15,16) is intriguing, leading to the question of which major taxon is the most likely candidate for the filovirus reservoirs. No conclusive evidence based on a sufficiently broad survey exists for any of these groups as a filovirus reservoir. What little evidence exists, however, suggests that mammals may constitute an excellent first candidate for detailed consideration: 1) results of efforts to infect plants and arthropods with filoviruses have been negative

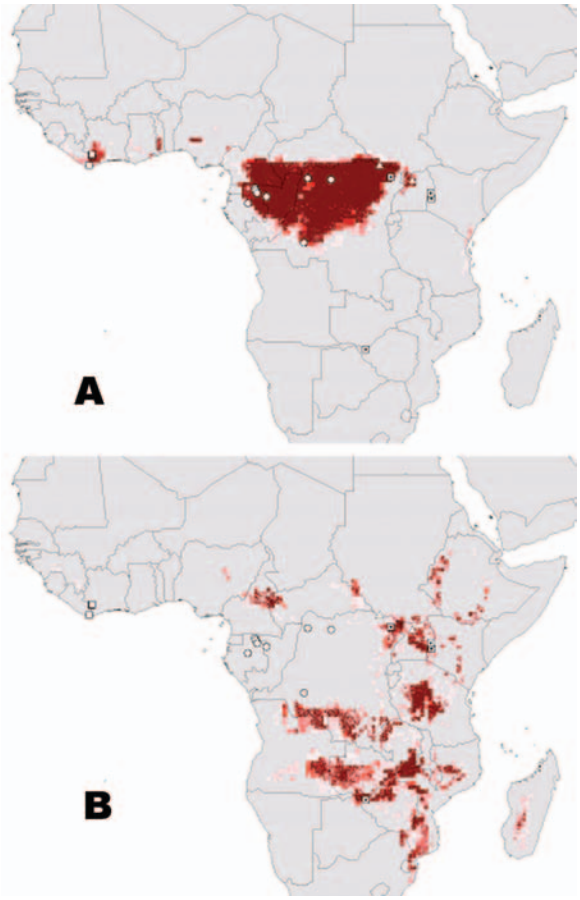

Figure 1. Distributional summary of A) Ebola and B) Marburg viruses, with predicted distributions based on ecologic niche models of outbreak coordinates (13). Darker shades of red represent increasing confidence in prediction of potential presence. Disease outbreaks attributed to various filovirus species are represented as follows: open square, Ebola Ivory Coast; open circle, Ebola Zaire; open triangle, Ebola Sudan; dotted square, Marburg.

(9); 2) small mammals (particularly bats) can sustain infections and even amplify virus (9); 3) certain small mammal species may have encountered filoviruses in the course of their evolutionary history (17); and 4) bats and other small mammals are known to serve as reservoirs for other viruses (18). Hence, a first assumption of this article, or a first step in the application of this approach to the challenge of detecting filovirus reservoirs, is to focus on mammals as candidate taxa.

Persistent, Largely Asymptomatic Filovirus Infections Associated with Reservoir

Ample theoretical bases exist for the idea that in most cases a "good parasite" will evolve toward avirulence among members of a reservoir taxon with which it has a long-term evolutionary relationship (19), although some recent discussions suggest the contrary (20). Garnet and Antia (21) demonstrated that such coevolution results in a trade-off between host death and probability of virus transmission. Empiric evidence from studies of other viral hemorrhagic fever reservoirs provides support for the idea of evolved avirulence: the best example is the growing body of information regarding coevolution of hantaviruses and arenaviruses and their rodent hosts (22). In these systems, long-term associations have apparently resulted in, or at least maintained, typically asymptomatic infections of hantaviruses and arenaviruses in host rodents (23).

This line of reasoning suggests that the reservoir taxa will not likely include species such as the vervet monkey (*Cercopithecus aethiops*) or other primates known to experience high death rates after filovirus infections because the virus kills so quickly and efficiently that maintenance in such taxa is not likely (7). Hence, a second premise of this analysis is that a reservoir taxon should exist that has minimal negative effects of filovirus infection. For this reason, we eliminate primates from consideration since laboratory filovirus infection is known to result in fatal disease in both African and New World primates (24,25).

Virus Restricted to Range of Reservoir

The range of each filovirus and the disease it causes is assumed to be restricted to the distributional range of the reservoir taxon. If the viruses exist in a natural reservoir, then their occurrence outside of the distribution of that reservoir taxon would be only fleeting, as long-term maintenance is not feasible.

Coevolution of Filoviruses and Their Hosts

The phylogenetic structure of filovirus species has fairly clear geographic patterns (26,27). The distinct filoviruses have likely coevolved with their specific host species, implying that each virus is likely associated with a distinct host species. Therefore, we consider as unlikely reservoirs those mammal species with distributions that overlap the combined distribution of all filoviruses, although we use caution in eliminating some groups, given taxonomic arrangements in need of modern revision. However, given the preceding assumptions, reservoir species likely belong to a single genus or subfamily, with members occurring throughout the entire range of filoviruses.

Other recently described host-virus systems among small mammals have followed this pattern, especially among the hemorrhagic fever viruses. Several rodent species in the family Muridae, for example, serve as hosts for hantaviruses and arenaviruses, and patterns of cospeciation are clear when phylogenies of hosts and viruses are compared (28,29). A similar pattern may be developing for paramyxoviruses (henipaviruses), whose hosts are pteropodid bats in the southwest Pacific (30). We thus assume that a single, monophyletic group (genus or

subfamily) of reservoir taxa associated with the filovirus lineages should exist (either for filoviruses as a whole or for the Ebola viruses and Marburg virus separately). Because the phylogenetic distance between Marburg virus and the Ebola viruses is much greater than the distance among the Ebola viruses, we provide separate reservoir candidate lists for Marburg virus, to allow for the possibility that the reservoir for Marburg virus falls within a separate reservoir taxon.

Small Body Size of Reservoir Species

We further, if provisionally, eliminate from consideration those species of large body size. We base this assumption on two facts: large-bodied species would be eaten frequently by local people, and transmission to humans would likely be more frequent; also, contact with, or killing of, such a large animal would likely be memorable enough that either the animal would have been brought home or comment would have been made of it. For example, cases acquired by contact with infected chimps were quickly characterized (2,3). On this basis, we eliminated from consideration a variety of taxa, using an approximate cut-off of raccoon (*Procyon lotor*) size, including ungulates, Manidae, Felidae, and others.

Reservoir Not a Commensal Species

Human filovirus infection index patients, when detailed information is available, have most frequently been men who work in the field, particularly in forests, excavations, caves, or mines (8,31,32). Commensal species (e.g., *Mus musculus*, *Rattus* spp.), on the other hand, might be expected to come into contact with persons working around the home. For this reason, we omit from consideration species known to be commensals with human.

Methods

We used the following procedure and the above rationale to narrow the list of potential reservoir species for filoviruses. First, we reviewed the mammal species of the world, following the taxonomy and known distributions as summarized in Wilson and Reeder (33). Each species was tallied as to its approximate co-occurrence with distributions (13) of each filovirus (Ebola Ivory Coast in West Africa, Ebola Zaire in central Africa, and Ebola Sudan and Marburg in East Africa), as well as Ebola Reston, for which we used the entirety of the Philippines, given uncertainty as to the virus's geographic origins. Because of general geographic coincidence between Ebola Sudan and Marburg outbreaks, these two distributional areas were considered equivalent for the purpose of this first-pass, coarse-scale review.

Next, to the limits of the resolution of knowledge of phylogeny of mammals of Africa and Asia, we sought clades with geographic distributions that coincided with those of the African filoviruses. In particular, we identified genera and subfamilies with component taxa distributed in all of the filovirus distributional areas. Here, although individual species were often endemic to small areas, we detected genera or subfamilies (ostensibly monophyletic clades) with distributions that cover the entire distributional area of African filoviruses. We then noted which of these clades either also include species distributed in the Philippines, or for which related clades (i.e., same subfamily or same family) are present in the Philippines.

Finally, we reduced our genus and subfamily level lists in several ways on the basis of the assumptions outlined above. We removed clades with species known to experience high death rates from exposure to filovirus infection (e.g., primates). We removed clades with species that are frequently hunted for food or that have large body size (larger than raccoons). We removed genera for which all species are commonly commensal with humans (e.g., *Mus*). For Ebola virus, we removed species that occur exclusively in savannah habitats because Ebola disease outbreaks have occurred only in forested or ecotonal habitats (13); savannah-living genera were not eliminated from consideration in the lists for taxa coinciding with Marburg disease occurrences. Lists were developed for the *Filoviridae* in general, as well as for Marburg virus only; again, at the crude geographic scale of this review, owing to rough distributional coincidence between Ebola Sudan and Marburg virus outbreaks, Ebola virus distributions are more or less coincident with those of the entire family, and so the two are considered together at this point.

To assess how these lists based on coarse-scale biogeography relate to those taxa tested in epidemiologic studies to date, we reviewed all studies known to us that have involved testing of wild African mammals for filoviruses (10,11,34,35), including recent unpublished analyses (R. Swanepoel and D. Carroll, unpub. data). These lists were organized in spreadsheets, and queries were developed to establish the degree to which such studies have assessed priority taxa.

Results

Thirty-eight genera were encountered that include species with distributions coinciding with those of all African filoviruses (Table 1). Seven of these genera, containing species of large body size, were eliminated from further consideration (*Panthera*, *Helogale*, *Herpestes*, *Aonyx*, *Genetta*, *Phacochoerus*, *Manis*). Of the remaining 31 genera, 10 either include species occurring in the Philippines or are in the same subfamily as genera occurring in the Philippines.

Considering coincidence of generic distributions with only Marburg hemorrhagic fever occurrences (Table 2), an

Table 1. Genera for which component species coincide distributionally (coarse-scale) with distributions of disease outbreaks associated with all four known African filoviruses[a]

Genus[b]	Species
Insectivora: Soricidae (shrews)	
Sylvisorex+	*johnstoni*, *megalura*
Crocidura[c]	**Many species**
Suncus[c]	*etruscus*, *infinitesimus*, *lixus*, *remyi*, *varilla*
Chiroptera: Pteropodidae (fruit bats)	
Epomophorus+	***labiatus***, *minimus*
Epomops+	*buettikoferi*, *dobsoni*, ***franqueti***
Rousettus[c]	*aegyptiacus*, *angolensis*, *lanosus*
Chiroptera: Emballonuridae (sac-winged bats)	
Taphozous[c]	*hamiltoni*, *hildegardeae*, ***mauritianus***, *nudiventris*
Chiroptera: Nycteridae (slit-faced bats)	
Nycteris–	*arge*, *gambiensis*, *grandis*, ***hispida***, *intermedia*, *macrotis*, *major*, *nana*
Chiroptera: Rhinolophidae (horseshoe bats)	
Rhinolophus[c]	*adami*, *alcyone*, *deckenii*, *denti*, *eloquens*, *fumigatus*, *guineensis*, *hildebrandti*, *landeri*, *maclaudi*, *silvestris*, *simulator*
Hipposideros[c]	*beatus*, *caffer*, *camerunensis*, *commersoni*, ***cyclops***, *fuliginosus*, *jonesi*, *lamottei*, *marisae*, ***ruber***
Chiroptera: Vespertilionidae (vesper bats)	
Kerivoula[c]	*argentata*, *cuprosa*, *lanosa*, *phalaena*
***Chalinolobus*+**	*alboguttatus*, *argentatus*, *beatrix*, *egeria*, *gleni*, *poensis*, *superbus*, *variegatus*
Eptesicus+	*brunneus*, *capensis*, *guineensis*, *hottentotus*, *platyops*, *rendalli*, ***somalicus***, ***tenuipinnis***
Myotis[c]	***bocagei***, *tricolor*
Pipistrellus[c]	*crassulus*, *eisentrauti*, *inexspectatus*, *musciculus*, *nanulus*, ***nanus***, *rusticus*
Scotoecus+	*albofuscus*, ***hirundo***
Scotophilus[c]	***dinganii***, *nigrita*, *nux*, *robustus*, *viridis*
Miniopterus[c]	*inflatus*, ***minor***, *schreibersi*
Chiroptera: Molossidae (free-tailed bats)	
Chaerephon[c]	*aloysiisabaudiae*, ***ansorgei***, *bemmeleni*, *bivittata*, *chapini*, *gallagheri*, ***major***, *nigeriae*, ***pumila***, *russata*
Mops+	*brachypterus*, ***condylurus***, ***congicus***, *demonstrator*, *midas*, ***nanulus***, *niangarae*, ***niveiventer***, *spurrelli*, ***thersites***, ***trevori***
Myopterus+	*daubentonii*, ***whitleyi***
Tadarida+	*aegyptiaca*, *fulminans*
Rodentia: Sciuridae (squirrels)	
Funisciurus+	***anerythrus***, *bayonii*, *carruthersi*, ***congicus***, ***isabella***, ***lemniscatus***, *leucogenys*, ***pyrropus***, *substriatus*
Heliosciurus+	*gambianus*, *mutabilis*, *punctatus*, ***rufobrachium***, *ruwenzorii*, *undulatus*
Protoxerus+	*aubinnii*, ***stangeri***
Rodentia: Muridae (mice and rats)	
Dendromus–	*insignis*, *kahuziensis*, *kivu*, *melanotis*, ***mesomelas***, *messorius*, ***mystacalis***, *nyikae*
Grammomys+	***aridulus***, *buntingi*, *caniceps*, ***dolichurus***, *dryas*, *gigas*, *ibeanus*, *macmillani*, ***rutilans***
Lemniscomys+	*barbarus*, *bellieri*, *hoogstraali*, *linulus*, *macculus*, *rosalia*, ***striatus***
Lophuromys+	*cinereus*, ***flavopunctatus***, *luteogaster*, *medicaudatus*, *nudicaudus*, *rahmi*, ***sikapusi***, *woosnami*
Malacomys+	*cansdalei*, *edwardsi*, *longipes*, ***lukolelae***, *verschureni*
Rodentia: Anomaluridae (scaly-tailed squirrels)	
Anomalurus	***beecrofti***, ***derbianus***, *pelii*, *pusillus*
Rodentia: Myoxidae (dormice)	
Graphiurus	*christyi*, *crassicaudatus*, *hueti*, *lorraineus*, *microtis*, *murinus*, *parvus*

[a]Component African species are listed (only those coinciding with one or more specific filovirus distributional areas); species that have been tested in epidemiologic studies are indicated in **boldface**.
[b]Genera for which related genera occur in the Philippines are indicated by + for same subfamily and – for same family.
[c]Genera including species occurring in the Philippines.

initial list included 63 genera; 22 of these were omitted because their species had a large body size or were primates (*Perodicticus*, *Galago*, *Gorilla*, *Leptailurus*, *Atilax*, *Dologale*, *Mungos*, *Crocuta*, *Lutra*, *Civettictis*, *Ceratotherium*, *Orycteropus*, *Potamochoerus*, *Litocranius*, *Taurotragus*, *Tragelaphus*, *Cephalophus*, *Sylvicapra*, *Oryx*, *Kobus*, *Redunca*, *Manis*). Of the remaining 41 genera, 3 include species occurring in the Philippines, 18 have consubfamilials occurring in the Philippines, and 29 have confamilials occurring in the Philippines.

Table 2. Genera that coincide distributionally with the geographic distribution of disease outbreaks associated with Marburg virus[a]

Genera[b,c]	Species coinciding with Marburg virus
Insectivora: Chrysochloridae (golden moles)	
Chrysochloris	*stuhlmanni*
Insectivora: Soricidae (shrews)	
Suncus[b]	*etruscus, infinitesimus, lixus, varilla*
Chiroptera: Pteropodidae (fruit bats)	
Epomops–	***franqueti**, dobsoni*
Epomophorus–	***gambianus**, grandis, crypturus, **labiatus**, minimus, wahlbergi*
Chiroptera: Emballonuridae (sac-winged bats)	
Saccolaimus[b]	***peli***
Chiroptera: Megadermatidae (false vampire bats)	
Lavia+	*frons*
Chiroptera: Rhinolophidae (horseshoe bats)	
Cloeotis+	*percivali*
Chiroptera: Vespertilionidae (vesper bats)	
Mimetillus+	*moloneyi*
Rodentia: Sciuridae (squirrels)	
Xerus+	*erythropus, inauris, rutilus*
Rodentia: Muridae (mice and rats)	
Acomys+	*kempi, spinosissimus, ignitus, percivali, wilsoni*
Aethomys+	*namaquensis, kaiseri, hindei, chrysophilus*
Arvicanthis+	*niloticus, nairobae*
Beamys–	*hindei, major*
Cricetomys–	***gambianus***
Dasymys+	***incomtus***
Delanymys–	*brooksi*
Deomys–	***ferrugineus***
Gerbillurus–	*paeba*
Hybomys+	***univittatus**, lunaris*
Hylomyscus+	***stella**, denniae*
Lophiomys–	*imhausi*
Mastomys	*erythroleucus, pernanus, hildebrandtii*
Mylomys+	*dybowskii*
Oenomys+	***hypoxanthus***
Otomys–	*anchietae, typus, tropicalis, angoniensis, denti*
Praomys+	*misonnei, **jacksoni***
Rhabdomys+	*pumilio*
Steatomys–	*parvus, **pratensis***
Stochomys+	*longicaudatus*
Tachyoryctes–	*naivashae, annectens, ankoliae, spalacinus, ruddi, ruandae*
Tatera–	*robusta, leucogaster, inclusa, boehmi, nigricauda*
Taterillus–	*harringtoni, emini*
Thallomys+	*loringi, paedulcus*
Uranomys+	*ruddi*
Zelotomys+	*hildegardeae*
Rodentia: Anomaluridae (Scaly-tailed Flying Squirrels)	
Idiurus	*zenkeri*
Rodentia: Pedetidae (springhare)	
Pedetes	*capensis*
Rodentia: Thryonomyidae (cane rats)	
Thryonomys	***swinderianus**, gregorianus*
Lagomorpha: Leporidae (rabbits and hares)	
Poelagus	*marjorita*
Macroscelidea: Macroscelididae (elephant shrews)	
Petrodromus	***tetradactylus***
Rhynchocyon	*chrysopygus, **cirnei**, petersi*

[a]Genera that have been tested in epidemiologic studies are indicated in **boldface**.
[b]Genera, including species occurring in the Philippines.
[c]Genera for which related genera occur in the Philippines are indicated by +, same subfamily; or –,same family.

Table 3. Subfamilial- or familial-level taxa that hold genera with distributions that coincide (coarse-scale) with distributions of disease outbreaks associated with African filoviruses[a]

Subfamily or family
Coincides with Marburg occurrences only
Chrysochloridae
Otomyinae
Petromyscinae
Rhizomyinae
Lophiomyinae
Pedetidae
Bathyergidae
Macroscelididae
Coincides with African filovirus occurrences
Crocidurinae[b]
Potamogalinae
Pteropodinae[b]
Macroglossinae[b]
Emballonuridae[b]
Megadermatidae[b]
Rhinolophinae[b]
Hipposiderinae[b]
Kerivoulinae[b]
Vespertilioninae[b]
Miniopterinae[b]
Molossidae[b]
Nycteridae
Procaviidae
Sciurinae[b]
Murinae[b]
Cricetomyinae
Dendromurinae
Gerbillinae
Anomalurinae
Zenkerellinae
Graphiurinae
Thryonomyidae
Leporidae

[a]Taxa including species that have been tested are indicated in **boldface**.
[b]Genera including species occurring in the Philippines.

Finally, we considered clades recognized at the subfamilial (or familial, if no subfamilies were recognized; Table 3) level that coincided with all African filovirus occurrences or Marburg occurrences only. Of these 65 clades, we eliminated 33 because their members had large body size or were primates (Loridae, Galagonidae, Cercopithecinae, Colobinae, Hominidae, Canidae, Acinonychinae, Felinae, Pantherinae, Herpestinae, Hyaeninae, Protelinae, Lutrinae, Mellivorinae, Mustelinae, Nandiniinae, Viverrinae, Elephantidae, Rhinocerotidae, Orycteropodidae, Aepycerotinae, Alcelaphinae, Antilopinae, Bovinae, Cephalophinae, Hippotraginae, Reduncinae, Phacochoerinae, Suinae, Hippopotamidae, Tragulidae, Giraffidae, Manidae). Of the 32 remaining subfamilies, 8 coincided with Marburg virus occurrences only, and 11 with all African filovirus or all African Ebola virus disease occurrences; 13 not only coincided with African filovirus disease occurrences but also included distributional areas in the Philippines (Table 3).

Of the 134 species that have been tested in previous studies (11,12,34,35) (R. Swanepoel and D. Carroll, unpub. data), only 58 are from genera that coincide with African filovirus disease occurrences. Overall, of 4,709 mammals tested, only 2,545 were from clades with distributions coincident at some taxonomic level with that of African filovirus disease outbreaks.

Of the taxa that geographically cooccur with filovirus disease occurrences (Tables 1–3), variable numbers have been tested (Appendix online; available from http://www.cdc.gov/ncidod/EID/vol10no12/04-0346_app.htm): genera coinciding with filovirus disease occurrences, 26 (81.2%) of 32; genera coinciding with Marburg, 14 (40.0%) of 35; subfamilies coinciding with filovirus occurrences, 17 (70.8%) of 24; and subfamilies coinciding with Marburg occurrences, 2 (25%) of 8. Hence, considering the lists presented in Tables 1 to 3, a significant diversity of taxa remains to be tested even a single time. If prevalences are anything other than high, testing greater numbers of mammals from some clades will also be necessary (Figure 2); the list of clades not tested satisfactorily thus becomes quite long.

Discussion

Searches for the filovirus reservoirs have been conducted periodically since filovirus diseases were first recognized. Nevertheless, until the present, very little practical or theoretical information was available to help researchers plan trapping expeditions, choose sites and trapping techniques, or focus on particular species. This analysis should help in these aspects of planning and may reduce the number of hours that researchers spend sampling low-priority habitats or species.

The preliminary, coarse-filter analyses presented here rely on a series of explicit assumptions drawn from past studies of filovirus disease outbreaks and from biologic principles and theory. Regarding the first two assumptions—that reservoirs exist and that they are mammals—filovirus disease outbreaks could conceivably be maintained through rapid and efficient transmission among highly susceptible taxa such as primates. No historic evidence for this hypothesis has been assembled, but a recent epidemic among nonhuman primates in Gabon and the Democratic Republic of the Congo has been intense and continuous; some believe this outbreak may be being transmitted among primate populations and may be pushing chimpanzee and gorilla populations towards extinction (36,37).

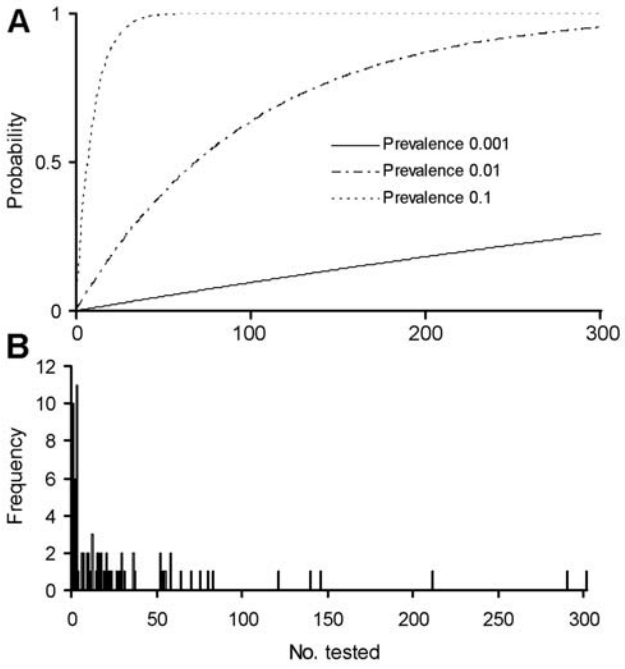

Figure 2. Summary of numbers per species that have been tested in studies seeking filovirus infections in wild mammals (See Appendix online, available from http://www.cdc.gov/ncidod/EID/vol10no12/04-0346_app.htm). A) Theoretical probabilities of detecting the reservoir in samples of particular sizes, given prevalences of 0.1%, 1%, and 10%. B) Frequency (1–11 species) with which species have been tested for filoviruses.

That the reservoir is a mammal is a more tenuous assumption. Numerous suggestions have been made regarding potential nonmammal reservoirs, including arthropods and plants, but no experimental evidence has documented either successful infection of these taxa or long-term infection that suggests maintenance of virus in the host (9). Although many possible alternatives exist, given successful laboratory infections of several mammal taxa, this clade seems a logical starting point. These procedures are feasibly applicable only to taxa that are reasonably well-known, both taxonomically and distributionally, such as mammals and birds. Recent analyses of filovirus nucleic acids, however, suggest that "filoviruses are more closely related to paramyxoviruses, particularly human respiratory syncytial virus" (15). As such, other vertebrates that host paramyxoviruses—birds, reptiles, and amphibians—may also merit study (15,16).

The assumption that the reservoir should be relatively nonsusceptible to negative effects of filovirus infection is based in large part on theory regarding host-parasite coevolution (19,20). A reservoir that is coevolved with the virus and experiences less severe effects of infection would lend greater long-term stability and lower probability of extinction to the pathogen populations.

The assumption of distributional coincidence between reservoir and virus is key to our inferences. Viruses not uncommonly escape from their natural reservoirs and are maintained at least temporarily in an alternate host (e.g., Nipah virus, influenza viruses, West Nile virus, even Ebola viruses in primate populations). However, these events do not go unnoticed, and we assume that such an escape from natural maintenance would be detected, as it has been in the past. That filoviruses could not persist long-term without the coevolved reservoir taxon's being present is fairly clear. This effect should ensure that virus distributions will be coincident with or nested within host distributions. The converse, however, is not necessarily clear: reservoir taxa can exist in areas in which the virus is not present (18).

Our assumption that the reservoir would be a species with a small body size is perhaps the most tenuous. However, we suspect that large-sized species would be hunted more often, which would provide more frequent opportunities for human infections. Also, contact with larger-sized species would be more notable and would likely be mentioned to family members before symptoms appeared. Finally, phylogenetic patterns among filoviruses suggest cospeciation within a similarly diverse clade (probably a genus or subfamily) of host species, and highly diverse genera or species complexes are less common among larger bodied mammals.

The clades identified in our analyses represent a broad swath of African mammal diversity. Dominant are bats, rodents, and insectivores. When Ebola or all African filovirus outbreaks are considered, bat clades dominate the lists, whereas when only Marburg outbreaks are considered, rodents enter the picture more broadly, reflecting the greater diversity of rodents in the arid habitats that characterize the known distributional area of Marburg virus.

Where do these explorations take us? We suggest four important adjustments to the epidemiologic studies that accompany most filovirus disease outbreaks. First, attention should focus on species that are spatially coincident with the aggregate distribution of outbreaks attributed to a given filovirus and that are from clades coincident with the distribution of filoviruses in general. Second, rather than testing species that are most common proportionally, collectors should attempt to sample each species and clade that co-occurs with known ranges of filovirus disease outbreaks, particularly given the rarity of reservoir-to-human transmission events. Third, emphasis should be placed on testing samples of each species large enough to give a reasonable probability of actual detection. For example, if the prevalence of filoviruses in the reservoir were 1%, then a sample of ≈60 to 70 would provide only a 50% chance of detecting the virus, and a sample of >200 would be needed to have a 90% chance of detection (Figure 2) (38). Fourth, publication of negative results in testing for

filovirus infections will be important in guiding future studies and making them still more efficient. These recommendations imply the need to bring specialized expertise on mammals to bear on the collecting challenge. Capturing animals of some taxa will require specialized equipment and techniques, as well as carefully planned strategies. Knowledge of the natural history and ecology of each of the key taxa will be important to successful sampling. Enlisting the assistance of local hunters and trappers who have specific experience with native species has proven very helpful in previous investigations (J. Mills and D. Carroll, unpub. data). We believe that concentrating sampling efforts on the taxa listed in Tables 1 to 3 will improve the chances of discovering filovirus reservoir species.

Laboratory inoculation studies also should focus on candidate taxa identified herein and should attempt to identify species that support persistent and largely asymptomatic infections with filoviruses. Such studies will serve to further direct field sampling efforts toward the most likely reservoir candidates. Investigators should be cognizant of new regulations regarding the importation of African rodents or rodent tissues and obtain all required permits (39).

In summary, our aim has been to use a series of biologic principles to guide reasoning towards narrowing the list of potential mammal reservoirs for filoviruses. These lists can guide sampling efforts, even when disease outbreaks are not ongoing. We do not imply certainty that the reservoir is among the species on our lists. We do, however, suggest that, under explicit assumptions, we have identified a suite of clades with a higher probability of being filovirus reservoir hosts than remaining African mammal clades. These lists can serve as a guide to future mammal surveys, allowing investigators to focus sampling efforts on high-probability taxa. In future studies, we will apply tools from ecologic niche modeling to refine and reduce these lists still further.

Acknowledgments

We thank numerous colleagues with whom we have discussed and refined these ideas, particularly E. Martínez-Meyer, D.H. Clayton, D.A. Vieglais, T. Ksiazek, D. Bausch, J. Montgomery, and P. Rollin. S. Reeder and E. Jentes provided helpful reviews of the manuscript.

This work was supported by the U.S. National Science Foundation.

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Nipah Virus Encephalitis Reemergence, Bangladesh

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We retrospectively investigated two outbreaks of encephalitis in Meherpur and Naogaon, Bangladesh, which occurred in 2001 and 2003. We collected serum samples from persons who were ill, their household contacts, randomly selected residents, hospital workers, and various animals. Cases were classified as laboratory confirmed or probable. We identified 13 cases (4 confirmed, 9 probable) in Meherpur; 7 were in persons in two households. Patients were more likely than nonpatients to have close contact with other patients or have contact with a sick cow. In Naogaon, we identified 12 cases (4 confirmed, 8 probable); 7 were in persons clustered in 2 households. Two *Pteropus* bats had antibodies for Nipah virus. Samples from hospital workers were negative for Nipah virus antibodies. These outbreaks, the first since 1999, suggest that transmission may occur through close contact with other patients or from exposure to a common source. Surveillance and enhancement of diagnostic capacity to detect Nipah virus infection are recommended.

Nipah virus is a recently described zoonotic paramyxovirus that causes a highly fatal encephalitis in humans (1,2). The only previously reported outbreaks of Nipah virus occurred in Malaysia and Singapore from September 1998 to May 1999. In Malaysia, 265 encephalitis cases, primarily among pig farmers, and a 40% death rate were reported (3–5). Concurrent outbreaks of a respiratory and neurologic illness caused by Nipah virus occurred among pigs in the affected areas, and close contact with pigs, especially sick pigs, was the major risk factor for human infection (3,6). Person-to-person transmission of Nipah virus was not documented (7). In Singapore, 11 cases and 1 death were reported among abattoir workers who slaughtered pigs imported from affected areas of Malaysia (3,6). The outbreak was contained by the mass culling of >1 million pigs, and since then, no other outbreaks of Nipah virus have been reported in Malaysia (8). Subsequent investigations identified *Pteropus* bats as a possible natural host for Nipah virus (9–11). *Pteropus* bats are also believed to be the natural host for Hendra virus, a zoonotic paramyxovirus that is genetically related to Nipah virus and has been associated with fatal respiratory and neurologic illness among persons in Australia (12).

In April and May 2001, a cluster of febrile neurologic illnesses with nine deaths was reported in a village in Meherpur District, Bangladesh. Preliminary investigations by the Bangladesh Ministry of Health and the World Health Organization (WHO) excluded a diagnosis of Japanese encephalitis, dengue fever, or malaria, but 2 of 42 serum specimens obtained from village residents in May 2001 showed reactive antibodies to Nipah virus antigen in tests performed at the U.S. Centers for Disease Control and Prevention (CDC). However, a comprehensive investigation of this outbreak was not conducted. In January 2003, a cluster of febrile illnesses with neurologic features and eight reported deaths occurred in adjoining villages in Naogaon District, ≈150 km from the village in Meherpur District. Similarities in the clinical manifestations observed among patients in Naogaon and Meherpur raised the question of whether the outbreaks were caused by the same agent.

In March 2003, we conducted a detailed retrospective investigation to describe the outbreaks in Meherpur and Naoganon, characterize their clinical features, and determine the etiologic agents, presence of asymptomatic infection, risk factors for infection and disease, and possible animal reservoirs.

Methods

The field investigation took place March 6–16, 2003, and consisted of separate outbreak investigations in

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Meherpur and Naogaon districts, a cross-sectional study among healthcare workers in Meherpur Hospital, and an assessment of possible animal reservoirs in the outbreak regions. Because of the substantial time lapse between the outbreak period and the field investigation in Meherpur, approval for the Meherpur portion of the study was obtained from the appropriate ethical review committees. Informed consent was obtained from all participants, except for children <16 years of age, for whom consent was obtained from the parent or guardian. Approval for participation was obtained for children ≥7 years of age. In both outbreak investigations, the surveys were conducted among residents ≥2 years of age, which consisted of an oral interview and collection of 10 mL of blood by venipuncture. Field research assistants used a standardized data collection instrument to collect information on demographics, symptoms of illness, exposure to ill patients, exposure to animals in the surrounding area, and other possible risk factors. When persons were deceased, an interview was conducted by proxy with a household member. Interviews were typically completed within 30 minutes. We attempted to verify clinical information on hospitalized patients from medical records, but records were either not found or contained incomplete clinical data.

Meherpur Outbreak Investigation

A population census was performed before the study by field research assistants, who visited each household and obtained information on the age and sex of each household member. Surveys took place in the villages of Chandpur (population 604), where persons who died or were hospitalized had resided, and Sishipara (population 237), an adjacent village located ≈1/2 km south of Chandpur. We defined the outbreak period as April 1 through May 31, 2001. Potential patients were identified from lists compiled by the initial WHO investigation and from self-reports of illness by village residents during the outbreak period. Household members were also surveyed as potential patients and assessed for clinically compatible illness or asymptomatic infection. To assess risk factors for infection, we enrolled controls by using simple random sampling of the numbered population census of remaining residents to select twice as many controls for each potential patient.

Naogaon Outbreak Investigation

Surveys were conducted in the adjacent villages of East Chalksita (population 529) and Biljoania (population 481); suspected deaths and hospitalizations caused by Nipah virus infection were reported from both villages. The outbreak period was defined as January 1–31, 2003. Because the scope of the outbreak was not well-defined, emphasis was given to case finding, which consisted of a household-to-household search by the field research assistants to identify potential cases. A sample of asymptomatic household members and other village residents, selected by simple random sampling from an available government population census, were surveyed for illness and serologic evidence of infection.

Healthcare Worker Study

A cross-sectional survey was performed at Meherpur District Hospital, where most of the ill residents from Chandpur were admitted with encephalitis symptoms. We interviewed and collected serum samples from healthcare workers whose job descriptions involved close contact with patients, such as physicians, nurses, orderlies, and nursing assistants. Information was obtained on demographics, symptoms of illness, the degree of contact with the patient, and type of barrier precaution used during patient care.

Serum Sample Collection

Blood specimens were centrifuged on site, transported on wet ice, and stored at –20°C. Serum samples were shipped frozen at –70°C to CDC and tested with an immunoglobulin (Ig) M capture enzyme immunoassay (EIA) for detection of Nipah/Hendra IgM antibodies and an indirect EIA for Nipah/Hendra IgG antibodies (13). Nipah (Malaysia prototype) virus antigen was used in both assays.

Data Analysis

Interview data were entered into Epi Info 6.04 (CDC, Atlanta, GA) and validated and analyzed by using SAS version 9 (SAS, Cary, NC). Based on serologic results, we defined a confirmed case as a case in a village resident with fever, headache, or altered level of consciousness within the specific outbreak period with antibodies reactive with Nipah antigen. A probable case was defined as a case in a resident with onset of fever plus headache or altered level of consciousness during the outbreak period who died before serum samples could be collected for testing. For the case-control study, univariate analysis was performed for each risk factor variable. Potential cases, including those in household members who did not have cases that met definition for confirmed or probable cases, were defined as noncases and analyzed together with the control group.

Assessment of Animal Reservoirs

In both districts, attempts were made to obtain representative samples from domestic and wild birds and mammals. Collections were based in part on relative abundance, suggestions of any history of ill animals indicated by village reports, and the likelihood for human

contact. Domestic species were restrained manually, when possible, or sedated by the intramuscular administration of ketamine hydrochloride (≈5–10 mg/kg). Traps were set near village residences for small mammals, such as rodents and insectivores. Mist netting or hand collections for bats and birds occurred in and around homes, suspected flyways, roosts, such as abandoned buildings, and likely foraging areas, such as fruit plantations. In addition, samples were obtained from bats captured or killed by local villagers because they were suspected of feeding on fruit trees in nearby orchards. In Naogaon, villagers reported that a herd of pigs was in the vicinity of the village ≈2 weeks before the outbreak. Pigs owned by the same herder (but not from the same herd) were bled for serologic testing. Animal serum samples and tissue were shipped frozen to CDC, and all specimens were tested using an indirect EIA employing protein A/G conjugate for mammals and an antibird conjugate for avian species.

Results

Meherpur Outbreak Investigation

In Meherpur, 13 residents, all from Chandpur (attack rate 2.1%), met the case definition: 4 had confirmed cases and 9 had probable cases. The outbreak, which spanned 1 month, began with the index patient, a 33-year-old farmer who had onset of symptoms on April 20, 2001 and died 6 days later. The outbreak ended with the last case which occurred in a 60-year-old woman, a neighbor of the index patient, with onset of symptoms on May 20 (Figure A). All nine persons with probable cases were hospitalized and died as a result of their illness before laboratory specimens could be collected (case-fatality rate = 69%). The average length of illness from onset to death was 6 days (range 3–10 days). All four persons with confirmed cases had IgG antibodies reactive to Nipah virus antigen (including two persons that had previous positive results from initial testing in May 2001). IgM antibodies were not detected in any of the specimens. Six (46%) of 13 patients were male; their ages were 4–60 years of age (median 38 years). A cluster of five cases occurred in persons from the same household as the index patient; the cluster consisted of the index patient's wife, son, brother, and sister. Eight separate households were affected, and 9 of the 13 persons with probable or confirmed cases were relatives of the index patient either by blood or marriage. Although patients with probable or confirmed cases lived in the western half of the village, no other obvious geographic clustering was noted; households with no cases were located in between those with cases.

Of 119 surveys completed in Meherpur, 96 (81%) were from Chandpur residents. Of these, 15 (16%) were initially identified as potential cases, 28 (29%) were their household members, and the remaining 53 (55%) were randomly selected as controls. All the patients came from the group identified as persons with potential cases in Chandpur; no patients were found among their household members or the randomly selected controls. The case-control analysis was restricted to Chandpur residents. With respect to baseline characteristics, patients (n = 13) did not differ from nonpatients (n = 83) by sex or occupation, but patients were older than nonpatients (mean age 40 vs. 27 years of age, $p < 0.001$). We examined close contact with patients and contact with animals as potential risk factors for illness. Persons who lived with or cared for patients during the time of their illness were more likely to become patients themselves (odds ratio [OR] 4.80, 95% confidence interval [CI] 1.23–18.8) (Table 1). In a subanalysis among those who lived with or cared for patients, no significant difference was found between patients and nonpatients with regard to sharing items of a personal nature, such as toothbrush or utensils, but patients were more likely to have touched secretions, such as urine or saliva, of other patients (OR 5.7, CI 1.0–32.7). Among all interviewed residents, patients were more likely than nonpatients to have had contact with an ill cow (OR 7.9, CI 2.2–27.7). Although >90% of villagers reported that bats were frequently seen near their homes, patients and controls showed no differences in contact with bats or other animals, whether ill or well.

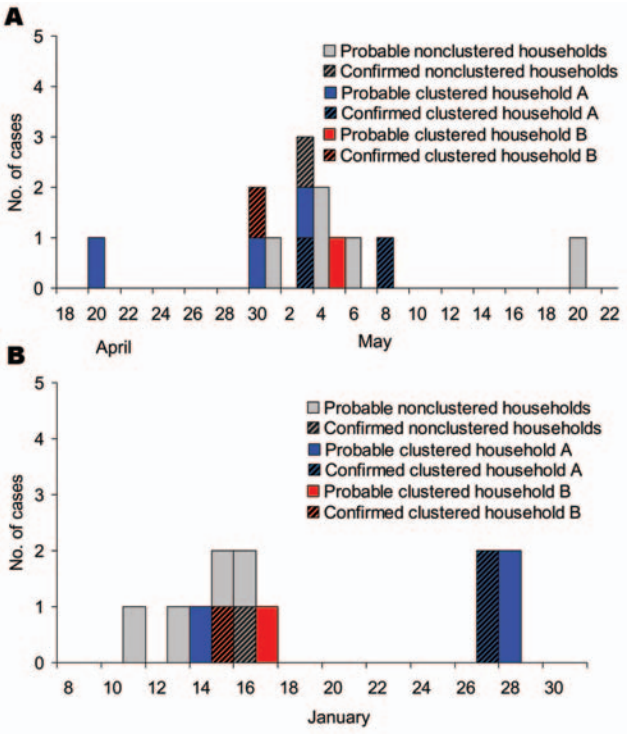

Figure. Illness onset of probable and confirmed cases of encephalitis. (A) Meherpur District, 2001. B) Naogaon District, 2003.

Table 1. Risk factors for illness among patients and nonpatients, Chandpur village, Meherpur, Bangladesh, 2001

Characteristic	Patients	Nonpatients	OR (95% CI)[a]
Caring for or living with a person with a case	10/13	34/83	4·80 (1.23–18.8)
Shared personal items	7/10	24/29	0·49 (0.09–2·56)
Contact with secretions from a person with a case	7/9	11/29	5·73 (1.00–32·7)
Any animal contact			
Chickens	13/13	83/83	Undefined
Cows	13/13	68/83	6.11 (0.34–108.4)
Dogs	12/13	78/83	0.76 (0.08–7.16)
Goats	10/13	64/83	0.98 (0.25–3.97)
Ducks	9/13	58/83	0.97 (0.27–3.45)
Bats	3/13	36/83	0.39 (0.10–1.53)
Sick animal contact			
Chicken	7/13	44/ 83	1.03 (0.32–3.34)
Cow	8/13	14/83	7.89 (2.24–27.7)
Dog	0/13	2/83	1.20 (0.05–26.5)
Goat	5/13	23/83	1.63 (0.48–5.50)
Duck	1/13	24/83	0.20 (0.03–1.66)

[a]OR, odds ratio; CI, confidence interval.

Naogaon Outbreak Investigation

In Naogaon, 12 persons from both villages met the case definition (attack rate 1.1%): 4 cases were confirmed, and 8 were probable. All cases were found through the reports of initial deaths and hospitalizations; no patients were found among asymptomatic family members or randomly sampled village residents. The index case occurred in a 12-year-old boy with symptom onset on January 11, 2003, and the last case occurred in 12-year-old girl on January 28 (Figure B). All but one of the patients were hospitalized (one died before hospital admission), and all eight patients with probable cases died (case-fatality rate = 67%); mean period from onset of symptoms to death was 4 days (range 2–7 days). No diagnostic specimens were available from the deceased patients. Of the four confirmed patients, all had IgG antibodies and three had IgM antibodies reactive with Nipah virus antigen. Eight (67%) patients were male, and their ages were 4–42 years of age (median 12 years of age). Clustering of patients occurred in one household, in which the head of household became ill on January 14 and later died. Symptoms developed in his wife and three daughters 2 weeks later; only the two younger girls survived. Altogether, eight households were affected. As with Meherpur, no geographic clustering of affected households was seen, but in contrast to the outbreak in Meherpur, members of affected households had no blood relationship to those in other affected households.

Clinical Features of Cases

The typical clinical course was similar in both outbreaks and began with onset of fever, followed by headache and varying degrees of diminishing consciousness. In both outbreaks, a fever was found in all patients, followed by an altered level of consciousness in 22 (88%) and headache in 18 (72%) (Table 2). Cough (16 [65%]) and difficulty breathing (16 [65%]) were also common. Vomiting occurred in half of the patients, but seizures and diarrhea were uncommon. Although a significant difference was seen between patients with confirmed and probable cases only with regard to dyspnea (25% vs. 82%, p = 0.01), patients with probable cases (all of whom died) tended to have a higher proportion of all symptoms compared to patients with confirmed cases, all of whom survived.

Healthcare Workers Study

A total of 46 healthcare workers (6 physicians, 20 nurses, 20 ward assistants) participated in the survey; 32 (70%) reported having contact with at least one of the encephalitis patients through the course of direct patient care. Of those who had direct patient contact, 12 (40%) used barrier precautions, such as gloves, masks, or gowns. One worker had unprotected mucous membrane contact with secretions of ill patients with encephalitis, and one reported a needlestick injury. Six workers reported an illness characterized by fever and headache in the period from the outbreak onset through June 30, 2001, but none reported mental status changes. None of the participating healthcare workers had antibodies reactive with Nipah virus antigens.

Assessment of Animal Reservoirs

No cluster of ill animals was observed or reported in either district. In Meherpur, serum samples were collected from two pigs and 31 bats, including 25 *P. giganteus*. None had antibodies reactive with Nipah virus antigens. In Naogaon, 50 animals were tested for evidence of Nipah-like virus infection: 10 birds, 4 pigs, 4 dogs, 2 shrews, 5 rodents, and 25 bats, including 19 *P. giganteus*. Antibodies reactive to Nipah virus antigen were detected in two *P. giganteus* adult females. Serum specimens from all other animals were negative.

Table 2. Clinical characteristics of patients with probable and confirmed encephalitis cases, by case classification, Meherpur and Naogaon, Bangladesh, 2001 and 2003

Symptom	Patients with		p value	Total patients n (%)
	Confirmed cases, n (%)	Probable cases, n (%)		
Fever	8 (100)	17 (100)	1.00	25 (100)
Altered level of consciousness	6 (75)	16 (94)	0.17	22 (88)
Headache	4 (50)	14 (82)	0.12	18 (72)
Cough	3 (38)	13 (76)	0.08	16 (64)
Dyspnea	2 (25)	14 (82)	0.01	16 (64)
Vomiting	4 (50)	9 (53)	0.61	13 (52)
Seizures	1 (13)	5 (29)	0.34	6 (24)
Diarrhea	0 (0)	3 (18)	0.30	3 (12)

Discussion

In these two outbreaks, antibodies reactive with Nipah virus antigen were found in seriously ill persons with encephalitis and antibodies were absent in asymptomatic persons or those without serious illness. These findings strongly suggest that Nipah, or a related virus, is the cause of both outbreaks. Nipah virus–associated illness has not been previously reported outside of Malaysia and Singapore. However, in contrast to the outbreaks in Malaysia, where animal illnesses were reported and close contact with pigs was strongly associated with Nipah virus infection in Bangladesh, no obvious zoonotic source has been identified. Pigs are infrequently found in Bangladesh, and no animal illnesses or die-offs in or around the affected villages were reported. Although case-control results indicated that patients were more likely to have contact with an ill cow, no such cow was available for testing, and the associations may have been due to chance. However, such potential risk factors need to be explored in future outbreak settings. Because antibodies reactive with Nipah virus were identified in local *Pteropus* bats, which reinforces previous findings, this genus may serve as the reservoir for this group of viruses (9–11). A possible explanation for acquisition of infection without an obvious domestic reservoir may be inadvertent direct contact with bats or bat secretions.

Human-to-human transmission of Nipah virus was not shown in the Malaysia and Singapore outbreaks (7), but several findings from the Bangladesh outbreaks suggest that close contact may have resulted in transmission. In Meherpur and in Naogaon, clusters of cases occurred within family households, with dates of symptom onset occurring over a range of time. In Meherpur, relatives with close contact with patients became ill, and handling or exposure to secretions of patients was found to be a risk factor for illness. Nipah virus has been detected in respiratory secretions and urine of patients, which suggests that person-to-person transmission is possible (14). However, we cannot rule out the possibility that a common source within households and among relatives may have been responsible for infection. In contrast, we found no evidence for transmission of Nipah virus from patients to healthcare workers. Contact between secretions or blood of healthcare workers and patients was reported in only two instances, which is an insufficient number to assess transmissibility through these routes. However, the lack of symptoms and lack of detectable antibody to Nipah virus in all hospital staff we evaluated suggest that transmission from patients to healthcare workers is uncommon.

The major clinical characteristics described in Bangladesh were generally similar to the characteristics described during the Nipah virus outbreaks in Malaysia and Singapore, with most persons having fever, headache, and an altered level of consciousness (2). In Bangladesh, a higher proportion of patients had an altered level of consciousness than those in Malaysia, although the results in our study relied on self-reporting, and objective descriptions of symptoms were not systematically documented. The absence of antibodies to Nipah/Hendra virus in asymptomatic persons suggests that subclinical infection did not occur or was an uncommon event, although subclinical infection has been previously documented (15,16). Among patients with probable or confirmed cases, patients in Naogaon tended to be younger (median age 12 years vs. 38 years of age) and to have a shorter interval from symptom onset to death (4 days vs. 6 days), compared to patients in the outbreak in Meherpur. Whether younger age is associated with a more fulminant course is uncertain, but the experience in Naogaon suggests that children appear to be as susceptible to infection as adults.

We restricted our case definition to confirmed or probable cases and did not include suspected cases in classification, defined as a surviving resident with onset of fever plus headache or altered level of consciousness, without serologic evidence for Nipah virus infection. In Meherpur, five nonpatients would have had suspected cases; in Naogaon, 44 patients would have had suspected cases. In the absence of objective clinical and serologic findings, persons with suspected cases are more likely to be patients with false-positive test results. On the other hand, the sensitivity of the Nipah virus EIA is limited (13); determining whether suspected cases represented true Nipah virus

infection versus another process (such as hysteria or a different clinical syndrome) is difficult. Another limitation, especially of the Meherpur investigation, was the difficulty of obtaining an accurate recollection of activities that took place almost 2 years before the study.

Two features of this outbreak of Nipah virus encephalitis are distinct from previous outbreaks. A clear history of exposure to a specific species of animals was lacking, although bats in the region had serologic evidence of infection, and person-to-person spread may have been an important mode of transmission. Two independent clusters of cases suggest that this virus may sporadically infect humans. From January through April 2004, two new clusters of fatal Nipah virus encephalitis have been reported in Bangladesh. These outbreaks further underscore the need for enhancing regional surveillance for Nipah virus and clarifying transmission patterns. Also, increasing the capacity to conduct surveillance for new cases may add to our understanding of the disease and guide development of effective prevention strategies.

Acknowledgments

We thank Mohammed Eskander Ali, Mohammed Abdur Rashid Khan, Bikash Chandra Swar, and members of the Bangladesh investigation team, for performing interviews and collecting serum samples; Sunity Acharya, George Kombo-Kono for assistance in planning the outbreak investigation; James A. Comer and Thomas Stevens for laboratory testing of serum samples; Larry Anderson for reviewing the manuscript; and K.R. Talukder for assistance in planning the outbreak investigation.

The study was funded by CDC.

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Risk Factors for Alveolar Echinococcosis in Humans

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We conducted a case-control study to investigate risk factors for acquiring autochthonous alveolar echinococcosis in Germany. Forty cases and 120 controls matched by age and residence were interviewed. Patients were more likely than controls to have owned dogs that killed game (odds ratio [OR] = 18.0), lived in a farmhouse (OR = 6.4), owned dogs that roamed outdoors unattended (OR = 6.1), collected wood (OR = 4.7), been farmers (OR = 4.7), chewed grass (OR = 4.4), lived in a dwelling close to fields (OR = 3.0), gone into forests for vocational reasons (OR = 2.8), grown leaf or root vegetables (OR = 2.5), owned cats that roamed outdoors unattended (OR = 2.3), and eaten unwashed strawberries (OR = 2.2). Sixty-five percent of cases were attributable to farming. Measures that prevent accidental swallowing of possibly contaminated material during farming or adequate deworming of pet animals might reduce the risk for alveolar echinococcosis.

Human alveolar echinococcosis is caused by the larval stage (metacestode) of the fox tapeworm *Echinococcus multilocularis*, which usually develops in the liver of infected persons. Slow larval growth results in an asymptomatic phase of several years before diagnosis. When left untreated, the condition is lethal (1). Although modern treatments have considerably improved survival, complete cure is rare (2–4).

E. multilocularis occurs worldwide in many arctic and temperate zones of the northern hemisphere (5). Its life cycle is predominantly sylvatic; several carnivorous species such as the fox, wolf, and coyote serve as definitive hosts that excrete the eggs in their feces. Several small rodent species, such as the vole, lemming, and muskrat, serve as intermediate hosts; they become infected by oral intake of the eggs, and the larvae develop in their liver. In Europe, the red fox (*Vulpes vulpes*) is the main definitive host. In Germany, the parasite is endemic in many regions; the prevalence in red fox populations is <1%–>60% (6). Dogs and cats can also become infected as definitive hosts, but their infection rates are low (7).

Human infections follow accidental ingestion of infective eggs. From 1982 to 2000, a total of 126 alveolar echinococcosis patients with autochthonous infections reportedly received treatment in German clinics (8). In spite of these low case numbers, the disease is an important public health problem because of the high frequency of infections in specific geographic clusters (8), the severity of organ damage in cases of infiltrative parasitic growth or hematogenous spread, and the necessity for costly long-term treatment and follow-up (3). Current hypotheses of possible routes of transmission of the eggs to humans include infection by hands contaminated from the fur of infected animals (foxes, dogs, or cats) or from soil while gardening or during field work; eating contaminated uncooked food from fields or gardens; drinking contaminated spring water; or inhaling dust containing tapeworm eggs, possibly during field work. Only three published case-control studies have assessed risk factors for human infections. In Alaska, dog ownership, living in houses directly built on the tundra, and keeping dogs tethered near the house were identified as important risks (9). In Austria, cat ownership and hunting were associated with alveolar echinococcosis, while farming and dog ownership were not (10). In Japan, persons with a clinical diagnosis of alveolar echinococcosis or a positive serologic result were more likely than controls to have reared cattle or pigs or to have used well water (11). We investigated possible risk factors for acquiring human alveolar echinococcosis in Germany.

Methods

We conducted a matched case-control study. Cases were selected from the European Echinococcosis Registry at the University of Ulm (8). An eligible case-patient was

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defined as a person 1) with positive histopathology of alveolar echinococcosis, or with positive morphologic findings by imaging techniques (ultrasound, computer tomography, magnetic resonance imaging) compatible with alveolar echinococcosis with or without serologic findings for the disease, 2) who was first diagnosed from 1990 to 2000, and 3) who lived in Germany and was still alive. The time frame of diagnosis was restricted to reduce possible recall bias, and only live patients were included to avoid possible information bias introduced by interviewing the relatives of cases. Fifty-three patients were eligible; of these, 40 participated in the study, 11 refused participation, and 2 gave consent after the study was completed.

Controls were individually matched to case-patients by age and place of residence. Matched residences were those in which the patients had lived during the 10 years before diagnosis. If the patients had moved during this time, the residence where they had lived for the longest period was chosen. Potential controls were contacted by random-digit telephone dialing. For every residence (5-digit postal code), 100 randomly selected numbers from the electronic version of the telephone directory were provided by ZUMA (Centre for Survey Research and Methodology, Mannheim, Germany). Eligible controls were persons who had lived in the municipality during the same period as the patients for at least 1 year, and who were of the same age (±5 years). Three controls for each of the 40 cases were chosen to detect an odds ratio of 3.0, assuming a frequency of a single exposure of 20% among controls and 43% among patients, with a power of 80% and a two-sided significance level of 5%.

Exposure information was obtained with a standardized questionnaire administered by telephone from February to August 2000. Specific behavior and activities during the 10 years preceding the diagnosis of an individual case were assessed. Persons were considered to be dog or cat owners or to have farmed if the duration exceeded 1 year. Deworming of dogs and cats was rated as an effective prophylactic anthelmintic measure only when performed at monthly intervals. For dwellings, gardens, and meadows, a close vicinity to possibly contaminated areas was defined as being ≤100 m from meadows, forests, fields, or rivers. Eligible patients were asked for their written informed consent; controls were asked for their oral informed consent before the interview. All data were processed without personal identifiers. The ethical committee of the University of Ulm approved the study protocol.

Statistical data were analyzed with SAS Version 8.2 (SAS Institute Inc., Cary, NC). For variables that might influence the occurrence of alveolar echinococcosis, the crude odds ratio (OR), the 95% confidence interval (CI), and the p value were calculated from simple conditional logistic regression (12). For each risk factor with a p value ≤0.05, the attributable risk was calculated by multiplying the proportion of exposed among the cases by (OR–1)/OR. All exposure factors strongly associated with the disease (p values ≤0.05) and independent of each other (Cramers's V <0.5) were combined in a specific risk score. Of factors with high interdependencies, only one was chosen according to its contextual relevance as compared to the other variables. The score was computed for each participant by adding 1 point for each specific exposure when this factor was present. The distribution of the score points in cases and controls was described by a boxplot. A stratified analysis for farmers and nonfarmers was performed; the regression models, including interaction terms of farming with the 10 other exposure factors of the risk score, showed that the effects of none of the factors were clearly distinct between the two groups (results not shown).

Results

Forty cases and 120 controls took part in the study. The gender distribution differed between patients and controls. Only 22% of the patients were <50 years of age, 73% were 50–79, and 5% were >79 (range 15–82 years). The educational status was similar among patients and controls. Most study participants lived in small villages (Table), and most in southern Germany, only 10% lived in central and northern Germany. Of the patients, 36 had lived in these places for >20 years; 4 had moved during the possible exposure time.

Simple conditional logistic regression analyses indicated 22 possible risk factors that were more common among patients than controls (p values ≤0.05) (online Appendix Table 1 available from http://www.cdc.gov/ ncidod/eid/ vol10no11/03-0773_app.htm). Patients were more likely than controls to have owned dogs (OR = 4.2), and several characteristics, such as leaving the dog in the garden unattended (OR = 6.1) or killing game (OR = 18.0), were more common among dogs belonging to patients (online Appendix Table 1). Patients were also more likely to have dewormed their dogs at infrequent intervals (OR = 5.6). Six persons in the study population reported hunting; one patient and two controls had hunted foxes, all for long periods (18–45 years). Owning cats that roamed outdoors unattended (OR = 2.3) and cats that ate mice (OR = 2.3) were more common factors for patients than controls.

Patients were more likely to be farmers (OR = 4.7); attributable risk calculations suggested that farming could account for almost two thirds of the infections. Specific farming activities were more common among patients than controls (online Appendix Table 1). Of all garden-related activities, only growing leaf or root vegetables was more common among patients (OR = 2.5). The location of the garden showed no remarkable influence. Patients were also more likely to enter forests for vocational reasons than

were controls (OR = 2.8) and were more likely to have collected wood (OR = 4.7).

Eating unwashed or uncooked vegetables, salads, herbs, berries, or mushrooms did not appear to be an important risk factor for alveolar echinococcosis; only eating unwashed strawberries or chewing grass was more common among patients than controls (OR = 2.2 and 4.4, respectively), and attributable risk calculations suggested these exposures could at most account for only a quarter of the overall risk for alveolar echinococcosis (online Appendix Table 1). Drinking water from natural sources had no identifiable association with the disease.

In order to describe, simply, persons at risk among the study population, a specific risk score was derived from the 22 factors with p values ≤0.05; we chose only those factors with low interdependencies. Eight of the 22 factors were not strongly associated with any of the other variables (online Appendix Table 2). The remaining variables with high interdependencies were selected as follows: living in a farmhouse was chosen instead of haymaking since including a three-level variable would have required weighing this factor; leaving the dog in the garden unattended was favored instead of six other dog-related factors (dog ownership, allowing the dog into the house, playing with the dog, walking the dog without leash, having a dog that ate mice, infrequent deworming of the dog) since it was a more relevant risk than dog ownership alone and was more reliably observed by the owners than the other factors. Cats left outdoors unattended was chosen as a risk factor instead of cats eating mice for the same reasons; being a farmer was chosen since it best represents the factors with which it was correlated (working in fields, pastures, grain fields).

Thus, the score was composed of 11 variables (online Appendix, Table 1): owning dogs that kill game, living in a farmhouse, owning dogs that roam outdoors unattended, collecting wood, being a farmer, chewing grass, living in a dwelling close to fields, going into forests for vocational reasons, growing leaf or root vegetables, owning cats that roam outdoors unattended, and eating unwashed strawberries. The score (range 0–11 points) was computed for 141 participants (37 patients, 104 controls); 19 participants had missing values in at least 1 exposure factor. The distribution among patients had a median of 6 score points (range 2–10); the distribution among controls had a median of 3 score points (range 0–9) (Figure). Of the patients, 81% had score values ≥4, but only 39% of the controls had score values ≥4.

Table. Characteristics of the study population

	Patients N = 40	Controls N = 120
Demographic characteristics	n (%)	n (%)
Age (in y) at time of interview[a]		
<20	1 (2)	
20–29	2 (5)	
30–39	4 (10)	
40–49	2 (5)	
50–59	10 (25)	
60–69	10 (25)	
70–79	9 (23)	
>79	2 (5)	
Sex		
Male	18 (45)	41 (34)
Female	22 (55)	78 (65)
Data not available	–	1 (1)
School education		
Secondary school	28 (70)	74 (62)
Intermediate level	7 (18)	29 (24)
Grammar school	4 (10)	14 (11)
Left school early	0	1 (1)
Still in school	1 (2)	1(1)
Data not available	0	1(1)
Completed vocational training		
Yes	27 (68)	87 (73)
No	13 (32)	30 (25)
Data not available		3 (2)
Population of hometown[a]		
<200	4 (10)	
200–<600	10 (25)	
600–<7,000	21 (53)	
7,000–<20,000	2 (5)	
≥20,000	3 (7)	
House at town outskirts		
Yes	26 (65)	85 (71)
No	14 (35)	34 (28)
Data not available		1 (1)
Duration of residence at assumed place of exposure		
≥30 y	31 (78)	
20–29 y	5 (13)	
10–19 y	3 (7)	
<10 y	1 (2)	

[a]Controls were matched to patients by age and hometown.

Discussion

This study identified several possible important risk factors for acquiring alveolar echinococcosis. Farming was perhaps the most important risk factor identified; more than three quarters of patients were farmers, and attributable risk calculations suggested that almost two thirds of the cases could be accounted for by farming. The apparent risk with farming supports the view that substantial environmental contamination can be expected in open areas. The parasite's eggs can survive and remain infective for months under favorable conditions (high humidity, low temperatures) (13); thus, soil-related exposures are plausible. The finding that haymaking in meadows adjacent to streams or rivers bears a higher risk than haymaking in other areas agrees with the finding that more infected foxes are found close to water than in other habitats (14).

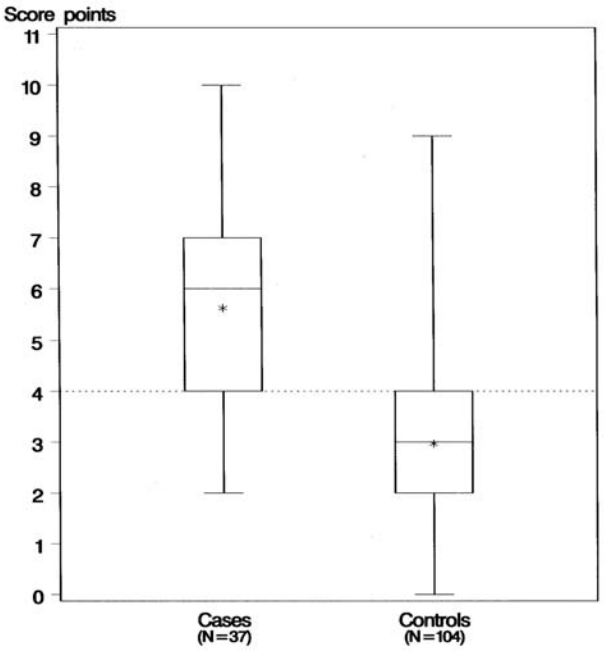

Figure. Risk score for alveolar echinococcosis in cases and controls. The plot presents minimum, 25th percentiles, median, 75th percentiles, and maximum values of the score points in cases and controls. The asterisk indicates the mean.

Although farming was an important risk factor, having a garden was not. An explanation may be that gardens usually cover a small area, and working in a garden requires less time, thus reducing exposure. Growing leaf or root vegetables was the only garden-related risk factor for A alveolar echinococcosis. The risk potential of growing specific garden produce may be interpreted in light of the greater amount of care and activity required for annual plants (leaf or root vegetables, salad vegetables), the fact that they are usually grown on larger patches than perennial herbs and strawberries, and the intense soil contact that occurs during harvesting.

Pet animals might pose a risk because of their close contact to humans and their contamination of soil around houses and in gardens. We found an association of dog ownership with acquisition of alveolar echinococcosis, and a lower but still relevant relationship with owning cats that roam freely outdoors or eat mice. The factor with the strongest association with the disease was "dogs that killed game," which is a rare disobedient behavior of individual dogs. Therefore, the attributable risk was lower than for the other variables related to dog ownership. Several other studies have indicated that dogs and cats are important risk factors for alveolar echinococcosis, although findings have been inconsistent (9,10). In China, an extensive inquiry with >2,500 participants including 86 patients with alveolar echinococcosis found that the number of dogs owned over time and the degree of dog contact were the most important risk factors (15).

Our results attach greater importance to ownership of dogs than of cats, particularly when the dog had activities possibly resulting in increased contact with soil or game. This finding is supported by experimental infection studies in which dogs proved to be susceptible to *E. multilocularis* eggs to the same high extent as foxes, and high worm loads developed; by contrast, cats had lower susceptibility and a slower maturation of the parasite (6,16). In the light of these findings, dogs and cats likely become a risk factor mainly by being infected themselves, in addition to transferring the eggs from fox feces or soil in their fur. Natural infections of pets have rarely been investigated systematically. The largest study on live cats and dogs from disease-endemic areas found coproantigen rates of 0.8% for both species (17).

In Austria, a strong association was found between hunting and the risk of acquiring alveolar echinococcosis (OR = 7.8) (10); however, a similar association was not shown in our study. Only 1 of 40 patients reported hunting. In Alaska, where hunting activities are more frequent than in Germany, no association between hunting and alveolar echinococcosis was observed (9). In China, no association was found between fox hunting and the disease (15).

Of all activities in the woods, only collecting wood was a likely important risk factor for alveolar echinococcosis, as indicated by the high OR and attributable risk calculations. Possibly, collecting wood posed a risk through contact with contaminated soil when a person picked the wood up from the ground, or the wood itself became contaminated when stacked in places accessible to roaming animals (clearings, forest perimeters, exterior parts of walls, open barns).

Chewing grass and eating unwashed strawberries were the only two variables of food consumption associated with alveolar echinococcosis. This risk may be attributable to ingestion of eggs from contaminated plant parts or from soil-contaminated hands. Other garden produce and mushrooms from fields and meadows were only rarely consumed raw and unwashed. Berries from the woods were more frequently consumed raw and unwashed than strawberries. The reasons why only strawberries constitute a risk include the fact that forest areas may be less likely to be egg-contaminated or that strawberries are eaten in larger quantities. The two case-control studies of Alaska and Austria found no association of alveolar echinococcosis with picking and eating raw produce from gardens, or berries and mushrooms from fields and forests (9,10).

This study had several important limitations. First, the long latent period for alveolar echinococcosis precluded determining the exact period relevant for an exposure. We restricted the assessment of most variables to the 10 years

preceding the diagnosis of a case; we also restricted eligibility to diagnoses since 1990, which had the advantage that diagnoses were probably ascertained "early" after the patients' infection owing to improved diagnostic technology and greater awareness over time. The case-control studies on alveolar echinococcosis published previously included cases irrespective of diagnosis dates. Furthermore, in Austria, the observation period spanned the 20 years preceding diagnosis, and the study included data about deceased patients (10). In Alaska the time frame encompassed the whole lifetime of the participants (9). Second, many possible risk factors were correlated with each other, and eliminating possible confounding factors was not possible. In our analyses, we omitted multiple logistic regression because of the multicollinearity of the factors. In such a situation, variable selection procedures in multiple logistic regression might lead to the arbitrary removal of important factors from the final model. In our opinion, interpreting such a reduced risk model might be misleading, especially if recommendations for preventive measures were derived from these models alone. Instead, we considered different degrees of exposure between cases and controls. We constructed an unweighted risk score from high risk variables that were not strongly dependent on each other. Patients were more likely to have been exposed to a greater variety of potential risks during the defined exposure time, which speaks for a possible cumulative effect of potentially hazardous activities. Third, the matching of case-patients and controls by location could have selected for similar behavior among them, and thus falsely reduced the observed strength of associations of possible risk factors.

We conclude that farmers, compared to persons in other occupations, are at high risk for alveolar echinococcosis in endemic areas in Germany. The disease should be strongly suspected in farmers living in these areas who have symptoms suggestive of this disease. Since no single farming-related activity alone likely accounts for this risk, general measures to reduce possible exposure during farming (e.g., wearing gloves when handling soil, plants, or wood; washing hands before taking meals after farming) might best reduce this risk. The risk observed with haymaking suggests a need to evaluate a possible role of inhalation; although evidence is lacking, wearing protective masks in very dusty conditions during such work may minimize risk. Our data also suggest that dogs and cats may pose a risk and that an adequate anthelmintic prophylaxis (praziquantel at monthly intervals) may possibly reduce this risk. Finally, our data suggest that cleaning produce from fields or gardens may help to reduce the risk for this disease.

Until the early 1980s, human alveolar echinococcosis was known to occur in four countries of western and central Europe: Austria, France, Germany, and Switzerland (5). Since the 1990s, sporadic cases have been found in Belgium, Poland, and Greece (8); a first case report from Slovakia dates from 2000 (18). These cases suggest that the disease is spreading. Since eliminating the parasite is unfeasible, the population in the disease-endemic areas should be advised to adhere to personal cautionary measures to prevent new infections.

Acknowledgments

We thank the patients and controls who voluntarily participated in the study.

The work of the European Echinococcosis Registry is financially supported by the University of Ulm, the Paul-Ehrlich-Gesellschaft e.V., and GlaxoSmithKline GmbH&Co. KG, Munich.

Dr. Kern is a research assistant at the Department of Biometry and Medical Documentation at the University of Ulm, Germany. She is responsible for the data collection of human cases of alveolar echinococcosis, data control, and analysis in the European Echinococcosis Registry.

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Exposure to Nonhuman Primates in Rural Cameroon

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Exposure to nonhuman primates has led to the emergence of important diseases, including Ebola hemorrhagic fever, AIDS, and adult T-cell leukemia. To determine the extent of exposure to nonhuman primates, persons were examined in 17 remote villages in Cameroon that represented three habitats (savanna, gallery forest, and lowland forest). Questionnaire data were collected to assess whether persons kept wild animal pets; hunted and butchered wild game; had experienced bites, scratches, or injuries from live animals; or had been injured during hunting or butchering. While all villages had substantial exposure to nonhuman primates, higher rates of exposure were seen in lowland forest sites. The study demonstrates that exposure is not limited to small groups of hunters. A high percentage of rural villagers report exposure to nonhuman primate blood and body fluids and risk acquiring infectious diseases.

Closely related species generally share susceptibility to the same groups of microorganisms (1). The anthropoid primates, which include humans, and to a lesser degree simian primates share broadly similar physiologic and genetic characteristics and thus susceptibility to many viruses, bacteria, fungi, protozoa, helminths and ectoparasites (2,3). Members of the family *Hominidae*, which includes humans, chimpanzees, bonobos, and gorillas, share an even greater similarity in susceptibility to microorganisms (3). Our closest relatives, chimpanzees and, most likely, bonobos, share with us the potential for infection with virtually the same set of microorganisms.

A range of activities involves direct contact between humans and nonhuman primates and allow for the transmission of microorganisms. Such behavior can facilitate transmission of microorganisms from nonhuman primates to humans (4), with consequences for human health, as well as from humans to nonhuman primates, with consequences for wildlife conservation (5). Care for captive nonhuman primates has lead to the transmission of a range of infections, including simian foamy virus (6), herpesvirus B (HBV) (7), primate malaria (8), and tuberculosis (9). Nonhuman primate ecotourism (e.g., gorilla watching) has been associated with the possible transmission from humans to nonhuman primates of diseases that include scabies (*Sarcoptes scabiei*) (10), intestinal parasites (11), and measles (12). Laboratory handling of tissues or fluids of nonhuman primates has lead to transmission of a range of infections to humans, including simian immunodeficiency virus (SIV) (13) and SV40, which was subsequently distributed through oral polio vaccine to millions of people (14). Additionally, keeping nonhuman primate pets has been linked to transmission of a variety of microorganisms (15). Finally, hunting and butchering nonhuman primates have been linked to the transmission of Ebola (16,17), monkeypox (18), and simian foamy virus (19). Because of the broad range of fluid and tissue types involved with hunting and butchering, this mechanism of transmission may be particularly important in cross-species transmission (1), although other behavior, such as wildlife necropsy, has similar risks (20).

A number of important human diseases, including AIDS (HIV), adult T-cell leukemia (HTLV-1) and malaria (*Plasmodium* spp.), are believed to have emerged as the results of ancient or contemporary cross-species transmission from nonhuman primates. While the emergence of malaria was presumably the result of vector-borne transmission, the mechanisms which led to the emergence of HIV and HTLV remain unknown. One of the current primary hypotheses to explain the origins of HIV is that hunting and butchering nonhuman primates led to cross-species transmission (21,22). This hypothesis is strengthened by recent evidence suggesting that hunted

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nonhuman primates have a high rate of SIV infection (23) and evidence of hunting-associated cross-species transmission of a nonhuman primate retrovirus, simian foamy virus, in central African hunters (19).

While some groups are at risk for contact with nonhuman primates, the frequency of behavior involving exposure to nonhuman primates remains largely unknown. The objective of the present study was to use behavioral tools to examine the frequency and extent of exposure to nonhuman primates among persons living in rural village sites in a region of high primate biologic diversity.

Materials and Methods

Participants

Seventeen village sites in Cameroon were selected for this study (Table 1, Figure 1). Sites were selected in highly rural areas, often at the end of small, unpaved roads. Sites were chosen to obtain different habitats, including 2 savanna sites, 2 gallery forest sites, and 13 lowland forest sites, and based on their proximity to regions supporting wild game populations. The sites selected are all in the southern part of Cameroon, a region that includes extensive lowland rainforest (Figure 1). The 17 sites include 2 in each of the Southwest, Northwest, West, Littoral, and Central Provinces, 3 in the South Province, and 4 in the East Province. All 17 sites in the study participated in commercial sales of hunted wild game.

Studies were conducted in the context of a community-based HIV prevention campaign designed to provide information and decrease transmission. Participation in the study was voluntary. Persons who participated in the HIV prevention campaign were asked if they would like to hear more about a research study, and the study was described to those who were interested. Study description, the informed consent procedure, and questionnaire administration were all done orally in English or French, which are widely spoken as second languages in the study villages. Participants were offered compensation approximately equivalent to 1 day of work, since participation often precluded farm work on that day. The study protocol was approved by the Johns Hopkins Committee for Human Research, the Cameroon National Ethical Review Board, and the HIV Tri-Services Secondary Review Board. In addition, a single project assurance was obtained from the Cameroonian Ministry of Health and accepted by the National Institutes of Health Office for Protection from Research Risks.

Behavioral Data

After the informed consent process, participants were asked to respond to a behavioral questionnaire. The questionnaire was administered individually by trained interviewers, without regard to the sex of the participant. The questionnaire, which was linked, was designed to provide basic demographic information as well as information on behavior either directly or indirectly associated with exposure to the blood or body fluids of nonhuman primates. The questionnaire was pretested in Cameroon before use. Behaviors considered indirectly associated with exposure to blood or body fluids include butchering, hunting, and keeping pets. Behavior considered to be directly associated with exposure to blood or body fluids includes having been scratched or bitten by a nonhuman primate or having been injured while hunting or butchering. Following pretests, locally appropriate taxonomic categories were derived from accepted local terms for animals. For example, "monkey" was identified as an appropriate taxonomic

Table 1. Characteristics of the 17 rural Cameroonian villages

Site no.	Latitude	Longitude	Altitude (m)	Habitat type	River basin (tributary)	Language group	Located at end of road
I	5.8°	10.7°	1,180	Savanna	Sanaga (Noun)	Shu-pamen	Yes
II	5.3°	11.0°	730	Gallery forest	Sanaga (Mbam)	Shu-pamen	No
III	4.8°	10.8°	800	Lowland forest	Wouri/Sanaga	Tunen	Yes
IV	4.2°	12.7°	680	Lowland forest	Nyong	Beti-fang	Yes
V	3.4°	10.6°	120	Lowland forest	Nyong/Lokoundje	Kwasio	No
VI	2.3°	10.4°	400	Lowland forest	Ntem	Beti-fang	Yes
VII	2.4°	11.8°	560	Lowland forest	Ntem	Beti-fang	No
VIII	2.2°	14.1°	630	Lowland forest	Congo (Dja)	Kawzime	No
IX	2.4°	15.0°	330	Lowland forest	Congo (Dja)	Mpo	Yes
X	6.1°	9.8°	1,500	Gallery forest	Calabar/Niger (Cross)	Esimbi	Yes
XI	6.3°	10.8°	1,700	Savanna	Niger	Limbum	Yes
XII	5.2°	9.4°	350	Lowland forest	Calabar (Cross)	Kenyang	No
XIII	4.9°	8.9°	100	Lowland forest	Ndian	Oroko	No
XIV	4.5°	10.3°	200	Lowland forest	Wouri (Dibamba)	Bassa	No
XV	3.7°	9.7°	0	Lowland forest	Sanaga	Duala	Yes
XVI	3.4°	12.7°	650	Lowland forest	Congo (Dja)	Beti-fang	Yes
XVII	5.2°	13.6°	640	Gallery forest	Sanaga	Gbete	Yes

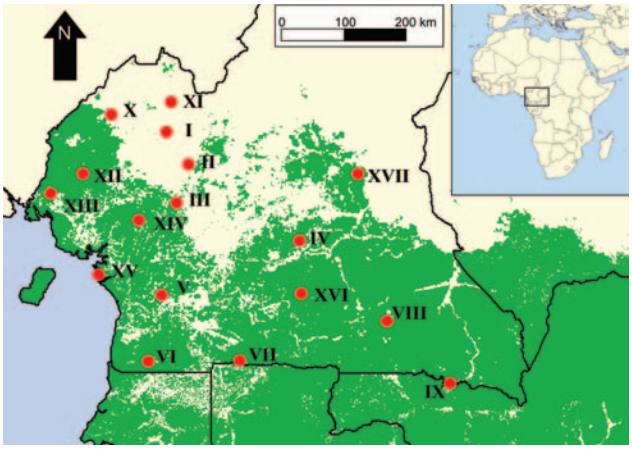

Figure 1. Map of study sites in southern Cameroon in relation to the distribution of lowland tropical forest in central Africa (in green).

category, and participants were not asked to distinguish between monkey species. Other taxonomic categories used include chimpanzee, gorilla, and rodent. Rodents were included because they are perhaps the most commonly hunted and eaten type of forest animal and serve as a useful comparison with nonhuman primates. Participants were asked to identify which of these four taxa they had consumed, hunted, butchered, or kept as pets. Participants were asked to estimate their monthly frequency of consumption of each of the wild taxa and of the wild game overall. Participants were also asked to report on direct contact with wild taxa, including the taxa involved, having been scratched or bitten, or having been injured while hunting or butchering.

Results

A total of 3,971 persons were interviewed. Both men and women participated and were represented approximately equally in the sites, with 46.3% female participants and 53.7% male participants. These aggregate results were similar to those found within the sites. Participants' ages ranged from 16 to 97 years. Participants were not equally distributed with regards to age; younger age groups were more represented. Participants were classed into four age groups: 16–30 years, 31–45 years, 46–60 years, and >60 years. Ages 16–30 made up 42% of participants, ages 31–45 made up 27% of participants, ages 46–60 made up 21% of participants, and ages >60 made up 10% of participants.

Participants reported having kept pets in all four taxonomic categories. Monkeys were kept as pets more frequently than other types of wild animals. The overall percentage of participants keeping pets from all sites combined was 0.6% keeping gorillas, 1.5% keeping chimpanzees, 9.9% keeping monkeys, and 1.8% keeping rodents. Sites in the study differed in their tendency to keep pets; persons in lowland sites reported keeping pets more frequently than people in gallery and savanna sites did (Figure 2).

In addition to keeping wild animal pets, three additional contact-associated activities were examined in this study, including hunting, butchering, and eating. Participants in all sites reported having hunted, butchered, and eaten animals from the four wild game taxa examined in this study. A higher percentage had eaten wild game than had butchered wild game, and a higher percentage had butchered wild game than hunted wild game (Figure 2). Hunting, butchering, and eating wild game were more common in forest sites than in other sites. For hunting, butchering, and eating, participants in the study had greater contact with rodents and monkeys than chimpanzees and gorillas (Figure 3).

While no significant departure was seen from expected proportions of women and men reporting eating of wild game of all types ($\chi^2 = 0.046$, nonsignificant) or of rodents ($\chi^2 = 0.001$, nonsignificant), fewer women than expected and more men than expected reported eating monkeys ($\chi^2 = 6.762$, $p < 0.01$), chimpanzees ($\chi^2 = 102.216$, $p < 0.001$), and gorillas ($\chi^2 = 0.046$, $p < 0.001$). Of persons reporting hunting nonhuman primates, a higher proportion than expected by chance were men (53.7% expected, 98.7% observed) and lower proportion than expected were women (46.3% expected; 1.3% observed) ($\chi^2 = 1{,}119.130$, $p < 0.001$). However, of participants reporting butchering nonhuman primates, a significantly higher proportion than expected were women (46.3% expected, 50.9% observed) and a lower proportion than expected were men (53.7% expected, 49.1% observed) ($\chi^2 = 61.376$, $p < 0.001$). The average number of nonhuman primate meals differed between the three habitat types ($F = 201.273$, $p < 0.001$): it

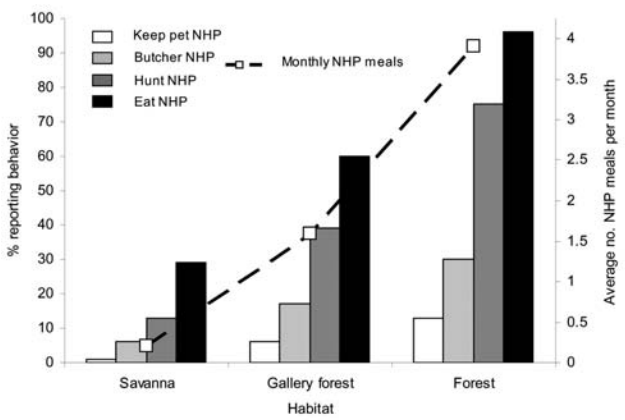

Figure 2. Percentage of participants in rural villages reporting exposure to wild game (monkeys, chimpanzees, and gorillas combined) by keeping pets, hunting, butchering, and eating, with average monthly frequency of wild game meal consumption for all species examined.

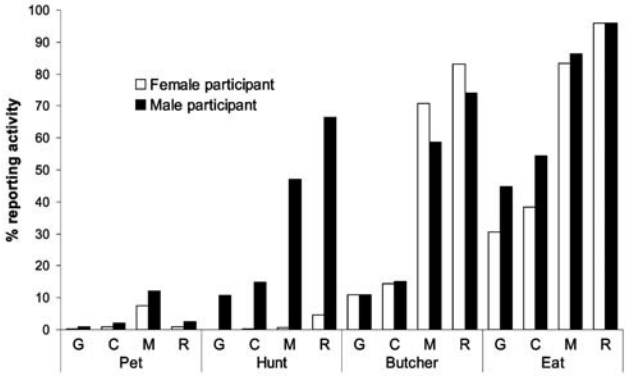

Figure 3. Percentage of male and female participants reporting exposure to wild game taxa (gorilla [G], chimpanzee [C], monkey [M], and rodent [R]) through keeping pets, hunting, butchering, and eating.

was significantly higher in lowland forest than in gallery forest (Bonferroni posthoc test $p < 0.001$) and significantly higher in gallery forest than in savanna (Bonferroni posthoc test $p < 0.001$) (Figure 2).

Data were also examined for evidence of direct contact with nonhuman primate blood and body fluids. Two types of evidence for direct exposure were examined: self-reports of scratches or bites from live nonhuman primates and self-reports of injuries involving body fluids associated with hunting and butchering nonhuman primates (Table 2). Injuries associated with hunting and butchering occurred in 14 of the 17 villages and in all three habitation types, with a total of 1.67% of participants reporting such injuries (online Appendix available at http://www.cdc.gov/ncidod/EID/vol10no12/04-0062_app.htm). Scratches or bites from nonhuman primates occurred in 14 villages in gallery forest and forest but did not occur in savanna sites. A total of 2.64% of participants reported such injuries. Of the participants who reported direct contact with nonhuman primate blood and body fluids through scratches, bites, or injuries, 91.2% reported butchering nonhuman primates, 73.0% reported hunting nonhuman primates, and 43.1% reported keeping a nonhuman primate as a pet. Men made up 82.5% of participants reporting direct contact, and women made up 17.5%. Most reports of direct contact involved monkeys (73.7%), although some direct contact was reported with gorillas (16.7%) and chimpanzees (9.6%).

Discussion

Hunting nonhuman primates is a biologically ancient behavior that we share with our closest living relatives, the chimpanzees (24). Human hunting techniques and patterns, however, have changed substantially in contemporary times. During the 20th century, firearms increased the efficiency and frequency of hunting. Both subsistence and commercial hunting with wire snares and firearms are widespread activities throughout the forests of central Africa (1,25,26). In addition, road networks and increasing opportunities for transporting hunted game have led to an increase in sales and the rate of hunting (27).

Hunting and butchering nonhuman primates has been linked to the emergence of infectious disease (1,4). Hunting a red colobus (*Procolobus badius oustaleti*) has been implicated in a localized epidemic of monkeypox that continued for four generations of human-to-human contact (18). In addition, an outbreak of Ebola hemorrhagic fever in Mayibout, Gabon, in January 1996 was linked to butchering and eating a chimpanzee that had been found dead; 29 of 37 identified cases involved exposure to the chimpanzee (16). A number of subsequent epidemics in Gabon and Congo have also been linked to hunting and butchering apes (17).

Not only humans are at risk for diseases transmitted from nonhuman primates through hunting and butchering. Chimpanzees are regular hunters of monkeys and other forest vertebrates, and a study of Ebola hemorrhagic fever among chimpanzees in the Tai forests showed that the primary risk factor for contracting Ebola among wild chimpanzees was hunting behavior, which showed a stronger association with infection than other acknowledged risk factors, such as “touching dead bodies” (28).

More recent research suggests that hunting and butchering nonhuman primates resulted in the emergence of HIV (i.e., after cross-species transmission of SIV and subsequent spread) (21,22). While reconstructing the history of viral emergence is a substantial challenge, one possibility is that transmission of SIV associated with hunting and butchering is an ongoing process and that contemporary hunters may yet be found with SIV infection. This hypothesis has been strengthened by recent evidence suggesting that hunted nonhuman primates have a high rate of SIV infection (23) and evidence of hunting-associated cross-species transmission of another nonhuman primate retrovirus, simian foamy virus (19).

Table 2. Frequency of persons in 17 Cameroonian villages reporting direct contact with nonhuman primate body fluids

Village location	Persons reporting direct nonhuman primate body fluid contact	
	Scratch or bite, n (%)	Injury during hunting or butchering, n (%)
Savanna (N = 364)	0 (0)	2 (0.55)
Gallery forest (N = 564)	8 (1.42)	7 (1.24)
Lowland forest (N = 3,043)	97 (3.19)	57 (1.87)
Overall (N = 3,971)	105 (2.64)	66 (1.67)

These results show that at least some rural villagers have a high level of exposure to nonhuman primates. While officially hunting of wild animals is forbidden, it is nonetheless widely accepted and permitted for personal use, so while the data may contain some bias, we do not feel that it is substantial. Our results show butchering to be the most common activity associated with contact with nonhuman primate blood and body fluids. More than 60% of the participants in the study reported having butchered a nonhuman primate, compared with ≈30% of participants who had hunted nonhuman primates. The higher frequency of persons reporting butchering as compared to hunting is expected, since those who hunt will often participate in some sort of butchering, generally including some preparation of wild game (e.g., disembowelment). Approximately 11% of the persons in the study reported keeping nonhuman primate pets. Because pets are usually young, the prevalence of chronic diseases in this population may be less than that among adult prey to which hunters and butchers are exposed. Nevertheless, because of the potential for regular contact with pet animals, even a low frequency of infections among pets may be important.

Villages from different habitats differed with regards to their reported exposure activities (Figure 2). Reported monthly consumption was significantly higher in the lowland forest sites. This finding may be due to the higher density and diversity of wildlife located close to these regions. Men and women both had high levels of contact with primate body fluids (Figure 3). While men were more likely than women to hunt wild animals, women were more likely than men to butcher. Because of differential participation in risk activities by men and women, these data suggest that gender-based interventions may be appropriate to decrease potential exposures to nonhuman primate blood and body fluids in central Africa.

The number of persons who reported direct contact with nonhuman primate blood or body fluids through scratches and bites from live primates or injuries during hunting and butchering was low. A total of 66 participants reported being in contact with nonhuman primate blood and body fluids through an injury associated with hunting or butchering wild game (online Appendix). These persons often had multiple risk factors associated with nonhuman primate contact. Most of these persons reported a history of hunting nonhuman primates; eight persons reported only butchering. Results suggest that injuries associated with butchering may be less frequent than those associated with hunting, although injuries associated with butchering may be less severe and therefore less memorable than injuries associated with hunting and therefore underreported.

The results of this study show that contact with nonhuman primates, through both keeping wild animal pets and hunting and butchering nonhuman primates, is not confined to a small segment of the rural population. Rural central Africans are more highly exposed to microorganisms present in nonhuman primates than was previously considered. Persons in industrialized countries who have regular contact with nonhuman primates, such as laboratory workers, are known to risk contracting infectious diseases from nonhuman primates, such as herpesvirus B, SIV, and simian foamy virus. These occupationally exposed persons are the subject of extensive public health interventions, aimed at controlling zoonotic transmission (29,30). While poor rural villages depend on wild game for animal protein, education on the risks associated with contact with nonhuman primate blood is an essential step for these communities, as is work to develop economic alternatives to hunting. Studies in such villages can provide further insights into behavioral links with disease emergence. Behavioral interventions aimed at decreasing exposure to nonhuman primates in villages with high exposure rates may provide an opportunity to prevent both zoonosis on an individual level, as well as emergence events that have the potential for global effects.

Acknowledgments

We thank the government of Cameroon for permission to undertake this work; A. Boupda, K Long, the EICAM Project Staff, and the U.S. Embassy in Yaounde for assistance in carrying out the work; and W. Sateren and T. Folks for valuable comments.

This work was supported by an award from the U.S. Military HIV Research Program (to D.B.); an International Research Scientist Development Award from the National Institutes of Health, Fogarty International Center (K01 TW00003-01 to N.W.); and an award from the Johns Hopkins Bloomberg School of Public Health, Center for a Livable Future (to A.P. and N.W.).

The views and opinions expressed herein do not necessarily reflect those of the U.S. Army or the Department of Defense.

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Origin of the Amphibian Chytrid Fungus

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The sudden appearance of chytridiomycosis, the cause of amphibian deaths and population declines in several continents, suggests that its etiologic agent, the amphibian chytrid *Batrachochytrium dendrobatidis*, was introduced into the affected regions. However, the origin of this virulent pathogen is unknown. A survey was conducted of 697 archived specimens of 3 species of *Xenopus* collected from 1879 to 1999 in southern Africa in which the histologic features of the interdigital webbing were analyzed. The earliest case of chytridiomycosis found was in a *Xenopus laevis* frog in 1938, and overall prevalence was 2.7%. The prevalence showed no significant differences between species, regions, season, or time period. Chytridiomycosis was a stable endemic infection in southern Africa for 23 years before any positive specimen was found outside Africa. We propose that Africa is the origin of the amphibian chytrid and that the international trade in *X. laevis* that began in the mid-1930s was the means of dissemination.

One of the biggest threats facing amphibian species and population survival worldwide is the disease chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (1,2). Chytridiomycosis was proposed as the cause of death in frog populations in the rain forests of Australia and Panama and was associated with the decline of frog populations in Ecuador, Venezuela, New Zealand, and Spain (3–6). Evidence for a countrywide decline in frog populations in South Africa is lacking (7), and local declines of several species have been ascribed to two main threats, habitat destruction and pollution (8). Chytridiomycosis is known in South Africa from infections in *Xenopus laevis*, *Afrana fuscigula*, and *Strongylopus grayii* (9–11). Through surveys of extant and archived specimens, *Batrachochytrium* has been found in every continent that has amphibians, except Asia (6,9,12,13). Since *B. dendrobatidis* has been recognized as an emerging pathogen, whose spread is facilitated by the international and intranational movement of amphibians (1), identifying its origin will be useful.

Some emerging infectious diseases arise when pathogens that have been localized to a single host or small geographic region go beyond previous boundaries (14). If *B. dendrobatidis* emerged in this fashion, we hypothesize that the source would meet the following criteria: 1) the hosts would show minimal or no apparent clinical effects, 2) the site would be the place of the earliest known global occurrence, 3) the date of this occurrence would precede any amphibian declines in pristine areas (i.e., late 1970s), 4) the prevalence in the source host or hosts would be stable over time, 5) no geographic spreading pattern would be observed over time in the region, 6) a feasible means of global dissemination of *Batrachochytrium* from the region of origin would be identified, and 7) *B. dendrobatidis* would show a greater genetic variation in the host region than in more recently invaded regions.

B. dendrobatidis is common in African frogs from Ghana, Kenya, South Africa, and Western Africa (12,15) and declines in frog populations are poorly documented in Africa (7,16). These factors, combined with the global trade in *X. laevis* and *X. tropicalis*, prompted us to investigate the likelihood that Africa was the origin of *Batrachochytrium* and that the trade in *Xenopus* spp. played a key role in its global dissemination. Within the *Xenopus* genus, *X. laevis* is distributed over the greatest area in sub-Saharan Africa. *X. laevis* occupies most bodies of water in savannah habitats from the Cape of Good Hope to Nigeria and Sudan (17,18).

We report the earliest case of the amphibian chytrid found in any amphibian and present epidemiologic evidence to support the hypothesis that *B. dendrobatidis* originated in Africa. In this article, chytridiomycosis refers to infection of amphibians by *B. dendrobatidis*.

Materials and Methods

A retrospective survey was conducted on archived specimens of the genus *Xenopus* housed in five southern Africa institutions, Bayworld (Port Elizabeth), Natal

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Museum (Pietermaritzburg), National Museum (Bloemfontein), South African Museum (Cape Town), and Transvaal Museum (Pretoria). Specimens in these museums had been collected for archiving by a large number of persons for various purposes and had not been selected for a systematic survey of amphibian disease. Specimens were collected mainly from South Africa, Lesotho, and Swaziland. A piece (3 x 3 mm) of the interdigital webbing was removed from one hind foot of each specimen of *X. gilli*, *X. muelleri*, and *X. laevis*. Tissue was prepared for histologic examination with routine techniques (19). Sections were cut at 6 μm and stained with hematoxylin and eosin. Chytridiomycosis was diagnosed by using described criteria (20). Sections from the two specimens diagnosed as having chytridiomycosis with hematoxylin and eosin before 1971 (one collected in 1938, the other in 1943) were confirmed with the more specific immunoperoxidase test (21) to increase the confidence of the diagnosis. Measurements of sporangia were performed with a calibrated eyepiece and expressed as mean ± standard deviation (SD). Histologic slides were examined "blind," without reference to dates that the frogs were collected, to decrease any opportunity for bias in diagnosis.

Exact versions of chi-square tests were used to analyze bivariate associations between chytridiomycosis prevalence and host species, region in South Africa (southwestern, eastern, and central), and season. Bivariate time trends of prevalences were analyzed by exact chi-square tests for trend. Multivariate logistic regression models were applied to assess potential confounding effect of species, region, and season on the time trend of chytridiomycosis prevalence. Confidence intervals (CI) were calculated by using exact binomial probabilities. Longitudinal and latitudinal historical patterns of spread were analyzed with linear regression models.

Results

Zoosporangia with a diameter (mean ± SD) of 5.2 ± 0.72 μm (maximum 6 μm) were seen in the stratum corneum of the digital webbing of infected frogs (Figure 1). Most sporangia were empty spherical structures, but occasional sporangia were observed with developing stages, septa, or discharge papillae. The structures stained brown (indicating positivity) in the immunoperoxidase test with the specific anti-*Batrachochytrium* antibody (Figure 1). Lesions usually associated with chytridiomycosis, including hyperplasia of the epidermis and hyperkeratosis of the stratum corneum, were mild and localized to areas of infection.

Overall, chytridiomycosis prevalence from the survey was 2.7% (19 positives out of 697 specimens) and did not differ significantly across species (p = 0.7; Table 1). The earliest date for a chytridiomycosis-positive specimen was

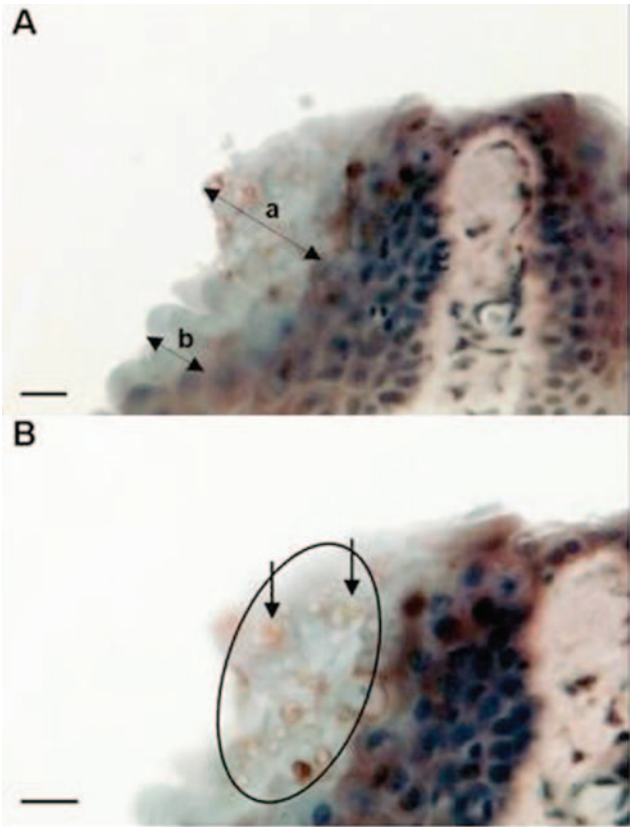

Figure 1. Micrographs of immunoperoxidase stained sections through the interdigital webbing of *Xenopus gilli*, showing the morphologic features and size of zoosporangia consistent with *Batrachochytrium dendrobatidis*. A) Arrow a indicates localized hyperplastic epidermal response; arrow b indicates an uninfected region of the epidermis. B) Arrows indicate two zoosporangia with internal septa. Circle indicates location of the infection in the stratum corneum. Bar, 10 μm.

1938 in an *X. laevis* collected from the Western Cape coastal lowland. This specimen is housed in the South African Museum, Cape Town (SAMZR 18927). The next earliest positive specimen detected was an *X. gilli* from 1943 (specimen number NMB 112, National Museum, Bloemfontein). The distribution of dates specimens were collected was greatly skewed to the latter half of the 20th century (Table 2). The breakdown for the time interval 1871–1940 is presented in order (decade, number of frogs infected/number of frogs examined) as follows: 1871–1880, 0/1; 1881–1890, 0/0; 1891–1900, 0/6; 1901–1910, 0/6; 1911–1920, 0/4; 1921–1930, 0/2; 1931–1940, 1/37. No statistically significant change of chytridiomycosis prevalence occurred over the decades since the 1940s (p = 0.36), or when the broader interval of pre-1971 is used as the baseline for the calculations (p = 0.22; Figure 2). No evidence for any trend in prevalence over time could be found using multivariate modeling where the odds ratios for the time intervals were adjusted

Table 1. Prevalence of chytridiomycosis in archived *Xenopus* spp. from southern Africa[a]

Species	No. examined	% positive (95% CI)	Earliest positive detected	Country
Xenopus laevis	583	2.6 (1.5–4.2)	1938	South Africa
X. meulleri	53	3.8 (0.5–13.0)	1991	Swaziland
X. gilli	61	3.3 (0.4–11.4)	1943	South Africa
Total	697	2.7		

[a]p = 0.7; CI, confidence interval.

for the potential confounders of species, season, and region. The multivariate odds ratios in these models were not significant and very similar to the bivariate findings, which indicate no confounding effects. The prevalence of chytridiomycosis in South Africa showed no significant change over time after 1940. No significant change of the geographic distribution of chytridiomycosis was detected after 1973. By 1973 the distribution of chytridiomycosis, as proved by positive specimens, covered already the area from 27° to 34° latitude and 18.25° to 32.5° longitude. This finding implies that positive specimens were detected from all regions of southern Africa by 1973. Infected frogs were found in 5 of the 9 provinces in South Africa, including the Western Cape (5 of 171), Northern Cape (2 of 22), Free State (6 of 141), Kwazulu-Natal (3 of 152), and Eastern Cape (1 of 137), as well as in Swaziland (2 of 42). Prevalence of *B. dendrobatidis* did not differ (p = 0.24) between the designated three broader regions with prevalences of 3.0% in the southwest, 3.8% in the central region, and 1.5% in the eastern region. Overall, the seasons (wet versus dry) when the specimens were collected were not significantly associated with prevalence (p = 0.22). Only in the eastern region, was a significantly higher prevalence found in the wet season than the dry season.

Discussion

Our study has extended the date for the earliest case of chytridiomycosis in wild amphibians by 23 years. The next earliest case outside South Africa was found in *Rana clamitans* from Saint-Pierre-de-Wakefield, Québec, Canada, in 1961 (22). After the case in Canada, the earliest cases from other countries follow sequentially over a period of 38 years from 1961 to 1999 (Figure 3).

X. laevis in the wild does not show clinical signs, nor has it experienced any sudden die-offs. Moreover, only subclinical chytrid infections have been observed among captive colonies of *X. laevis* (26,27). A frog of a related species, *X. tropicalis*, died in captivity from chytridiomycosis, it was suspected of having contracted the fungus from *X. laevis* (27). An ideal host for transmission of chytridiomycosis through international translocation would be a species of amphibian that does not become diseased or die from the infection; hence, *X. laevis* could take on the role of a natural carrier.

The sudden appearance of chytridiomycosis can best be explained by the hypothesis that *B. dendrobatidis* was recently introduced into new regions and subsequently infected novel host species (1). Dispersal of *B. dendrobatidis* between countries is most likely by the global transportation of amphibians (1,2,23,28,29). The World Organization for Animal Health has recently placed amphibian chytridiomycosis on the Wildlife Diseases List in recognition of this risk. If Africa is the source of *B. dendrobatidis*, a feasible route of dissemination by infected amphibians needs to be identified. Some members of the family *Pipidae* have been exported, in particular *Hymenochirus curtipes* and *X. laevis*, to North America and Europe (30).

In terms of a most likely candidate for spread from Africa, the number of frogs and geographic dissemination favor *X. laevis*. Soon after discovery of the pregnancy assay for humans in 1934 (30), enormous quantities of the species were caught in the wild in southern Africa and exported around the world. The pregnancy assay is based on the principle that ovulation in *X. laevis* is induced by injection with urine from pregnant women because of high levels of gonadotropic hormones in the urine (31,32). *X. laevis* was selected as the most suitable amphibian for investigating the mechanism of the mating reflex because of the relative ease with which the animal can be maintained in captivity (33). For 34 years, the trade in *X. laevis* in South Africa was controlled by the then Cape of Good

Table 2. Prevalence of chytridiomycosis in archived *Xenopus*, by time intervals[a]

Time interval	No. examined	No. positives	% positive (95% CI)
1871–1940	56	1	1.8 (0.0–9.6)
1941–1950	16	1	6.3 (0.2–30.2)
1951–1960	63	0	0.0 (0.0–5.7)
1961–1970	17	0	0.0 (0.0–19.5)
1971–1980	230	6	2.6 (1.0–5.6)
1981–1990	145	3	2.1 (0.4–5.9)
1991–2001	170	8	4.7 (2.0–9.0)
Total	697	19	2.7 (1.7–4.2)

[a]p = 0.36; CI, confidence interval.

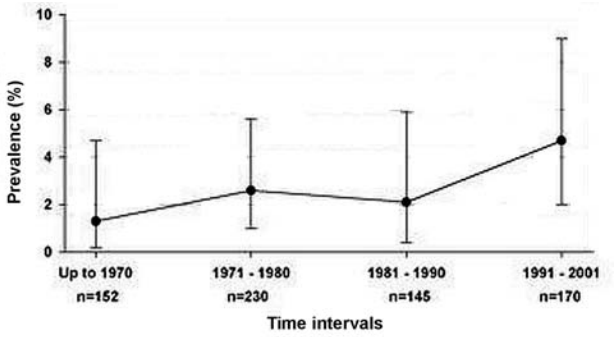

Figure 2. Historical time-trend of chytridiomycosis prevalence in southern Africa. No significant change was shown in the prevalence over time (p = 0.22, 95% confidence interval).

Hope Inland Fisheries Department (Western Cape Nature Conservation Board) at the Jonkershoek Fish Hatchery. As an indication of the numbers involved in this trade, 10,866 frogs were distributed in 1949, of which 3,803 (35%) were exported, and of the 20,942 frogs distributed in 1970, a total of 4,950 (24%) were shipped abroad (34,35). After the introduction of nonbiologic pregnancy tests, *X. laevis* became important as a model for the scientific study of immunity and later embryology and molecular biology. *X. laevis* could have carried the disease globally, particularly if the prevalence was similar to that seen in wild-caught *X. laevis* today. In the importing country, escaped frogs, the water they lived in (36), or both, could have come into contact with local amphibian species, and subsequent transmission of the disease could have followed. The establishment of feral populations of *X. laevis* in Ascension Island, the United Kingdom, the United States, and Chile in 1944, 1962, the 1960s, and 1985 (37), respectively, show that transmission could have become ongoing if these feral populations were infected.

Although we have demonstrated that *B. dendrobatidis* was in southern Africa since 1938, our studies provide no indication regarding whether this region was the original source within Africa. *B. dendrobatidis* has been found in wild frogs in Kenya and in frogs (*X. tropicalis* and *X. laevis*) wild-caught in Western Africa and detected after importation into the United States (12,26,27,38), which indicates that *B. dendrobatidis* is widely disseminated in Africa. *Xenopus* consists of 17 species that are found in sub-Saharan Africa, with a varying degree of sympatry between species (17). The overlap in the distribution and, in some cases, the sharing of habitats could facilitate transmission of *B. dendrobatidis* between these species. This finding would imply that chytridiomycosis could have originated elsewhere in Africa and spread within multiple host-region combinations. More detailed historical studies of archived African amphibians may indicate whether *B. dendrobatidis* was originally present in a small area of Africa from which it emerged to occupy large areas of the continent. Until the deficit in distribution data and comparative genetic studies is remedied, locating the source of the origin of *B. dendrobatidis* within Africa remains speculative. The relationship appears to have coevolved within an anuran host, and the opportunity to disseminate across the globe existed for *B. dendrobatidis* in southern Africa.

If *X. laevis* did carry *B. dendrobatidis* out of Africa as we propose, other amphibian species subsequently could have distributed it between and within countries. The American bullfrog, *Rana catesbeiana*, has been proposed as an important vector, mainly through international trade as a food item, but also within countries as populations established for the food trade escape and spread (29). The earliest current record for the occurrence of chytridiomycosis in *R. catesbeiana* is 1978 in South Carolina (38), 40 years after the first record in southern Africa, but details on the intensity of the search for chytridiomycosis in archived bullfrogs are not available. The transmission of chytridiomycosis globally may involve a series of key steps: 1) occurrence of *B. dendrobatidis* in an amphibian vector in southern Africa that is relatively resistant to disease (*X. laevis*), 2) sudden rise in 1935 of export trade in this vector because of technologic advances (*Xenopus* pregnancy test), 3) escape of the pathogen from the exported *Xenopus* to establish new foci in other countries (possibly expedited in some countries by establishment of feral populations of *X. laevis*), 4) transmission into other vector amphibians (food and pet trade), and 5) further transmission to other countries along different trade routes in key amphibian vectors that move in high numbers and become established in commercial populations and closely interact with wild

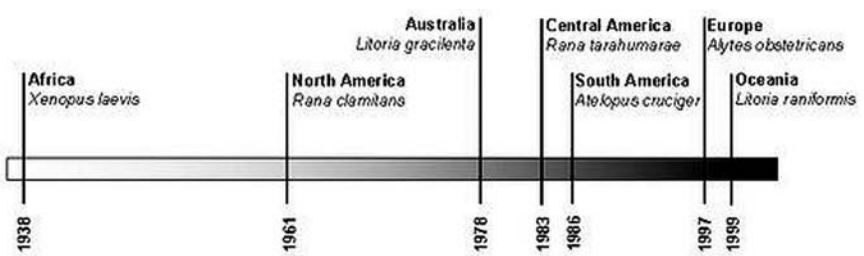

Figure 3. Time bar indicating when chytridiomycosis first appeared in the major centers of occurrence in relation to each other. Following a 23-year interruption in occurrences after the *Xenopus laevis* infection in 1938, records outside Africa appear with increasing frequency up until the present; North America (22), Australia (2,23), South America (5), Central America (24), Europe (6), Oceania (New Zealand) (25).

frogs, which likely leads to feral populations (food frogs *R. catesbeiana*). Spread through native amphibian populations with epidemic disease in some species could have occurred at any point after *B. dendrobatidis* entered a naïve native species.

We have provided epidemiologic evidence that Africa is the origin of the amphibian chytrid fungus. Support for six of the seven criteria proposed for the source of *B. dendrobatidis* has been demonstrated: 1) the major host (*X. laevis*) shows minimal or no apparent clinical effects, 2) site of the earliest global occurrence (1938), 3) this date precedes any amphibian declines in pristine areas, 4) the prevalence in the source host or hosts (*Xenopus* spp.) has been stable over time, 5) no geographic spreading pattern could be observed over time, and 6) a feasible means of global dissemination exists via the international trade in wild-caught *X. laevis*, which commenced in 1935 and continues today. Criterion 7, greater genetic diversity of *B. dendrobatidis* at the source, has not been investigated. A low level of genetic variation was shown for 35 strains of *B. dendrobatidis* and suggested that *B. dendrobatidis* was a recently emerged clone (39). The strains had been collected in North America, Australia, Panama, and Africa from wild and captive amphibians. Three strains isolated from captive *X. tropicalis* in United States had been imported from Ghana. Although these showed no significant differences from the U.S. strains (39), their assignment to Africa assumes no cross-infection had occurred within the importing facility. Future work on the genetic diversity of *B. dendrobatidis* in Africa compared with strains from regions outside Africa will add weight to the hypothesis if greater genetic diversity is found in African strains.

Acknowledgments

We thank Bayworld (Port Elizabeth), Natal Museum (Pietermaritzburg), National Museum (Bloemfontein), South African Museum (Cape Town), and Transvaal Museum (Pretoria) for making the material available.

This work was supported by the National Research Foundation (South Africa) and the Declining Amphibian Populations Task Force.

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Nonsusceptibility of Primate Cells to Taura Syndrome Virus

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Taura syndrome virus (TSV), a pathogen of penaeid shrimp and member of the family *Dicistroviridae*, was recently reported to have the ability to infect primate cells. We independently retested this hypothesis. Three lines of primate cells FRhK-4, MA-104, and BGMK, which are highly susceptible to infection by human picornaviruses, were challenged with TSV. Viral replication was assayed by real-time reverse transcription–polymerase chain reaction using cell media samples collected on days 0, 4, and 7 postchallenge. By day 7, genome copy numbers had decreased 25%–99%. No cytopathic effect was observed after 7 days. An in situ hybridization assay, with gene probes specific for detection of TSV, was negative for TSV in challenged cells. The infectivity of residual virus in the cell culture media at day 7 was confirmed by bioassay using TSV-free indicator shrimp (*Litopenaeus vannamei*). TSV did not infect the primate cells tested, and no evidence of zoonotic potential was found.

The general assumption is that the viral agents that cause disease in penaeid shrimp do not infect vertebrates. Supporting this assumption is the absence of documented cases of any shrimp virus causing disease in any animal species other than crustaceans. In a recent article, Taura syndrome virus (TSV), exclusively a pathogen of penaeid shrimp, was attributed a zoonotic potential because of its reported ability to infect cultured human and monkey cells (1). Aside from the food safety issues raised by this report, we were very interested in confirming those results because of the practical value of this new option for growing TSV in vitro. To date, TSV (or any other of the known viruses of the penaeid shrimp) has not yet been successfully cultured in any invertebrate or vertebrate cell-culture system. Hence, if viable, the use of primate cells for propagation of TSV would prove to be an important advancement in the study of TSV, and perhaps of other crustacean viruses. While the experiment reported in this study did not include all of the cell lines used by Audelo-del-Valle et al. (1), namely human rhabdomyosarcoma (RD), human larynx carcinoma (Hep-2), and Buffalo green monkey kidney (BGMK), the three cell lines that we used are also routinely used for virus isolation and diagnosis of diseases caused by human enteroviruses that belong to the family *Picornaviridae* (2–7). TSV, is a member of the family *Dicistroviridae* (closely related to *Picornaviridae*), genus *Cripavirus* (8,9). Other than TSV, which only infects penaeid shrimp, members of the genus *Cripavirus* are known to infect only insects (9). We report the results obtained after performing an experiment to test the hypothesis proposed by Audelo-del-Valle et al. (1).

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Materials and Methods

Source of TSV

Rather than using TSV-infected shrimp originated from shrimp farms as a source, TSV infection was induced under laboratory conditions by injecting specific pathogen free (SPF) shrimp (10) *Litopenaeus vannamei* with purified TSV reference isolate Hawaii-94 (11). The use of SPF shrimp ensured that no contamination with other viral pathogens would interfere with the experiment. Hemolymph and hepatopancreas were obtained from moribund shrimp during the acute phase of Taura syndrome and used to prepare the viral inocula.

Preparation of Inocula

Hemolymph was drawn from a moribund shrimp with acute-phase Taura syndrome and the hepatopancreas was excised by using aseptic technique. The hemolymph was diluted 1:10 with Eagle's balanced salts minimum essential medium (EMEM), without fetal bovine sera (FBS), and filtered through a syringe filter of 0.22-µm pore size. The hepatopancreas was homogenized in 10 mL of EMEM without FBS, centrifuged at 125 x *g* for 2 min to eliminate coarse material and the supernatant filtered with a syringe filter of 0.22-µm pore size. Samples of hemolymph and hepatopancreas from SPF shrimp were processed in identical manner and used as a negative control.

Cell Culture

The lines and cell cultures used were African green monkey kidney (BGMK), Monkey Rhesus female kidney embryonic (FRhK-4), and Monkey African green kidney (MA-104). Other than the report by Audelo-del-Valle et al. (1), TSV culture or CPE has not been reported in any invertebrate or vertebrate cell line. Hence, no positive control for TSV-induced CPE in cell culture was included in this study.

TSV Injection

Each of four 75-cm^2 flasks of confluent monolayers of each cell line (BGMK, FRhK-4, and MA-104) was injected with 0.1 mL of either of the four inocula: 1) hemolymph from shrimp with acute-phase Taura syndrome, 2) hepatopancreas from shrimp with acute-phase Taura syndrome, 3) hemolymph from SPF shrimp, or 4) hepatopancreas from SPF shrimp. After injection, the standard volume (15 mL) of fresh EMEM with 2% FBS was added without removing the inoculum. The cells were incubated at 37°C and monitored once a day for 7 days for cytopathic effect (CPE). As an additional negative control, one flask of each of the three cell lines was left untreated but monitored once a day alongside the TSV-injected flasks.

As an additional test to determine if a productive TSV infection occurred, representative samples of cells at day 7 were collected with a sterile pipette and pelleted at 130 x *g*. The pellet of cells was fixed in Davidson's AFA (alcohol, formaldehyde, and acetic acid) fixative and processed by using conventional techniques for paraffin embedding and sectioning. Paraffin sections were subjected to in situ hybridization with a mixture of two gene probes, P15 and Q1, specific for detection of TSV (12), according to protocols published elsewhere (13,14).

TSV Quantification

RNA Extraction

A total of 0.2 mL from each of the original inocula (inocula prepared from the hemolymph and hepatopancreas of infected and noninfected shrimp) and 0.2 mL of cell culture media from each of the three different cell line cultures collected at days 0, 4, and 7 postexposure, were subjected to RNA extractions using a High Pure RNA tissue kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's recommendations. The concentration of extracted RNA was estimated by measuring optical density, OD_{260nm}, with an Eppendorf spectrophotometer.

Real-Time TSV RT-PCR

The real time TSV RT-PCR assays were performed using an ABI GeneAmp 5700 with TaqMan One-Step RT-PCR master mixture (Applied Biosystems, Foster City, CA). The reaction mixture contained no more than 10 ng of extracted RNA, with each primer at a concentration of 0.3 μmol/L, and the TaqMan probe at a concentration of 0.1 μmol/L in a final volume of 25 μL. The cycling consisted of 30 min at 48°C for reverse transcription and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The data acquisition and analysis were carried out with GeneAmp 5700 Sequence Detector Software (Applied Biosystems). The real-time RT-PCR primers and TaqMan probe for the detection of TSV had been previously designed from ORF1 region of the TSV genomic sequence (15). Serial dilutions from a previously constructed TSV plasmid were used to determine a standard linear relationship for quantification with a correlation of the serial dilutions >0.99.

Confirmation of TSV Infectivity

Bioassay

To confirm the infectivity of the virus, a 6-day bioassay was performed by injecting groups of four to six SPF indicator shrimp (*L. vannamei*) with approximately 200 μL of either of the following: 1) inoculum prepared from hemolymph of infected shrimp; 2) inoculum prepared from hemolymph of noninfected shrimp; 3) cell media collected at day 7 from TSV-challenged cell culture flasks; or 4) culture media collected at day 7 from SPF shrimp tissue–treated cell culture flasks (negative control). The shrimp were monitored once a day for signs of disease. Moribund shrimp were collected when observed, preserved in Davidson's fixative and transferred into 70% ethanol after 24 h (14,16). All surviving shrimp at termination of the bioassay (day 6) were preserved in the same manner. Shrimp tissue samples were processed according to conventional techniques for paraffin embedding and sectioning (16). Paraffin sections were stained with Mayer-Bennett's hematoxylin/eosin-phloxin and subjected to histologic evaluation to determine the presence of TSV diagnostic lesions. Selected specimens were subjected to a confirmatory assay by in situ hybridization (ISH) with a mixture of two gene probes, P15 and Q1, specific for detection of TSV (12–14).

Results

Cytopathic Effect (CPE)

No CPE was observed in any of the three cell lines injected with TSV infected hemolymph, TSV infected hepatopancreas, SPF shrimp hemolymph, or SPF shrimp hepatopancreas (Figure 1). The BGMK cell line showed normal fibroblastic structure throughout the 7-day period of exposure to the different treatments. The BGMK

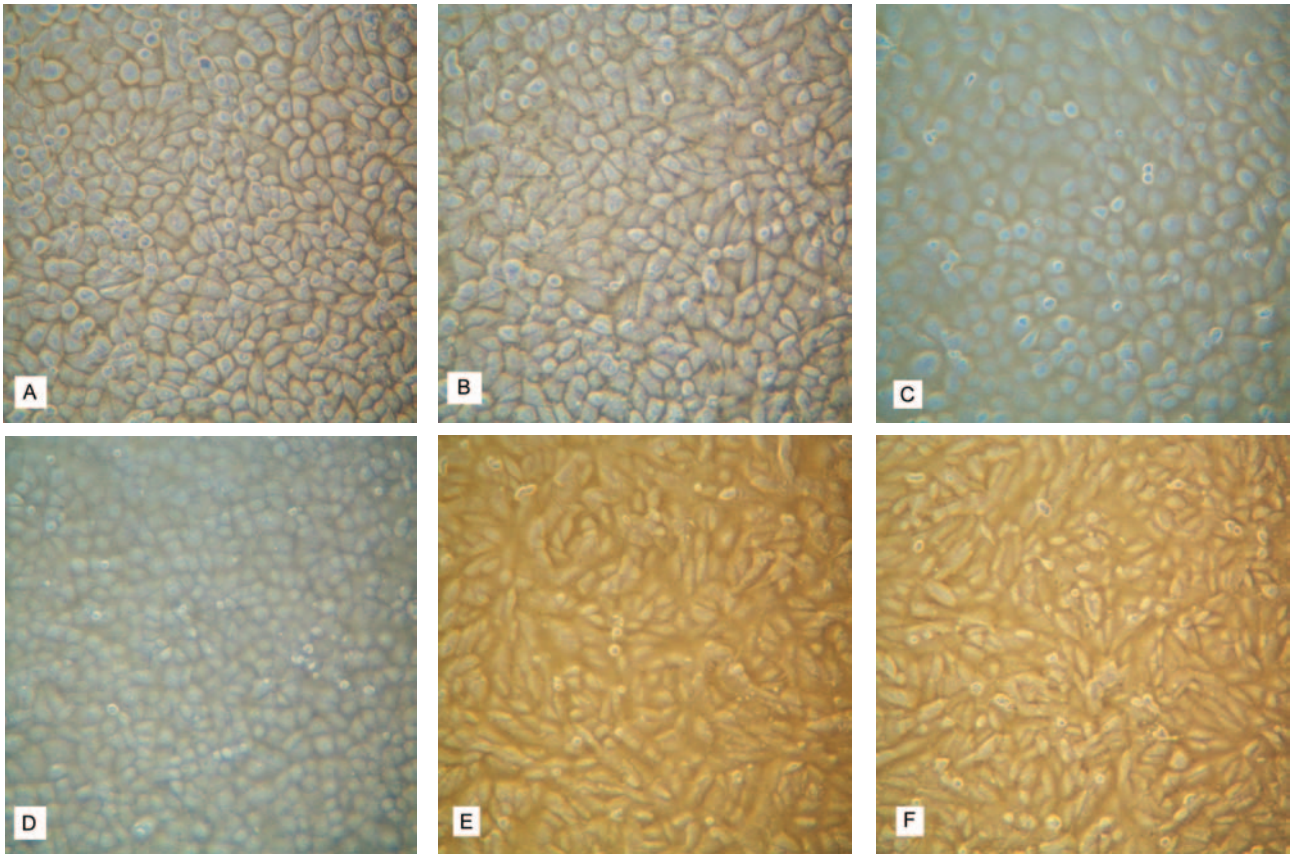


Figure 1. Absence of cytopathic effect after a 7-day exposure of three lines of primate cells (FRhK-4, MA-104, and BGMK) to an inoculum prepared with hemolymph from Taura syndrome virus (TSV)-infected shrimp (*Litopenaeus vannamei*) during the acute phase of the disease compared to a control inoculum containing hemolymph from specific pathogen free (SPF) shrimp. A) FRhK-4 cells exposed to SPF hemolymph; B) FRhK-4 cells exposed to TSV hemolymph; C) MA-104 cells exposed to SPF hemolymph; D) MA-104 cells exposed to TSV hemolymph; E) BGMK cells exposed to SPF hemolymph; F) BGMK cells exposed to TSV hemolymph (no stain; 25x).

monolayer remained confluent with no evidence of cell detachment or lysis. Similarly, the FRhK-4 and the MA-104 cell lines retained their typical epithelial structure for the 7-day period after exposure to TSV, with confluent monolayers and no evidence of cell detachment or lysis.

Virus Quantification

Approximately 1.3 x 10^5 to 2.7 x 10^6 viral copies/µL were detected at day 0 in the tissue cell flasks exposed to TSV-infected hemolymph. In the case of the tissue cell flasks exposed to TSV-infected hepatopancreas, ≈1.2 x 10^4 viral copies/µL were detected (Table). TSV was not detected by real time RT-PCR in the inoculum prepared from SPF shrimp hemolymph and hepatopancreas, nor in the tissue cell culture flasks exposed to these inocula.

At day 7, real time RT-PCR showed a decrease of 25% to 99% of the TSV genome copy number in the tissue cell culture flasks exposed to TSV (Figure 2), which suggests that no viral replication had occurred but that some residual virus remained from the inoculum.

Bioassay

Samples of cell-culture media from tissue culture flasks injected with TSV-infected hemolymph were collected at day 7 and used to inject SPF indicator shrimp *L. vannamei* to determine the infectivity of the residual virus. Moribund shrimp from these bioassays were examined by conventional hematoxylin/eosin-phloxin histology and by in situ hybridization with the gene probes specific for detection of TSV. TSV infection was diagnosed in all of the moribund shrimp, which indicates that at 7 days after injection, the tissue culture media contained sufficient residual TSV to produce infections in challenged shrimp (Figure 3). Paraffin sections from known TSV-infected and noninfected shrimp were used as ISH positive and negative controls, respectively (results not shown).

During this study, a relationship was observed between the concentration of TSV in the inocula prepared from day 7 tissue culture media (from cells exposed to TSV-infected hemolymph) and the severity of TSV infection in the challenged SPF indicator shrimp. The shrimp that had been injected with tissue cell culture media with the highest

Table. Mean TSV quantification in tissue cell culture media[a,b]

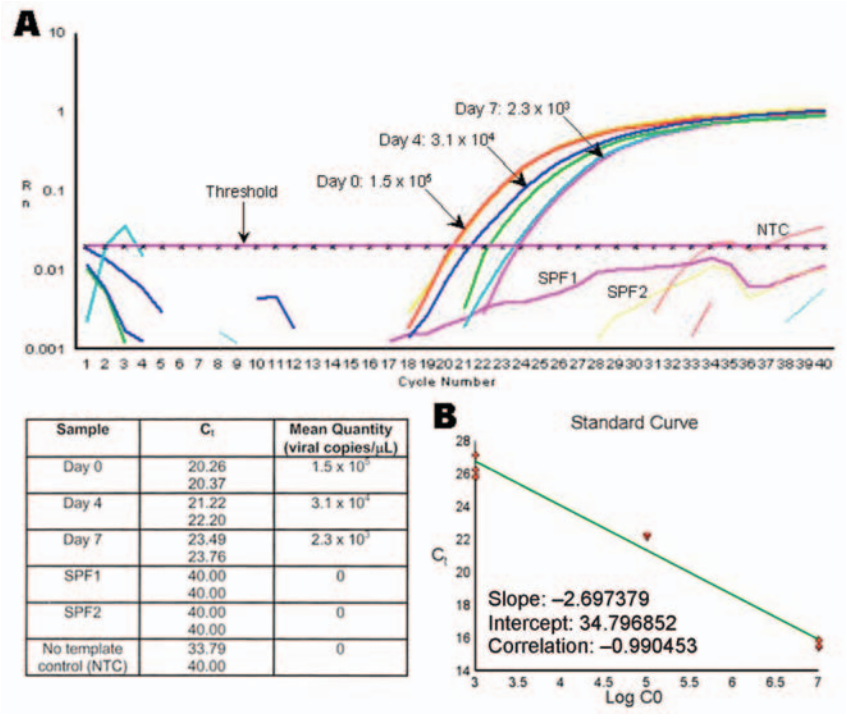
Cell type	Source of inoculum	Inoculum type	Mean viral quantification (viral copies/μL) postexposure		
			Day 0	Day 4	Day 7
BGMK	Hemolymph	TSV-infected	2.7×10^6	2.4×10^5	2.5×10^4
		SPF[c]	0	0	0
	Hepatopancreas	TSV-infected	1.2×10^4	5.0×10^3	5.0×10^3
		SPF	0	0	0
FrhK-4	Hemolymph	TSV-infected	1.3×10^5	1.9×10^4	4.7×10^3
		SPF	0	0	0
	Hepatopancreas	TSV-infected	1.3×10^4	8.4×10^3	3.7×10^3
		SPF	0	0	0
MA-104	Hemolymph	TSV-infected	1.5×10^5	3.1×10^4	2.3×10^3
		SPF	0	0	0
	Hepatopancreas	TSV-infected	1.2×10^4	1.3×10^4	9.2×10^3
		SPF	0	0	0

[a]Estimated by real time reverse transcription–polymerase chain reaction at different intervals postexposure.
[b]BGMK, Buffalo green monkey kidney; FrhK-4, monkey *Rhesus* female kidney embryonic; MA-104, monkey African green kidney; TSV; Taura syndrome virus; SPF, specific pathogen free.
[c]Inoculum originated from SPF penaeid shrimp.

TSV concentration (≈2.5×10^4 viral copies/μL) developed an acute (overt) infection within 3 days postchallenge, whereas shrimp injected with tissue cell culture media with the lowest viral concentration (≈2.3×10^3 viral copies/μL) developed only a subacute (covert) infection. Both overt and covert infections were confirmed by histologic analysis and by ISH with gene probes specific for detection of TSV (Figure 4).

Additional ISH Test

As an additional test to further confirm the absence of viral replication, the accumulation within the cells, or both, a sample of cells at day 7 was obtained from the BGMK cell line and subjected to ISH with TSV-specific gene probes. The BGMK cell line was selected for this assay because, among all three lines, it had the highest initial (day 0) concentration of viral particles and a 2 log reduction at day 7, suggesting either degradation or internalization of the virus. The ISH assay gave negative results (Figure 5), which indicates degradation as the more likely explanation for the reduction in virus content of the cell media. As in previous ISH assays, paraffin sections from known TSV-infected and noninfected shrimp were used as ISH-positive and -negative controls, respectively (results not shown).



Sample	C_t	Mean Quantity (viral copies/μL)
Day 0	20.26 20.37	1.5×10^5
Day 4	21.22 22.20	3.1×10^4
Day 7	23.49 23.76	2.3×10^3
SPF1	40.00 40.00	0
SPF2	40.00 40.00	0
No template control (NTC)	33.79 40.00	0

Figure 2. Example of the decrease on Taura syndrome virus (TSV) genome copy number within tissue cell culture flasks exposed to TSV. A) Real time reverse transcription–polymerase chain reaction plots and mean quantity of TSV copies/μL from tissue cell culture media collected at days 0, 4, and 7 postinfection from MA-104 cell flasks injected with TSV-infected shrimp hemolymph. Samples of tissue cell culture media collected from FrhK-4 and BGMK cell culture flasks inoculated with TSV-infected hemolymph or hepatopancreas also decreased by >1 log in concentration of viral copies as a function of time. The value of 33.79 obtained for one of the no template control (NTC) replicates is considered an artifact. B) Standard curve of TSV copy number versus threshold cycle (C_t) value. Purified TSV plasmid was serially diluted and used as templates in real-time polymerase chain reaction. The resulting C_t values are plotted against the logarithm of their respective copy numbers (C0). R_n, fluorescence signal; SPF 1 and 2, specific pathogen free.

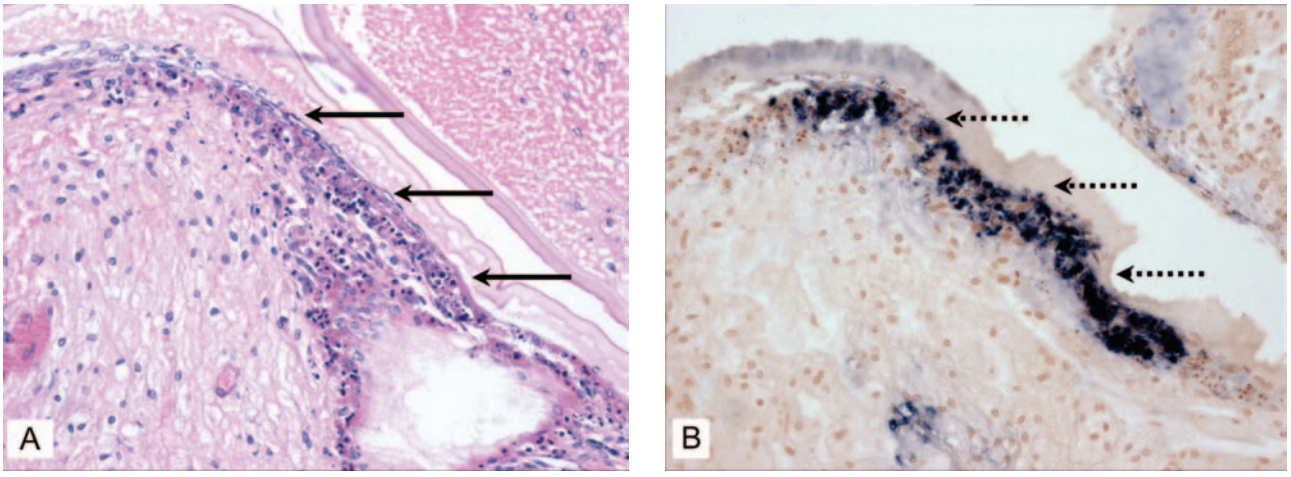


Figure 3. Histologic section through the anterior gastric chamber of a moribund juvenile *Litopenaeus vannamei* that was injected with an inoculum prepared with tissue cell culture media from BGMK cells exposed to Taura syndrome virus (TSV) (day 7 postexposure). A) The arrows point to a portion of cuticular epithelium displaying diagnostic acute-phase TSV lesions (hematoxylin/eosin-phloxin stain; 50x). B) The dashed arrows point to a portion of the stomach epithelium from the same shrimp, where digoxigenin (DIG)-labeled TSV-specific gene probes were reacted by in situ hybridization (ISH), resulting in the deposition of a black precipitate on areas where the probe hybridized with target TSV (Bismarck Brown counterstain; 50x).

Discussion

The real-time RT-PCR results (Table) show that the number of TSV genome copies in the cell culture media did not increase for any of the three cell lines challenged with TSV. While differences were observed in the estimated number of viral copies in each flask at day 7, the number of viral copies present was from one to two logs less than that of the day 0 values, which were determined immediately after cells were injected. The apparent plateau of TSV counts at day 7, regardless of the concentration of viral particles in each flask at day 0, may have been due to a protective effect of the cell-culture media, specifically the fetal bovine serum (FBS). This protective effect of FBS on viruses has been documented by other researchers (17–20). Studies on viral transport media for the preservation of virus viability have concluded that the best transport media for specimens at risk of being delayed by long transit times and exposed to significant temperature variations en route, are those that contain 2% FBS (i.e., CVM-Copan Diagnostics, Corana, CA, and M4-Multi-Microbe, Micro Test, Inc., Snellville, GA).

To further confirm the absence of viral replication or accumulation within the cells, a sample of cells at day 7 was obtained from the BGMK cell line and subjected to

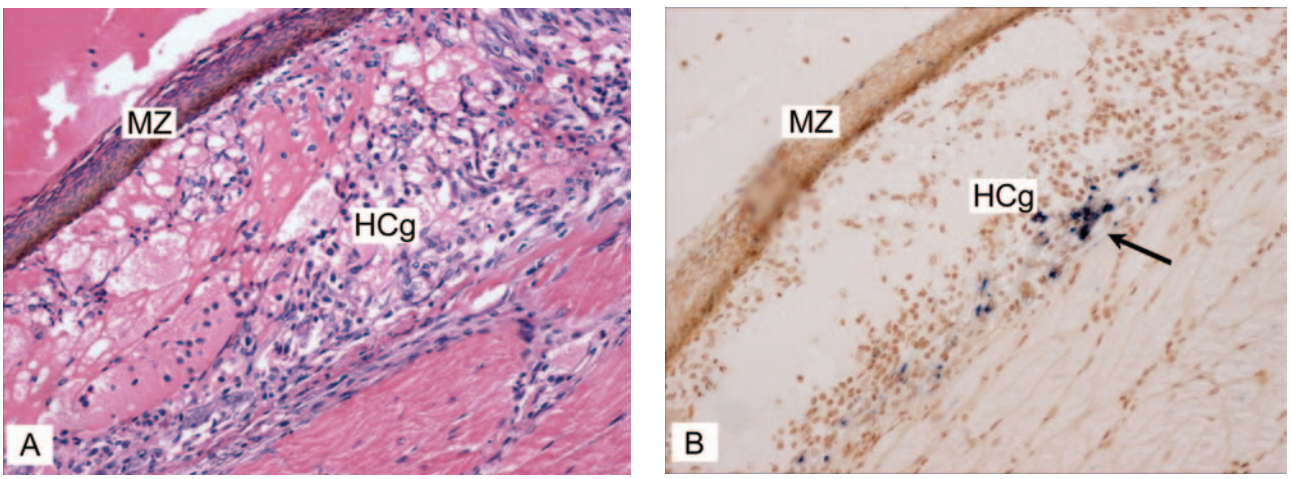


Figure 4. Covert Taura syndrome virus (TSV) infection (transition/chronic phase of TS disease) in indicator specific pathogen free-*Litopenaeus vannamei* shrimp was confirmed by in situ hybridization (ISH) with digoxigenin-labeled gene probes specific for detection of TSV. A) Histologic section through the dorsal cuticular epithelium showing a melanized resolving lesion (MZ) and hemolytic congestion (HCg), indicative of the transition phase of TSV infection (hematoxylin/eosin-phloxin stain; 50x) . B) TSV ISH on the consecutive section to that shown in 5A, where binding of the TSV probes is shown by the black precipitate (arrow) indicating the presence of TSV within the cytoplasm of cells of the cuticular epithelium (Bismarck Brown counterstain; 50x).

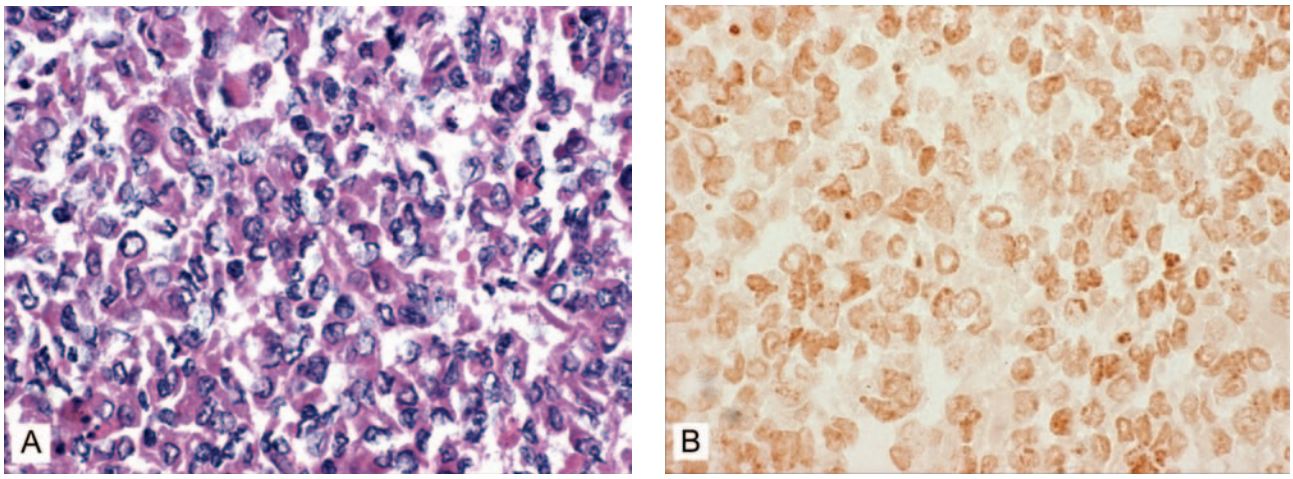


Figure 5. Absence of reaction by in situ hybridization (ISH) to the digoxigenin (DIG)-labeled Taura syndrome virus (TSV) probes within the BGMK cells harvested at day 7 postinjection with TSV. A) No cytopathic effect suggestive of TSV infection was evident by conventional hematoxylin/eosin-phloxin (H&E) histology (H&E stain; 100x). B) Consecutive histologic section to that shown in Figure 4A, but subjected to ISH with DIG-labeled TSV probes specific for TSV. No reaction to TSV is apparent in the challenged cells (Bismarck Brown counterstain; 100x).

ISH with TSV-specific gene probes. The absence of reaction to the TSV probes and the absence of CPE indicate that viral replication did not occur in the BGMK cells and that if any viral particles were internalized, they were degraded.

The relationship observed between the concentration of TSV in the inocula prepared from day 7 tissue culture media (from cells exposed to TSV-infected hemolymph) and the severity of TSV infection in the challenged SPF indicator shrimp agree with the results obtained during a previous study in the authors' laboratory, in which it was concluded that a minimum concentration of ≈ 1.0 x 10^4 viral copies/μL is necessary to induce an acute infection (21). In this case, those shrimp that had been injected with tissue cell culture media with the highest TSV concentration (≈2.5 x 10^4 viral copies/μL) developed an acute (overt) infection within 3 days postchallenge, whereas shrimp injected with tissue cell culture media with the lowest viral concentration (≈2.3 x 10^3 viral copies/μL) developed only a subacute (covert) infection.

BGMK, FRhK-4, and MA-104 cell lines are often used for isolation and research purposes for enteroviral meningitis (3), hepatitis A virus (7), polioviruses, coxsackie A, and coxsackie B (22) because of their marked susceptibility to infection by these members of the *Picornaviridae*. When exposed to any of these agents, these cell lines develop conspicuous CPE within ≈5 days (3,7,22,23). However, no CPE in any of the three cell lines (BGMK, FRhK-4, and MA-104) challenged with TSV was observed in this experiment, even at day 7 postinjection. These results contradict those of Audelo-del-Valle et al. (1), who reported the development of CPE within 19–23 hours. The average incubation time required for CPE development (induced by enteroviruses) in human or monkey cells at 37°C is 5 days, although detection time may be reduced to ≈3 days by use of the shell vial method (23). Shorter incubation times of <24 hours for CPE development could be more suggestive of a toxicity problem rather than of virus induced CPE.

As mentioned above, SPF shrimp (*L. vannamei*) were used to amplify a reference strain of TSV to prepare the inocula. We used SPF shrimp for three reasons. First, pond-reared or wild shrimp may harbor human or other mammalian picornaviruses. Shrimp and other decapod crustaceans have been shown to internalize and passively carry certain fish viruses (24–26) and human enteroviruses (27, C. Gerba, unpub. data). Hence, wild or pond-reared shrimp may be passive carriers of human or other mammalian pricornaviruses or other viruses which could produce CPE in studies such as that reported by Audelo-del-Valle et al. (1). Second, by using a commercially available line of SPF shrimp, the experiment can be standardized; therefore, other researchers can repeat or confirm the present study. Third, the SPF shrimp used are produced in closed biosecure systems with controlled water sources, which preclude chance contamination of the stocks with human or other animal viruses.

BGMK cells were the only cell type in common between our study and that of Audelo-del-Valle et al (1), who also used RD and Hep-2 however, BGMK, FRhK-4, and MA-104 cells were selected for use in our study because these cell types have a marked susceptibility to infection by members of *Picornaviridae* (28), which makes them as adequate as RD or Hep-2 cells for determining the possible infectivity of TSV to primate cells. We conclude that TSV did not infect the primate cells challenged with TSV in our study.

The lack of CPE in any of the three different cell lines tested, the negative ISH results with TSV specific gene probes assay of TSV challenged BGMK cells, and multilog reduction in TSV number in the cell-culture media as determined by real time RT-PCR indicate that TSV did not replicate in the primate cell lines used in our study. That TSV infection had occurred in SPF indicator shrimp after injection with media collected from day 7 cell culture flasks indicates that sufficient residual TSV remained in the media to infect the challenged shrimp and to cause acute disease or subacute disease as a function of relative concentration of residual TSV present.

Funding for this research was provided by the USDA CSREES Marine Shrimp Farming Program, grant number 2002-38808-01345, and a special grant from the National Fishery Institute.

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Venezuelan Equine Encephalitis Virus, Southern Mexico

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Equine epizootics of Venezuelan equine encephalitis (VEE) occurred in the southern Mexican states of Chiapas in 1993 and Oaxaca in 1996. To assess the impact of continuing circulation of VEE virus (VEEV) on human and animal populations, serologic and viral isolation studies were conducted in 2000 to 2001 in Chiapas State. Human serosurveys and risk analyses indicated that long-term endemic transmission of VEEV occurred among villages with seroprevalence levels of 18% to 75% and that medical personnel had a high risk for VEEV exposure. Seroprevalence in wild animals suggested cotton rats as possible reservoir hosts in the region. Virus isolations from sentinel animals and genetic characterizations of these strains indicated continuing circulation of a subtype IE genotype, which was isolated from equines during the recent VEE outbreaks. These data indicate long-term enzootic and endemic VEEV circulation in the region and continued risk for disease in equines and humans.

Venezuelan equine encephalitis (VEE) epidemics or epizootics involving hundreds of thousands of equine and human cases have occurred in the Americas since the 1930s (1,2). In Mexico, human VEE was first recognized during the 1960s along the Atlantic coast (3–5). In 1962, a total of 13 human cases were detected by serologic testing in Campeche and Champoton, state of Campeche (3,6); 5 deaths occurred (38% case-fatality rate), and 3 patients exhibited neurologic disease (3). A more extensive serosurvey in 1962–1964 in four southeastern states found 23 of 770 serum specimens had antibodies against VEE virus (VEEV; *Togaviridae*: *Alphavirus*) (4). Although clinical cases were not detected during that study, the findings implied extensive VEEV circulation. During 1963, VEEV subtype IE was recovered from a sentinel hamster and mosquitoes collected in southeastern Veracruz State (7). In 1965, a fatal human case occurred in the village of Jaltipan, Veracruz State (5). Almost simultaneously, equine epizootics were reported in 1966 in Tamaulipas State and in northern Veracruz State (8). Although no virus isolations were made, a VEEV etiology was suggested by serosurveys.

In 1969–1972, a major VEE outbreak began on the Guatemala-El Salvador border soon after an epizootic occurred in Ecuador and Peru. The Central American outbreak affected tens of thousands of equines and humans as it spread northward into Mexico. The first equine deaths were reported in 1969 in mountainous areas in La Trinitaria and La Concordia, Chiapas State, close to the Guatemalan border (8). By 1970, the epidemic and epizootic had caused 10,000 equine deaths and many unconfirmed human cases in the states of Chiapas and Oaxaca. By the end of 1972, nearly 50,000 equine deaths and 93 confirmed human deaths, as well as several hundred nonfatal human cases, had occurred in Mexico (9,10). The epidemic and epizootic eventually reached southern Texas, where ≈1,500 horses died and several hundred human cases occurred (11). This epizoodemic was caused by a subtype IAB strain and may have been halted in Texas by equine vaccination, insecticide spraying (12), and possibly by preexisting natural immunity to other alphaviruses in the equine population (13). Between 1973 and 1992, no VEE outbreaks were reported anywhere, prompting speculation that epizootic strains of VEEV had become extinct (12). However, reemerging VEEV activity in the early and mid-1990s in Venezuela and Colombia (13,14) and on the Pacific coast of southern Mexico (15) underscores the continued threat of VEE in the Americas.

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Two equine epizootics occurred on the Pacific coast of Mexico in 1993 and 1996. In the summer of 1993 in coastal areas of Chiapas State, an outbreak affecting125 horses, with 63 deaths, was documented. Three years later, during the summer of 1996 in the adjacent State of Oaxaca, another equine epizootic involved 32 horses with 12 deaths (15). Both outbreaks were caused by a subtype IE VEEV strain. However, no human cases were documented during either outbreak. VEEV strains isolated from encephalitic horses during 1993 and 1996 produced little evidence of viremia in experimentally infected horses, although the strains had caused encephalitis (16). Viremia titers were similar to those generated by enzootic VEEV strains, which indicates that equines were probably not important amplifying hosts during either Mexican epizootic.

Two hypotheses could explain the sudden appearance of equine encephalitis on the Pacific coast of Mexico in 1993: 1) an equine-virulent VEEV variant was introduced into the region or 2) an enzootic variant circulating previously in the region became more virulent or began circulating at a higher level in 1993. To test these hypotheses, to determine whether VEEV has persisted since 1996, and to determine whether humans are affected by these viruses, we conducted surveillance during 2000–2001 in various coastal villages involved in the 1993 epizootic. Our results indicate that enzootic and endemic VEEV have been circulating in the region and that persons and horses face a continuing risk for disease.

Methods

Selection and Description of the Study Area

During the summer of 1999, the Mexican Agricultural Ministry was notified of a suspected VEE equine outbreak in coastal areas of Mapastepec and Pijijiapan Municipalities in the state of Chiapas. A total of 26 equine cases showing syndromes compatible with equine VEE were reported, with 23 deaths. All of the affected horses were 8–14 months of age with no VEEV vaccination history. Although necropsies were carried out on some equines, no viruses were isolated. However, three of the dead horses showed histopathologic changes suggestive of a nonrabies viral etiology, with VEEV hemagglutination inhibition (HI) antibody titers from 20 to 5,120 (R. Navarro-Lopez, unpub. data). Bovine serosurveys also indicated recent VEEV circulation in the area.

On the basis of these preliminary data, we selected several locations for further study within the La Encrucijada preserve, a coastal ecosystem of mangrove estuaries and mangrove forest located along the Pacific coastal plain in the southwestern portion of the State of Chiapas. The preserve is about 357,824 acres (1,440 km^2) and is located from 14°43′ to 15°40′N and from 92°26′ to 93°20′W. The area is composed of coastal lagoons, swamps, and marshes forming the largest mangrove forest on the North American Pacific coast and is important for its biodiversity and flora, including the only zapotonal woodland (*Pachira acuatica*) of Mesoamerica. The flora include mangrove, zapote forest, marshes, evergreen seasonal forest, deciduous seasonal forest, coastal dune vegetation, and palm forest. The area supports a large variety of threatened wildlife, including 11 species of amphibians, 34 of reptiles, 294 of birds (94 migratory), and 73 of mammals (www.ramsar.org/profiles_mexico.htm).

About 30,000 persons live within 64 settlements in the preserve, and the main activities are commercial fishing, slash and burn agriculture, and extensive cattle ranching. We selected seven villages serologically implicated in VEEV circulation, all located between N 15°46′07″–15°02′00″ and W 93° 4′92″–92°43′00″ and within the municipalities of Pijijiapan, Mapastepec, and Acapetahua (Figure 1). The villages included Las Coaches, Isla Morelos, Buenavista, 10 de Abril, Francisco Sarabia, Roberto Barrios, and Las Palmas (Table 1). Two other villages, Cintalapa and Jamaica, were selected as negative controls because they are well outside the area affected by the recent VEE epizootics in the foothills of the Sierra del Soconusco Mountains, 30–50 km from La Encrucijada. As part of ongoing dengue surveillance, serum samples from 434 persons from these different locales were tested for VEEV antibodies.

Animal Trapping and Collection of Serum Specimens

Recommended biosafety methods were used in the field to minimize the risk for infection of workers by rodentborne zoonoses (17). Marsupials and rodents were collected in Tomahawk and Sherman traps. Based on data provided by the animal exotic disease division of the Mexican Agricultural Ministry (CPA), the traps were placed in the periphery (usually next to fences) or inside farms that recently reported equine encephalitis. Trapped

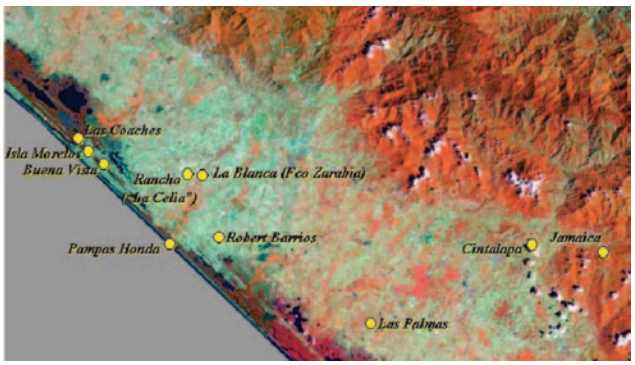

Figure 1. Satellite image of the Pacific coastal areas studied for Venezuelan equine encephalitis virus activity (Landsat thematic mapper). Bands 4, 5, and 1 are displayed as a red-green-blue false-color composite. The villages sampled are indicated in yellow.

Table 1. Communities in the La Encrucijada Preserve studied for VEEV circulation and seroprevalence[a]

Community	Coordinates	Population size	Surveillance methods
Las Coaches	93°07′24″ x 15°27′03″	362	1,2,3
Isla Morelos	93°07′12″ x 15°26′00″	391	1
Buenavista	93°08′17″ x 15°26′53″	440	1
10 de Abril	93°02′04″ x 15°21′16″	81	1
Francisco Sarabia	92°59′51″ x 15°25′09″	358	1
Roberto Barrios	92° 58′48″ x 15°20′51″	372	1,3
Las Palmas	92°45′14″ x 15°34′51″	832	1

[a]VEEV, Venezuelan equine encephalitis virus; 1, human serosurvey; 2, small mammal collections; 3, sentinel hamster exposure; data from Instituto Mexican del Seguro Social, Comision para las Areas Marginadas (rural clinics) as of July 2001.

mammals were collected each morning and transferred to the Agricultural Animal Health Laboratory in Mapastepec, Chiapas, for processing. After being anesthetized with halothane, trapped rodents and marsupials were bled by cardiac puncture, and ≈1 mL of blood was transferred to vacutainers (Becton Dickinson, Franklin Lake, NJ) for serum separation. Some rodents were also bled from the retroorbital sinus with heparinized capillary tubes. Serum specimens were stored in liquid nitrogen for the subsequent analysis at the biosafety level 3 facilities of CPA in Mexico City or at the University of Texas Medical Branch in Galveston, Texas. Dog blood samples were obtained by venipuncture and processed as above. Domestic bird blood samples were collected from the brachial vein. Cattle blood samples were collected from the jugular veins.

Sentinel Animals

Based on human serosurvey data and to maximize chances for viral isolations, we selected two locations for exposure of sentinel animals: Las Coaches (75% seroprevalence) and Roberto Barrios (54% seroprevalence) (Figure 1). In Las Coaches we placed ten, 6- to 8-week-old Syrian hamsters, 1–2 per cage in hamster-baited mosquito traps as described previously (18). The traps were located adjacent to marshes at the eastern part of the village, ≈75 m from human dwellings and close to cattle pastures, swamps, and mangrove forest. In Roberto Barrios, we exposed four sentinel hamsters in open cages, one hamster per cage, at four locations at the western tip of the village, adjacent to marshes and mangrove forest. Hamsters were monitored daily for 7 consecutive days, and brain, spleen, heart, and lung were dissected from moribund (euthanized by halothane overdose) or dead animals and stored in liquid nitrogen.

Serologic Tests

Initial screening of human serum specimens for VEEV-reactive antibodies was conducted by using 80% plaque reduction neutralization tests (PRNT) with subtype IE strain 68U201, shown previously to be very closely related to VEEV strains circulating on the Pacific coast of Mexico (19). Positive samples (≥1:20 titer) were further analyzed for immunoglobulin (Ig) M by using an antibody capture enzyme-linked immunoassay (MAC-ELISA) (20–22). The University of Texas Medical Branch Institutional Review Board approved screening of all human sera.

Virus Isolation

Heart and brain tissues from sentinel hamsters were triturated in Eagle's minimal essential medium (MEM) containing gentamicin and 20% fetal bovine serum to generate 10% suspensions. After centrifugation at 10,000 x *g* for 5 min, the supernatant was added to Vero cell monolayers and incubated for 4 days or until cytopathic effects were evident. VEEV antigen was identified in the Vero cells by using monoclonal antibodies (23), and VEEV RNA was detected in the supernatant by using reverse transcription–polymerase chain reactions (RT-PCR) as described previously (19).

Viral Sequencing and Phylogenetic Analyses

Partial or complete VEEV genomic sequences were determined (19,24). Viral RNA was extracted from the first BHK cell passage by using Trizol (Gibco BRL, Bethesda, MD), according to the manufacturer's protocol, and subjected to reverse transcription with a primer of sequence T25V designed to anneal to the poly (25) tract of the genomic and subgenomic RNA. Complementary DNA was subjected to PCR by using primers to amplify either the complete PE2 envelope glycoprotein precursor gene or the entire viral genome (24). PCR amplicons were purified with the Qiagen (Valencia, CA) kit according to the manufacturer's protocol and sequenced directly by using the ABI BigDye kit (Applied Biosystems, Foster City, CA) and an ABI377 automated sequencer. The sequence of the 5′ terminal 20 nucleotides of the genome was not determined because the PCR amplicons incorporated primers corresponding to this region. Sequences were aligned to homologous GenBank sequences for subtype IE VEEV strains subtypes, and other VEE complex sequences were used as outgroups for phylogenetic analyses. Aligned nucleotide and deduced amino acid sequences were analyzed by using the maximum parsimony, neighbor-

joining, and maximum likelihood programs in the PAUP 4 package (26). Bootstrap values were determined to assess the robustness of topologies with 1,000 replicates (27). A relative rates test was used to estimate the rate of evolution for VEEV in Mexico and elsewhere.

Statistical Methods

Statistical analyses were performed with Stata (Stata corp2001. STATA Statistical software release 7.0. College Station, TX) and EpiInfo 86 (Centers for Disease Control [CDC]-World Health Organization version 6.04 July 1996, CDC, Atlanta, GA). We estimated 95% confidence intervals (CI) and tested risk factors by age, sex, location, and occupation. Where appropriate, chi-square tests and other statistical approaches were used to test differences in risk among populations.

Results

Seroprevalence in Wild Animals and Bovines

Animals captured with Tomahawk and Sherman traps were tested for VEEV antibodies by using hemagglutination inhibition (HI), PRNT, or both, depending on the capabilities of the laboratory testing the samples. The results are shown in Table 2. Most notable were opossums, with an overall seropositivity of 25%, cotton rats (*Sigmodon hispidus*) with 67%, and rice rats (*Oryzomys alfaroi*) with 17% seropositivity. Cotton rats were suspected to be the most important reservoir hosts in enzootic VEEV foci studied in other parts of Mexico and Central America (28).

Simultaneously, we conducted a bovine sentinel serosurvey by testing serum specimens from 20 calves (6–18 months old) from several different farms in and around La Encrucijada. Of these samples, 14 were VEEV-positive by PRNT (titers 20–640); 8 of the calves lived within La Encrucijada Preserve. Subsequently, we bled an additional 110 calves from the same region, and 50 had HI titers from 20–320. Thirty of these also came from La Encrucijada within a radius of ≈15 km of the mouth of the river Novillero. The overall 49% seroprevalence in cattle ≤18 months of age (lifelong residents of the same ranches, as reported by owners) indicated that bovines are exposed to VEEV in southern Mexico and are excellent sentinels because they are fed on by large numbers of mosquitoes, are susceptible to benign VEEV infection, seroconvert (29), and are never vaccinated against VEEV.

Human Seroprevalence

Serum samples from 434 persons who resided in La Encrucijada were tested for VEEV antibodies. The PRNT results are summarized by age cohort in Figure 2. The overall PRNT seropositivity in the region was 37.6%; 38.5% of women and 36.4% of men. Among the different communities sampled, seropositivity ranged from 4% (Jamaica) to 75% (Las Coaches; see Table 3). Most notable was the distribution of seropositivity by age, with rising rates in the older age groups (Figure 2). The only exceptions to this trend were in age groups with small sample sizes (e.g., 0–5 years of age, 4/10 positive). Assuming that infection leads to PRNT antibodies lasting ≥25 years (e.g., human antibody responses to the TC-83 live-attenuated VEEV vaccine can last >30 years (R.E. Shope, pers. comm.), these data are consistent with a steady rate of endemic exposure to VEEV during the past 70 years; there is no suggestion of a disproportionately higher rate in persons >30 years of age, which would be expected from exposure during the 1969–1971 Mexican VEE epizootic.

Table 2. VEEV seroprevalence in wild animals from coastal Chiapas State, Mexico[a]

Species	Locality	No. Collected	Month (2000)	% pos	Titers (test)
Philander opossum	Pampa Honda	2	Apr	0	0
(Marsupialia; Didelphidae)	Las Coaches	5	Nov	20	640 (HI)
Didelphis marsupialis	La Providencia	1	Jun	100	160 (HI)
(Marsupialia; Didelphidae)					
Orizomis alfaroi	Pampa Honda	2	Apr	50	20 (PRNT)
(Rodent: Muridae)	Pampa Honda	4	Aug	0	
Orizomis couesi (Rodent; Muridae)	Pampa Honda	2	Apr	0	
	Pampa Honda	1	Aug	0	
	Santa Olga	6	Nov	0	
Sigmodon hispidus (Rodent; Muridae)	Pampa Honda	1	Apr	0	
	La Providencia	6	Aug	100	20–160 (HI)
	San Pedro	2	Jun	0	
Rattus rattus (Rodent; Muridae)	Pampa Honda	2	Aug	0	
Bovines (1.5 mo age)	Las Coaches	20	Nov	70	20–640 (PRNT)
Canis familiaris (dog)	Pampa Honda	3	Jun	33	20 (PRNT)
Chicken	Pampa Honda	5	Jun	20	20 (PRNT)
Meleagris gallopavo (turkey)	Pampa Honda	3	Jun	33	20 (PRNT)
Anser cinereus (goose)	Pampa Honda	1	Jun	100	320 (PRNT)

[a]VEEV, Venezuelan equine encephalitis virus; HI, hemagglutination inhibition; PRNT, plaque reduction neutralization test; pos, positive.

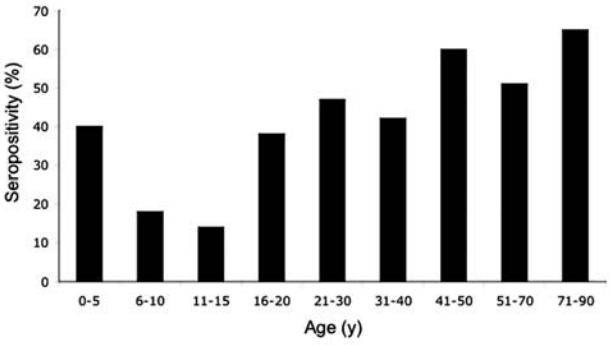

Figure 2. Rates of Venezuelan equine encephalitis virus (VEEV) seropositivity by age group for persons living in the La Encrucijada region. Positive samples had 80% plaque reduction neutralization test (PRNT) titers of ≥1:20. Numbers on bars indicate the total number of serum specimens tested for each age group.

These data indicate long-term endemic circulation of VEEV in the La Encrucijada region.

Of the PRNT positive human serum specimens, 8 (2%) were positive by IgM capture ELISA, which indicates VEEV infection during the past several months. As was the case for overall seropositivity, the IgM-positive samples were relatively evenly distributed among age groups and sexes (Table 4), which suggests that infections were recent. The low PRNT titers of some of the IgM-positive samples could have reflected differences between antigens on the circulating VEEV strains and those used in the neutralization test, or the titers could have decreased between the time of acute infection.

Risk Analysis for Human Infection

The risk factor analysis conducted on the inhabitants of the nine sampled villages showed varying results, according to different characteristics of the inhabitants and the geographic location of their households. Statistically significant associations for past VEEV exposure (seropositivity) were linked to occupation, age, and geographic location of the village. Analysis of occupations showed that medical personal had the highest risk (odds ratio [OR] 95.75, 95% CI 10.23–106.93), followed by fisherman (OR 14.39, 95% CI 4.20–52.22), housewives (OR 5.79, 95% CI 2.20–16.10), and farmers (OR 5.10, 95% CI 1.78–15.32). Results for these categories were compared to the results for the high school student population. Additional occupations not statistically associated with VEEV exposure included junior high school and primary students and preschool children. With regard to age, the groups at higher risk of past VEEV exposure were persons 71–90 years of age (OR 8.44, 95% CI 2.18–34.19), followed by persons 51–70 years (OR 4.69, 95% CI 2.13–10.41), 41–50 years (OR 7.37, 95% CI 3.2–17.18), 31–40 years (OR 3,41, 95% CI 1.59–7.36), and 21–30 years (OR 4,54, 95 % CI 2.06–10.11). We compared all the above categories to the age group of children 11–15 years of age. The youngest persons (≤10 years of age) had the lowest risk for past exposure, which is consistent with endemic VEEV circulation.

The geographic location or address of people also had a significant association with past VEEV infection, including the physical location of the village with respect to the mangrove forests and marshes. The closer the household was to these habitats, the higher the risk for their inhabitants being seropositive (OR 11.8, 95 % CI 4.03–38.85).

Isolation of VEEV Strains

Of a total of 14 hamsters exposed in La Encrucijada during July, 2001 (98 hamster-days), 10 became ill or died. Deaths began on the third day after hamster exposure, and VEEV was isolated from the hearts of five animals (Table 5). These viruses were confirmed antigenically as VEEV subtype ID/E by using enzootic subtype-specific monoclonal antibodies and immunofluorescence (23). Additionally, several isolates of Group C arboviruses (*Bunyaviridae*: *Orthobunyavirus*) were made.

Two of the VEEV strains (MX01-22, MX01-32) from two locations in Las Coaches were selected to assess markers of the epizootic phenotype and genetic relationships to Mexican VEEV isolates from the 1993 and 1996 outbreaks. The partial (856 nucleotides) PE2 envelope glycoprotein

Table 3. VEEV human seroprevalence in 9 localities of Coastal Chiapas sampled from October to December, 2000

Location	No. inhabitants	No. sampled	No. (%) positive	Estimated no. persons exposed
Las Coaches, Pijijiapan	362	36	27 (75)	221
Isla Morelos, Mapastepec	391	43	25 (58)	226
Roberto Barrios, Mapastepec	372	69	37 (54)	201
10 de Abril, Mapastepec	81	16	5 (31)	25
Francisco Sarabia, Mapastepec	354	76	14 (18)	67
Buena Vista, Pijijiapan	440	83	52 (63)	277
Las Palmas, Acapetahua	832	44	8 (18)	151
Jamaica, Escuintla[b]	703	26	1 (4)	28
Cintalapa[b]	632	41	3 (7)	47
Total	4,167	434	172 (40)	1,666

[a]VEEV, Venezuelan equine encephalitis virus.
[b]Cintalapa and Jamaica were the negative controls for our "unexposed" VEEV zone.

Table 4. Titers of IgM, IgG and PRNT in humans positive for VEEV IgM during the sampling period, October–December, 2000[a]

Community	Sex	Age	IgM titer (ELISA)	IgG titer (ELISA)[b]	PRNT titer
Roberto Barrios	Female	55	100	400	40
10 de Abril	Female	17	1,600	<100	80
10 de Abril	Male	14	400	100	20
Isla Morelos	Female	25	400	400	80
Buena Vista	Female	43	6,400	100	20
Buena Vista	Male	37	400	100	40
Buena Vista	Male	29	100	400	160
Buena Vista	Female	66	100	400	160

[a]Ig, immunoglobulin; PRNT, plaque reduction neutralization tests; VEEV, Venezuelan equine encephalitis virus; ELISA, enzyme-linked immunosorbent assay.
[b]ELISA titers were determined by using fourfold serum dilutions; others were determined by using twofold dilutions.

precursor gene was sequenced from both isolates (GenBank accession numbers AY823298 and AY823299) and both sequences were identical and most closely related to subtype IE VEEV isolated from the 1996 Oaxaca outbreak. As in previous analyses (19), the Mexican and Pacific Coastal Guatemalan subtype IE isolates grouped independently of the Atlantic/Caribbean Central American and Mexican isolates, as well as the Panama genotype (Figure 3). However, relationships among the Mexican strains from the Pacific coast were not well resolved. Therefore, the complete genome of one strain, MX01-22, was sequenced. The MX01-22 sequence had only 29 (0.26%) different nucleotides and three different deduced amino acids when compared to its closest relative, the 1996 equine strain OAX142; phylogenetic reconstruction showed that the MX01-22 strain is closely related to all of the 1996 equine isolates (Figure 4). Relative rate analyses indicated sequence divergence of 2–2.9 x 10^{-4} subst/nt/year for this Mexican lineage, similar to estimates of other enzootic VEEV lineages in South America (30). These data are consistent with the continuous circulation of a single major lineage of subtype IE VEEV in coastal Chiapas and Oaxaca states since 1993. The Guatemalan enzootic lineage represented by the 1968 and 1980 isolates from La Avellana on the Pacific coast had a slightly lower estimated sequence divergence rate of 6.8 x 10^{-5} subst/nt/year.

Discussion

Endemic and Enzootic VEE in Coastal Chiapas State

Using a combination of human serosurveys, wild and domestic animal serosurveys, and detection of virus circulation with sentinel hamsters, we obtained evidence that VEEV circulated in the La Encrucijada area of coastal Chiapas State for at least several decades before the 1993 epizootic and continues to circulate in an undescribed transmission cycle. Satellite imagery (Figure 1) and visual inspections indicate that lowland tropical forest habitats characteristic of VEEV enzootic foci, including of subtype IE viruses in other areas of Mexico and the nearby Pacific coast of Guatemala (31,32), have been almost completely destroyed for the purposes of cattle ranching and other agricultural activities. Mosquito collections in La Encrucijada have indicated an extremely low abundance of *Culex* (*Melanoconion*) *taeniopus*, the proven enzootic vector in a coastal Guatemalan subtype IE VEEV focus (33). This indicates that VEEV is likely using a different mosquito species as its enzootic vector in coastal Chiapas State. Experimental studies indicate that adaptation of the Mexican VEEV strains for efficient infection of *Ochlerotatus taeniorhynchus* mosquitoes, through a mutation in the E2 envelope glycoprotein, may have contributed to epizootic transmission (34).

Seroprevalence in wild animals suggests a possible role for cotton rats (*S. hispidus*), implicated previously as a VEEV reservoir host in other parts of Mexico (28) and in Panama (35,36). Although the number of animals tested was small, our data also suggest a possible role for rice rats (*Oryzomys alfaroi*) and opossums (*Didelphis marsupialis* and *Philander opposum*) as vertebrate reservoir hosts required to maintain horizontal transmission or amplification hosts involved in increased circulation, resulting in equine cases. Other domestic animals, including dogs, cattle, and birds, are also exposed to VEEV in Chiapas State. Previous experimental studies of dogs (37) and cattle (38) indicate that low levels of viremia or none develops in those animals after VEEV infection and, therefore, they are probably not important as enzootic hosts. Larger sample sizes and experimental infections to assess viremia levels are needed before conclusions can be drawn regarding the relative importance of different animals as reservoir or amplification hosts of VEEV in Mexico.

Table 5. Results of Sentinel hamster exposure in La Encrucijada Preserve, July, 2001[a]

Site	No. hamsters exposed	Hamster-days of exposure	No. moribund or dead hamsters	VEEV isolations
Las Coaches	10	70	7	5
Roberto Barrios	4	28	0	0

[a]VEEV, Venezuela equine encephalitis virus.

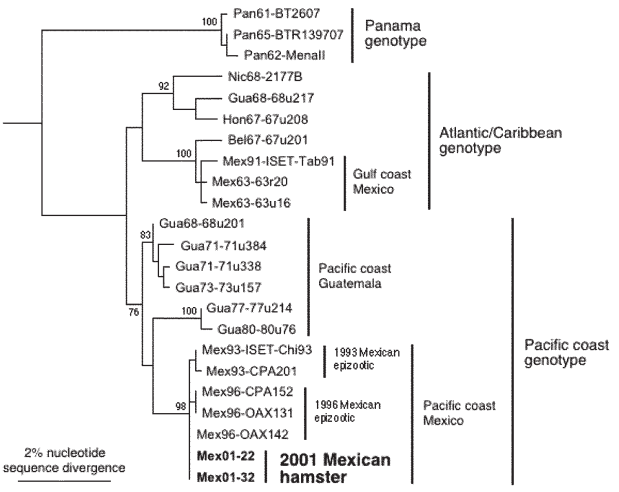

Figure 3. Maximum parsimony phylogenetic tree derived from partial PE2 envelope glycoprotein precursor gene sequences showing relationships of the newly isolated Venezuelan equine encephalitis virus (VEEV) strains from sentinel hamsters (Mex01-22 and Mex01-32) to other subtype IE strains sequenced previously (19). Strains are designated by country abbreviation followed by year of isolation and strain designation. Numbers indicate nucleotide substitutions assigned to each branch.

The vertebrate amplification hosts responsible for increased circulation of VEEV and its transmission to equines during 1993 and 1996 remain unidentified, since equines inefficiently amplify the etiologic VEEV strains of the subtype isolated during the outbreaks (16). One possibility for an alternative amplification host is humans, in whom high levels of viremia develop after infection by subtypes IAB and IC VEEV (14,39). Our results indicate that persons are regularly exposed in coastal Chiapas, and their migratory work habits could enhance spread during outbreaks. Estimation of viremia titers from infected persons in southern Mexico is needed to test this hypothesis. Another possibility is that increased populations of wild vertebrate hosts such as *S. hispidus* resulted in increased amplification of the virus and its transmission to equines during the summer of 1993.

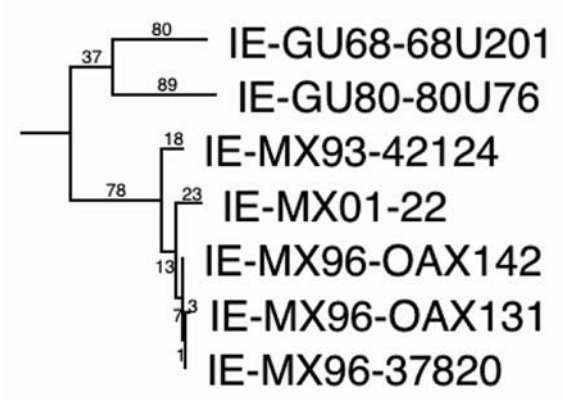

Figure 4. Maximum parsimony phylogenetic tree derived from complete genomic sequences showing relationships of the newly isolated Venezuelan equine encephalitis virus (VEEV) strain (MX01-22) to other strains from Mexico and Guatemala. Numbers indicate nucleotide substitutions assigned to each branch. All nodes had bootstrap values of 100%, except the OAX131-37820 (62%) and GU68-GU80 (<50%) groupings. Relative rates tests applied to the branches indicated a rate of nucleotide substitution in Mexico of 2.0–2.9 x 10^{-4} subst/nt/y since 1993, and 6.8 x 10^{-5} for the Guatemalan lineage from 1968–1980. These data suggest continuous circulation of VEEV in Mexico since 1993.

Human Risk and Disease

Although our data indicate that persons are regularly infected with VEEV in coastal Chiapas State, the effect of endemic transmission on human health is unknown. Most undifferentiated febrile illness in the region is clinically diagnosed as dengue or flulike febrile illness, and currently no diagnostic tools are in place to test for human VEEV infections. We identified the occupations of La Encrucijada inhabitants and the ecologic habitats where they live and work as risk factors for VEEV infection. We detected no risk differences between the sexes. A possible explanation is that, in this region, female homemakers often accompany male farm laborers to sites where VEEV may circulate.

The spatial risks for VEEV infection apparently do not include proximity to human habitations. Thus, VEEV is distinct from dengue viruses, which circulate peridomestically (40). The higher risk for medical personnel for VEEV infection suggests the possibility of aerosol exposure in clinical settings. Epidemiologic data on the seasonality of flulike febrile illness in coastal Chiapas State (Instituto Mexicano del Seguro Social, unpub. data) indicate peak incidence from June to November, coincident with the rainy season and peak mosquito populations. These data suggest that many flulike illnesses may be caused by VEEV and possibly other arboviruses such as group C orthobunyaviruses.

Acknowledgments

We thank Amelia Travassos da Rosa and Robert Tesh for assistance with serologic testing and Irene Lopez, Yolanda Barrera, Antonio Cossio, and Enar Galicia for support in the field.

This research was supported by grants AI39800 and AI48807 and contract N01-AI25489 from the National Institutes of Health and from grants provided to J.G.E.F. by the Interamerican Institute for Cooperation on Agriculture and the Pan American Health Organization in Washington D.C. and Mexico.

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Opisthorchiasis from Imported Raw Fish

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Liver fluke infection caused by *Opisthorchiidae* is a major public health problem in many parts of the Far East, Southeast Asia, and eastern Europe. However, with the growing volume of international travel and population migration, the infection is increasingly diagnosed in countries where the disease is not endemic, particularly in North America. We report an outbreak of acute opisthorchiasis in a family that was infected in a non–disease-endemic area after eating raw carp illegally imported from a highly disease-endemic area in Siberia. With the growing numbers of former Soviet Union citizens immigrating to other countries, western physicians need to be alert regarding *Opisthorchis*-associated pathology in this population. The epidemiology and biology of *Opisthorchiidae* in the former Soviet Union are reviewed.

Liver fluke infection caused by trematodes belonging to the family *Opisthorchiidae—Opisthorchis viverrini*, *O. felineus*, and *Clonorchis sinensis*—is a major public health problem in many parts of the Far East, Southeast Asia, and eastern Europe. An estimated 17 million persons worldwide are infested: 7 million with *C. sinensis*, 9 million with *O. viverrini*, and 1.6 million with *O. felineus*. *O. viverrini* is prevalent in Thailand, Lao People's Democratic Republic, and Cambodia; *C. sinensis* is widespread in Korea, China, Taiwan, and Vietnam; and *O. felineus* is found in the Russian Federation and eastern Europe. Migration and global tourism are responsible for cases diagnosed in areas where the disease is not endemic (1).

The adult worms are flat, leaf-shaped, transparent, and hermaphroditic flukes that reproduce by self-fertilization. They live in the biliary and pancreatic ducts and occasionally in the gallbladder. The eggs are passed with the feces of the definitive natural host (cats, dogs, pigs, and many other fish-eating mammals) and are mature at excretion. The embryonated eggs are ingested by the intermediate host, a suitable freshwater snail, which varies geographically and according to the parasite species (2). In the digestive tract of the snail, the eggs hatch and become miracidia that go through several developmental stages and multiply asexually into thousands of tailed, free-swimming cercariae. The cercariae penetrate under the scales of a susceptible fish, which serves as the second intermediate host; they encyst as metacercaria, mainly in the fish body muscles. Fish belonging to the family *Ciprinidae* (carp) are the major intermediate host of *Clonorchis sinensis* and *Opisthorchis* spp. (2). However, a wide range of species of freshwater fish can be naturally infected by liver flukes, and more than one fish species in any aquatic environment can become infected (2). Humans, as incidental definitive hosts, are infected by ingesting a raw fish containing metacercariae. After excysting in the duodenum, the metacercariae migrate through the ampulla of Vater into the bile ducts, where they mature into adult worms within 4 weeks and deposit yellow, operculated eggs**.** The parasites may live for up to 45 years in a human host, producing 1,000–2,500 eggs per day (2).

The infection is associated with a number of hepatobiliary diseases. The pathologic and clinical consequences of opisthorchiasis are related to the intensity and duration of cumulative infestations. The flukes cause mechanical injury to the bile ducts, and their metabolic products irritate the biliary epithelial cells, leading to cell desquamation, hyperplasia, dysplasia, and eventual fibrosis or cancer. Chronic infestation can result in obstruction of the biliary tract, dilatation of intrahepatic ducts, and subsequent cystic and saccular formations. The gallbladder may enlarge and become nonfunctional, containing muddy bile (3). Because adult flukes are long-lived, they can produce eggs and symptoms long after the human host has emigrated from the area (4). The acute symptoms of *O. felineus* infection consist of high-grade fever, malaise, anorexia, diarrhea or constipation, dull pain and discomfort in the upper right quadrant of the abdomen, arthralgia, lymphadenopathy, and urticarial skin rash. Subacute and chronic complications include suppurative cholangitis,

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liver abscess, and cholangiocarcinoma (2). Acute infestation with *C. sinensis* is usually asymptomatic, although some patients may have fever, rash, malaise, and abdominal discomfort in the right upper quadrant. Chronic clonorchiasis may be complicated with gallbladder and intrahepatic duct stones, recurrent pyogenic cholangitis, cholecystitis, liver abscess, and cholangiocarcinoma (5). Most persons with *O. viverrini* infection have no symptoms. Only 5%–10% of heavily infected persons have nonspecific chronic symptoms, such as right upper quadrant abdominal pain, flatulence, and fatigue. Cholangiocarcinoma is a known complication (5).

We report herein on a familial outbreak of liver fluke infection due to eating raw fish personally imported from Siberia. While ample information is available on the biology and epidemiology of liver fluke infection in Southeast Asia (recently summarized in a special issue of Acta Tropica [6]), reports in the English language literature on the situation in the former Soviet Union are scarce (2). Because so many persons have emigrated from the former USSR to Western countries in recent years, physicians in these countries should be more familiar with the condition; thus, review of the epidemiology of opisthorchiasis in former USSR is appropriate.

Patients and Methods

A 46-year-old woman and her 47-year-old husband, who immigrated from Siberia to Israel 7 years earlier, were admitted because of gastrointestinal complaints of 10 days' duration. The symptoms included nausea, vomiting, yellow sclera, diffuse arthralgia (in the woman), weakness, rigors, and fever up to 39°C. On admission, the woman was afebrile, and results of her physical examination were normal. The husband's temperature was 38.4°C; his enlarged, nontender liver was palpated 3 cm below the right costal margin, and his sclera were jaundiced. Laboratory findings in both patients (Table) consisted of marked leukocytosis with notable eosinophilia and elevation of liver enzymes. Ultrasonographic examination of the abdomen showed a slightly enlarged spleen in both patients, and an enlarged liver in the husband.

Results

The triad of abdominal symptoms, eosinophilia, and liver enzyme impairment evoked the possibility of a helminthic infection. On further questioning, the couple recalled having eaten a smoked carp 10 days before becoming sick. The fish was bought in Nizhnevartovsk, Siberia, and was brought to Israel by the couple's son. The other members of the family, the couple's 23-year-old son and 17-year-old daughter, and a friend had also eaten the imported fish. They were asymptomatic at the time of the investigation, although the son reported a short febrile episode that resolved spontaneously, and the daughter had transient abdominal pain in the right upper quadrant. Their leukocyte counts and liver enzyme test results were normal (Table). The wife also recalled having had similar symptoms 15 years earlier, while still living in Siberia. A diagnosis of opisthorchiasis was made on the basis of ova identified in the bile from the wife.

Stool samples from the five persons were examined for ova after concentration with the formaldehyde-ether technique. *Opisthorchis/Clonorchis* eggs (Figure 1) were found in all stool samples with the exception of that belonging to patient 5, the friend. The fish was not available for examination.

Treatment consisting of praziquantel, 25 mg/kg orally three times daily for 1 day, was administered to the infect-

Table. Main symptoms and laboratory findings in persons who ate raw fish imported from Siberia[a,b]

Relationship	Time	GI symptoms	Temp, °C	WBC, x10^3/μL	Eosin, %	ALT/AST, U/L	ALP, U/L	LDH, U/L	Bili, mg/dL
Wife	Pretherapy	Vomiting, jaundice	38.4	58.3	78	125/96	294	528	1.69
	Posttherapy[c]	None	36.0	19.2	50	44/29	178	355	1.09
	Follow-up[d]	None	36.4	7.2	6.2	45/37	64.6	ND	0.7
Husband	Pretherapy	Nausea	39.0	16.9	50	188/164	1147	531	3.17
	Posttherapy	None	36.8	13.4	45.6	170/65	555	512	1.9
	Follow-up	None	36.6	6.85	4.5	48/35	134	390	1.17
Son	Pretherapy	None	36.5	8.2	5.7	28/31	110	368	0.77
	Posttherapy	None	ND	ND	ND.	ND	ND	ND	ND
	Follow-up	None	36.6	ND	ND	ND	ND	ND	ND
Daughter	Pretherapy	RUQ pain	36.0	7.23	4.7	33/32	53	377	0.49
	Posttherapy	None	ND	ND	ND	ND	ND	ND	ND
	Follow-up	None	36.6	ND	ND	ND	ND	ND	ND
Friend	On admission	None	36.6	8.08	1	15/14	111	372	0.64

[a]GI, gastronintestinal; Temp., temperature; WBC, leukocyte count; eosin, eosinophils; ALT/AST, alanine aminotransferase/aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; ND, not done; RUQ, right upper quadrant.
[b]Abnormal laboratory results are underlined. Normal values: WBC (leukocyte) count, 4–10 x 10^3/μL; eosinophils, 1%–6%; ALT, 0–41 U/L; AST, 0–38 U/L; ALP, 39–117 U/L; LDH, 240–480; bilirubin, 0.3–1.0 mg/dL.
[c]Posttherapy, 4 days after therapy.
[d]Follow-up, 2 weeks after therapy.

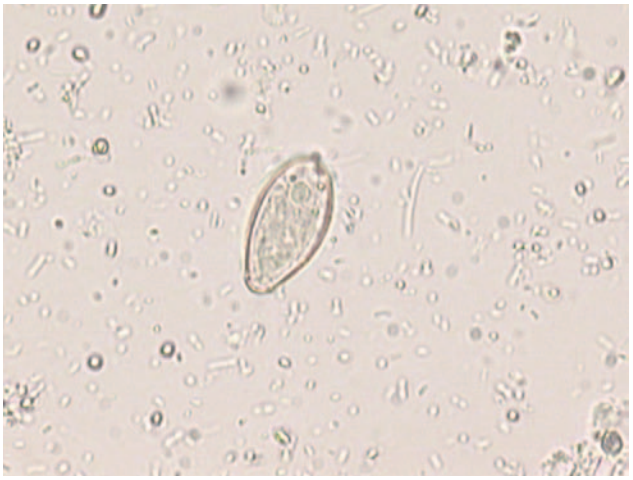

Figure 1. *Opisthorchis-Clonorchis* egg detected in the stool of one patient.

ed patients (with positive stools for ova). The symptomatic patients (wife and husband) improved promptly, both clinically and as evidenced by laboratory values. On follow-up 2 weeks later, leukocyte count and liver enzyme levels had returned to normal (Table).

Discussion

The diagnosis of liver fluke infection in this outbreak was confirmed by identifying ova in stool. Determining correctly the species of the causative parasite on the basis of egg form and shape is more challenging because eggs of *O. viverrini*, *O. felineus*, and *C. sinensis* are morphologically similar, and the differentiation is difficult even for experts (3,7). Only the identification of adult worms will confirm the species (3). It has been suggested that *O. felineus* differ from the other two flukes in the ratio of the length to the width of the egg, which is 1:3 in the former and 1:2 in the latter (3). However, we could not find additional evidence in the literature in support of this statement (4). Although the ova identified in our patients had a ratio of 1:2, we believe that the infection in this outbreak was caused by *O. felineus* on the basis of the source of the consumed fish and the acute symptoms in two of the four infested family members. In fact, Nizhnevartovsk is located in the Ob River basin, where *O. felineus* infection is hyperendemic (8). In addition, acute serum sickness–like symptoms are much more common after *O. felineus* infection than after infection with *O. viverrini* or *C. sinensis* (4,5). Moreover, *O. viverrini* is not endemic in the Russian Republic, while *C. sinensis* is found only in the Amur River area on the Russian-Chinese border (2).

O. felineus infection is the most prevalent foodborne liver fluke infection of humans in Russia, Ukraine, and Kazakstan (Figure 2). Infestation usually follows consumption of raw, slightly salted, and frozen fish ("stroganina"). The parasite is endemic in an area that covers nearly all the territory of the Russian Federation with the exception of the northern parts of Siberia and the far-eastern regions. The largest parasite-endemic area is in western Siberia, namely the Ob and Irtysh River valleys and their tributaries (9–12). In the central part of this area, the Tyumen and Tomsk Districts, the mean prevalence of human infection is 40%–95%. Prevalences of 45% to 65% were reported in the Komi-Permiak national district, and infection rates up to 46% have been documented in some communities in Omsk District. Other districts and territories where opisthorchiasis is endemic include the Yekaterinburg (formally Sverdlovsk) District (13), Altai territory, Voronezh District (14), Volga River valley (15) and Archangelsk District in western Russia, and the Angara River (16), Krasnoyarsk territory, and Irkutsk District in eastern Siberia (2). In Ukraine, opisthorchiasis is limited to the Sumy, Poltava, and Chernigov Districts of the Dnieper River basin (17,18), where the prevalence is 5%–40%. In Kazakstan, opistorchiasis is endemic in the Aktyubinsk, Dzhezkazgan, Karaganda, Pavlodar, Tselinograd, and Turgay Districts. Foci of opisthorchiasis have also been found in the Brest, Gomel, and Grodno provinces of Belarus (2). Limited endemic foci of opisthorchiasis in some areas of the Baltic States, eastern Germany, and Poland were described before the Second World War; however, no recent information on the occurrence of the infection in humans in these countries is available.

The correlation between *O. felineus* infection and cholangiocarcinoma was studied in the Tyumen region, Russia. In the southern part of the region, where 0.5% of the population was infected with *O. felineus*, the prevalence of cholangiocarcinoma was 4.4 per 100,000 population. In the central area of Tyumen with 45% prevalence of *O. felineus* infestation, the rate of cholangiocarcinoma was 10-fold higher than in the south (49.8 per 100,000 population) (2).

C. sinensis infection is endemic in the Amur River valleys and Khabarovsk territory, situated in the far eastern part of the Russian Federation. The prevalence of the infection in the native Nanai population is 24% in the most affected villages (2,19).

The major snail hosts for *O. felineus* are *Codiella (Bithynia) inflata*, *C. troscheli*, and *C. leachi*. *C. sinensis* is transmitted by a wide range of operculate snails, *Parafossarulus manchouricus* being the main one (2). Twenty-two species of 17 genera of the family *Ciprinidae* are infected by *O. felineus*; the most important are *Leuciscus idus*, *L. leuciscus*, and *Rutilus rutilus*; five species host the *C. sinensis* fluke (2).

The growing volume of international travel and population migration, facilitated by increasing availability of air transportation, is responsible for cases of opisthorchiasis

Figure 2. *Opisthorchis* (solid lines) and *Clonorchis* (broken lines) endemic areas in the former USSR. Original map was obtained from the United Nations Development Programme Web site (www.undp.org).

and clonorchiasis diagnosed in non–disease-endemic countries, mainly in North America. Most reports from the United States and Canada describe the detection of *Opisthorchis/Clonorchis* ova in immigrants from Southeast Asia with chronic infection (20–27). The infection was also documented in North American residents who contracted the disease during long-term or short visits to disease-endemic areas (22). In the United States, liver fluke infection continues to be an active health problem for hundreds of thousands of Southeast Asian refugees who have immigrated since 1975. *Clonorchis* infestation was documented in 26% of 150 Chinese immigrants in New York City (21). Stool examinations of 186 Indochinese refugees in California have detected *C. sinesis* eggs in 13% (23). In another report, the prevalence of *Opisthorchis* eggs among 226 asymptomatic adult Southeast Asian immigrants to the United States was 11% (24). In Montreal, Canada, *Clonorchis* infestation was documented in 15.5% of 400 Chinese immigrants (20).

We have identified only two reports (in German) of patients of Russian origin whose conditions were diagnosed in western Europe. A 58-year-old woman, who emigrated from Tomsk a year earlier, was seen in Wiesbaden, Germany, for right upper abdominal and flank pain, reduced appetite, and weight loss; *O. felineus* eggs were detected in stool and duodenal aspirate (28). For two patients from Siberia with suspected eosinophilic leukemia and carcinoma of the gallbladder, respectively, opisthorchiasis was diagnosed in Hamburg (29).

Unlike previous reports of opisthorchiasis diagnosed in non–disease-endemic countries, which included patients infected in areas endemic for disease, the patients in the present series were infected outside an endemic region by food imported illegally from a country where the disease was highly prevalent. An estimated 2 million citizens from the former USSR have moved since the Soviet collapse in 1989, mostly to North America, western Europe (mainly Germany), and Israel (30). Most of these immigrants continue to maintain strong cultural ties with their countries of origin, including through eating delicatessen food from the "old country." Thus, both chronic and acute infections can be diagnosed in this population. Physicians providing care to immigrants from the former Soviet Union should be aware of the potential presence of liver fluke infection in these patients and consider the entity in the differential diagnosis, when appropriate.

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Identifying Rodent Hantavirus Reservoirs, Brazil

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We describe the genetic analysis of samples from hantavirus pulmonary syndrome (HPS) patients from southern and southeastern states of Brazil and rodents captured at the presumed site of infection of these patients. A total of 65 samples that were antibody-positive for Sin Nombre or Laguna Negra virus by enzyme-linked immunosorbent assay were processed by nested reverse transcription–polymerase chain reaction (RT-PCR) by using several primer combinations in the M and S genome segments. PCR products were amplified and sequenced from samples from 11 HPS patient and 7 rodent samples. Phylogenetic analysis of nucleotide sequence differences showed the cocirculation of Araraquara and Juquitiba-like viruses, previously characterized from humans. Our genetic data indicate that Araraquara virus is associated with *Bolomys lasiurus* (hairy-tailed Bolo mouse) and the Juquitiba-like virus is associated with *Oligoryzomys nigripes* (black-footed pigmy rice rat).

Hantaviruses are mainly rodentborne viruses of the family *Bunyaviridae* (1). Two clinical forms of infections by hantaviruses are known: hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome (HPS) in the American continent (2–4). Hantaviruses are enveloped, single-stranded, negative-sense RNA viruses, with a genome with three segments, designated small (S), medium (M), and large (L). The S segment encodes the nucleocapsid protein N, the M segment encodes a glycoprotein precursor that is processed into the envelope glycoproteins G1 and G2, and the L segment encodes the RNA polymerase (5,6).

The hantaviruses that cause HPS are associated with wild rodents species of the subfamily *Sigmodontinae*. They are transmitted mainly by contact or through aerosols of excrete and secretions of infected rodents (7–9). Person-to-person transmission has been reported in the 1996 outbreak in Argentina, involving the Andes (AND) virus (10,11). In Chile, this kind of transmission is suggested by clusters of cases in household contacts (12).

In Brazil, during the 1980s and 1990s, virologic and serologic studies conducted in humans and urban rodents showed the circulation of a hantavirus related to Seoul virus (13–16). In 1993, cases of an acute respiratory illness were detected in a family cluster in Juquitiba County, approximately 80 km from São Paulo City, in southeastern Brazil. Three brothers were affected by the infection, and two of them died (17,18). Necropsy material from one of them allowed the genetic characterization of a new hantavirus, later named Juquitiba (JUQ) virus, by sequencing a fragment of 139 nucleotides (nt) of the M genomic segment G2 encoding region (19). During 1995 and 1996, three more cases of HPS were confirmed by enzyme-linked immunosorbent assay (ELISA); one patient was from the central western county of Vilarejo de Castelo dos Sonhos, in Mato Grosso State, and the remaining two patients were from Araraquara and Franca counties in São Paulo State. Molecular studies carried out on samples from those HPS patients identified two novel genetic lineages of hantaviruses, Castelo dos Sonhos (CAS) and Araraquara (ARA) viruses (20). In 1998, new cases of HPS were detected: two in Minas Gerais, four cases in Rio Grande do Sul, and five in São Paulo State. Since then, an increasing number of HPS cases have been diagnosed annually in many states of Brazil. By March 2004, 342 HPS cases had been diagnosed on the basis of characteristic clinical syndrome, epidemiologic data, and Ig (immunoglobulin) M and IgG serologic response against Sin Nombre (SN), Laguna Negra (LN), or AND virus antigens by ELISA (9,21). Some of these cases were also diagnosed by immunohistochemistry. Most of the HPS cases occurred in the southern and southeastern states of Brazil (177 and 113, respectively). Paraná reported the highest number of cases (n = 92), followed by São Paulo (n = 59), Minas Gerais (n = 54), Santa Catarina (n = 50), Rio Grande do Sul (n = 35), Mato Grosso (n = 33), Maranhão (n = 7), Pará

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(n = 4), Goiás (n = 3), Rio Grande do Norte (n = 1), and Bahia (n = 1) (M. Elkhoury, pers. comm.).

This study describes the genetic analysis carried out on samples from HPS-case patients from southern and southeastern states of Brazil and rodents captured at the presumed site of infection of the human case-patients. The primary aims were to identify the hantavirus lineages causing HPS in that area, because few reports were available on this topic, and to identify the potential rodent host reservoirs because genetic data were not available from hantavirus-positive rodents. Genetic analysis of the nucleotide sequences indicates that ARA and JUQ-like viruses are circulating in the studied area. We report the genetic identification of the putative primary rodent reservoirs for these viruses.

Material and Methods

Area of Study

The studied areas included two kinds of natural ecosystems: the Atlantic rainforest and "cerrado" at the southern states of Paraná, Santa Catarina, and Rio Grande do Sul and at the southeastern states of Minas Gerais and São Paulo (Figure 1). Basically, the Atlantic rainforest extends along the Brazilian Atlantic Coast, and it is found as umbrofilous tropical forest of hillside or as its regional variation known as Araucaria forest. The cerrado occurs in the Brazilian central plateau and part of northeastern region, and it is characterized by small trees, and grass vegetation, adapted to climates with long dry periods. Both kinds of ecosystems are found in São Paulo and Minas Gerais States.

Patient and Rodent Samples

We studied samples from HPS patients and from rodents captured at the potential sites where HPS exposures occurred. All samples included in the present study had tested positive to hantavirus by ELISA (9) by using SN virus and LN virus antigens (provided by T.G. Ksiazek, Centers for Disease Control and Prevention, Atlanta, GA).

Patients

A total of 40 blood and serum samples of HPS patients from five different states of Brazil were processed by nested RT-PCR: 6 samples from Minas Gerais (Patrocinio, Uberaba, Araxá, and Passos); 10 samples from São Paulo (Flórida Paulista, Batatais, Franca, São Carlos, Jaú, Cotia, Barra do Turvo, and Tupi Paulista); 7 samples from Paraná (General Carneiro, Bituruna, Ponta Grossa, Catanduvas, Curitiba, and Guarapuava); 7 samples from Santa Catarina (Seara, Arroio Trinta, and Lindóia do Sul); 10 samples from Rio Grande do Sul (Vacaria, Pelotas, Marcelino Ramos, São Lourenço do Sul, Capão Canoa, Santana do Livramento, Santa Cruz do Sul, Novo Hamburgo, and Arvorezinha). Of seven samples from HPS patients from Santa Catarina, five samples were from a family cluster reported by Seara (22).

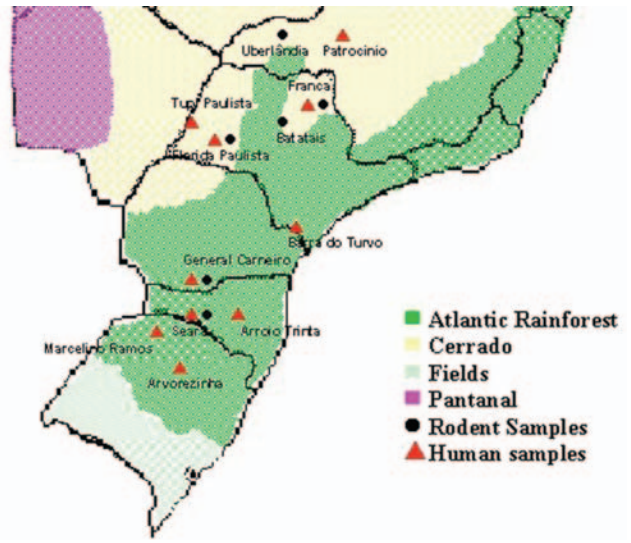

Figure 1. Distribution of natural ecosystems in Brazil. Red triangles and black circles indicate the location of hantavirus pulmonary syndrome cases and rodent capture, which originated the studied DNA sequences, respectively.

Rodents

Rodents were captured by using Sherman live-capture traps (Sherman Traps Inc., Tallahassee, FL) set in rural or sylvan environments, around the presumed sites of HPS infection. The rodents were processed in the field; biologic samples (blood, liver, kidneys, spleen, heart, and lung) were obtained according to established biosafety guidelines (23) and stored in liquid nitrogen for further processing. The carcasses of the rodents were brought to the laboratory; the skins and craniums were used for further identification of the positive specimens. Samples of carcasses were deposited at Museu de Zoologia—Universidade de São Paulo, São Paulo, São Paulo State, and Museum of Southwestern Biology, University of New Mexico, Division of Mammals, Albuquerque, New Mexico, and most of the specimens were deposited in the Vertebrates Collection at Instituto Adolfo Lutz— Seção de Vírus Transmitidos por Artrópodos in São Paulo, SP.

A total of 25 rodent samples (subfamily *Sigmodontinae*) were studied by nested RT-PCR: 3 samples were from Uberlândia (2 *Bolomys lasiurus*) and Uberaba (1 *B. lasiurus*), in Minas Gerais; 13 samples were from Araraquara (1 *B. lasiurus*), Batatais (2 *B. lasiurus)*, Franca (1 *B. lasiurus* and 1 *Calomys tener*), Cassia dos Coqueiros (1 *Oximycterus rutilans*), Cravinhos (1 *B. lasiurus*), Fartura (1 *Akodon* sp.), Mariápolis (2 *B. lasiurus*),

Nuporanga (1 *B. lasiurus* and 1 *Oligoryzomys nigripes*), and São Carlos (1 *B. lasiurus*), in São Paulo; 3 samples from General Carneiro (2 *O. nigripes* and 1 *Akodon* sp.) in Paraná; 3 samples from Seara (2 *O. nigripes* and 1 *Bolomys* sp.) in Santa Catarina; 3 samples from Marcelino Ramos (1 *O. nigripes* and 1 *Akodon* sp.) and São Lourenço do Sul (1 *Akodon* sp.), in Rio Grande do Sul.

RNA Extraction and Nested RT-PCR

Total RNA was extracted from human blood samples and rodent lung samples by using the RNaid (PLUS) Kit (BIO 101 Inc., La Jolla, CA) as described elsewhere (24). Briefly, approximately 100 mg of tissue was mixed with 300 μL of cell lysis solution containing guanidine thiocyanate extracted with phenol/chloroform and purified with RNA matrix beads. From some tissue samples, RNA was extracted with Trizol LS reagent (Invitrogen Co., Carlsbad, CA), following the manufacturer's recommendations. When human serum specimens were used as source of viral RNA, the QIAmp Viral RNA Kit (Qiagen, Chastworth, CA) was used according to the manufacturer's instructions. Amplification of virus RNA was performed by "RT-PCR-one step," followed by a second PCR amplification as described previously (4). Numerous primer combinations in the M and S segments were used in nested RT-PCR reactions, including oligonucleotide sequences published (4,25,26) and unpublished that were designed to amplify conserved fragments of the S and M genome segments of South American hantaviruses.

Genetic and Phylogenetic Analysis

The DNA products of the nested PCR reactions were separated from an agarose gel, and bands of the correct predicted size were purified from gel slices with a GeneClean kit (BIO 101 Inc.) or GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The nucleotide sequence of these products was determined on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Foster City, CA.) using the dydeoxy cycle sequencing technique (4). Sequences were aligned with those of previously described hantaviruses by using BioEdit version 5.0.9 (North Carolina State University, Raleigh, NC) and the computer software package Clustal W 1.4 (27). Primer sequences were removed from sample sequences before being aligned**.** Phylogenetic analysis was carried out on the multiple nucleotide and amino acid sequence alignments by using maximum parsimony (PAUP* version 4.0b4a Macintosh computer software programs) (28) and the distance-based neighbor-joining method. Phylogenetic analysis by maximum parsimony was obtained by the heuristic search method. Pairwise genetic distances were computed by using the Kimura-2 parameter, as implemented in the computer program MEGA version 2.1 software (29). The bootstrap support for the results of the phylogenetic analysis was based on 500 replicates. GenBank accession numbers of the previously published sequences of the hantaviruses used in this study are listed in figure legends.

Results

PCR products of the expected size were amplified from 11 of 40 HPS patient samples (Table 1) and 7 of 25 rodent samples studied (Table 2). A 303-nt fragment of the G2 gene was amplified and sequenced (bases corresponding to position 2807 to 3109 of Lechiguanas [LEC] virus). Phylogenetic analysis of nucleotide sequence differences showed the cocirculation of two genetic hantavirus lineages previously characterized from humans only: ARA virus and a genotype compatible with the previously identified JUQ virus. ARA virus sequences were derived from eight samples: from three HPS patients and three rodents (*Bolomys lasiurus*) from the state of São Paulo, and 1 HPS case-patient and one rodent (*B. lasiurus*) from Minas Gerais. Pairwise comparisons of the sequences of ARA virus strains from HPS patients and *B. lasiurus* showed an 85.1%-99.7% nt and 95%-99% amino acid (aa) identity. The viral sequence from the HPS patient Hu237251 from Patrocínio, Minas Gerais, the northernmost location included in this study, was most divergent from the other members of this group (16.7%). The

Table 1. Sequenced viral RNA samples and epidemiologic data of hantavirus pulmonary syndrome case-patients, Brazil

Specimen	Age (y)	Onset of symptoms	Sample date	County/State	Outcome
Hu193054	25	Oct 18, 2000	NA	Seara, SC[a]	Survived
Hu193256	22	Nov 5, 2000	NA	Seara, SC	Survived
Hu196618	NA	Mar 5, 2001	NA	Flórida Paulista, SP	Died
Hu199084	39	Apr 8, 2001	Apr 13, 2001	Batatais, SP	Survived
Hu201444	28	NA	May 8, 2001	Arvorezinha,RS	Survived
Hu205597	40	Aug 28, 2001	Sep 2, 2001	Tupi Paulista, SP	Survived
Hu206776	NA	NA	Nov, 2001	Arroio Trinta, SC	Died
Hu237251	22	Jul 1, 2002	Jul 17, 2002	Patrocínio, MG	Survived
Hu238063	39	NA	Aug 12, 2002	General Carneiro, PR	Died
Hu239727	32	Oct 8, 2002	NA	Barra do Turvo, SP	Died
Hu206102	52	NA	Sep 27, 2001	Marcelino Ramos, RS	Died

[a]SC, Santa Catarina; SP, São Paulo; RS, Rio Grande do Sul; MG, Minas Gerais; PR, Paraná; NA, not available.

Table 2. Rodent samples hantavirus positive by RT-PCR according to location and original ecosystem, Brazil[a]

Specimen code	Rodent species	Original ecosystem	County/state
On193576	*Oligoryzomys nigripes*	Atlantic rainforest	General Carneiro, PR[a]
Bl194307	*Bolomys lasiurus*	Cerrado	Franca, SP
Bl235018	*B. lasiurus*	Cerrado	Uberlândia, MG
Bl235518	*B. lasiurus*	Cerrado	Mariápolis, SP
Bl236546	*B. lasiurus*	Cerrado	Nuporanga, SP
On238341	*O. nigripes*	Atlantic rainforest	Seara, SC
On238477	*O. nigripes*	Atlantic rainforest	Seara, SC

[a]RT-PCR, reverse transcription–polymerase chain reaction; PR, Paraná; SP, São Paulo; MG, Minas Gerais; SC, Santa Catarina.

second hantavirus genetic lineage identified was closely related to JUQ virus. Samples from seven HPS patients and three *Oligoryzomys nigripes* from the states of Rio Grande do Sul, Santa Catarina, Paraná, and São Paulo fall into this group (Figure 2).

Sequence comparison with JUQ virus, limited to an overlapping piece of 139 nt of the G2 encoding region, the only available from this virus (19), showed 85.7%-97.7% nt identity (Figure 3). The maximum identity with the prototype JUQ virus was found to correspond to the viral RNA from the patient Hu239727 (97.7%) from Barra do Turvo, São Paulo, the closest location to the site of infection of the fatal HPS case from which JUQ virus was originally characterized in Juquitiba in 1993. Sequences in JUQ-like clade group in two subclades, one including JUQ prototype strain and Hu239727, and the other one comprising six human and the three *O. nigripes* sequences (distance 0.273). The distance observed between these two subclades is intermediate between in-group and between group distances (Table 3).

Comparison of the 303-nt G2-encoding region sequences derived from HPS-patient and *O. nigripes* samples of this group showed 86.6%-99.7 % nt identity. Sequence comparison with the corresponding to ARA group showed 20.8% nt and 4.9% amino acid (aa) divergence. Genetic distances between the sequences studied are shown in Table 3. The general time reversible model (GTR) with a 0.2479 proportion of invariable sites and $\gamma = 0.4121$, was used in analysis. AND virus resulted in ARA closest sequence (mean distance = 0.516) and Hu39694, JUQ-like closest sequence (0.723). Mean distances between ARA and JUQ-like sequences further support that they are different viruses.

A longer fragment of 1,239 nt of the G2 encoding region of the M segment (Figure 4), as well as a fragment of 259 nt of the nucleoprotein encoding region of the S segment, was generated by one representative strain of each hantavirus group (Bl194307 for ARA virus and On193576 for JUQ-like virus). Comparison of viral RNA from *B. lasiurus* sequences with ARA virus showed a 96.8%-nt and 99.3%-aa identity for the 1,239-nt G2-M piece, and 98.8% nt and 100 % aa identity for the S segment piece. When RNA viral sequences from *B. lasiurus* were compared with those from *O. nigripes*, 77.8% nt and 93.4% aa identity for the 1,239-nt G2-M piece, and 84.1% nt and 98.8% aa identity for the S segment piece were observed.

The phylogenetic relationship of ARA and JUQ-like genotypes to other hantaviruses of South America was

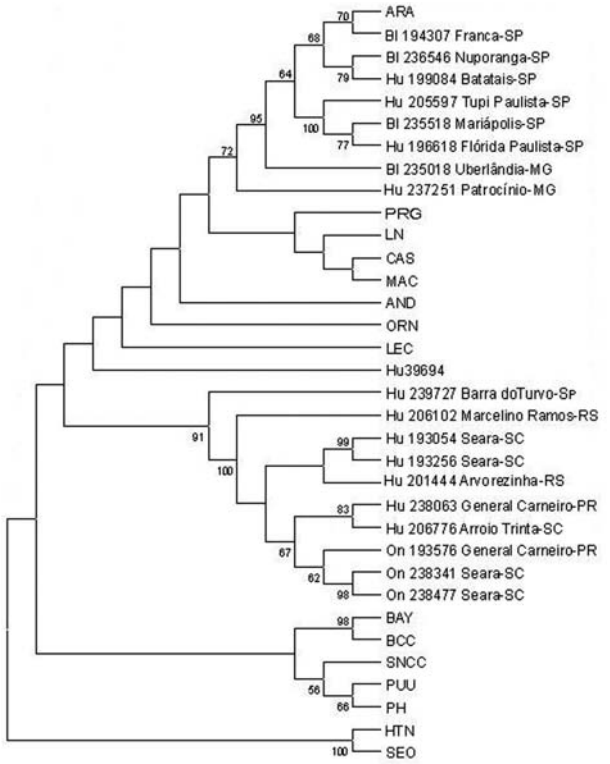

Figure 2. Phylogenetic relationships among Brazilian and previously characterized hantaviruses. Maximum parsimony analysis of the nucleotide sequence of 303-nt fragment of the G2 gene was performed with the heuristic search option. Bootstrap values of >50%, obtained from 500 replicates of the analysis are shown. Abbreviations and GenBank accession numbers of the previously published sequences of the hantaviruses used in this study: Andes, AND-AF324901; Araraquara, ARA-AF307327; Bayou, BAY-L36930; Bermejo, BMJ-AF028025; Castelo dos Sonhos, CAS- AF307326; Hu39694-AF028023; Lechiguanas, LEC-AF028022; Laguna Negra, LN-AF005728; Maciel, MAC-AF028027; Oran, ORN-AF028024; Pergamino, PRG-AF028028; Prospect Hill, PH-X55129; Puumala, PUU-X61034; Sin Nombre, SN-CC74L33684; Hantaan, HTN strain 76/118-Y00386; Seoul, SEO-M34882; Black Creek Canal, BCC-L399500.

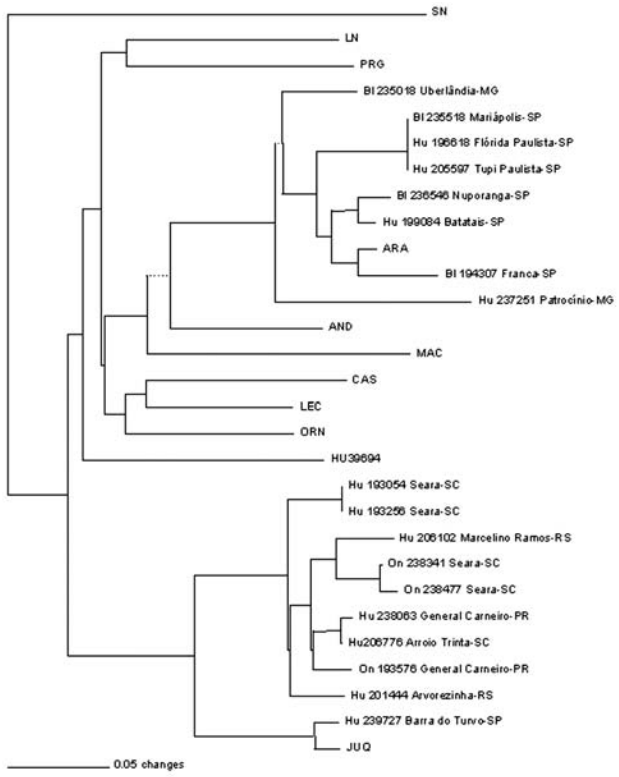

Figure 3. Phylogenetic relationship between newly and previously characterized Brazilian hantaviruses, using a 139-nt region of the M genomic segment G2 encoding region. Maximum parsimony analysis was performed by using the heuristic search option. Bootstrap values of ≥50%, obtained from 500 replicates of the analysis are shown. Abbreviations and GenBank accession numbers of the previously published sequences of the hantaviruses used in this study: Araraquara-AF307327 and Castelo dos Sonhos-AF307326; Juquitiba, JUQ (19).

determined on the nucleotide sequences of the 303-nt and 1,239-nt G2-encoding region of the M segment genomes by using maximum parsimony and neighbor-joining analysis. Both trees showed a similar topology, except for the altered placement of certain genotypes that displayed low bootstrap support. Phylogenetic analysis performed on the 303-nt sequence fragment (Figure 2) showed that all samples from *B. lasiurus* fell into the ARA virus group, whereas all samples from *O. nigripes* fell into the JUQ-like genetic group. The nodes separating these groups had high bootstraps values (72% and 91%, respectively); however, the exact branching order among the Brazilian and the other hantaviruses cannot be resolved by the present phylogenetic analysis. The phylogenetic tree, based on the analysis of the 1,239-nt M segment sequence differences, showed the same topology of the 303-nt sequence, data-based tree. Both maximum parsimony (Figure 2) and neighbor-joining (data not shown) analysis demonstrated that the divergent ARA and JUQ-like viruses form a unique monophyletic clade with the other South American hantaviruses (71% bootstrap support).

Within this clade the ARA virus forms a subclade with other two akodontine-borne Argentinean hantaviruses, Maciel (MAC) from *Necromys benefactus* and Pergamino (PRG) from *Akodon azarae*, although with a low bootstrap support (66%). The JUQ-like virus from *O. nigripes* 193576 is ambiguously placed within the South American viruses, together with CAS virus from Brazil and the rest of the *Oligoryzomys*-borne Argentinean hantaviruses (LEC, Orán, Hu39694, Bermejo, AND virus), as well as LN virus from Paraguay. A slight difference in the topology of this 1,239-nt database tree was observed when neighbor-joining analysis was used; the grouping of this *Oligoryzomys*-borne Argentinean hantavirus clustered in a subclade with a bootstrap support of 79% (data not shown).

Discussion

Eleven (27.5%) of 40 human samples and 7 (28.0%) of 25 rodent samples studied tested positive for hantavirus by RT-PCR. Previous studies have characterized three different hantavirus genetic lineages associated with HPS cases in Brazil: CAS, ARA, and JUQ (20,30,31).

Previous serologic studies detected IgG antibodies in rodent biologic samples: in *B. lasiurus* and *Akodon* spp. captured in the state of São Paulo (32), as well as *B. lasiurus* from São Paulo and Minas Gerais States, and *O. nigripes* from São Paulo, Rio Grande do Sul, Santa Catarina, and Paraná States (33).

The data we report on the phylogenetic analysis of viral M and S genome segment fragments, from HPS patients and rodent samples from different locations of southern and southeastern Brazil, showed the circulation of two distinct hantaviruses, closely related to ARA and JUQ viruses, previously characterized only from humans. Our data on the phylogenetic analysis of a 303-nt G2 encoding region of the M genome segment represents the first genetic evidence of the role of *B. lasiurus* as rodent host reservoir for ARA virus, as well as *O. nigripes* as rodent host reservoir for the JUQ-like virus in the region under study. The nodes separating each group of virus within the South American hantaviruses clade were highly supported (72% and 91% bootstrap for ARA and JUQ-like lineages, respectively). The two subclades observed in Figure 3 (i.e., 139-nt tree), and the intermediate distance between them suggested the possibility of some geographic isolation. More extensive sequencing of JUQ prototype and JUQ-like sequences is needed to clarify this point.

Comparison of the sequences of ARA virus strains obtained from rodents (*B. lasiurus*) and HPS patient samples showed an identity of up to 99.7% at the nucleotide level, while within the JUQ-like virus group,

Table 3. Mean distance between sequences[a]

	ARA clade		JUQ-like clade	
Distance from	Mean	SD	Mean	SD
Laguna Negra	0.683	0.037	1.135	0.060
Oran	0.642	0.077	0.785	0.071
Pergamino	0.608	0.019	0.770	0.056
Maciel	0.747	0.042	1.105	0.127
Castelo dos Sonhos	0.555	0.047	0.970	0.046
Hu39694	0.742	0.100	0.723	0.057
Lechiguanas	0.642	0.081	0.798	0.069
Andes	0.516	0.054	0.862	0.044
JUQ-like clade	0.924	0.138	–	–
ARA clade	–	–	0.924	0.138
Inside clade distances	0.149	0.087	0.111	0.089

[a]ARA, Araraquara virus; SD, standard deviation; JUQ, Juquitiba virus.

the comparison between *O. nigripes* and HPS patient virus strains showed an identity of up to 97.7%. However, the phylogenetic relationship of JUQ-like hantavirus to other members of the South American hantavirus lineages, determined on the nucleotide sequences of the 303-nt sequence, as well as on a longer fragment of a 1,239-nt piece of the M segment performed for one representative strain derived from one *O. nigripes*, systematically failed to resolve the branching order.

In South America, human illnesses associated with hantaviruses have been linked to viruses from the Oryzomyini and Phyllotini tribes. Known akodontine-borne hantaviruses have not been associated with human illnesses. Thus, the data reported here on ARA rodent reservoir constitute the first evidence that a hantavirus associated with an akodontine rodent can cause HPS. Phylogenetic tree based on a 1,239-nt G2 sequence fragment places together the akodontine-borne ARA virus from *B. lasiurus* in Brazil, MAC from *N. benefactus*, and PRG from *A. azarae* in Argentina (34). Although the bootstrap support displayed was low (66%), this finding is in accordance with previous observations based on the S genome phylogeny of Argentinean hantaviruses (35). This would support the hypothesis of cospeciation of hantaviruses with their specific rodent hosts. As it has been described with other hantaviruses, biogeographic factors are also involved in the evolution of hantavirus lineages (36,37). The human- and rodent-derived ARA strains analyzed in the current study were distributed at a distance of approximately 650 km. As expected, ARA virus strains originated from the more distant localities displayed the highest genetic divergence, as shown between samples from Patrocínio (Hu237251) and Uberlândia (Bl235018) in Minas Gerais State, and Flórida Paulista (Hu196618) in São Paulo (16.7%-nt difference). Similarly, the divergent JUQ-like virus sample Hu239727 originated from Barra do Turvo, São Paulo, in relation to the rest of the human and rodent JUQ-like virus samples included in this group from locations in Santa Catarina, Paraná, and Rio Grande do Sul may be associated with the geographic distance between them (500 km on average).

Although the data from serologic testing by ELISA indicated four positive *Akodon* spp., one *C. tener*, and one *Ox. rutilans*, specific viral sequences could not be amplified from those specimens. Thus, additional studies are needed to determine the possible role of these species in the epidemiology of hantavirus in Brazil.

The habitats and behavior of the rodents are important aspects to consider in elucidating the reservoirs of etiologic agents. ARA virus was recovered mostly from HPS patients as well as *B. lasiurus* samples from the ecosystem called cerrado, while JUQ-like virus was recovered mostly from human and *O. nigripes* samples from the ecosystem called Atlantic rainforest. Geographic distribution of *B. lasiurus* in Brazil includes the original areas of cerrado and "caatinga." This environment is typical in northeastern

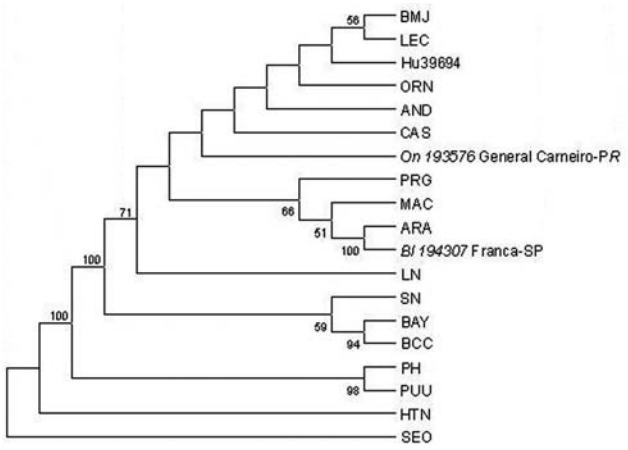

Figure 4. Phylogenetic relationships between Brazilian and previously characterized hantaviruses. Abbreviations and GenBank accession numbers of the previously published sequences of the hantaviruses used in this study are listed in the legend of Figure 2. Maximum parsimony analysis of the nucleotide sequence of the 1,239-nt fragment of the M segment was performed with the heuristic search option. Bootstrap values of >50%, obtained from 500 replicates of the analysis, are shown.

Brazil, characterized by deciduous trees and cactus and an extremely prolonged dry season. The *B. lasiurus* distribution shows its ability to adapt to anthropic environments, especially grasses (*Brachiaria*) and sugar cane cultures. These rodents are aggressive and usually dominate the areas they infest (38); they do not colonize human dwellings, although occasionally they can invade houses.

O. nigripes is adapted to live in the primary and secondary forests, especially in the Atlantic rainforest and Araucaria forest. It is primarily found in anthropic environment, such as the lineal natural habitats bordering cultivated areas, especially those with corn, where it is the most abundant species. These rodents can easily invade dwelling houses and barns to search for food, and they can nest in the domestic habitats.

Among the rodents captured in the cerrado, *B. lasiurus* was the most abundant species (44%) and showed the highest prevalence of antibodies to hantavirus (11%). *Akodon* spp. and *O. nigripes* were the two most abundant among those rodents captured in the transition area between cerrado and the Atlantic rainforest, but the highest prevalence of antibodies to hantavirus was found in *O. nigripes* specimens (8%) (33). These data help incriminate *B. lasiurus* and *O. nigripes* in the transmission of ARA and JUQ-like viruses, respectively, to humans.

Acknowledgments

We thank the staff of Seção de Vírus Transmitidos por Artrópodos, São Paulo State Health Secretary and A. Degleue, S. Bourda, and V. Fasciani for their laboratory help. We also thank the personnel of Brazilian Ministry of Health, State and Municipal Health Secretaries in the States of São Paulo, Minas Gerais, Paraná, Santa Catarina, and Rio Grande do Sul for their efforts in the surveillance of notifiable diseases.

This work was supported in part by VIGISUS/FUNASA/MS–Project no. 914/BRA/02. The work was done at Instituto Adolfo Lutz–Secretaria de Estado da Saúde de São Paulo, São Paulo/SP, Brazil, and Instituto Nacional de Enfermedades Virales Humanas Dr. Julio I. Maiztegui, Pergamino, Argentina.

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West Nile Virus Outbreak in North American Owls, Ontario, 2002

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From July to September 2002, an outbreak of West Nile virus (WNV) caused a high number of deaths in captive owls at the Owl Foundation, Vineland, Ontario, Canada. Peak death rates occurred in mid-August, and the epidemiologic curve resembled that of corvids in the surrounding Niagara region. The outbreak occurred in the midst of a louse fly (*Icosta americana*, family *Hippoboscidae*) infestation. Of the flies tested, 16 (88.9 %) of 18 contained WNV RNA. Species with northern native breeding range and birds >1 year of age were at significantly higher risk for WNV-related deaths. Species with northern native breeding range and of medium-to-large body size were at significantly higher risk for exposure to WNV. Taxonomic relations (at the subfamily level) did not significantly affect exposure to WNV or WNV-related deaths. Northern native breeding range and medium-to-large body size were associated with earlier death within the outbreak period. Of the survivors, 69 (75.8 %) of 91 were seropositive for WNV.

Since its initial detection in the New York City area in 1999 (1), West Nile virus (WNV) has emerged as a health risk for humans and has been associated with illness and death in a wide variety of North American birds, mammals, and reptiles (2,3). In addition, serologic evidence of exposure to this arthropodborne flavivirus (family *Flaviviridae*) has been reported in many other North American species in which disease had not been previously recorded (4–6).

While severe clinical disease develops in a minority of humans and horses with WNV infection (7,8), the situation in birds appears to be different in North America. Some North American birds are highly susceptible to this virus, as shown by large-scale local deaths of American Crows (*Corvus brachyrhynchos*, family *Corvidae*) (1,9). This finding was further demonstrated recently by experimental infection (10). The factors that make some species highly susceptible to WNV remain largely unknown.

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Studying the effect of taxonomic, geographic, and demographic background on susceptibility to WNV is potentially useful for predicting and modeling the effect of WNV on host populations. So far, taxonomy alone has offered limited help in predicting susceptibility to WNV, as some closely related species (e.g., within the same genus) show different susceptibility patterns. For example, Fish Crows (*C. ossifragus*) appear less susceptible to WNV than do American Crows (10).

Geographic distribution could explain susceptibility patterns if a species has had previous exposure to the same or similar agent. Exposure to St. Louis encephalitis virus (SLEV) has been suggested as a possible mechanism of acquired immunity against WNV (2). In addition, species that have evolved in areas where other flaviviruses, such as SLEV, are common may have undergone selection for an innate immune response that may offer protection against WNV.

Age-related differences in susceptibility to WNV have been described for chickens (2) and domesticated geese (11). These age-related differences have not been reported in wild birds, perhaps because of the difficulty determining their age.

From July to September 2002, high death rates occurred in captive owls (family *Strigidae*) kept at the Owl Foundation, Vineland, Ontario, Canada. At the time, many of the birds were infested with adult hematophagous louse flies (order Diptera, family *Hippoboscidae*); some had loads >400 flies per bird. Initially, the deaths were attributed to this infestation. On August 9, the authors examined three dead owls. Necropsy findings included marked hepatomegaly, splenomegaly, and cerebral hemorrhage. A rapid antigen-capture assay for WNV detection (Vec Test, Medical Analysis Systems, Camarillo, CA) was used for initial screening of oropharyngeal swabs from these birds; however, all samples were negative. On August 16, tissue samples from eight owls, including the initial three, were found to be positive for WNV by reverse transcription–polymerase chain reaction (RT-PCR) test.

Beginning August 23, the remaining birds were vaccinated with a killed WNV vaccine (West Nile Innovator Vaccine, Fort Dodge Animal Health, Fort Dodge, IA). However, from July 26 to September 28, a total of 108 (44%) of 245 owls died.

To our knowledge, the outbreak at the foundation is the largest WNV outbreak in captive wildlife collections in North America since 1999 (12–14) and the first in Canada. These outbreaks offer a unique and transient opportunity to study the effect and epidemiologic features of WNV infection in multiple species under quasinatural conditions. With the implementation of preventative measures against WNV (e.g., vector control and vaccination), this opportunity will disappear.

The objective of this study was to describe the epidemiologic features of the WNV outbreak at the foundation in 2002. Specifically, we studied the effect of outdoor housing, age, body size, taxonomy, and native breeding range on exposure to WNV and on WNV-related deaths.

Materials and Methods

Study Site

The Owl Foundation specializes in breeding and rehabilitating North American owls. Its facility in the Niagara region (Vineland, Ontario; 43°10′ N, 79°20′ W) has ≈3,340 m^2 of specially designed outdoor cages and a few indoor cages.

Records and Observations

The Owl Foundation maintains detailed records of all birds in the facility, which includes each bird's history, date admitted, species, cage in which it is housed, movements, and medical history. These data were used in the epidemiologic analysis of this outbreak. For most birds in this study, sex had not been determined.

Data summarizing dead corvid sightings in the Niagara region (1,896 km^2 in size, map available at http://www.regional.niagara.on.ca/exploring/pdf/regional-niagara.pdf) were obtained from the Canadian Cooperative Wildlife Health Center national WNV surveillance database. These data were gathered by the Niagara Region Health Unit from May 14 to October 12, 2002.

Sample Collection

Complete diagnostic necropsies were performed at the Ontario Veterinary College on 94 owls and one falcon (family *Falconidae*) that died at the Owl Foundation from April 15 to December 25, 2002. This broad time frame was to facilitate detection of the first and last WNV-related deaths in the 2002 outbreak. All carcasses were kept frozen at –20°C from shortly after death and were allowed to thaw at 4°C for 24 to 48 h before examination (March–August 2003). For each bird, samples of brain, lung, liver, spleen, and kidney were pooled and refrozen at –70°C until analyzed by real time RT-PCR. Small core samples of brain, kidney, and liver were collected with a 20-gauge spinal needle from three additional carcasses that had not been available for necropsy.

During the outbreak, several hundred louse flies were collected at the foundation and frozen at –20°C. These flies were collected off sick or dead birds, as well as from birds that appeared healthy. Six flies were submitted for species identification, while whole-body homogenates of 23 flies, including 18 that were removed from dead or sick owls, were tested for WNV by real time RT-PCR.

From January 22 to May 1, 2003, a serologic survey of all outbreak survivors was conducted. Blood samples were collected from the jugular or cutaneus ulnar vein and placed in heparinized tubes. Plasma was then separated by centrifugation and frozen at –70°C until analyzed.

Real-time RT-PCR

Real-time RT-PCR assay was used to detect WNV RNA as previously described (15). Samples consisted of homogenates prepared from pooled brain, lung, liver, spleen, and kidney (approximately 1 mm^3 of each tissue). RNA was extracted by using the RNeasy 96 viral isolation kit (Qiagen, Inc.,Valencia, CA). The ABI Prism 7700 Sequence Detection System and the TaqMan One Step PCR Master Reagents Kit (PE Applied Biosystems, Foster City, CA) were used for the assay. Positive controls consisted of three 10-fold dilutions of Egypt 101 (Eg 101) strain of WNV. Three sets of negative (water) controls were used, two during the extraction procedure and one during amplification (i.e., no template). Extracts were screened with the generic 3′ NC primer set. Positive samples underwent a second RNA extraction and were then tested with both the 3′ NC and WNV primer sets. Primer sequences were as described (15). Samples that had threshold cycle $\leq$37 with both primer sets were considered positive. Homogenates prepared from whole louse flies were tested with the same procedures described for owl tissues.

Serologic Testing

We used an enzyme-linked immunosorbent assay (ELISA) recently shown to detect avian anti-WNV immunoglobulin (Ig) G in 23 avian species of 12 orders, including a Barred Owl (*Strix varia*) (6). The assay was performed as described, with slight modifications. Briefly, the inner 60 wells of a 96-well plate were coated with the monoclonal antiflavivirus antibody 4G2. After the wells were treated with blocking buffer, WNV recombinant COS-1 viral antigen (Centers for Disease Control and Prevention, Atlanta, GA) and a control recombinant COS-1 antigen (Centers for Disease Control and

Prevention) diluted to 1:100 were added to the top and bottom 48 wells of the plate, respectively. Plasma samples diluted 1:400 were added, as were positive and negative controls. Horseradish peroxidase-conjugated goat anti–wild bird IgG (Bethyl Laboratories, Inc., Montgomery, TX) was added and allowed to bind to anti-WNV antibodies before the substrate tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added. The reaction was stopped after 30 min by H_2SO_4 and read by a microtiter plate reader at 450 nm. Samples showing positive and negative optical density ratios >2 at the 1:400 dilution were considered positive.

A subset of 20 plasma samples representing eight species of owls and four species of raptors was tested by plaque reduction neutralization tests (PRNT). The assay was performed as described (4). Titers were expressed as the highest dilution that produced >90% reduction in plaque number. Titers ≥40 were considered positive. The controls used were back titrations of WNV (NY strain) at 2.5×10^7 PFU/mL diluted to 100, 10, and 1 PFU and a negative control (media only). PRNT assays were performed in a biosafety level 3 facility at the National Microbiology Laboratory, Winnipeg, Manitoba.

Study Population

The period between the first and last WNV-related deaths at the Owl Foundation was determined from real-time RT-PCR results and defined as the West Nile outbreak period. The study population included 245 birds that were at the foundation on the first day of the outbreak period. These birds represented 16 species of North American owls, one species of Eurasian owl, and two species of falcons (Table 1). Most were permanently disabled birds of wild origin; some had spent many years at the Owl Foundation, while others were recent additions or hatchlings. Ten birds were housed indoors, and 235 were kept outdoors.

Statistical Analysis

Logistic regression was used to test the effect of outdoor placement on WNV-related deaths. Once this effect was established, birds kept indoors were excluded from further analysis. Logistic regression was then used to test the effect of taxonomy, native breeding range, age, and species body size on exposure to WNV and on WNV-related deaths (among exposed birds). Species were considered northern if their reported native breeding range was largely north of latitude 48°N or "other" if it was not so (16–19). Affiliation (at the subfamily level) followed previously published taxonomic classifications (20). Species were further classified as small, medium, or large if their average body weight was <250 g, 250–500 g, or >500 g, respectively (20). The two age groups compared were <1 year and >1 year. A general linear model was used to test the effect of the same factors on the date of death (i.e., the number of days to death from when the index bird died). Regression procedures were performed using the SAS software (version 8.2, SAS Institute Inc., Cary, NC). When the odds ratio (OR) could not be calculated directly

Table 1. Deaths at the Owl Foundation property during a WNV outbreak (July 26–September 28, 2002) and results of real-time RT-PCR on tissues from dead birds[a]

Species	At risk	Died	Tested	Positive n (%)	Crude DR %	WNV-related DR %
Snowy Owl (*Bubo scandiacus*)	20	20	11	11 (100)	100	100
Northern Hawk Owl (*Surnia ulula*)	26	26	17	17 (100)	100	100
Great Gray Owl (*Strix nebulosa*)	27	27	23	21 (91.3)	100	91.3
Boreal Owl (*Aegolius funereus*)	11	11	11	10 (90.9)	100	90.9
Northern Saw-whet Owl (*Ae. acadicus*)	13	12	12	12 (100)	92.3	92.3
Northern Pygmy Owl (*Glaucidium gnoma*)	6	1	1	1 (100)	16.7	16.7
Short-eared Owl (*Asio flammeus*)	16	2	2	2 (100)	12.5	12.5
Flammulated Owl (*Otus flammeolus*)	9	1	1	1 (100)	11.1	11.1
Long-eared Owl (*A. otus*)	13	3	3	1 (33.3)	23.1	7.7
Great Horned Owl (*B. virginianus*)	22	2	1	1 (100)	9.1	4.5
Barn Owl (*Tyto alba*)	10	0	0	–	0	0
Barred Owl (*S. varia*)	8	1	1	0 (0)	12.5	0
Burrowing Owl (*Athene cunicularia*)	10	0	0	–	0	0
Eastern Screech Owl (*Megascops asio*)	36	0	0	–	0	0
Elf Owl (*Micrathene whitneyi*)	1	0	0	–	–	–
Spotted Owl (*S. occidentalis*)	1	1	1	1 (100)	–	–
Tawny Owl (*S. aluco*)	2	1	1	1 (100)	–	–
American Kestrel (*Falco sparverius*)	2	0	0	–	–	–
Peregrine Falcon (*F. peregrinus*)	2	0	0	–	–	–
Total	235	108	85	79 (92.9)	46	43

[a]WNV, West Nile virus; RT-PCR, reverse transcription–polymerase chain reaction; DR, death rate (calculated when n > 6).

because of a zero value, an approximation of the OR was calculated by adding 0.5 to each cell (21). For the purpose of this analysis, eight moribund birds that were euthanized during the outbreak were considered to have died spontaneously. Seven birds that died during the outbreak but were negative for WNV, were excluded from all statistical analysis. The κ statistic (Quickcalcs, GraphPad Software, Inc., San Diego, CA) was used to test for agreement between ELISA and PRNT results.

Results

A total of 138 birds died in the Owl Foundation from April 15 through December 30, 2002. Thirteen of these died due to known causes other than WNV, leaving 125 that died of unknown cause. Of these, 98 were tested by real-time RT-PCR for WNV, and 80 (81.6%) were positive. Based on these results, the outbreak period was July 26–September 28, 2002. The first five cases occurred during an 11-day period in five different cage complexes scattered throughout the facility. Further analysis of spatial patterns was not attempted because the birds frequently moved between cages during the outbreak period. Daily deaths at the Owl Foundation and dead corvid sightings in the Niagara region from July 5 through October 4, 2002, are shown in Figure 1. Within the outbreak period, 79 (92.9 %) of 85 birds that died were positive for WNV based on RT-PCR. Table 1 summarizes the results of birds tested by species and shows species-specific death rates.

A total of 91 outbreak survivors, which were kept outdoors during the entire outbreak period and not vaccinated against WNV, were tested by ELISA for anti-WNV IgG. Of these, 69 (75.8%) were seropositive. Agreement between ELISA and PRNT results was good with $\kappa = 0.857$ (0.58–1.13). The two tests produced conflicting results for 1 of 20 samples; ELISA results are shown in Table 2. Species-specific exposure rates are shown in Table 3. The overall exposure rate was 84.3%.

Being kept outdoors during the outbreak period was found to be a highly significant risk factor ($p < 0.0001$) for WNV-related death. None of the 10 birds kept indoors died during the outbreak period, despite the fact that 8 of 10 belonged to species that otherwise had very high death rates (four Northern Hawk Owls, three Boreal Owls, and one Northern Saw-whet Owl). These birds were excluded from further analysis.

Species' northern native breeding range and large-to-medium body size were significant risk factors for exposure to WNV ($p < 0.05$), with OR = 52.56 (95% confidence interval [CI] 3.13–881.84) and OR = 16.82 (95% CI 3.79–74.67), respectively. Age and taxonomy at the subfamily level were not significant risk factors for exposure. Among exposed birds, northern native breeding range was a highly significant risk factor for WNV-related death

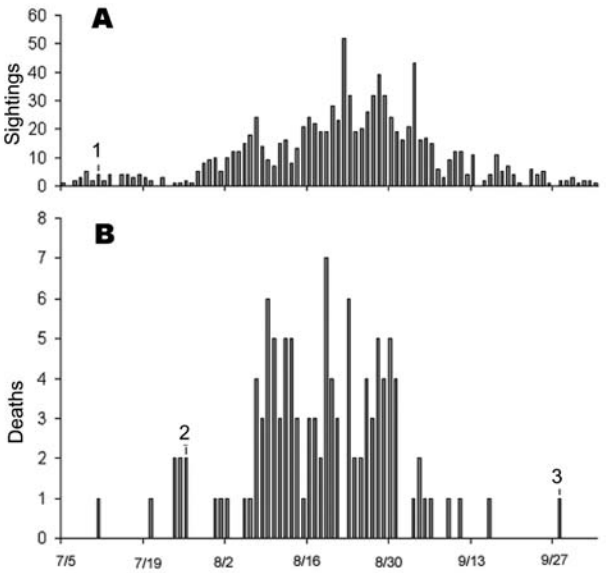

Figure 1. A) Dead corvid (family *Corvidae*) sightings at the Niagara region, July 5–October 4, 2002, and B) daily death rates at the Owl Foundation during the same period. 1) First WNV-positive crow, 2) first and 3) last WNV-related deaths at The Owl Foundation are shown.

($p < 0.0001$), with OR = 1,507 (95% CI 85.51–26,557). Birds >1 year of age were also more likely to die of WNV ($p < 0.05$) with OR = 4.87 (95% CI 2.46–9.62). Size and subfamily were not found to be significant risk factors for WNV-related death.

Native breeding range was significantly associated with the date of death ($p < 0.01$). Northern species died earlier during the outbreak period (mean and standard deviation 23.8 ± 9.8 days, $n = 93$) compared to other species (35.0 ± 12.9 days, $n = 8$). Large or medium birds also died earlier during the outbreak period (21.9 ± 8.5 days, $n = 77$) compared to small species (33.9 ± 11.2 days, $n = 24$) ($p < 0.05$). Subfamily and age were not significantly associated with the date of death.

The six louse flies examined were identified as *Icosta americana* (order Diptera, family *Hippoboscidae*). WNV RNA was detected by real-time RT-PCR in 16 (88.9%) of 18 flies collected from dead or sick owls during the outbreak period. Five adult flies collected from birds that appeared healthy were WNV negative.

Discussion

WNV RNA was present in most owls that died at the Owl Foundation during the outbreak period, and antibodies against WNV were detected in most outbreak survivors (overall exposure rates 84.3%). The similarity between the epidemiologic curve of the outbreak at the Owl Foundation and that of dead corvid sightings in the Niagara region (Figure 1) and the fact that the initial cases occurred in

Table 2. Results of enzyme-linked immunosorbent assay for detection of anti-WNV IgG in survivors[a] of a WNV outbreak, the Owl Foundation, 2002[b]

Species	Tested	No. seropositive (%)
Barn Owl (*Tyto alba*)	10	8 (80)
Short-eared Owl (*Asio flammeus*)	8	8 (100)
Barred Owl (*Strix varia*)	2	2 (100)
Great Horned Owl (*Bubo virginianus*)	12	12 (100)
Burrowing Owl (*Athene cunicularia*)	10	9 (90)
Flammulated Owl (*Otus flammeolus*)	8	1 (12.5)
Eastern Screech Owl (*Megascops asio*)	33	24 (72.7)
Northern Pygmy Owl (*Glaucidium gnoma*)	5	2 (40)
Elf Owl (*Micrathene whitneyi*)	1	1 (100)
Peregrine Falcon (*Falco peregrinus*)	2	2 (100)
Total	91	69 (75.8)

[a]Birds kept outdoors and not vaccinated against WNV.
[b]WNV, West Nile virus; Ig, immunoglobulin.

several cages scattered throughout the Owl Foundation property, suggest that the outbreak was part of a regional WNV activity rather than a point-source introduction (e.g., by admitting a viremic bird). As none of the birds kept indoors were affected, the major route of WNV transmission likely was vector-borne.

Northern native breeding range and large-to-medium body size were significant risk factors for exposure to WNV, while age and subfamily were not. Birds that attract more vectors have a higher risk for exposure to WNV, and the host's body size may be an important determinant of vector attraction, as reported for mosquitoes and sandflies (22–24). Why species' breeding range should affect exposure is less obvious. However, northern species may attract more feather-dwelling arthropods, such as louse flies, because of their thick feathering.

WNV RNA was detectable in 88.9% of *I. americana* louse flies collected off dead or sick birds. If this parasite can transmit WNV, the overall high exposure rates seen at the Owl Foundation would be explained in light of the louse fly infestation. Subjective observations made by the Owl Foundation staff suggest that northern owl species had the heaviest infestation. If confirmed, these findings could explain why northern species had higher exposure rates; however, this subject requires further investigation.

Looking at the species-specific death rates (Table 1), the distribution of WNV-related deaths was uneven. Given the high overall ER, this finding suggests marked differences in species susceptibility to the virus, with species falling into one of three groups (for scientific names see Table 1): death rates >90% (Snowy Owl, Great Gray Owl, Northern Hawk Owl, Boreal Owl, and Northern Saw-whet Owl), death rates <20% (Long-eared Owl, Short-eared Owl, Great Horned Owl, Flammulated Owl, and Northern Pygmy Owl), and death rates = 0% (Barn Owl, Burrowing Owl, and Eastern Screech Owl).

Affiliation at the subfamily level did not significantly affect death rates. Susceptibility to WNV-related death crossed taxonomic lines and was strongly related to native breeding range. This finding together with the serologic data suggest that death rates were not determined by the ability of WNV to infect different owl species but rather by

Table 3. Exposure[a] rates and WNV-related death rates for 12 species of owls kept outdoors at the Owl Foundation property during WNV outbreak, 2002[b]

Species[c]	Exposure rate (%)	WNV-related DR (%)
Snowy Owl (*Bubo scandiacus*)	100	100
Northern Hawk Owl (*Surnia ulula*)	100	100
Northern Saw-whet Owl (*Aegolius acadicus*)	92.3	92.3
Great Gray Owl (*Strix nebulosa*)	100	91.3
Boreal Owl (*Ae. funereus*)	90.9	90.9
Northern Pygmy Owl (*Glaucidium gnoma*)	50	16.7
Short-eared Owl (*Asio flammeus*)	100	12.5
Flammulated Owl (*Otus flammeolus*)	22.2	11.1
Great Horned Owl (*B. virginianus*)	100	4.5
Burrowing Owl (*Athene cunicularia*)	90	0
Eastern Screech Owl (*Megascops asio*)	72.7	0
Barn Owl (*Tyto alba*)	80	0
All species	84.3	43

[a]Exposure defined as positive reverse transcription–polymerase chain reaction result (for birds that died during the outbreak) (Table 1) or positive serologic results (for birds that survived the outbreak) (Table 2).
[b]WNV, West Nile virus; DR, death rate.
[c]Only species for which $n \geq 6$ are shown.

the ability of each species to survive the infection. All owl species showed either serologic or pathologic evidence of WNV infection.

Immunity to WNV, as for other pathogens, can be either innate or acquired. If immunity to WNV in this case was acquired, one could expect that northern owl species that spent years at the Owl Foundation would be just as resistant as the locally breeding Eastern Screech Owls. Furthermore, if this supposition was the case, young age should have been a significant risk factor, as juvenile birds regardless of species would have been less likely to have acquired immunity. Our data show the opposite; birds >1 year were at a significantly higher risk for WNV-related death. The reason for this finding is unclear and may suggest that juvenile owls have some resistance to WNV infection.

Innate immunity could have evolved through selection if some of the species coexisted for long periods of time with agents similar to WNV. Based on their native breeding range, northern owl species may have little or no exposure to SLEV (Figure 2). Indeed, species in the high (>90%) death rates group are all northern species, most of which breed north of latitude 48°N, with some exceptions. Great Gray and Boreal Owls have southern extensions to this range, which follow the distribution of high-altitude conifer forest. The Northern Saw-whet Owl has a complex distribution, but all Northern Saw-whet Owls at the Owl Foundation have likely originated in the species' boreal population. Species that showed no WNV-related deaths have relatively southerly distributions, while species in the low (<20%) death rate group have intermediate or very widespread native breeding range (Figure 2).

The link between native breeding range and susceptibility to WNV is intriguing. A similar relation between northern distribution and susceptibility to aspergillosis has been documented in a variety of avian species with northern native breeding range, including owls (27). T-cell–mediated immunity is essential for fighting fungal pathogens (28) and is also believed to play an important role in the immune response against flaviviruses (29,30). Northern species may have a less effective cell-mediated immune response to pathogens that are scarce or nonexistent in their natural environment.

Northern native breeding range and large-to-medium body size were significantly associated with earlier death during the outbreak period. This finding could be a result of infection at an earlier date, shorter incubation, shorter disease course, or a combination. Northern owl species had high death rates and may have had a more acute form of the disease. Larger birds, by attracting more vectors, may have been infected earlier in the outbreak period or received a higher dose of WNV.

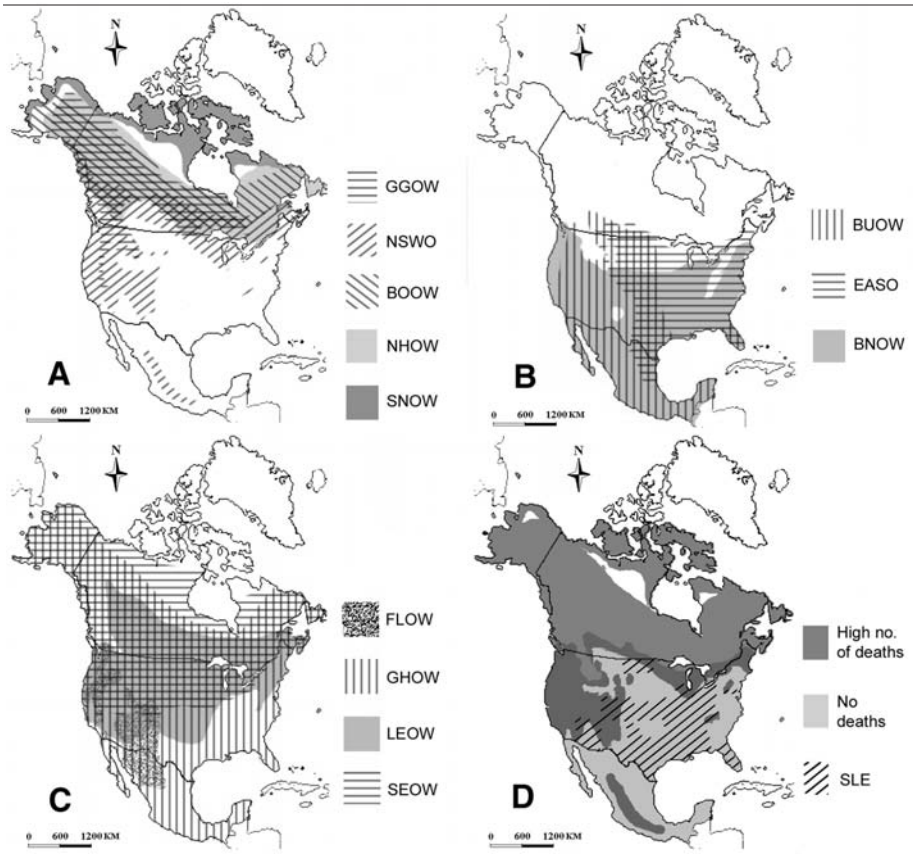

Figure 2. A) Native breeding range of owl species showing high death rates (>90%), B) no deaths, C) low death rates (<20%), and D) the combined distributions of species in the high and no mortality groups with that of Saint Louis encephalitis (SLE) in the United States and Canada. The distribution maps have been redrawn based on maps previously published (16–19). The distribution of SLE is based on human cases reported in the United States and in Canada from 1964 to 2000 (25,26). Only states and provinces that had 1 case per 100,000 capita during this period were included. GGOW, Great Gray Owl; NSWO, Northern Saw-whet Owl; BOOW, Boreal Owl; NHOW, Northern Hawk Owl; SNOW, Snowy Owl; BUOW, Burrowing Owl; EASO, Eastern Screech Owl; BNOW, Barn Owl; FLOW, Flammulated Owl; GHOW, Great Horned Owl; LEOW, Long-eared Owl; SEOW, Short-eared Owl.

While concluding that the differences observed in WNV-related death rates are the result of differential innate immunity of the various owl species, and that this pattern follows a north-south distribution based on host-pathogen coevolution is tempting, this finding does not appear to be the case for other North American species. American Crows, for example, have a wide native breeding range (16–19) and yet appear to be very susceptible to WNV (1,9,10). In addition, contributing factors cannot be ruled out. The outbreak occurred during an exceptionally dry and hot summer concurrently with an *I. americana* infestation. These conditions may have been especially harsh on the northern species, likely causing stress and immune suppression.

Conclusion

A strong link exists between native breeding range and susceptibility to WNV in North American owls. This relationship crosses taxonomic lines at the subfamily level and may be related to differential immunocompetence. Factors such as size and age may, to a lesser magnitude, affect exposure and susceptibility, respectively. Louse flies, common avian hematophagus parasites, may play a role in transmitting WNV; however, this role requires further examination. As WNV continues to spread, free-ranging populations of susceptible owl species may be affected.

Acknowledgments

We thank Annick Gionet, Kara Kristjanson, and Jody Crossingham for their assistance in data collection and organization and handling birds; Chris Enright and Amanda Low for assisting in sample collection and performing necropsies; Heather White for technical assistance; Douglas Hodgins and Hillary Voet for their assistance in the statistical analysis; Nathalie Lemieux for help in documenting and preparing distribution maps; Dale A. Smith for critically reading the manuscript; Nixon Wilson for assistance with species identification of hippoboscid flies; and Rob Lanciotti for providing monoclonal antiflavivirus antibody 4G2

This study was funded in part by the Rathlyn Foundation.

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Crimean-Congo Hemorrhagic Fever, Mauritania

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From February to August 2003, 38 persons were infected with Crimean-Congo hemorrhagic fever (CCHF) virus in Mauritania; 35 of these persons were residents of Nouakchott. The first patient was a young woman who became ill shortly after butchering a goat. She transmitted the infection to 15 persons in the hospital where she was admitted and four members of her family. In Nouakchott, two disease clusters and 11 isolated cases were identified. The case-fatality ratio was 28.6%. Of the patients not infected by the first case-patient, almost half were butchers, which suggests that the primary mode of animal-to-human transmission was direct contact with blood of infected animals. The hospital outbreak alerted health authorities to sporadic cases that occurred in the following weeks, which would have probably gone otherwise unnoticed. Studies must be conducted to determine the potential risk for continued sporadic outbreaks of CCHF in humans and to propose prevention measures.

Crimean-Congo hemorrhagic fever (CCHF), an acute viral disease in humans, is characterized by extensive ecchymoses, bleeding, and hepatic dysfunction and is associated with a 30% case-fatality ratio (1–3). It is caused by CCHF virus (genus *Nairovirus*, family *Bunyaviridae*).

CCHF is a zoonosis transmitted to large and small mammals and birds by ticks. Although the virus has been isolated from several genera and species of ixodid ticks, the main group of vectors involved in CCHF virus transmission appears to be ticks of the genus *Hyalomma* (1,4–6). Immature ticks acquire the virus by feeding on infected small vertebrates. Once infected, they remain infected throughout their development and, when they are mature, transmit the infection to large animals, such as livestock. Transovarian transmission has also been demonstrated (7,8).

Hyalomma ticks are widespread throughout Europe, Asia, the Middle East, eastern Asia, and Africa, and evidence of CCHF virus has been found in all these regions. The virus is transmitted to humans by the bite of infected ticks, direct contact with blood or infected tissues from viremic animals, and direct contact with the blood or secretions of an infected person. Animals are viremic for ≈1 week after infection but have only a moderate fever, which often goes unnoticed (9). The incubation period is usually 5–6 days after contact with blood (1). As with other hemorrhagic fevers, such as Ebola fever, several nosocomial CCHF outbreaks have been described (3,10–12). A lack of resources and hygiene in medical facilities plays a role in amplifying transmission (10,11). Hospitalized patients often bleed and are highly viremic; in overcrowded hospitals, where no isolation measures are taken, these patients can infect attending medical personnel as well as other patients who come in contact with their blood or vomit.

Epidemics of CCHF were first recorded in the Balkans in 1944 (4) and in Africa in 1956 (13). The first human case of hemorrhagic fever due to CCHF virus in West Africa was identified and serologically confirmed in Mauritania in 1983 (14) in a patient from Selibaby (Guidimakha region) (Figure 1).

In February 2003, six persons, including one physician and two nurses, were admitted to Nouakchott National Hospital for fever and hemorrhage. Three persons died. Serum samples from these patients tested positive for CCHF by immunoglobulin (Ig) M detection by enzyme-linked immunosorbent assay (ELISA), reverse transcription–polymerase chain reaction (RT-PCR), or isolation. We report on an investigation of the magnitude and conditions of emergence of this first urban CCHF outbreak in Mauritania.

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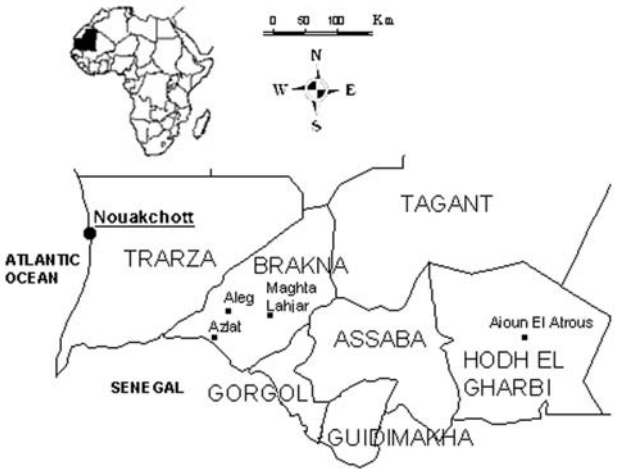

Figure 1. Map of southern Mauritania.

Materials and Methods

Case Definitions

In the field, a probable case was defined as occurring in someone who had an unexplained fever and acute hemorrhagic symptoms, such as petechiae, epistaxis, gingival hemorrhages, hematemesis, or melena, or who had an unexplained fever and contact with another case-patient. For the purpose of this report, a case-patient was defined as someone who fit the definition of a probable case and who had a positive laboratory result or who had fever and hemorrhagic signs, was in contact with a virologically confirmed case-patient, and died before sampling. One healthy person with no obvious recent history of disease, who was investigated as a contact, had anti-CCHF virus IgM and was included as case-patient.

Case Findings and Management

Once the first cases of CCHF were confirmed, information on the disease was sent to all health facilities in the country. Persons with probable cases who had hemorrhagic signs and reported to health facilities were isolated. In Nouakchott, an isolation ward was opened, where strict measures of hygiene were followed. Blood was drawn from each patient on the day of admission after an initial interview and drawn again 1 week later. Blood samples were sent to the Institut Pasteur laboratory in Dakar (Senegal) for testing. In addition, home visits were conducted. Relatives and neighbors were interviewed, and patients with probable cases were identified and sent to the isolation ward. Blood samples were obtained from contacts of patients, and these persons were monitored. A blood sample was taken from patients on day 21, and they were discharged when IgG was detected in this sample.

Animals

Serum samples were collected from domestic sheep and goats living near the patients in Nouakchott and in Azlat (Brakna region), where the family of the first identified patient was living. Ticks were collected from domestic animals (goats, sheep, and dogs) living in close proximity to patients in Nouakchott and from animals in livestock markets. Ticks collected from each animal were kept alive in separate vials covered with gauze. The collections were frozen in liquid nitrogen on site and taken to the Institut Pasteur of Dakar. Ticks were sorted on a cold table and then pooled according to stage, sex, host, species, and geographic origin.

Diagnostic Testing

Diagnosis of CCHF virus infection in humans, animals, and ticks was made at the Institut Pasteur of Dakar (WHO Collaborative Centre for arboviruses and viral hemorrhagic fever) by serologic testing (IgM capture and IgG indirect ELISA) (15), RT-PCR on S segment (16) with Titan One-Step RT-PCR System (Roche Diagnostics, Mannheim, Germany), according to the recommendations of the manufacturer, or viral isolation.

Serum or tick supernatant was injected into the cerebrum of 2- to 3-day-old mice and into Vero cell culture. The mice were observed for 2 weeks. If mice died or became sick, their brains were removed for injection into Vero cells and for virus identification. CCHF virus was confirmed by indirect immunofluorescence antibody test, with polyclonal and monoclonal antibodies. Identity of virus isolates was confirmed by complement fixation.

The PCR product (538 base pairs) was purified on agarose gel and directly sequenced by Genome Express (Meylan, France). We compared the resulting sequence with those available in the GenBank database, with BLAST tool.

Statistical Analysis

Data were anonymously analyzed with Stata software version 6.0 (Stata Corporation, College Station, TX). Median and range of quantitative variables were calculated. For qualitative variables, proportions were calculated. To compare qualitative variables, the chi-square test was used. A p value < 0.05 was considered significant.

Results

From February to August 2003, the field case definition was met by 63 persons, 59 of whom had blood samples collected for diagnostic testing. Among them, 33 had a positive laboratory test for CCHF virus infection. Four additional patients met the case definition but died before samples were obtained.

During the acute phase of the epidemic, 84 asymptomatic case contacts were interviewed and sampled. Only one (1.2%) was found positive and was considered to have a case of CCHF. The distribution of the 38 cases, according to the laboratory test results, is shown in Table 1.

Table 1. Distribution of Crimean-Congo hemorrhagic fever cases that were confirmed by serologic test, Mauritania, February–August 2003

Laboratory test[a]	No. positive (%)[b]
ELISA-IgM	22 (64.7)
RT-PCR	1 (2.9)
Isolation	2 (5.9)
ELISA-IgM + RT-PCR	6 (17.6)
ELISA-IgM + RT-PCR + isolation	3 (8.8)
Total	34 (100.0)

[a]ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription–polymerase chain reaction; Ig, immunoglobulin.
[b]An additional 4 persons met the criteria for having a probable case but died before sampling, so their cases could not be confirmed with serologic tests; according to the case definition, these persons were considered case-patients, which brings the total number of cases to 38.

Human Outbreak

First Patient and Initial Outbreak Cluster

The first patient to be identified (patient 1) was a 30-year-old pregnant woman who became ill on February 12, 2003, 7 days after she had butchered a goat. She was taken to the Nouakchott National Hospital by her relatives on the night of February 17. She had a severe nosebleed, which did not respond to treatment. She was extremely agitated, and her blood was spread across the small room where she was hospitalized, in the presence of other patients and their relatives. She died on February 18, 2003.

The doctor and the nurse who examined patient 1, one nursing student, and two hospital workers who were working in the emergency ward at the time were infected, and all had fatal cases. Of the 10 hospital patients and visitors infected in the ward where patient 1 was treated, 1 died. Four family members of patient 1 were directly infected. From these infected persons, two secondary cases occurred.

During the investigation, serum samples were collected from the three surviving goats from the same flock as the goat that the first case-patient had butchered. Anti-CCHF virus IgG was detected in the serum of one of the goats. These animals had come from Azlat, in the Brakna region, the native village of the index case-patient's family, where the investigation continued. Serologic evidence for CCHF virus infection was found in 4 of 25 sampled sheep (CCHF IgG-positive).

Evolution of the Epidemic

In Nouakchott, an outbreak of 35 cases of CCHF occurred between February 12 and August 24, 2003 (Figure 2). Two clusters and 11 isolated cases were identified (Appendix Figures, available online at http://www.cdc.gov/ncidod/EID/vol10no12/04-0535-G.htm). The main cluster (cluster 1), made up of 22 persons, was caused by contact with patient 1; this cluster included patient 1, members of her family, the hospital staff, and patients in the emergency ward. Cluster 2 comprised two persons who were infected after slaughtering a sheep.

All of these patients were living in Nouakchott during the month preceding their illness onset. The male-to-female ratio of patients was 1.7 (22/13); the mean age was 35.7 years (median = 31 years, range = 19–60 years). Of the 13 persons who did not belong to cluster 1 and for whom information was recorded, 6 (46.2%) were butchers, and 1 was in the habit of carrying animals in his truck. Of the nine women of childbearing age, two were pregnant.

The overall case-fatality ratio was 28.6%, 42.9% among patients likely infected by an animal and 19.0% among patients likely infected through person-to-person transmission. Death occurred 3–11 days after onset (median = 4 days). The male-to-female ratio (2.3) and median age (30 years) for deceased persons were comparable to those of survivors, but the time between infection and disease onset was shorter (80% of deceased patients had incubation periods <6 days before onset, compared to 29% of survivors; $\chi^2 = 3.997$, $p = 0.046$) (Table 2). In other regions, three case-patients were detected, including two housewives from the Brakna region (Aleg and Maghta Lahjar), one of whom died, and one butcher from Aioun El Atrous, in the Hodh El Gharbi region.

Of 14 confirmed patients tested on days 4 and 5, 10 (71%) tested positive for IgM; these antibodies were systematically detected in samples taken on day 6 or later. RT-PCR was performed for 16 confirmed cases. The results were positive for 10 patients for whom the median delay between the date of onset and the sampling date was 5 days (range 3–7 days). The five patients with negative results

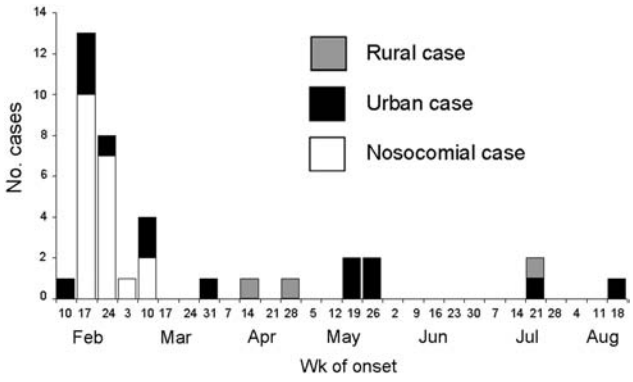

Figure 2: Distribution of CCHF cases by week of onset, Mauritania, February–August 2003.

Table 2. Incubation period (date of infection to clinical onset) among Crimean-Congo hemorrhagic fever patients, Mauritania, February–August 2003

	Outcome		
No. days	Survivor	Dead	Total
4	2	0	2
5	2	4	6
7	4	1	5
8	1	0	1
9	1	0	1
10	1	0	1
11	1	0	1
21	1	0	1
22	1	0	1
Total	14	5	19

and known delay (between date of onset and sampling) had been sampled on days 4 and 5 after onset. Isolation was successful on samples taken days 4–7 after onset (four samples with known dates of onset and sampling). For one patient who died on day 4, the results of ELISA and RT-PCR tests performed on blood samples taken the same day were negative, but virus isolation was successful.

Animals

Serum samples from 72 animals living near case-patients in Nouakchott were obtained and tested. In addition, serum samples from 25 animals belonging to the family of patient 1 in their native village, Azlat, Brakna, were analyzed. In Nouakchott, samples were obtained from animals living near patient 1, patients who were contaminated at the hospital (case-patients 13, 14, and 16), and patients with a separate source of infection (case-patients 23, 24, and 27). Of the 72 animals, 13 (18.1%) were positive for CCHF virus IgG by ELISA (Table 3). No animal was positive for IgM.

Tick Survey

The local department of public health treated the domestic animals in patients' homes with acaricidal treatments soon after cases were confirmed. As a result, we were not able to collect a sufficient number of ticks in these locations, and we extended our tick collection to include the animals belonging to the patients' neighbors. We collected 119 ticks from 70 domestic animals living near patients.

In addition, 259 ticks were collected from animals in livestock markets. Two genera and six species of ticks were collected (Table 4). Members of the genus *Hyalomma*, the principal vector of CCHF virus, were found in the same proportion as genus *Rhipicephalus*. *Hyalomma* ticks were the main species collected in the market places, whereas *Rhipicephalus* were mostly found in patients' homes. None of the ticks collected in the patients' neighborhoods were positive for CCHF virus. The presence of CCHF virus or genome was detected on *Rhipicephalus evertsi evertsi* ticks collected on three sheep from the markets. Two of these three sheep had been imported from the Hodh el Gharbi region (Figure 1).

Isolated Strains

The positions of nucleotides in the entire S segment of the CCHF virus isolated from patients infected in Nouakchott National Hospital are presented in Figure 3. The strain HD 168662, which is representative of human isolates obtained from this study, shows 82.1 % nucleotide identity with the strain HD 49199, which was isolated from a human patient in Mauritania in 1987. All strains isolated from patients infected during this outbreak had 100% homology.

Discussion

This study is the first to report CCHF virus in Nouakchott, the capital of Mauritania. The circulation of CCHF virus and the high prevalence of infected animals and ticks have been well documented in Mauritanian farming areas since 1983 (5,17,18). However, the disease had not yet been reported in Nouakchott, despite the fact that livestock are regularly transported there from farming areas.

The index case-patients came from six of the nine districts in Nouakchott, which suggests that a large part of the city was affected by the disease. In Mauritania, as in other developing countries, a demographic transition occurred in recent years, characterized by massive rural-to-urban migration. In Nouakchott, in one generation, from 1977 to

Table 3. Serologic results for animals sampled in areas surrounding patients' homes, Mauritania, February–August 2003

			Livestock species (no. positive/no. tested)		
Case	Probable source of infection	Place of sampling	Sheep	Goats	Total
1	Animal	El Mina, Nouakchott	0/7	1/2	1/9
1	Animal	Azlat, Brakna	4/25	0/0	4/25
13	Hospital	Arafat, Nouakchott	3/10	2/19	5/29
14	Hospital	Arafat, Nouakchott	0/2	0/4	0/6
16	Hospital	Tevragh Zeina, Nouakchott	3/9	0/0	3/9
23, 24	Animal	El Mina, Nouakchott	4/16	0/0	4/16
27	Animal (?)	Teyarett, Nouakchott	0/1	0/2	0/3
Total			14/70	3/27	17/97

Table 4. Distribution of ticks collected during the investigation of the Crimean-Congo hemorrhagic fever outbreak, Nouakchott, Mauritania, March 2003

Tick species/host	No. ticks		Positive pools/total pools tested
	Home	Market	
Hyalomma dromedarii			
Cattle	0	49	0/30
Camels	0	39	0/21
Floor	0	54	0/54
Total	0	142	0/105
H. marginatum rufipes			
Cattle	0	8	0/2
Camels	0	3	0/1
Sheep	6	9	0/10
Total	6	20	0/13
H. impeltatum			
Cattle	0	11	0/6
Sheep	8	8	0/7
Floor	0	3	0/3
Total	8	22	0/16
Hyalomma sp.			
Sheep	1	0	0/1
Rhipicephalus evertsi evertsi			
Sheep	97	75	4/56
Goats	2	0	0/1
Total	99	75	4/57
R. sanguineus			
Dogs	5	0	0/2
Total	119	259	4/194

2003, the population increased from 135,000 to 600,000. Nomadic habits, such as possessing domestic animals, have been maintained. In cities with a high population density, especially in areas where zoonoses are prevalent, this practice represents a major risk for human populations.

Wilson (19) and Gonzalez (9) demonstrated that West African sheep play a central role in the maintenance cycle of CCHF virus in disease-endemic areas because they serve as host for both the virus and the tick vector. These researchers also showed that even sheep that were infected previously and had anti-CCHF virus IgG can be reinfected and transmit the virus. In Nouakchott, sheep and goats are the most numerous domestic animals, and they live in close proximity to humans.

In Nouakchott, direct contact with blood of an infected animal seems to have been the primary mode of transmission from animals to humans. During the outbreak, half of the index patients were butchers, and a number were housewives, which suggests that handling freshly cut meat is a risk for infection. This hypothesis seemed to be confirmed by a survey conducted in June 2003 by the Centre National d'Hygiène in Mauritania. During this survey, anti-CCHF virus IgG was detected among 20 (7.0%) of 287 abattoir workers in Nouakchott (unpub. data).

The outbreak was initially observed in a hospital emergency ward, where the index patient infected five hospital staff members and 10 patients and visitors. The risk for nosocomial transmission of CCHF virus has been previously reported in Albania, Pakistan, Iraq, South Africa, and Dubai (3,11–12,20–24). In all of these reports, infected persons were heavily exposed to the blood of a patient. The same observation was made during this outbreak: the 10 patients and visitors who spent the night in the same room as the index patient, as well as the five health workers who died, had close contact with her blood. Secondary cases occurred only among her family and hospital contacts. In that cluster, two secondary contacts tested positive for CCHF virus. No other secondary cases occurred. This observation confirms other reports (11,12) that suggest a heavy exposure is needed for infection to occur. This finding is consistent with the hypothesis that subpopulations of virus adapted to a host are selected after passage through another vertebrate host. According to Gonzalez (25), these subpopulations seem to be less virulent and might have an altered capacity of transmission.

Although the difference between the two groups was not significant, probably due to lack of statistical power, we observed that the case-fatality ratio among patients contaminated by an animal was higher than the case-fatality ratio among secondary cases. This result could be due to a decrease in virulence after passage to humans, but it could also be explained by the fact that, while all patients

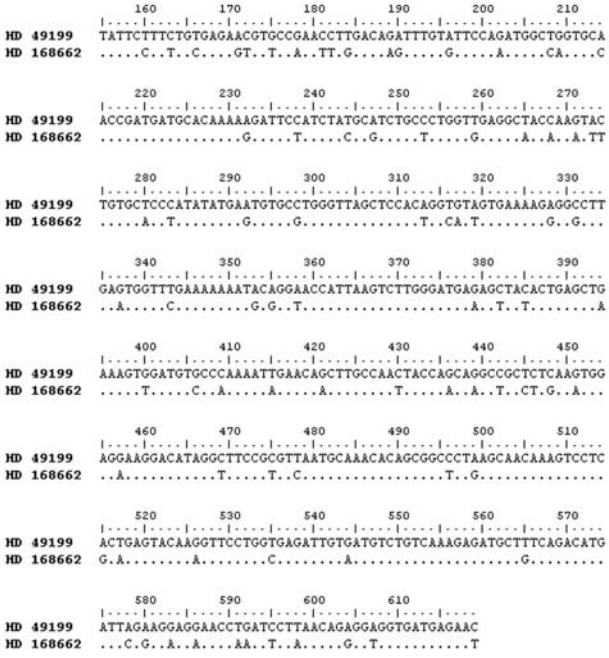

Figure 3. Comparison of partial sequences (465 base pairs) of the S segment of Crimean-Congo hemorrhagic fever virus isolated in Mauritania. The BLAST tool was used and positions of nucleotides in the entire S segment are shown. The strain HD 168662, which is representative of human isolates obtained from this study, shows 82.1% nucleotide identity with the strain HD 49199, isolated from a human case-patient in Mauritania in 1988.

in cluster 1, where the person-to-person transmission was observed, were detected, only the most severe cases resulting from contact with animals were reported.

We also observed that the incubation period was shorter for patients with fatal cases, which suggests that viremic load was higher. Half of the deaths occurred before day 4 after onset, when one third of seroconversions had not yet been observed, which confirms that ELISA cannot be used alone to diagnose CCHF (26,27). More than 5 days after onset, ELISA (IgM capture) systematically diagnosed infection. During the first 5 days, CCHF infection was confirmed by RT-PCR or isolation.

We investigated four patients who had most likely been infected by animals. One of the patients came from the Brakna region, where the presence of infected animals was confirmed. Animals suspected of infecting the other three patients had already been slaughtered and could not be investigated. In addition, none of the animals in the neighborhoods surrounding these patients' homes tested positive for IgM against CCHF virus, despite the fact that IgM remains elevated for 40 days after infection (9). However, this finding could be explained by the fact that very few ticks were found, and horizontal transmission from animal to animal does not occur in the absence of tick vectors. During our investigation, we found anti-CCHF virus IgG in 8 of 44 animals (2 of 23 goats, 6 of 21 sheep) that lived near the case-patients for whom person-to-person transmission at the hospital was well documented. These animals were therefore not involved in human infection. This finding suggests that the CCHF virus has widely spread among animal populations in Nouakchott.

Ticks were collected in neighborhoods surrounding the patients' homes and in marketplaces. During these investigations, *R. evertsi evertsi* was the only species found to be infected by the CCHF virus. Even if *Hyalomma* is the main vector for CCHF, *R. evertsi evertsi* may play a role in CCHF virus transmission (6).

Genetic analyses indicated that viruses isolated from case-patients linked to the nosocomial outbreak, the sporadic cases that occurred in the following weeks, and ticks all belonged to the same cluster (data not shown). Only two strains that caused fatal infections have been isolated from humans in Mauritania. The strain isolated during the 2003 outbreak was different from the strain previously responsible for human cases, but it was closely related to a virus previously isolated from ticks in Mauritania. The stability of the strain structure and the high prevalence CCHF antibodies in abattoir workers indicate that the CCHF virus is well established in Mauritania.

The 2003 epidemic was probably discovered because the outbreak occurred in a hospital. The hospital setting amplified the severity of transmission—with 19 secondary and 2 tertiary cases connected to the hospitalized index case-patient. This factor, in addition to the simultaneous death of a doctor and a nurse working in the same ward, alerted the medical authorities. The sporadic cases that occurred in Nouakchott in the following weeks (13 cases, 5 deaths) would have probably gone unnoticed if health personnel had not already been alerted because of the hospital outbreak.

CCHF may have emerged recently in Nouakchott, however. The rainy season normally lasts from June to September, but in 2002, the rains were scarce (<200 mm in farming areas) and pastures were difficult to find. As a result, farmers had to lead their flocks near large cities to feed them with imported food, increasing human exposure to infected animals and therefore the risk for infection. Because urban populations can access health facilities relatively easily, the risk for nosocomial transmission in overcrowded hospitals, where basic hygiene measures are not followed, was high.

Regardless of whether CCHF was recently imported or has been long established in Nouakchott, no human case had been reported before 2003. Studies should be conducted to determine the potential risk for continued sporadic and clustered outbreaks of CCHF in humans and to identify prevention measures.

Acknowledgments

We thank Roughiétou Sylla, Magueye Ndiaye, Lang Girault, Mireille Mondo, Modou Diagne, and Ann Juergens-Behr for their technical assistance; Alioune Ould Ahmed Abeid and Mohamed Lémine Diakité for assistance with data collection; and Jean-Paul Gonzalez for his interest and for reviewing preliminary versions of this manuscript.

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Alligators as West Nile Virus Amplifiers

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Recent evidence suggests that American alligators (*Alligator mississippiensis*) may be capable of transmitting West Nile virus (WNV) to other alligators. We experimentally exposed 24 juvenile alligators to WNV parenterally or orally. All became infected, and all but three sustained viremia titers >5.0 $\log_{10}$ PFU/mL (a threshold considered infectious for *Culex quinquefasciatus* mosquitoes) for 1 to 8 days. Noninoculated tankmates also became infected. The viremia profiles and multiple routes of infection suggest alligators may play an important role in WNV transmission in areas with high population densities of juvenile alligators.

The primary enzootic cycle for West Nile virus (WNV) is between adult ornithophilic mosquitoes and birds, with these mosquitoes occasionally infecting incidental hosts such as horses and humans (1). Most research to date has focused on these endothermic vertebrate hosts. Other arboviruses infect a variety of ectotherms, including species of lizards (2–4), snakes (5–11), and turtles (12,13), but the knowledge of ectotherm involvement in the ecology of WNV is limited. In the lake frog (*Rana ridibunda*), West Nile viremia capable of infecting mosquitoes (14,15) develops, and antibodies develop in Nile crocodiles (*Crocodylus niloticus*) and other ectotherms after natural infection (16,17). Experimentally infected North American bullfrogs (*R. catesbeiana*) and green iguanas (*Iguana iguana*) sustain low viremia levels for a short period of time, which suggests that they do not transmit the virus to biting mosquitoes (18).

In North America, WNV infections in ectotherms were first reported in 2001 (19). In the years 2001 to 2003, U.S. alligator farms reported substantial economic losses and at least one human case of fever due to WNV outbreaks in juvenile American alligators (*Alligator mississippiensis*) (19, 20; L. Tengelsen, pers. comm.). These alligators were housed in crowded tanks at a constant temperature of 32°C. The mode of transmission, the risk posed to handlers, and role of alligators in secondary WNV transmission cycles are unknown. To assess the potential role of juvenile alligators in the ecology of WNV transmission, we evaluated routes of transmission, determined viremia profiles, evaluated viral persistence in organs, and examined the role of temperature on WNV replication in these animals.

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Materials and Methods

Acquiring and Housing Alligators

American alligators were transported to Fort Collins, Colorado, from two U.S. alligator farms: St. Augustine Alligator Farm, St. Augustine, Florida (N = 26, age = 1–2 years, weight = 1–3 kg) and Colorado Gator Farm, Mosca, Colorado (N = 22, age = 10 mo, weight = 200–400 g). Alligators were fed gator chow pellets (Burris Mill and Feed, Franklinton, LA) twice per week (food volume ≈5% of body weight) (20).

Alligators were divided between two rooms; one room was maintained at 32°C and the other at 27°C. Room temperature and humidity were monitored by HOBO data recorders (Onset, Bourne, MA). Within each room, alligators were placed in livestock tanks (2 m diameter) separated by plastic curtains to reduce cross-contamination between tanks. Each tank contained 15 cm of water at the corresponding temperature (27ºC or 32ºC) and an adequate basking surface. Water was heated with aquarium heaters and aerated with an aquarium water pump. Equipment was checked twice daily, and the water was changed and tanks were disinfected every other day. Rooms were kept dark to calm the alligators (a standard practice at some alligator farms).

Mouse Infection

The NY99-4132 strain of WNV, passaged 3–4 times in Vero cells, originally from crow brain provided by W. Stone, New York State Department of Environmental Conservation, Albany, New York, was used in this study. We injected 24 Swiss Webster mice (6–8 weeks of age) subcutaneously with ≈1,000–2,000 PFU of WNV. Mice that developed neurologic signs 7–8 days postinoculation were euthanized and frozen at –70ºC.

Alligator Infection

Six alligators in the 32°C room and six alligators in the 27°C room were subcutaneously injected behind the left front leg with ≈7,500 PFU of WNV with a volume of 0.15 mL. Another six animals from each room were fed WNV-infected mice (1/2 mouse per small alligator [<700 g] and 1 mouse per larger alligator [>700 g]). Two noninfected alligators were placed with each infected group to serve as tankmate controls. Eight noninfected alligators served as bleeding controls in each room.

WNV Isolation from Serum

Blood samples were collected from each alligator daily for 15 days postinfection for virus isolation (some tankmate alligators were bled daily through day 21). Blood (0.2 mL) was collected from the caudal vein and added to 0.9 mL of BA-1 diluent (composed of Hank's M-199 salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 U/mL penicillin, 100 mg/L streptomycin, 1 mg/L amphotericin B in 0.05 mol/L Tris, pH 7.6), producing an approximate 1:10 serum dilution. Blood samples were centrifuged at 3,750 rpm for 10 min to separate serum from clotted blood and stored at –70°C.

WNV viremia was quantified by plaque assay. Blood samples were serially diluted 10-fold with BA-1 through 10^{-8}, and 100 mL of each dilution was added in duplicate to Vero cell monolayers in six-well plates (Costar, Cambridge, MA). Samples were allowed to incubate on the cells for 1 h at 37°C. Cells were then overlaid with 3 mL per well of 0.5% agarose in M-199 medium, supplemented with 350 mg/L sodium bicarbonate, 29.2 mg/L L-glutamine, and antimicrobial drugs as in BA-1. After 48 h of incubation, a second 3-mL 0.5% agarose overlay containing 0.004% neutral red was added for plaque visualization. Plaques were counted on day 4 postinfection.

WNV Isolation from Other Samples

Cloacal swab samples were taken from each alligator daily for 15 days postinfection (some tankmate alligators were swabbed daily through day 21 postinfection). A cotton swab was inserted into the cloaca ≈2 cm, rotated, and then placed in a tube containing 1.0 mL BA-1. Virus content was quantified by plaque assay.

Nine alligators (two that died of infection and seven that recovered) were tested for virus in tissues. Tissue samples (≈0.5 cm^3 in size) were harvested from the lung, liver, spleen, heart, kidney, spinal cord, cerebrum, and cerebellum. Samples were trimmed as needed and ground in 1.5 mL BA-1 containing 20% fetal bovine serum with a Retsch MM300 mixer mill (Retsch GmbH & Co, Hann, Germany) (30 cycles/sec for 4 min). Each resulting homogenate was transferred to a 1.7-mL Eppendorf microcentrifuge tube and clarified by microcentrifugation at 7,500 rpm for 3 min. Each supernatant was transferred to a 1.8-mL cryovial (Nalge Nunc International, Rochester, NY) and stored at –70ºC. Virus content was quantified by plaque assay.

Water (0.5 mL) was taken from each tank daily (before cleaning) for 15 days postinfection and then twice per week through day 31 postinfection. Water samples were added to 0.5 mL BA-1 (containing 2x concentrations of antimicrobial drugs). Water samples were pooled according to tank. Half of each pool was used for virus isolation. Water samples were added to 25-cm^3 tissue culture flasks (Corning, Corning, NY) (1 mL per flask) containing Vero cell monolayers. Flasks were rocked every 15 min for 1 h at 37°C, and 10 mL of Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA), supplemented with 2% fetal bovine serum, was added to each flask. Flask media were replaced on day 6 postinfection. Flasks were checked daily for cytopathic effect (CPE) through day 10 postinfection. Remaining water samples were tested by Taqman reverse transcriptase–polymerase chain reaction (RT-PCR) (21).

Neutralizing Antibody Detection

Blood samples (0.4–0.6 mL) were collected from each alligator for neutralizing antibody detection twice per week from day 21 postinfection through day 31 postinfection. To detect neutralizing antibodies, 15-μL serum samples from day 21 to day 31 were mixed with 60 μL of BA-1 and 75 μL of a WNV preparation (200 PFU/0.1 mL) in a polypropylene 96-well plate (Costar, Cambridge, MA). The virus-serum mixtures were incubated at 37°C for 1 h to allow for virus neutralization. These mixtures were then tested by plaque assay. Controls employed BA-1 only (cell viability control), serum-free virus mixture with BA-1 only (to enumerate PFU in the challenge dose of virus), and West Nile hyperimmune mouse ascitic fluid (diluted 1:200) mixture with virus (to verify challenge virus identity). Specimens were considered positive for WNV neutralizing antibodies if they reduced a challenge dose of ≈100 PFU of WNV by at least 90% at a serum dilution of 1:10.

Results

Viremia after Parenteral Infection

Every alligator injected with WNV became viremic from days 1 to 3 postinfection (Figure A and B). Alligators housed at 32°C became viremic on day 1 or 2 postinfection, while those kept at 27°C became viremic on days 2 or 3 postinfection. Viremia in the 32ºC alligators persisted an average of 10 days with an average maximum WNV titer of 5.7 $\log_{10}$ PFU/mL (maximum 6.7 $\log_{10}$ PFU/mL). The alligators housed in 27°C conditions were viremic for ≈14 days and averaged a maximum WNV titer of 5.8 $\log_{10}$ PFU/mL (maximum 6.1 $\log_{10}$ PFU/mL). No injected alligators died of the infection.

Tankmates in the 32ºC injected group became viremic on days 10 and 12 postinfection, while the tankmates in the 27°C injected group failed to become viremic (Figure A and B). Infection of tankmates in the 32ºC injected group persisted for ≈10 to 12 days, and neither died of the infection.

Viremia after Oral Infection

Viremia developed in two alligators from the 32°C room and five alligators from the 27°C room 3–6 days after they ate WNV infected mice (Figure C and D). Alligators in the 32°C room remained viremic for ≥9 days, while the alligators in the 27°C room remained viremic for ≈14 days.

Every alligator in the 32°C orally infected tank eventually became viremic during the experiment, with an average maximum WNV titer of 5.6 $\log_{10}$ PFU/mL (max 6.2 $\log_{10}$ PFU/mL) (Figure C). Tankmate viremia onset ranged from 12 to 24 days after infection. Because we stopped routine daily bleeding after day 15 postinfection, the exact viremia onset days of two alligators in this group are unknown. Also, the average duration of viremia for these alligators cannot be calculated. Two alligators in this group died of WNV infection after 12 or 13 days of viremia.

Both tankmates from the 27°C orally infected group also became infected (Figure D). One tankmate came into contact with a viremic mouse but did not eat it; this alligator became viremic on day 4 postinfection, and the infection persisted for ≥14 days. Viremia developed in the other tankmate on day 16 postinfection. Because of the absence of daily bleeding, the duration of viremia is not precisely known.

Viral Loads of Cloacal Swabs

Of 29 viremic alligators, 24 had detectable viral loads in their cloacae (Table 1). All five remaining infected alligators became viremic on the last 1 to 2 days of swabbing or after daily swabbing ceased, so no positive swabs can be reported from them. Viral shedding was detected within 3 days of detectable viremia and, in some instances, was detected on the same day as viremia onset. Duration of shedding lasted 6 to ≥12 days, with an average maximum viral load of 5.2 $\log_{10}$ PFU/swab (maximum 6.2 $\log_{10}$ PFU/swab).

Viral Isolation from Other Samples

Of 29 infected alligators, 2 died, and WNV was detected in their tissues (Table 2). No virus was isolated from the seven alligators that recovered from infection. WNV neutralizing antibodies were detected in 100% of infected alligators within 25 days after virus detection. No infectious virus or viral RNA was detected in water samples. Sample volumes were each 0.00013% of the total tank water volume.

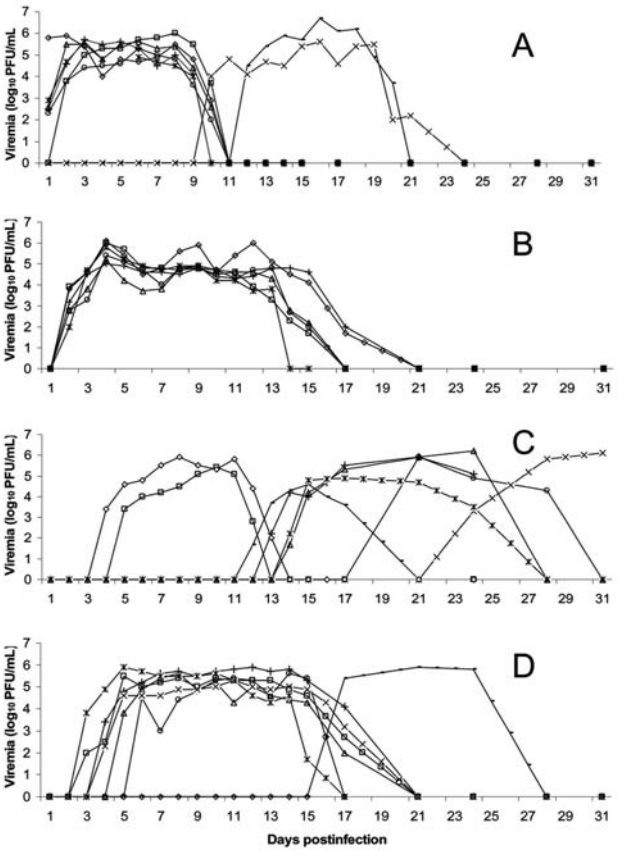

Figure. Daily viremia titers. A) Injected alligators held at 32°C (◇, *, ○, Δ, □, +) and their tankmates (x, –), B) Injected alligators held at 27°C (◇, ○, *, Δ, □, +). Tankmates did not become viremic. C) Orally infected alligators held at 32°F (Δ, □) and their tankmates (○,–, *, +, x, Δ). D) Orally infected alligators held at 27°C (◇, ○, *, Δ, □, +) and their tankmates (x, –). Blood samples were collected from each alligator for virus isolation once a day for 15 days postinfection. (Some tankmate alligators were bled daily through day 21 postinfection.) After day 15, alligators were bled biweekly through day 31 postinfection. West Nile viremia was quantified by using a Vero cell plaque assay. Plaques were counted after 4 days of incubation. The threshold of detection was 1.7 $\log_{10}$ PFU/mL of serum. Values $<10^{1.7}$ were considered to be zero.

Table 1. West Nile virus isolation from cloacal swabs of infected alligators[a]

Tank	Status	No. with WNV-positive swabs	Mean first day viral shedding[b]	Mean duration viral shedding (d)	Mean maximum viral load and range (log_{10} PFU/swab)
32°C parenteral	Infected (n = 6)	6	2	≥12	4.4 (3.5–4.9)
	Tankmate (n = 2)	2	12	≥9	5.9 (4.9–6.2)
32°C oral	Infected (n = 2)	2	6	≥8	4.9 (3.3–5.2)
	Tankmate (n = 6)[c]	4*	15	≥3	4.3 (2.0–4.8)
27°C parenteral	Infected (n = 6)	6	2	≥9	4.0 (1.9–4.4)
	Tankmate (n = 2)	0	NA	NA	NA
27°C oral	Infected (n = 6)	5	6	≥10	4.2 (1.9–4.7)
	Tankmate (n = 2)	1*	7	≥9	2.6 (NA)

[a]For some alligators (*), daily swabbing had stopped before or immediately after infection, so positive cloacal swabs were not detected.
[b]Days after injection or oral infection of the alligators; NA, not applicable.
[c]Four of six alligators were fed WNV-infected mice, but most likely became infected by tankmate transmission rather than oral transmission.

Discussion

In some southern states, alligator farms contribute to the economy as agricultural producers and tourist attractions. A typical operation raises 3,000 alligators each year. The market value of raw products (e.g., meat, hides) from an average adult alligator is ≈$300, and alligator meat typically fetches ≈$5 per pound. In Louisiana alone, the total value of farm-raised alligators is >$16 million (22). Beginning in 2001, alligator farms in at least four different states suffered substantial economic losses due to WNV outbreaks in young alligators. Public health risks involved in these large outbreaks and the eventual culling of thousands of young alligators are also substantial.

We have shown that sick juvenile alligators carry high viral loads in tissues, which poses a threat to handlers, processors, and consumers, although this risk has not been quantified beyond one reported case in Idaho of human West Nile fever in a handler of imported Florida juvenile alligators. Furthermore, all infected alligators in our study shed WNV from the cloaca, which poses another possible threat to other alligators and to handlers. Although tankmates in our study became infected at a high rate, we cannot conclude with certainty that cloacal shedding is the cause of this direct transmission.

Direct transmission likely plays an important role in the epizootiology of WNV infection in farmed alligators but has not been documented in wild alligators (19,20). However, we now know that high levels of viremia develop in young alligators, so WNV infection could likely lead to mosquitoborne transmission as well. In general, viremia reached titers considered to be infectious to *Culex quinquefasciatus* mosquitoes with the NY99 strain of WNV (5.0 log_{10} PFU/mL) in all but three infected alligators (23,24). *Cx. quinquefasciatus* is one of the principal vectors of WNV in the southeastern United States (25). Numerous species of mosquitoes feed on reptiles as well as birds and mammals and thus could be vectors from alligators to people (26). The primary WNV amplification cycle is believed to depend on birds and mosquitoes (1); however, the maximum duration of viremia in juvenile alligators was >2 weeks, which is longer than that observed in birds (maximum duration 7 days) (27).

Because most alligator farms raise juvenile alligators at a higher temperature (32°C) than older alligators, the effect of temperature on WNV infection was of interest. The 5°C difference in temperature that we tested did not significantly alter infection rates (Fisher exact test, p = 0.11). In general, alligators housed at 27°C maintained detectable viremia 4–5 days longer than the alligators housed at 32°C, which could be due to an enhanced immune function at the higher temperature. In 1969, Tait et al. discovered that lizards (*Egernia cunninghami*) housed at 30ºC produced higher titers of antibodies at a faster rate than those housed at 25ºC after injection with sheep red blood cells (28). In our study, WNV neutralizing antibodies developed in all infected alligators within a month of infection; these antibodies were detected in the alligators housed at 32°C an average of 5 days earlier than in the alligators housed at 27°C (data not shown). Although neutralizing antibody circulation is only one part of immune function, previous studies have suggested that multiple aspects of the ectothermic immune system may be affected by body temperature, which is directly affected by environmental temperature (29–31).

Transmission of WNV by means other than mosquitoes

Table 2. West Nile virus isolation from tissues of the two alligators that died[a]

Alligator	Tank	Day after viremia onset	Tissue (log_{10} PFU/0.5 cm^3)							
			Heart	Kidney	Spleen	Liver	Lung	Spinal cord	Cerebellum	Cerebrum
M0216 tankmate	32°C oral	12	5.8	<0.9	<0.9	1.4	6.1	2.1	2.7	1.6
M0228 tankmate	32°C oral	15	<0.9	2.2	2.5	1.6	3.5	NA	<0.9	<0.9

[a]No virus was detected in tissues from seven recovered alligators tested. [b]Temperature of tank and route of infection for alligators kept in tank.

has been shown in humans (32–34), mice (35), and birds (27,36), although some modes of transmission are poorly understood. In our study, alligators were successfully infected by parenteral and oral routes, although infection rates between the parenteral and oral groups differed significantly (Fisher exact test, $p < 0.05$). All 12 injected and 7 of 12 orally inoculated alligators became viremic. Furthermore, high viral loads in the cloacal samples indicate a possible fecal-oral route of transmission, although no viral RNA was detected in our water samples, probably because of the dilution effect of ≈400 L per tank (a 10^{-6} dilution factor). Other transmission routes could include bloodborne transmission, although wounds were observed on only two alligators during the experiment, or direct transmission by contaminated water droplets sprayed onto the conjunctiva or other mucous membranes. Although we apparently sampled water that was too dilute to detect WNV particles, at discrete moments, pockets of highly concentrated virus particles in the water could exist and lead to transmission. Infectious saliva could also contribute to direct transmission, but this factor was not examined in this study.

The only deaths observed in our study were two alligators housed at 32°C and infected by tankmate transmission. These data confirm the observations on the farms that WNV infection kills some alligators. Precise death rates on the affected farms are unknown, but we observed an overall death rate of 7% in this study (2 of 29 infected alligators).[1] Because of infectious virus in their tissues, these dead alligators represent a potential health threat to handlers, alligator meat consumers, and other alligators. Infectious virus was not isolated from tissues of seven alligators that recovered from infection, which suggests that surviving alligators do not pose a health threat after viremia and cloacal shedding cease (within 4 weeks postinfection).

In summary, juvenile alligators may be competent hosts for WNV. This study showed that juvenile alligators have adequate viremia levels (high-titer and long-lasting) for viral transmission by mosquitoes. Coupled with multiple routes of infection, alligators may play a role in WNV ecology, especially in areas where the density of young alligators is high.

Acknowledgments

We thank the St. Augustine Alligator Farm and the Colorado Gator Farm for the alligators used in this project; E. Jacobson and T. Campbell for their alligator expertise; T. Wilson for pathology support; and S. VandeVenter, P. Schneider, and K. Burkhalter for office and laboratory support.

This research was supported in part by NIH contract NO1-AI25489 and an appointment to the Research Participation Program at the Centers for Disease Control and Prevention (CDC), National Center for Infectious Diseases, Division of Vector-Borne Infectious Diseases, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC.

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[1]Of alligators infected by tankmate transmission, the death rate is 20% (2/10). Of alligators held at 32°C, the death rate is 13% (2/16).

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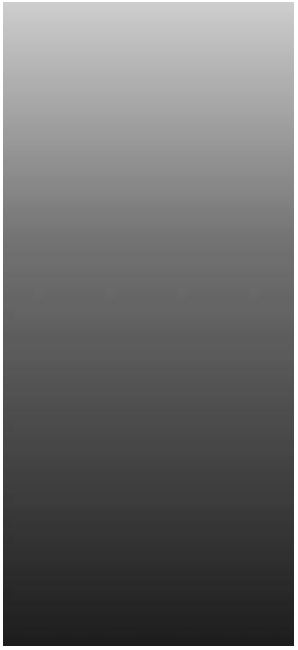

H3N2 Influenza Virus Transmission from Swine to Turkeys, United States

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In 1998, a novel H3N2 reassortant virus emerged in the United States swine population. We report the interspecies transmission of this virus to turkeys in two geographically distant farms in the United States in 2003. This event is of concern, considering the reassortment capacity of this virus and the susceptibility of turkey to infection by avian influenza viruses. Two H3N2 isolates, A/turkey/NC/16108/03 and A/turkey/MN/764/03, had 98.0% to 99.9% nucleotide sequence identity to each other in all eight gene segments. All protein components of the turkey isolates had 97% to 98% sequence identity to swine H3N2 viruses, thus demonstrating interspecies transmission from pigs to turkeys. The turkey isolates were better adapted to avian hosts than were their closest swine counterparts, which suggests that the viruses had already begun to evolve in the new host. The isolation of swine-like H3N2 influenza viruses from turkeys raises new concerns for the generation of novel viruses that could affect humans.

Influenza A virus is a highly infectious pathogen of a limited number of birds and mammals. Individual viruses are generally host-specific and are not readily transmitted between species. The species barrier reflects, at least in part, the different receptor preferences of mammalian and avian viruses. Researchers have suggested that human tracheal epithelial cells lack receptors for the attachment of avian influenza viruses and that avian tracheal epithelial cells lack the appropriate receptors for human viruses (1). Pigs, however, possess receptors for both avian and mammalian viruses and are postulated to be the host in which influenza viruses of different origins can genetically reassort (2–4).

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Currently, three subtypes (H1N1, H1N2, and H3N2) of influenza virus are commonly found in pigs worldwide. Depending on the location, these viruses are derived from mammalian viruses, avian viruses, or reassortants of the two (5). Until 1998, the classic H1N1 lineage was the only influenza virus circulating widely in the swine population in the United States (6). In 1998, H3N2 triple reassortants with genes derived from human (HA, NA, and PB1), swine (M, NS, and NP), and avian viruses (PA and PB2) were first isolated in the United States; they have since become endemic in swine populations (7–9). These viruses underwent further reassortment to create additional H3N2 viruses isolated from pigs (8), as well as H1N2 viruses isolated from pigs (10–12), turkey (13), and wild duck (14); this finding demonstrates that viruses containing this gene combination can cross the species barrier. We describe the isolation and characterization of H3N2 influenza viruses from domestic turkeys in the United States. Genetic and serologic characterization showed that these viruses have high homology to each other and to swine influenza viruses recently circulating among pigs in North America.

Materials and Methods

Virus Isolation

In 2003, influenza viruses were isolated from two geographically separate turkey farms: one in the Midwest (in February) and one in the eastern United States (in March). We isolated influenza viruses from each farm by using Madin-Darby canine kidney (MDCK) cells supplemented with 1 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. Briefly, the samples were add to monolayers of MDCK cells and incubated for 1 h at 37°C to allow viral adsorption to the cells. The inoculum was decanted, Eagle's minimum

essential medium supplemented with 0.2% bovine serum albumin was added, and monolayers were incubated for 3–5 days at 37°C. After cytopathic effects appeared, influenza virus was confirmed by using hemagglutination of chicken erythrocytes and reverse transcription–polymerase chain reaction (RT-PCR) against the HA gene as previously described (10).

Antigenic Analysis

To examine the antigenic relations between the newly isolated viruses and other avian influenza viruses (i.e., their serologic cross-reactivity), we conducted HI assays with a panel of reference antisera against all 15 HA subtypes and swine antisera to recent swine H3N2 viruses; A/Sw/NC/39615/01, A/Sw/MN/23062/02, A/Sw/TX/46710-35/02, A/Sw/TX/46710-37/03, A/Sw/NC/5854/02, and A/Sw/MO/22583/02. To determine the extent of turkey-to-turkey transmission on infected farms, we collected serum from 12 apparently healthy birds in one infected flock and used the HI assay (15) to test for antibodies to influenza virus surface glycoproteins.

DNA Sequencing

Viral RNA was extracted from supernatants of culture of infected cells by using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription and PCR amplification were carried out under standard conditions by using influenza-specific primers (16,17). PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital.

Sequence Analysis

DNA sequences were compiled and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Alignment of each influenza virus sequence was created by using the program Clustal_X (18,19). Rooted phylograms were prepared by using the neighbor-joining algorithm and then plotted by using NJplot (20). In this study, we used the following regions for phylogenetic analyses: PB2, 10-1262; PB1, 66-1368; PA, 8-1290; HA, 1-1002; NP, 55-962; NA, 41-1123; M, 1-889; and NS, 1-842.

Animal Experiments

Each virus was passaged in 10-day-old embryonated chickens eggs before infection of animals. Virus replication in chickens (specific pathogen–free white leghorn broilers, Charles River SPAFAS), quail (*Coturnix coturnix*, B&D Game Farm), turkey (commercial breeding operation), mice (Balb/C, Jackson Laboratories), and outbred pigs (Midwest Research Swine) was measured after intranasal inoculation with virus-infected allantoic fluid containing 10^6 EID_{50} (egg infectious dose 50) of virus, as previously described (21). All animals were 4–5 weeks old, and the numbers of each species used are listed in Table 1. Tracheal swabs were collected from chickens, quails, and turkeys on postinfection days 3–7, and virus was titrated in 10-day-old embryonated chicken eggs. Mice were weighed daily on days 0–7 postinfection, and animals were killed on day 3, 5, and 7 postinfection, and virus in lung tissue was titrated. Four-week-old pigs free of influenza antibody were intranasally infected with each virus (two pigs for each virus), and nasal swabs were collected on days 2–7 postinfection for virus titration in embryonated eggs. On days 5 and 7 postinfection, one of the two pigs infected with each virus was killed and subjected to pathologic examination and titration of virus in organs.

Results

Virus Isolation

Although not always present, clinical signs among turkeys at each farm included depression, coughing, sneezing, loss of appetite, and decreased egg production. Because of the susceptibility of turkeys to H1N1 swine influenza viruses (22), the flocks had been vaccinated with an autogenous vaccine against the H1N1 subtype. Four influenza viruses were isolated (in MDCK cells) from tracheal swabs of the affected birds. Only one isolate from each farm was selected for characterization, because preliminary antigenic and sequence analyses of the HA genes of isolates showed that all viruses in each farm were identical. These isolates were designated A/turkey/North Carolina/16108/03 (A/Tk/NC/16108/03) and A/turkey/Minnesota/764/03 (A/Tk/MN/764/03).

Antigenic and Genetic Characterization

The isolated viruses did not react with any of the reference antisera, even with early (1998) swine influenza virus antisera. We identified them as H3N2 viruses by using RT-PCR under conditions optimized for swine influenza viruses (16).

Sequence analysis of the PCR products demonstrated >98% nucleotide identity in each gene segment of the two isolates. According to the Influenza Sequence Database (23), the greatest sequence similarity was to genes of recent, triple-reassortant swine H3N2 (viruses containing gene segments derived from swine, avian, and human viruses) and swine H1N2 viruses from the United States (Table 2). A degree of similarity of >97% between the turkey and swine viruses indicated that interspecies transfer had occurred. This report is, to our knowledge, the first of transmission of swine H3N2 viruses to turkeys.

Table 1. Replication of turkey isolates and swine influenza viruses in various animals[a]

Animal	Isolate							
	A/Tk/MN/764/03		A/Tk/NC/16108/03		A/Sw/TX/4199-2/98		A/Sw/NC/29974/02	
	Positive/total[b]	$\log_{10}$ EID_{50}[c]	Positive/total[b]	$\log_{10}$ EID_{50}[c]	Positive/total[b]	$\log_{10}$ EID_{50}[c]	Positive/total[b]	$\log_{10}$ EID_{50}[c]
Mouse	6/6	4.3 (0.3)	6/6	4.7 (0.3)	6/6	5.3 (0.3)	6/6	5.0 (0.5)
Swine	2/2	2.7 (0.3)	2/2	3.3 (0.3)	2/2	3.7 (0.3)	NT	NT
Turkey	5/5	2.3 (0.3)	3/4	2.3 (0.3)	0/5	–	1/4	1.3 (0.0)
Quail	5/6	3.0 (0.5)	4/6	3.3 (0.5)	0/6	–	0/6	–
Chicken	0/6	–	0/6	–	0/6	–	0/6	–

[a] NT, not tested; –; negative
[b]Number of animals with a virus-positive lung homogenate (mice) or nasal swab at 3 days postinfection per number of animals infected.
[c]Average $\log_{10}$ EID_{50} per milliliter of homogenate (standard deviation).

Phylogenetic analysis of the HA and NA genes of the turkey viruses with those of a number of swine viruses collected in surveillance studies (unpub. data) demonstrated that A/Tk/NC/16108/03 and A/Tk/MN/764/03 are closely related to a swine H3N2 virus isolated in North Carolina in 2002 (A/swine/North Carolina/29974/02) (Figure). The GenBank numbers assigned to the sequences determined in this study are AY779253–AY779270.

Animal Infections

Serum taken from each of the 12 healthy birds in one of the infected flocks was highly reactive with the turkey H3N2 isolates (HI titer 1,280), demonstrating that transmission had occurred not only between pigs and turkey but also between turkeys. Therefore, these viruses may have had an opportunity to adapt to the avian host. To investigate this possibility, we compared replication of the two turkey isolates with that of A/swine/NC/29974/02 (the most genetically similar swine virus) and that of A/swine/TX/4199-2/98 (the index triple-reassortant H3N2 virus) in chickens, quail, turkey, mice, and pigs. Animals were infected intranasally with 10^6 EID_{50} of the respective influenza viruses, and virus was titered in lung homogenates and/or nasal swabs through 7 days after infection (Table 1). All viruses were recovered from pig and mouse lung homogenates (2 –5 $\log_{10}$ EID_{50}/gram of tissue); this titer demonstrated that viruses of the swine H3N2 triple-reassortant lineage could replicate in pigs and mice without adaptation. None of the viruses were recovered from infected chickens. Only the turkey isolates were reproducibly recovered from turkey and quail. A/swine/NC/29974/02, the most genetically similar swine virus, was isolated only from one of four infected turkeys on day 3 postinfection. Therefore, the turkey isolates appear to be better adapted to avian hosts than do their closest swine counterparts, but certain swine H3N2 viruses also have the potential to replicate in turkeys.

Discussion

We present the isolation of swinelike H3N2 influenza viruses from two geographically distinct turkey farms in the United States. These viruses are closely related to swine H3N2 viruses that emerged in pigs in the United States in 1998 and have since become endemic. Although infection of turkeys with H1N1 and H1N2 swine influenza viruses has been documented (13,22,24), to our knowledge, this report is the first of swinelike H3N2 infection in this host. The clinical signs of infection in these turkeys were not severe, but our findings have implications for influenza ecology and the possibility of further evolution of these viruses.

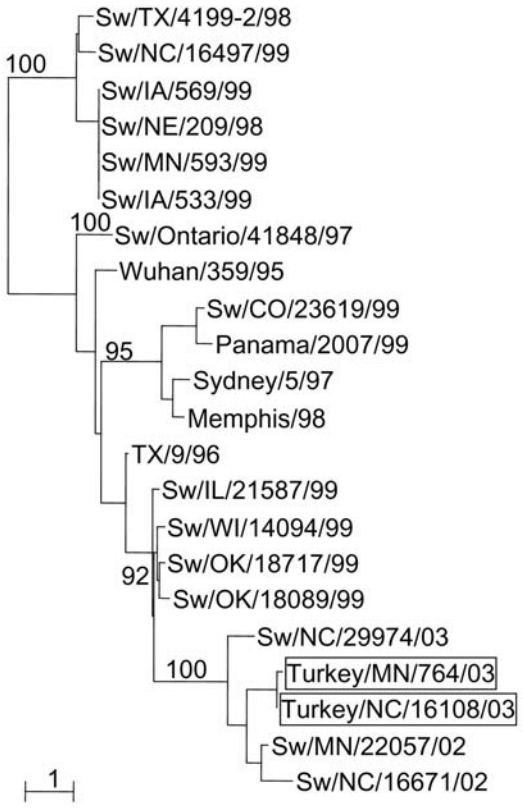

Figure. Phylogenetic tree representing the HA nucleotide sequences of two H3N2 influenza viruses isolated from turkeys on geographically distant farms in the United States and of selected swine influenza virus strains. Nucleotide sequences were aligned by using the Clustal_X (19) program, and phylograms were generated by the neighbor-joining method using the NJplot program (20). The scale is proportional to the numbers of substitutions per nucleotide.

Table 2. Type A influenza viruses with the highest nucleotide sequence identity to the turkey isolates

Gene	Nucleotide sequence identity (%)	Virus[a]	Subtype	Reference
HA	97.6	A/swine/NC/29974/02	H3N2	This report
NA	98	A/swine/NC/29974/02	H3N2	This report
PB2	98	A/swine/OH/891/01	H1N2	(11)
PB1	98	A/swine/MN/593/99	H3N2	(7)
PA	97	A/swine/NE/209/98	H3N2	(7)
NP	98	A/swine/OH/891/01	H1N2	(11)
M	98	A/swine/MN/34893/01	H1N2	(10)
NS	99	A/swine/IN/14810-T/01	H1N2	(10)

[a]Named with standard abbreviations of states in the United States.

Two properties of the swine-like H3N2 infection in turkeys raise concerns about the potential for further viral evolution. The first is that the H3N2 viruses have been successfully established in pigs and have demonstrated an ability to reassort with human (8), swine (11), and avian viruses (10). The second is that turkeys appear to be highly susceptible to infection by influenza viruses from aquatic birds (25), thus increasing opportunities for further reassortment. Although the fitness of such a swine-avian reassortant cannot be predicted, and the infected flocks showed no evidence of coinfection with avian viruses, continuing to monitor turkey populations for the presence of swine virus-like gene segments is prudent.

We found strong evidence that turkey-to-turkey transmission had occurred in at least one of the flocks, and the viruses were similar despite their geographically distinct origins. Although detailed information is not available, there are no obvious links between the infected flocks, and direct movement of turkeys between the two farms was unlikely. Further epidemiologic investigation of the flocks and surrounding swine herds is warranted. In a small scale retrospective screen of 125 turkey serum samples collected from 2000 to 2004, we were unable to detect any evidence of H3N2 infection using standard HI assays (data not shown), which suggests that such infections are not widespread. Although the most obvious explanation for the dual outbreak is a common source of infection, both flocks may have been infected by a swine virus circulating in pigs in both areas.

Although we recovered one swine isolate genetically similar to the turkey isolates from one of 4 infected turkeys, the ability of only the turkey isolates to replicate in quails suggests that the viruses may have already begun to adapt to the avian host. This adaptation to an avian host did not occur at the expense of the ability to replicate in pigs. Both turkey isolates were shed by experimentally infected swine for at least 5 days; this shedding pattern is consistent with that of genetically similar swine isolates. To speculate that the turkey isolates can replicate in quail and, possibly, other avian hosts as a result of their passage in turkeys is tempting. However, the traits that allowed the viruses to be transmitted to turkeys may also allow them to infect quail. The adaptation did not appear to be a result of egg passage, as we found no differences in HA sequence between MDCK and egg-grown virus. To investigate the possible mechanisms of avian adaptation, we compared the amino acid sequences of the HA1 proteins of the turkey isolates and the swine H3N2 viruses. The turkey isolates and A/swine/NC/29974/02 differed at only seven amino acids, two of which (residues 137 and 226) were in the receptor binding site. Although we found substitutions at these two sites in other swine isolates represented in sequence databases (data not shown), only the turkey isolates had both the Y137S and V226I substitutions. Ongoing reverse genetics experiments will help to identify the relative contributions of these amino acids to avian adaptation.

The introduction of H3N2 viruses to the U.S. swine population has significantly affected patterns of animal influenza in this country. Not only have the H3N2 viruses successfully established and reassorted in pigs, but H1N1 (22), H1N2 (13), and now H3N2 isolates carrying the internal genes of H3N2 viruses have been isolated from birds. Viruses of this genetic composition will likely continue to evolve and cause problems for animal and, potentially, human health. The swine population is likely a reservoir of yet another lineage of influenza viruses that have demonstrated the ability to reassort and be transmitted between species.

Repeated introductions of swine influenza viruses to turkeys, which may be coinfected with avian influenza viruses, provide opportunities for the emergence of novel reassortants with genes adapted for replication in pigs or even humans. Our studies emphasize the continuing need to monitor pigs and domestic birds to better understand interspecies transmission and the emergence of novel influenza viruses that have the potential to infect humans.

Acknowledgments

We thank Yolanda Sims and Jennifer Humberd for technical assistance and Janet R. Davies for editing the manuscript.

These studies were supported by grants AI-29680, AI-95357, and CA-21765 from the National Institutes of Health and

by the American Lebanese Syrian Associated Charities (ALSAC).

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Differential Virulence of West Nile Strains for American Crows

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Crow deaths were observed after West Nile virus (WNV) was introduced into North America, and this phenomenon has subsequently been used to monitor the spread of the virus. To investigate potential differences in the crow virulence of different WNV strains, American Crows were inoculated with Old World strains of WNV from Kenya and Australia (Kunjin) and a North American (NY99) WNV genotype. Infection of crows with NY99 genotype resulted in high serum viremia levels and death; the Kenyan and Kunjin genotypes elicited low viremia levels and minimal deaths but resulted in the generation of neutralizing antibodies capable of providing 100% protection from infection with the NY99 strain. These results suggest that genetic alterations in NY99 WNV are responsible for the crow-virulent phenotype and that increased replication of this strain in crows could spread WNV in North America.

West Nile virus (WNV, *Flaviviridae*: *Flavivirus*) is maintained in nature by transmission between mosquitoes and birds and has an extensive geographic range, including Europe, Africa, the Middle East, southern Asia, and Australia (1). In 1999, WNV was identified in North America (2) and has become the leading cause of arboviral encephalitis in humans and horses (3), as well as having been implicated in deaths of members of at least 198 bird species (4). Corvids, including the American Crow (*Corvus brachyrhynchos*), appear to be most susceptible (5,6), and corvid deaths have subsequently been used as a sentinel to track the spread of the virus (7).

Experimental injection of American Crows with the North American genotype of WNV (NY99 strain) has confirmed its highly pathogenic phenotype. Mean peak viremia titers in American Crows exceed 9 $\log_{10}$ PFU/mL in sera, with 100% deaths within 6 days postinfection (dpi) (5). With the exception of bird deaths in Israel (8), where a strain 99.8% similar to the NY99 genotype has circulated since 1997 (9), no bird deaths have been reported during numerous well-characterized WNV epidemics in North Africa (10), Europe (11–13), Russia (14), and South Africa (15). A closely related virus that circulates in Australia (Kunjin [KUN]) has never been associated with outbreaks of human or animal diseases, including bird diseases, nor have bird deaths been reported from enzootic transmission foci in Africa, where a virus that shares 96.5% nucleotide identity with the NY99 strain has previously been isolated (16,17). Possible explanations for the lack of reporting of bird deaths before 1998 include the following: failure to identify bird deaths in other regions, a higher susceptibility to WNV-induced disease among North American birds, or the fact that the North American WNV strain possesses increased avian virulence determinants. Additionally, the possible immunologic cross-protection of birds with lesser virulent strains could be a factor that has limited the identification of bird deaths outside the Middle East. Immunologically naïve bird populations in North America could be at an increased risk of acquiring severe disease.

The close genetic relatedness of the North American WNV genotype with the bird-pathogenic Israeli WNV strain suggests differential avian pathogenicity among WNV strains (9). To evaluate whether WNV-associated deaths in American Crows was due to infection by a more virulent genotype, we injected American Crows with NY99, a closely related WNV strain from Kenya (KEN) and a more distantly related WNV strain from Australia (KUN) and monitored viremia titers and illness. In addition, birds that survived challenge with the KEN or KUN viruses were challenged with a lethal dose of the NY99 strain to assess development of a cross-protective immunologic response.

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Materials and Methods

Viral Strains and Birds Used

The lowest passage WNV available were used for crow virulence studies to avoid incorporating confounding cell-culture–related genetic substitutions. The NY99 isolate used was originally isolated from an American Crow brain (strain NY99-4132) and was subsequently passaged once in Vero cells before being used for these studies. The Kenya-3829 (KEN) isolate was made from a pool of male *Culex univittatus* mosquitoes (16) and passaged twice in Vero cells. The Kunjin (KUN-6453) isolate was made from *Cx. annulirostris* mosquitoes and was passaged once in Vero cells and once in hamster kidney cells (Table 1). After-hatch-year American Crows were obtained by using net traps with the assistance of the Kansas Department of Wildlife Resources. The crows were banded and transported to Fort Collins, Colorado, where they were housed in the Colorado State University Animal Disease Laboratory in groups of two in 1-m^3 cages. Crows were fed a combination of ground corn and dried cat food and dog food.

Detection of Preexisting Flaviviral Antibodies

To confirm that crows had not previously been exposed to WNV or another endemic flavivirus, St. Louis encephalitis virus (SLEV), crows were bled before injection and serum-tested by plaque reduction neutralization assays (PRNTs) with WNV and SLEV viruses. Serum was diluted 1:5, heat inactivated at 56°C for 30 min, and incubated with an equal volume of virus (SLEV; strain TBH-28) and WNV (strain NY99-4132) to a final concentration of 100 PFU/0.1 mL. Samples were incubated at 37°C for 1 h, and 0.1 mL of each was added to a confluent monolayer of Vero cells in 6-well plates (Costar Inc., Cambridge, MA). After incubation for 1 h, cell monolayers were overlaid with 0.5% agarose; a second overlay containing 0.005% neutral red was added 48 h later. Plates were read 1–2 days after addition of the second overlay. A 90% reduction in PFU, as compared to the serum-negative control, was used as the determinant of neutralization. Detection of any neutralizing activity to either SLEV or WNV within the serum of any crow precluded use for experimental inoculation.

Virus Injection

Viral stocks were diluted to 3.2 $\log_{10}$ PFU/0.1 mL in minimal essential media (MEM) containing no fetal bovine sera (FBS). One hundred microliters of the diluted stocks was subcutaneously injected on the breast region of eight American Crows in four infection groups. Crows were injected with 1) NY99, 2) KEN, 3) KUN WNVs, or 4) with a media-only injection that served as a virus-negative control. In addition, a higher dose inoculum of 3.8 $\log_{10}$ PFU/0.1 mL was prepared for injection of a fifth group of crows with KEN WNV. All crows were examined for signs of disease twice daily for 14 days after injection and bled once daily from 1 to 7 dpi for characterization of viremia. Blood samples were collected from the jugular or brachial vein by using a 26-gauge needle; 0.2 mL of blood was added to 0.9 mL of MEM supplemented with 20% FBS to obtain approximately a 10^{-1} serum dilution. Coagulation was allowed to take place at room temperature for 30 min, at which point samples were placed on ice and spun at 3,700 x *g* for 10 min to pellet clotted cells. The supernatants from these samples were frozen at –80°C until samples were titrated for infectious units.

Assaying for Infectious Virus

Infectious virus was assayed by plaque formation on monolayers of Vero cells. Briefly, serial 10-fold dilutions of serum were added to Vero cells that were overlaid as described previously for PRNTs. PFU were enumerated at 3 dpi and multiplied by the dilution factor to determine viral titers per mL serum. Initial 1:10 dilution of serum as well as the use of 200 µL of the lowest dilution, resulted in a limit of viral PFU detection of 1.7 $\log_{10}$ PFU/mL serum. Inocula for all three viruses were back-titrated by plaque assay in order to confirm the uniformity of the doses administered.

Determination of Cross-Protection

Blood (0.6 mL) was drawn at 14 dpi to determine the levels of WNV-specific antibodies and cross-neutralization by using a 2-way β PRNT with homologous and heterologous WNV strains. Briefly, twofold dilutions of bird serum samples were incubated at 56°C for 30 min and mixed with either NY99, KEN, or KUN viruses. Samples were allowed to incubate for 1 h at 37°C, at which point the samples were injected onto Vero cells and overlaid as previously described for PRNT. Plaques were counted, and neutralization was reported as a 90% reduction in plaque formation as compared to the results for the serum-negative control.

Table 1. West Nile viral strains used for virulence studies in American Crows

Virus	Strain	Source	Passage history[a]	Location	Genetic lineage[b]
NY99	NY99-4132	American Crow (brain)	V1	USA	I
KEN	KEN-3829	*Culex univittatus*	V2	Kenya	I
Kunjin (KUN)	KUN-6453	*Cx. annulirostris*	V1, BHK1	Australia	I

[a]Viruses were propagated in Vero (V) or baby hamster kidney (BHK) cells. Numbers following passage source represent the number of viral passages.
[b]Genetic lineages as reported previously (9).

Crows that survived through 14 dpi were subsequently challenged with 3.2 $\log_{10}$ PFU of NY99 virus from the same seed that was used for the initial infection of the experimental NY99 infection group. Crows were bled daily through 7 dpi and were held through 11 dpi, at which point an additional 0.6 mL of blood was drawn to assess modulations in neutralizing activity after secondary challenge. Serum samples from the seven daily bleedings were diluted 1:10 in MEM diluent, spun, immediately assayed for the presence of infectious virus on Vero cells, and then stored at –80°C. Samples demonstrating virus were thawed and titrated on Vero cells as described above. Additionally, serum drawn at the end of the time course was assayed for antibody by PRNT.

Statistical Analyses

Statistical analyses were performed on peak viremia level, duration of viremia, day of viremia onset, and day of death. All analyses with the exception of day of death were performed by analyses of variance (ANOVA). Multiple comparisons, i.e., confidence intervals (CI) for the difference of means, were performed by using Tukey's highest significant difference (HSD) adjustment for comparisons of means. Because only two virus groups had birds that died, the day-of-death comparisons were analyzed by using a Student *t* test with Welch's modification for unequal variances. Proportions of illness and death were compared with the Fisher exact test.

Results

Flaviviral antibodies were not detected in any of the preinoculation serum samples assayed by PRNT. Therefore, all captured American Crows were used for experimental inoculation. Peak viremia titers ranging from 6.7 $\log_{10}$ to 10.7 $\log_{10}$ PFU/mL serum (mean peak viremia titers = 9.2 $\log_{10}$ PFU/mL serum, 95% CI 8.2 $\log_{10}$ PFU/mL serum–10.2 $\log_{10}$ PFU/mL serum) developed in all crows injected with the NY99 WNV genotype (Figure 1). Onset of viremia occurred within 24 h for three of the eight crows injected with NY99 and was present in all eight birds within 48 h postinjection (mean onset of viremia = 1.8 dpi, 95% CI 1.4 dpi–2.1 dpi) (Table 2). Mean onset of viremia and mean peak viremia titers differed significantly among the virus groups (mean onset, F = 31.6, df = 3,22, $p < 0.001$; mean peak viremia, F = 74.9, df = 2,21, $p < 0.001$). In contrast to the NY99-infected crows, detectable viremia ($\geq$1.7 $\log_{10}$ PFU/mL sera) developed in two crows infected with the KEN WNV. The onset of viremia in these two birds was delayed until 3 dpi and 4 dpi, and the mean peak viremia level was lower than that of the NY99 infection group (7.5 $\log_{10}$ PFU/mL) (difference of mean onset of viremia = 1.8 dpi, 95% CI 0.4 – 3.1). When the inoculum dose was increased to 3.8 $\log_{10}$ PFU for the KEN strain, viremia developed in all eight of the crows, with peak titers ranging from 4.2 $\log_{10}$ PFU/mL serum to 6.1 $\log_{10}$ PFU/mL serum (mean = 4.9 $\log_{10}$ PFU/mL serum, 95% CI 4.3–5.4 $\log_{10}$ PFU/mL serum). The onset of viremia was delayed in the higher dose KEN group compared to the NY99 infection group (mean = 4.5 dpi, 95% CI 3.9–5.1 dpi; difference of mean onset of viremia = 2.8 dpi, 95% CI 1.9–3.6 dpi). In all eight crows inoculated with 3.2 $\log_{10}$ PFU of KUN virus, peak viremia titers were 2.7–4.9 $\log_{10}$ PFU/mL serum (mean = 4.2 $\log_{10}$ PFU/mL, 95% CI 3.5–4.8 $\log_{10}$ PFU/mL serum). Onset of viremia relative to the NY99-infected crows was slightly delayed, with a mean onset at 2.4 dpi (95% CI 1.9–2.8 dpi) (difference of mean onset of viremia = 0.6 dpi, 95% CI 0.2–1.5 dpi) (Figure 1). Viremia developed in KUN-infect-

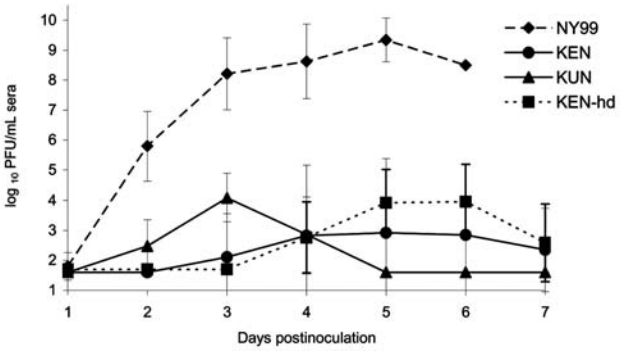

Figure 1. Viremia profiles for West Nile virus (WNV)–infected American Crows after injection of 1,500 PFU of KUN or KEN/NY99 WNV. Viral titers were determined by plaque formation on Vero cells and represented as geometric means. A detection limit of $\geq$1.7 $\log_{10}$ PFU/mL crow serum was determined. Bars represent standard deviations (SD) of the mean. hd, high dose.

Table 2. Clinical profile of American Crows infected with WNV strains NY99 (strain NY99-4132), KEN (strain KEN-3829), and KUN (strain KUN-6453)

Virus group	Mortality: no. died /N (%)	Morbidity: no. ill/N (%)	Mean day of death ± SD	Mean peak viremia[a] ± SD (mean duration ± SD) (n)	Mean day of peak viremia[a] ± SD
NY99	8/8 (100)	8/8 (100)	5.1 ± 0.6	9.2 ± 1.2 (4.2 ± 0.7) (8)	4.3 ± 0.9
KEN	1/8 (12.5)	2/8 (25)	9 ± NA	7.5 ± 0 (4.5 ± 0.7) (2)	5.0 ± 1.4
KEN-hd[b]	2/8 (25)	3/8 (38)	10.5 ± 2.1	4.8 ± 0.6 (3.1 ± 0.8) (8)	5.5 ± 0.9
KUN	0/8	0/8	NA	4.2 ± 0.8 (1.8 ± 0.5) (8)	3.3 ± 0.7
Control	0/8	0/8	NA	NA	NA

[a]Viral titers were expressed as the $\log_{10}$ PFU/mL of crow sera as determined by plaque assay on Vero cells.
[b]hd, high-dose group (6,000 [3.8 $\log_{10}$] PFU); NA, not applicable.

ed crows, lasting from 1 to 5 days with a mean duration of 3 days (95% CI 1.9–4.1 days). This finding differs qualitatively from the NY99- and KEN-infected birds, which sustained viremia for at least 4 days; viremia levels ceased only when the bleeding time course was halted or at time of death; the differences between the viremia durations for the KUN-infected crows and the NY99 and KEN groups were not statistically significant when adjustments were made for multiple comparisons.

All crows in the NY99 group died by dpi 6 (Figure 2). Signs of illness (unresponsiveness, anorexia, weight loss), signs of encephalitis (shaking, convulsion, ataxia), or both developed in all NY99-infected crows. In addition, hemorrhage from oral and cloacal cavities was evident in five (62.5%) of the eight crows in the NY99 group. One crow died of infection with NY99 at 4 dpi, five at 5 dpi, and the remaining two at 6 dpi (Figure 1). Only one crow (12.5%) died of infection with the KEN virus with the 3.2 $\log_{10}$ PFU injection. When the dose was increased to 3.8 $\log_{10}$ PFU, 2 (25%) crows did not survive the infection. Regardless of the dose administered, the crows infected with the KEN virus demonstrated a reduced mortality rate ($p < 0.001$), compared to that of the NY99 virus. Virus was isolated from the brains of the small subset of crows that died of infection with the KEN strain (data not shown). In addition to the three deaths from the KEN WNV genotype, an additional two crows showed signs of illness, yet survived through 14 dpi (Table 2). No illness or death was identified within the KUN infection group, yielding a significant difference from the NY99 infection group ($p < 0.001$), but the KUN group was not statistically differentiated from the KEN WNV infection groups ($p = 0.53$).

None of the eight crows previously challenged with KUN virus had detectable illness after secondary challenge with 3.2 $\log_{10}$ PFU of NY99 virus (Figure 3), which clearly indicates a cross-protective immune response against NY99; the lower 95% confidence limit on cross-protection probability was 0.63. In fact, viremia was not detected in any of the eight crows rechallenged with the

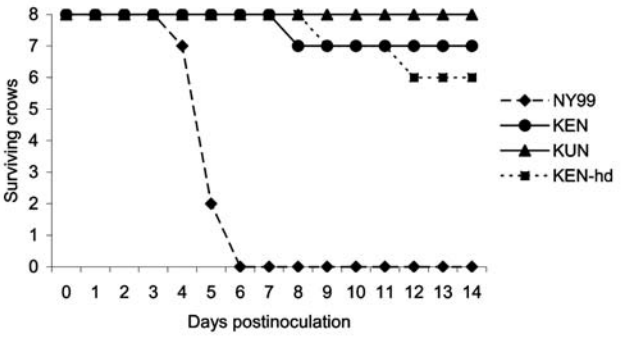

Figure 2. Survivorship of eight American Crows, each injected with 3.2 $\log_{10}$ PFU of NY99, KEN, or KUN virus. An additional eight crows were injected with a high dose (hd) of the KEN virus (3.8 $\log_{10}$ PFU). Crows were monitored daily for signs of disease through 14 dpi. No deaths were found within the control group (data not shown).

NY99 WNV on any of the 7 dpi (Figure 4). PRNTs demonstrated a homologous neutralization response in all eight of the crows for KUN virus (Table 3). Heterologous titers against NY99 virus were equal to or only twofold lower than those against KUN virus.

Only one of the seven crows from the lower dose (3.2 $\log_{10}$ PFU) KEN WNV inoculation group survived rechallenge with the NY99 strain (Figure 3). Sera drawn before the NY99 rechallenge from all crows within this group demonstrated that an immune response had developed in one crow (the single survivor). This crow demonstrated illness after the original KEN WNV challenge and was one of the two crows that had detectable viremia levels and subsequently exhibited a homologous protective antibody titer that was indistinguishable from its heterologous titer against the NY99 virus (1:640) (Table 4). The six KEN-infected survivors that did not become viremic from the original KEN viral challenge were devoid of detectable neutralizing antibody titers and had unmodified infections after the NY99 challenge. The viremia profile and clinical outcome (Figures 3 and 4) were indistinguishable from infection of naïve birds: five crows died on 5 dpi and an

Table 3. Cross-neutralization immune response of American Crows at 14 days postinfection with either KEN or KUN viruses

Sample no.	Inoculation	NY99	KEN	KUN	Difference
Crow 8	KEN	640[a]	**640**[b]	NT	0
Crow 1	KUN	80	NT[c]	**160**	2-fold
Crow 2	KUN	80	NT	**80**	0
Crow 3	KUN	160	NT	**320**	2-fold
Crow 4	KUN	40	NT	**80**	2-fold
Crow 5	KUN	40	NT	**40**	0
Crow 6	KUN	20	NT	**20**	0
Crow 7	KUN	80	NT	**80**	0
Crow 8	KUN	80	NT	**160**	2-fold

[a]Values represent the greatest reciprocal dilution in which ≥90% plaque inhibition was achieved as compared to sera-negative control cultures.
[b]Homologous titers are depicted in **bold** print.
[c]NT, not tested; KEN, West Nile virus strain from Kenya; KUN (Kunjin), West Nile virus strain from Australia.

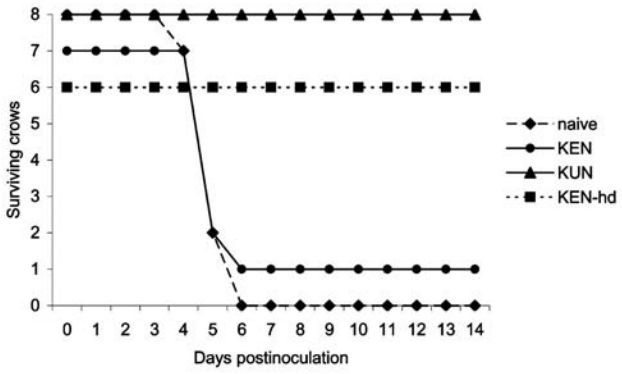

Figure 3. Survivorship of American Crows previously immunized with West Nile virus (WNV)-KUN or WNV-KEN viruses after injection with 1,500 PFU of NY99 WNV. hd, high dose.

additional crow died on 6 dpi. The single surviving crow that had demonstrated a 1:640 heterologous PRNT titer against NY99 WNV did not manifest a NY99 viremia level and had an unmodified 1:640 PRNT titer after the NY99 challenge. All crows from the group that received the higher dose of KEN generated KEN viremia titers and exhibited homologous PRNT titers (1:1,280–2,560) that were indistinguishable (less than fourfold difference) from heterologous titers against the NY99 virus. Neither clinical disease nor NY99 viremia levels were identified in these crows after secondary challenge with the NY99 virus, but neutralizing antibody titers increased up to 16-fold. The rise in PRNT titer was most likely the result of secondary infection or exposure; however, no control American Crows (to which a secondary challenge was not administered) were assayed for elevated PRNT titers at 24 dpi.

Sequence analyses of the coding differences between the NY99 and KEN viruses (Table 5) were performed on a NY99 virus (that had undergone an additional 2 Vero cell passages) to assess the possibility that limited cell-culture propagation could have resulted in attenuating genetic substitutions found between the KEN and NY99 genotype. These analyses did not demonstrate any genetic modification at any of the KEN or NY99 variable sites, further indicating that the genotype is stable for up to at least 3 passages and that the attenuated phenotype of the KEN or KUN viruses was unlikely to be the result of an additional tissue culture passage.

Discussion

Viremia levels observed in these studies confirm previous observations that American Crows have the potential to serve as amplification hosts for the NY99 genotype of WNV but suggest that corvids may not be important hosts for alternative WNV genotypes because of substantially reduced viremia titers that would not favor efficient virus transmission. Furthermore, these results demonstrate that viral-encoded determinants of avian pathology that are absent from KEN and KUN viruses exist in the NY99 virus. The viremia levels observed in crows inoculated with the KEN or KUN viruses were significantly lower than and delayed in their onset compared to those seen after inoculation with the NY99 strain. These data demonstrate that the differential pathogenic phenotypes of the WNV strains are the result of viral genetic differences that encode particular virulence determinants. Despite the finding that mouse virulence of the NY99 and KUN WNV strains (18) correlates well with the virulence phenotype identified in crow experiments here, experimental infection of mice with the KEN WNV strain did not demonstrate an attenuated phenotype (D.W.C. Beasley and A.D.T. Barrett, pers. comm.). This observation indicates that differential pathogenic mechanisms could modulate virulence in disparate vertebrate hosts.

Elevated viremia level could be a predominant factor for severe clinical outcome. KUN and KEN WNV-infected crows in which clinical signs did not develop did not manifest peak viremia titers >6 $\log_{10}$ PFU/mL; however, peripheral titers exceeded this level for the three crows in which neurologic symptoms and death occurred. Additionally, viremia levels of all crows injected with NY99 surpassed this level, which suggests that once a peripheral circulatory threshold titer is achieved, virus is capable of accessing the nervous system through a nonspecific mechanism. Intracerebral injection of mice with WNV strains differing in neuroinvasive capacity has demonstrated uniform lethality, indicating that the ability to enter the nervous system and not neurovirulence, is instrumental for virulence of WNV strains (18,19). If this phenomenon is true for WNV strains in crows, then the

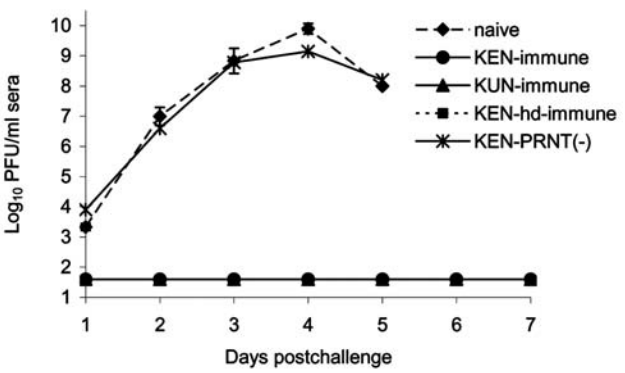

Figure 4. Viremia production of American Crows previously immunized with West Nile virus (WNV)-KUN or WNV-KEN viruses after injection with 3.2 $\log_{10}$ PFU of NY99 WNV. No detectable levels of viremia (≥1.7 $\log^{10}$ PFU/mL crow serum) developed in the KUN virus-immunized crows (0/8). Data points for the naïve (unexposed to WNV) crows challenged with the NY99 virus represent the mean of three samples chosen randomly. Bars represent standard deviations (SD) of the mean. hd, high dose; PRNT, plaque reduction neutralization assay.

Table 4. Cross-neutralization immune response of American Crows 24 days postinfection (dpi) with either KEN or KUN viruses[a]

Sample no.	Inoculation	NY99	KEN	KUN	Difference
Crow 8	KEN	640[b]	**640**[c]	NT	0
Crow 1	KUN	160	NT[d]	**320**	2-fold
Crow 2	KUN	320	NT	**320**	0
Crow 3	KUN	160	NT	**160**	0
Crow-4	KUN	160	NT	**320**	2-fold
Crow 5	KUN	160	NT	**160**	0
Crow 6	KUN	320	NT	**320**	0
Crow-7	KUN	640	NT	**640**	0
Crow 8	KUN	640	NT	**640**	0

[a]Following secondary NY99 challenge at 14 dpi.
[b]Values represent the greatest reciprocal dilution in which ≥90% plaque inhibition was achieved as compared to sera-negative control cultures.
[c]Homologous titers are depicted in **bold** print.
[d]NT, not tested; KEN, West Nile virus strain from Kenya; KUN (Kunjin), West Nile virus strain from Australia.

mechanism by which the crow-virulent genotype achieves extremely high peripheral titers must be elucidated. Viruses capable of replicating to higher titers could result from a unique access to cell types that facilitate high-titer replication through more efficient receptor-envelope interactions, viral replicase differences that increase replication efficiency within host cells, decreased sensitivity to host innate immunologic responses, or by altering the physiological host responses such as fever.

Immunologic status of a host can play an important role in limiting disease expression. WNV that are capable of inducing substantial levels of viremia and neuroinvasion of immunodeficient mice do not necessarily cause viremia or enter the neural tissues of mice with competent immune systems (19). Studies have demonstrated that previous infection with heterologous flaviviruses reduces the incidence of encephalitis and can provide protection from fatal WNV challenge in a hamster model for WNV pathogenesis (20,21). In contrast, a neutralization study performed with WNV strains of different lineages demonstrated that neutralizing antibodies against an Indian WNV strain provided poor protection against a South African WNV strain (22). Our results demonstrated that prior immunization with KUN virus can provide protection from lethal NY99 challenge in crows. Crows in which a detectable level of viremia did not develop from the initial KEN viral challenge exhibited viremia levels and death rates indistinguishable from NY99-infected naïve crows. Crows injected with the higher doses, which led to productive infections with the KEN virus, produced neutralizing-antibody titers that were protective against lethal NY99 challenge. The cross-neutralization of WNV strains suggests that areas in which WNV virus is endemic could be much less susceptible to invasion by the crow-virulent NY99 genotype.

Table 5. Amino acid differences between the NY99 and KEN West Nile virus strains[a,b]

Viral gene	Amino acid position	NY99	KEN
Capsid[a]	**3**	**Leu**	**Asn**
Capsid	**8**	**Val**	**Ala**
Envelope	**126**	**Ile**	**Thr**
Envelope	**159**	**Val**	**Ile**
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

[a]Source: (17).
[b]KEN, West Nile virus strain from Kenya; Leu, leucine; Val, valine; Ile, isoleucine; Ala, alanine; Thr, threonine; Pro, proline; Glu, glutamine; Asn, asparagine; Ser, serine; Asp, aspartic acid.
[c]Variable structural amino acid residues have been designated by **bold** text.

The effect that endemic flaviviruses such as SLEV has on the genetic stability of WNV in North America remains unclear; however, the fact that WNV and SLEV are distinguishable serologically through PRNT (23) and that WNV activity within the United States has occurred sympatrically within SLEV transmission foci (3) suggest that SLEV seroprevalence in birds has little impact on WNV transmission. Previous studies have demonstrated in a flaviviral pathogenesis hamster model that previous exposure to SLEV can significantly reduce WNV viral titers (21). Future experiments are warranted to determine if such protection is afforded in avian species.

Experimental inoculation with an Egyptian WNV strain has demonstrated deaths in sparrows and crows (24), providing evidence that bird deaths could result from natural infection with alternative WNV genotypes. Despite this fact, no bird deaths were reported during a well-described Egyptian epidemic involving the same viral strain used to experimentally inoculate these birds (10). Our results demonstrated that low numbers of deaths can occur from infection with alternative WNV strains, but the NY99 WNV genotype is significantly more virulent for American Crows. This result, coupled with the finding that similar pathogenicity was identified between the NY99 and KEN WNV in house sparrows (25), indicates the dual role of viral pathogenic phenotype and host susceptibility for the

expression of virulence in a particular bird species. Differential susceptibility of mouse strains for WNV infection has been identified and correlated with immunologic gene expression (26). Future experimental inoculation of Old World corvids with differential WNV genotypes would be useful to assess the role that host susceptibility has on the emergence of WNV genotypes in different geographic regions.

The mutations that encode the determinants for differential crow virulence are currently unknown. In crows inoculated with a recombinant virus containing WNV structural genes and nonstructural (NS) genes of yellow fever virus (YFV), viremia did not develop (27). The fact that the parental YFV-17D vaccine strain did not replicate to detectable levels in chickens (28) indicates that flaviviral NS gene regions could modulate viral replication in birds. Analysis of the complete genomes of the NY99 and KEN WNV has identified a maximum of 11 amino acids (Table 5) and 22 nucleotides from the 3'NCR that could mediate this phenotype (17). Seven (64%) of the 11 amino acid differences between these viruses resided with the NS gene region. The close genetic identity between the KEN and NY99 WNV genotypes makes this an optimal system for the systematic identification of genetic elements that encode viral pathogenic determinants. Studies are under way to identify the specific viral genetic determinants of crow virulence through the use of infectious cDNAs generated from both the NY99 and KEN WNV genotypes.

Acknowledgments

We thank Robert B. Tesh and David Beasley for providing the low-passage Kunjin isolate used in this study, Max Tehee and Paul Gordy for technical assistance, Charles Cope and Tom Janousek for invaluable assistance with crow trapping, and Ann M. Powers for critical review of the manuscript.

A.C.B. was supported by the American Society for Microbiology as a National Center for Infectious Diseases postdoctoral fellow. Trapping of American Crows was performed under U.S. Fish and Wildlife Scientific Collecting Permit number MB-032526. Experimental inoculations of crows were performed under Centers for Disease Control and Prevention IACUC protocol numbers 02-26-012-MSA and 03-13-013-MSA.

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Cats as a Risk for Transmission of Antimicrobial Drug–resistant *Salmonella*

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To determine whether cats were a risk for transmission of *Salmonella* to humans, we evaluated the excretion of *Salmonella* by pet cats. Rectal-swab specimens were taken from 278 healthy house cats, from 58 cats that died of disease, and from 35 group-housed cats. Group-housed cats were kept in one room with three cat trays and a common water and feed tray. Eighteen (51.4%) of 35 group-housed cats, 5 (8.6%) of 58 diseased cats (5/58), and 1 (0.36%) of 278 healthy house cats excreted *Salmonella*. *Salmonella* isolates were of serotypes Typhimurium, Enteritidis, Bovismorbificans and 4:i:-. Acquired antimicrobial resistance was found in serotype Typhimurium (resistance to ampicillin, chloramphenicol, and tetracycline; to ampicillin; and to chloramphenicol) and 4:i:- strains (resistance to ampicillin, chloramphenicol, sulfonamides, trimethoprim, and sulfamethoxazole/trimethoprim). Cats that excrete *Salmonella* can pose a public health hazard to people who are highly susceptible to *Salmonella*, such as children, the elderly, and immunocompromised persons.

Salmonella infections are still a leading cause of human foodborne infections in the world (1,2). These infections primarily originate from eating contaminated food, especially chicken eggs and egg products, and also meat products from pigs and chickens (3,4). Considering the high frequency of food contamination and the emergence of multidrug–resistant *Salmonella* strains, control of *Salmonella* in food-producing animals has become a worldwide challenge. Other environmental sources can lead to accidental human infections with *Salmonella* as well. The role of pet animals as a source of *Salmonella* has not been fully investigated, but severe human infections originating from reptiles, especially pet turtles, have been reported (5).

Cats and dogs are the most widely kept pet animals, yet the incidence of *Salmonella* in these animals is largely unknown, and the risk that these animals pose for transmission of *Salmonella* to humans is unclear. In particular, cats that can freely roam outside, and are therefore able to scavenge or hunt food of unknown quality, are potential candidates for *Salmonella* carriage. Most reports concerning *Salmonella* and cats are case studies of clinical salmonellosis, which resulted in septicemia and death (6,7). Subclinical infections and carrier animals, however, are much more important with respect to transmission to humans. In this study, rectal swabs from cats of different origin (house cats, group-housed cats, diseased cats) were cultured for *Salmonella*. The serotype and phage type of the *Salmonella* isolates were determined, and the isolates were characterized with respect to their antimicrobial drug–resistance pattern and interaction with human intestinal epithelial cells.

Methods

Collection of Fecal Samples

A total of 278 rectal swab samples from house cats of different age, sex, and breed were taken between July and November 2003. All house cats came from different owners. The animals came from all over the Dutch-speaking part of Belgium, i.e., north of Brussels. Rectal swab specimens were also taken from 58 cats that were submitted for autopsy to the Faculty of Veterinary Medicine, Ghent University. The latter died or were euthanized because of incurable disease. All cats came from different owners, except three cats that had feline immunodeficiency virus (FIV), which came from one owner. Finally, rectal samples of 35 kittens (all <4 months of age) were taken at a facility

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where the animals were group-housed, waiting to be adopted. These animals came from 16 different owners.

Bacteriologic Analysis

Bacteriologic analysis was performed by enrichment of the rectal swabs. The samples were first pre-enriched in buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK) overnight at 37°C, after which 1 mL of this suspension was added to 9 mL of tetrathionate brilliant green broth (Oxoid) (enrichment). After incubation overnight at 37°C, a drop of this suspension was spread on brilliant green agar (BGA) (Oxoid). Both the serotype and phage type of positive isolates were determined.

Antimicrobial Susceptibility Testing

Resistance to antimicrobial agents was tested by using the disk diffusion assay on Mueller-Hinton agar with commercial antimicrobial susceptibility disks (Oxoid) according to the international standards of the National Council for Clinical Laboratory Standards (NCCLS) (8). The following antimicrobial agents were tested: ampicillin (A, 10 μg), chloramphenicol (C, 30 μg), streptomycin (S, 10 μg), sulfonamide (Su, 300 μg), tetracycline (T, 30 μg), ciprofloxacin (Cip, 5 μg), kanamycin (K, 30 μg), gentamicin (Gn, 10 μg), sulfamethoxazole-trimethoprim (Sxt, 25 μg), cefotaxime (Cxt, 30 μg), nalidixic acid (Na, 30 μg), and amoxicillin-clavulanic acid (Amc, 30 μg). *Salmonella enterica* serovar Typhimurium 8420 (resistance type ACSSuT), 6237 (sensitive), 3520 (resistance type T), 2200 (resistance type ASSuT), and 5833 (sensitive) isolates from human patients in Belgium were used as control strains in antimicrobial susceptibility testing.

Polymerase Chain Reaction (PCR)

For PCR, a loop of bacterial culture was resuspended in 50 μL of water, and DNA was released from bacterial cells by boiling for 20 min. After the mixture was spun for 1 min in a microfuge at 14,000 x *g*, 2 μL of the supernatant was taken as a template DNA for PCR. PCR was carried out in 20-μL volumes by using PCR Master Mix from Qiagen (Hilden, Germany), according to the manufacturer's instructions. All the resistant strains were tested for the presence of the genes typical for particular resistance. The genes determined and primers used are listed in Table 1. Cycling consisted of 50-s incubations at 92°C, 55°C, and 72ºC, which were repeated 25 times. After PCR, amplification products were detected by electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Antimicrobial drug–sensitive strain *S.* Typhimurium F98 was used as a negative control in all the amplifications. *S.* Typhimurium strains 8420, 6237, 3520, 2200, and 5833 were used as positive controls.

All *Salmonella* strains were tested for the presence of the *SopB* gene. The primers were GATAGGAAAGATTGAGCACCTCTG and TACAGAGCTTCTATCACTCAGCTTC, and the PCR cycle consisted of 30 cycles of (30 s 95°C, 1 min 58°C, 1 min 72°C).

Pulsed-Field Gel Electrophoresis (PFGE)

The bacteria were grown while being shaken overnight at 37°C in Luria-Bertani broth (LB). The *Xba*I PFGE patterns were determined for all 21 *S.* Typhimurium strains by using previously described PFGE methods (16,17) with some slight modifications. The patterns were grouped in a dendrogram with GelCompar II software (Applied Maths, St.-Martens-Latem, Belgium) by using the Dice coefficient and the unweighted pair group method with an arithmetic averages clustering algorithm.

Invasion of the Human intestinal Epithelial Cell Line T84

The capacity of all cat *Salmonella* isolates and the human *S.* Typhimurium isolates 8420, 6237, 3520, 2200, and 5833 to invade human intestinal epithelial cells was determined. Cells of the human colon carcinoma cell line T84 were seeded in 96-well cell culture plates (Greiner, Frickenhausen, Germany) at a density of 5.10^5 cells/mL culture medium (DMEM + 10% fetal calf serum + 2% L-glutamine, without antimicrobial drugs) and grown for 24 h. Bacteria were grown for 20 h in LB-medium, after which the suspension was diluted 1:50 in fresh LB-medium. After 4 h of incubation at 37°C, suspensions were centrifuged and resuspended in DMEM with 10% fetal calf serum (FCS). The number of colony-forming units (CFU)/mL was determined by plating 10-fold dilutions on BGA. The suspensions were stored overnight at 4°C. The next day, 10^6 CFU in 200 μL were added to the T84 cell cultures, which were then centrifuged for 10 min at 1,500 rpm to make close contact between the bacteria and the colon cells. The plates were incubated for 1 h at 37°C and 5% CO_2. The cells were then rinsed three times with Hanks' Balanced Salt Solution (HBSS, Life Technologies, Paisley, Scotland). Cell culture medium with gentamicin (50 μg/mL) was added, and plates were incubated for 1 h at 37°C and 5% CO_2. Hereafter, the cells were rinsed three times with PBS and analyzed with 1% Triton X-100 (Sigma, St. Louis, MO) in distilled water. From this lysate, 10-fold dilution series were made. From each dilution, 6 x 20 μL was added to BGA, to determine the number of CFU *Salmonella* per mL. The assays were performed in triplicate. The percentage of intracellular bacteria, relative to the number of *Salmonella* bacteria, initially incubated with the cells, was calculated. The previously mentioned human isolates of *S.* Typhimurium were used for comparison

Table 1. List of primers used in the PCR reactions for detection of resistance genes

Resistance	Gene	Primer	Sequence (5′– 3′)	Size (bp)	Reference
Ampicillin	*blaPSE1*	PSEF	TAG CCA TAT TAT GGA GCC TC	321	AF261825
		PSER	TTA ACT TTT CCT TGC TCA GC		
	bla^{TEM}	TEMF	GCA CGA GTG GGT TAC ATC GA	310	9
		TEMR	GGT CCT CCG ATC GTT GTC AG		
	blaoxa1	oxa1F	AGC AGC GCC AGT GCA TCA	708	10
		oxa1R	ATT CGA CCC CAA GTT TCC		
Chloramphenicol	*floR*	floRF	GCG ATA TTC ATT ACT TTG GC	425	11
		floRR	TAG GAT GAA GGT GAG GAA TG		
	Cat	catF	CCT GCC ACT CAT CGC AGT	623	10
		catR	CCA CCG TTG ATA TAT CCC		
Streptomycin	*aadA1*	aad1For	CGA CTC AAC TAT CAG AGG TA	384	AY534545
		aad1Rev	CTT TTG TCA GCA AGA TAG CC		
	aadA2	aadA2F	CGG TGA CCA TCG AAA TTT CG	249	12
		aadA2R	CTA TAG CGC GGA GCG TCT CGC		
	strA	strAF	CCT ATC GGT TGA TCA ATG TC	250	11
		strAR	GAA GAG TTT TAG GGT CCA CC		
Tetracycline	*tetA*	tetAF	GCT ACA TCC TGC TTG CCT TC	210	13
		tetAR	CAT AGA TCG CCG TGA AGA GG		
	tetB	tetBF	TTG GTT AGG GGC AAG TTT TG	659	13
		tetBR	GTA ATG GGC CAA TAA CAC CG		
	tetC	tetCF	GCG GGA TAT CGT CCA TTC CG	207	14
		tetCR	GCG TAG AGG ATC CAC AGG ACG		
	tetG	tetGF	GCT CGG TGG TAT CTC TGC TC	468	13
		tetGR	AGC AAC AGA ATC GGG AAC AC		
Sulfonamide	*sul1*	sul1F	ATG GTG ACG GTG TTC GGC ATT CTG	841	15
		sul1R	GCT AGG CAT GAT CTA ACC CTC GG		
	sul2	sul2F	AGG GGG CAG ATG TGA TCG AC	249	11
		sul2R	GCA GAT GAT TTC GCC AAT TG		
Trimethoprim	*dfrA1*	dfrA1F	GTG AAA CTA TCA CTA ATG G	470	10
		dfrA1R	CCC TTT TGC CAG ATT TGG		
	dfrA10	dfrA10F	TTA ATT ACC AGA GCA TTC GG	374	AY049746
		dfrA10R	TAC ACA TCA GCA TGA ACA GG		
	dfrA12	dfrA12F	ACT CGG AAT CAG TAC GCA	463	10
		dfrA12R	GTG TAC GGA ATT ACA GCT		
Kanamycin	*aadD*	aadD¬F	ATA TTG GAT AAA TAT GGG GAT	161	12
		aadD¬R	TCC ACC TTC CAC TCA CCG GTT		
	aphA/aph(3′)¬Id	aphAaphIdF	ATG GGC GCC TAT CAC AAT TGG	257	12
		aphAaphIdR	TCG CCT CCA GCT CTT CGT AGA		
	aphAI¬IAB	aphAI¬IABF	AAA CGT CTT GCT CGA GGC	461	12
		aphAI¬IABR	CAA ACC GTT ATT CAT TCG TGA		
	aph(3′)¬IIa	KanAphF	GAG AAA GTA TCC ATC ATG GC	465	L19385
		KanAphR	GCT CAG AAG AAC TCG TCA AG		

between the cat isolates and human isolates. Statistical analysis was performed by analysis of variance methods using the SPSS 11.0 software.

Results

Characterization of *Salmonella* Isolates from Cats

Of 278 healthy house cats, 1 *Salmonella* strain was isolated, an *S.* Enteritidis phage type 21 strain, sensitive to all tested antimicrobial drugs. Five strains were isolated from cats that died from or were euthanized because of incurable disease. Feline AIDS (caused by feline immunodeficiency virus [FIV]) was diagnosed in three cats, one died due to feline panleukopenia parvovirus infection, and one was poisoned. Three isolates were identified as being ampicillin-resistant *S.* Typhimurium phage type 193, harboring the bla_{TEM} gene. They had the same pulsed-field gel electrophoresis (XbaI) pattern, indicating that the isolates were of clonal origin (Figure 1). The three cats came from the same owner. One isolate was an antimicrobial drug–sensitive *Salmonella* Bovismorbificans strain. One isolate was *Salmonella* 4:i:-, which was resistant to ampicillin, chloramphenicol, sulfonamides, tetracycline and sulfamethoxazole-trimethoprim (ACSuTSxt), harboring the bla_{TEM}, *cat*, *sul2*, *tet(A)*, and *dfrA1* antimicrobial drug–resistance genes. Eighteen strains were isolated from

Table 2. Characteristics of *Salmonella* isolates from cats[a]

Isolate no.	Source	Serotype	Phage type	PFGE pattern[b]	Resistance phenotype[b]	Resistance genotype
11	Diseased house cat	4:i:-	–	ND	ACSuTSxt	bla_{TEM}, *cat*, *sul2*, *tet(A)*, *dfrA1*
40	Diseased house cat	Bovismorbificans	–	ND	–	-
109	House cat	Enteritidis	21	ND	–	-
1145, 1147, 55	Diseased house cats	Typhimurium	193	II	A	bla_{TEM}
89, 165, 174, 198, 320, 326, 352, 355, 358, 359, 369, 380, 390, 392	Group-housed cats	Typhimurium	120/ad	Ia	ACT	bla_{TEM}, *cat*, *tet(A)*
161, 350	Group-housed cats	Typhimurium	120/ad	Ib	C	*cat*
220, 339	Group-housed cats	Typhimurium	120/ad	Ic	C	*cat*

[a]A, ampicillin; C, chloramphenicol; Su, sulfonamides, T, tetracycline; Sxt, sulfamethoxazole-trimethoprim; ND, not determined; PFGE, pulsed-field gel electrophoresis.
[b]Roman numerals indicate the major types of fragment patterns; lowercase letters indicate minor variations in the respective fragment pattern.

the group-housed cats. All of these were *S.* Typhimurium phage type 120/ad. Fourteen of these strains showed acquired resistance to ampicillin, chloramphenicol and tetracycline and harbored the bla_{TEM}, *cat*, and *tet(A)* antimicrobial drug–resistance genes, while four isolates were resistant to chloramphenicol only and only harbored the *cat* gene (Table 2). Pulsed-field gel electrophoresis showed that the isolates from the group-housed cats were of the same *XbaI* PFGE type, and that three subtypes within this type were present, indicating a clonal origin (Figure 1). One subtype contained the 14 strains that were resistant to the three mentioned antimicrobial drugs. All *Salmonella* strains harbored the *SopB* gene.

Invasion of the Human Intestinal Epithelial Cell Line T84

All isolates invaded T84 cells, with the cat isolates of *S.* Typhimurium PT193 (strains 1147, 1145, and 55, which belong to the same clone) and the human isolate *S.* Typhimurium strain 2200, the most invasive, yielding a percentage of invasion of 8% to 10%. The multidrug–resistant cat isolate *Salmonella* 4:i:- (strain 11) was the least invasive strain, having an invasion percentage of about 0.5%. Invasion percentages of the different isolates are shown in Figure 2. Of the strains of the same PFGE type, only one was shown in Figure 2, since no significant differences were detected between the invasion percentages of these strains. Statistically significant differences are shown in the figure.

Discussion

This study concluded that, although cats can transmit *Salmonella* strains, healthy house cats are generally safe. Earlier reports regarding isolation of pathogens from healthy cats showed low percentages (mostly around 1%) of *Salmonella*-positive rectal swabs (18,19). In our study, 1 of 278 healthy cats was found to be positive. Immunodeficiency and nonhygienic housing can be predisposing factors for cats to shed *Salmonella* in the feces, resulting in contamination of the environment. Rectal swabs from 18 of 35 group-housed kittens were *Salmonella*-positive in our study. The fact that the 35 kittens were derived from more than 10 different owners before being group-housed and that one PFGE type (three subtypes) of *S.* Typhimurium 120/ad was isolated, indicates spread of the *Salmonella* strain between the cats or a common source. The age of these animals may also play a role, since all animals in this group were <4 months. Young animals are more susceptible to *Salmonella* infection. Also immunodeficiency can result in *Salmonella* excretion. One outbreak of fatal salmonellosis in cats has been reported after mild immunosuppression induced by live panleukopenia virus vaccination (7). In our study, animals infected with FIV and one animal that had panleukopenia shed *Salmonella*. Three animals that were infected with FIV were derived from the same owner, which indicates that the animals were infected with *Salmonella* from the same source or that one animal contaminated the others.

In our study, serotypes Typhimurium, Enteritidis, Bovismorbificans, and 4:i:- were isolated from cats. The

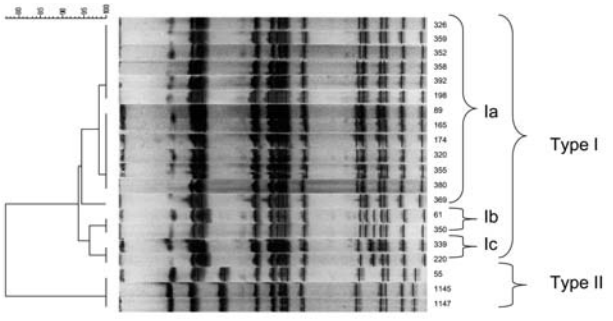

Figure 1. Dendrogram obtained by unweighted pair group method using arithmetic averages clustering of the pulsed-field gel electrophoresis-*XbaI* patterns of serovar Typhimurium strains isolated from cats on the basis of the Dice coefficient.

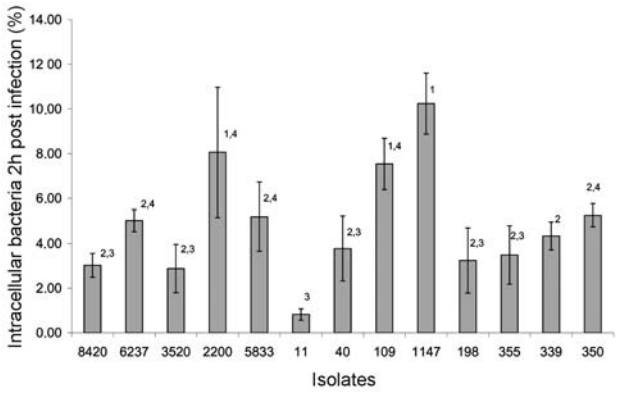

Figure 2. Invasion of *Salmonella* strains in the human intestinal epithelial cell line T84. The y-axis shows the percentages of intracellular bacteria 2 hours postinfection, relative to the initial number of bacteria, incubated with the cells. The x-axis shows isolate numbers. All isolates derived from the group-housed cats had the same invasion percentage as strains 198 and 355 (data not shown). Isolates 55 and 1145 had the same invasion percentage as strain 1147 (data not shown). Data not sharing superscript numbers indicate statistically significant differences ($p < 0.05$).

isolated serotypes indicate that the cats were infected from the same sources compared with other animals and man. Indeed, serotypes Typhimurium and Enteritidis are the most widespread serotypes and the serotype Bovismorbificans is not uncommon in other animals, including humans (2,20).

Generally, invasion in the human intestinal epithelial cell line T84 was comparable between the cat isolates and isolates from humans. Invasion in intestinal epithelial cells is the primary step in the pathogenesis of *Salmonella* that causes gastrointestinal problems (21). This finding implies that the cat isolates are potentially pathogenic for humans. Moreover, all cat isolates harbored the *SopB* gene, which is involved in blocking the closure of chloride channels in gut epithelium and thus in inducing diarrhea. As in most other animal species, the cat isolates of the serotype Typhimurium harbored antimicrobial drug–resistant genes, raising concerns about spreading antimicrobial drug–resistant strains to humans.

Since the 1990s, concerns have arisen about the emergence and spread of multidrug–resistant Typhimurium strains, especially the multidrug–resistant ACSSuT type, which is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (2). In our study, some *S.* Typhimurium isolates from cats were resistant to a single drug such as ampicillin or chloramphenicol, while most isolates from the group-housed cats (same clone) were resistant to ampicillin, chloramphenicol, and tetracycline. Resistance genes were found to be bla_{TEM} (ampicillin), *cat* (chloramphenicol), and *tet(A)* (tetracycline). The genes in the class 1 integron of the multidrug–resistant genomic island in ACSSuT type *S.* Typhimurium, required for the resistances to the above three mentioned antimicrobial drugs, are bla_{PSE1}, *floR*, and *tet(G)* (22). This illustrates that these isolates did not acquire their resistance genes from horizontal transfer from pentadrug–resistant ACSSuT type strains. The isolate *Salmonella* 4:i:- was resistant to ampicillin, chloramphenicol, sulfonamides, tetracycline, and sulfamethoxazole/trimethoprim (ACSuTSxt-type), encoded by bla_{TEM} (ampicillin), *cat* (chloramphenicol), *sul2* (sulfonamides), *tet(A)* (tetracycline), and *dfrA1* (trimethoprim). Also the resistance shown by this example had no relationship to the typical *S.* Typhimurium DT104 multidrug–resistant genomic island.

In conclusion, healthy house cats are generally safe with regard to excretion of *Salmonella* in the environment. Cats that are sick or are receiving medication resulting in immune deficiencies can potentially pose a threat to public health. Young children, the elderly, and immunocompromised persons are at risk because of their high sensitivity for the infection. All persons should follow good hygiene practices when keeping cats as pets.

Acknowledgments

We thank V. Eeckhaut, M. Foubert, and L. Winters for their excellent technical assistance.

Ivan Rychlik was supported by grant 1B44019 from the Ministry of Agriculture of the Czech Republic.

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VecTest as Diagnostic and Surveillance Tool for West Nile Virus in Dead Birds

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The VecTest antigen-capture assay for West Nile virus was performed on oral and tissue swabs from dead birds in New York State from April 2003 through July 2004. Results were compared with those from real-time reverse transcriptase–polymerase chain reaction of kidney or brain. Oral VecTest sensitivity is adequate for surveillance in American Crows (*Corvus brachyrhynchos*) (87%), Blue Jays (*Cyanocitta cristata*) (80%), and House Sparrows (*Passer domesticus*) (76%). Oral VecTest performed well for small samples of American Kestrels (*Falco sparverius*), Northern Cardinals (*Cardinalis cardinalis*), Common Grackles (*Quiscalus quiscula*), and House Finches (*Carpodacus mexicanus*). Poor sensitivity occurred in most raptors, Mourning Doves (*Zenaida macroura*), Fish Crows (*Corvus ossifragus*), and American Robins (*Turdus migratorius*). Specificity was excellent (98%), except for false-positive results that occurred mostly in Gray Catbirds (*Dumatella carolinensis*), Green Herons (*Butorides virescens*), and tests of blood and tissues. Feather pulp and kidney may be useful for VecTest assays in corvids.

After West Nile virus (WNV) was discovered in birds, horses, and humans in New York State in 1999 (1), the New York State Department of Health established a surveillance system to follow seasonal and geographic trends in WNV activity (2). An important part of this system is testing dead birds for WNV. Detection in bird species is used as an early warning system to measure the threat of the virus to humans and as a threshold indicator for mosquito control programs. Surveillance is also used to assess the impact of WNV on avian populations and to document geographic, seasonal, and annual trends.

In the dead bird testing program, birds are reported by the public and submitted (largely through county health departments) to the Wildlife Pathology Unit of the New York State Department of Environmental Conservation. The Wildlife Pathology Unit necropsies priority birds and collects tissues, which are then sent to the health department's Arbovirus Laboratory, where they are tested for WNV RNA by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) (3). Since WNV was detected in New York in 1999, the Wildlife Pathology Unit and the Arbovirus Laboratory have processed >19,000 specimens as part of the surveillance program. The elapsed time between the initial reporting of a dead bird and posting of the RT-PCR results on the surveillance system's Health Information Network, an Internet-based data and information tracking system (4), can be as long as 3 weeks. Along with faster processing of specimens, a simpler alternative to the RT-PCR test was desired to quicken the actual WNV testing and reporting and to reduce personnel time and expense.

In 2000, a rapid antigen-capture wicking assay in a dipstick format (VecTest, Medical Analysis Systems, Camarillo, CA) was introduced for detecting WNV in mosquito pools (5,6). The VecTest requires a minimal amount of supplies and equipment and is easy to use; its results are available 15 minutes after the dipstick is placed in the sample solution. After high WNV titers were demonstrated in the oral and cloacal cavities of corvids (7), the VecTest was evaluated relative to RT-PCR in saliva, feces, and tissue samples from a small sample of American Crows (*Corvus brachyrhynchos*) in Illinois (8), and oral and cloacal swabs from corvids in a larger study in Canada (9). The latter study reported a sensitivity of approximately 83% for American Crows.

The objective of this study was to determine whether the VecTest antigen assay would be useful for WNV surveillance in New York State. We compared the results of oral VecTests with RT-PCR of tissue for a large number of

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birds of various species. In addition, we explored use of the VecTest with swabs from the cloaca, feather pulp, and internal tissues to determine whether use of other antigen sources might improve the sensitivity of the test.

Methods

Birds included in this study were those found dead in New York State, from April 2003 to July 2004, and received at the Wildlife Pathology Unit for WNV testing from county health departments, veterinarians, wildlife rehabilitators, other organizations and agencies, and the general public. Some specimens were frozen before delivery to the Wildlife Pathology Unit. Although locally submitted birds were often examined and necropsied on the day of receipt, most specimens were not processed until the following day, or later. Carcasses were held at 4°C until necropsied. The selection of specimens for testing was not usually related to postmortem condition. The highest priority was placed on corvids, raptors, and House Sparrows (*Passer domesticus*), while low priority was given to Rock Doves (*Columba livia*), European Starlings (*Sternus vulgaris*), and Common Grackles (*Quiscalus quiscula*). At times, high priority was placed on birds from specific geographic areas for which local health departments had requested immediate testing.

At necropsy, oral swab samples were collected with sterile, polyester fiber-tipped plastic applicators by moving the tip vigorously around the entire oropharyngeal cavity and, by July 2003, the proximal esophagus. The swab was then twirled for 3 to 5 s in 1.0 mL of the VecTest buffer solution (provided with the VecTest kit) in 10-mL plastic tubes, pressing the tip against the side of the tube. The swab was then discarded.

For a number of different bird species, swabs were taken from kidney, liver, heart blood, cloaca, brain, and feather pulp, in addition to the oropharyngeal cavity. Swabs from kidney and liver were obtained by sticking the applicator tip into the parenchyma and rotating the tip to ensure capture of tissue. Heart blood swabs were obtained by immersing the tip in blood contained within the atria or right ventricle. Cloacal swabs were obtained by moving the tip vigorously against the mucosal lining. Brain swabs were taken by running the tip through cerebral gray and white matter. Feather pulp was taken by pulling a blood feather from the wing or tail and then expressing the pulp onto a swab. All swab samples were then mixed into separate VecTest buffer solutions as described above.

In a class II biosafety cabinet at the Wildlife Pathology Unit, 0.25 mL of each swab solution was transferred to a conical microfuge tube (supplied with the kit), the VecTest strip was inserted into the tube, and results were read in the fluorescent light of the safety cabinet 15–30 min later. According to manufacturer's instructions, the development of a reddish purple line, in both the test and control zones of the VecTest strip, was recorded as a positive result. Any test strip that did not develop a control line was discarded; then, 0.25 mL of the original solution was pipetted into another microfuge tube, and a new strip was used to test the solution. Observations on the intensity of positive results, as well as descriptions of any unusual features, were also recorded.

Swab samples in VecTest buffer solution that were not tested on the day of collection were refrigerated overnight at 4°C. If a delay of >24 h occurred before testing, solutions were frozen at –20°C. To determine the effects of refrigeration on the buffer solution, a limited number of swab samples were held at 4°C for intervals of 3 to 7 days. A similar study was conducted by freezing swab solutions at –20°C for 2 days to 7 months to determine if freezing would affect VecTest results. Most VecTest strips used throughout the study contained a single antigen-binding site specific for WNV; however, 500 test strips used during June and July 2003, and in July 2004, included additional test zones for eastern equine encephalitis and St. Louis encephalitis.

Tissues for RT-PCR testing were obtained during necropsy with single-use sterile disposable scalpels and stainless steel forceps and scissors. After use, forceps and scissors were placed in Promicidal disinfectant (Chemsearch Industries, Irving, TX) for later cleaning and steam autoclaving at 120°C for 20 min at 1.0 kg/cm^2. Harvested tissues were immediately placed in individual 1-oz plastic jars and capped. Jar lids were labeled with the individual specimen number, species name, and tissue type, and the jar sides with the specimen number. The plastic jars containing the tissues were then frozen at –80°C before transport to the state Arbovirus Laboratory for RT-PCR testing.

At the Arbovirus Laboratory, RNA was extracted from kidney or brain tissue by ABI Prism 6700 robotic workstation (Applied Biosystems, Foster City, CA) or RNeasy (Qiagen, Inc., Valenci, CA) and assayed for WNV by real-time RT-PCR using ABI Prism 7700 or 7000 sequence detectors, as described previously (3,10). Briefly, each sample was tested with two sets of primer probes, targeting the envelope or NS1 region of the WNV RNA. Controls consisted of a set of WNV RNA standards that ranged from 0.08 to 90 PFU per sample, and WNV-positive bird tissue that was prepared and RNA-extracted with the assay. The sensitivity of the real-time RT-PCR assay is 0.08 PFU or 40 copies of RNA. A sample was declared positive only if WNV was detected with both primer-probe sets. Differences in VecTest performance in data subsets of interest were assessed by chi-square analysis. Data are expressed as a percentage in the text and tables only where $n \geq 10$.

Results

Results from VecTests of oral swabs and RT-PCR of kidney or brain from 2,913 birds (116 species, 16 orders) were compared (Table 1); of these, 1,013 (35%) were positive for WNV by RT-PCR. The sensitivity of the oral VecTest in RT-PCR–positive birds was 87% in American Crows, 80% in Blue Jays (*Cyanocitta cristata*), and 76% in House Sparrows. WNV was detected by RT-PCR in small numbers (n = 1–16) of 29 additional species, and confirmed by oral VecTest in 11 of these species. In those 11 species, despite small sample sizes, results suggested some species-specific variability in sensitivity. The test detected

Table 1. Comparison of oral VecTest and RT-PCR results[a,b] for West Nile virus in dead birds, New York State, April 2003– July 2004

	No. of birds			
			VecTest results[c]	
Species (listed by order)	No. birds tested	RT-PCR–positive	True positive (%)[d]	False-positive (%)[e]
Ciconiiformes				
Great Blue Heron (*Ardea herodias*)	8	1	0	0
Anseriformes				
Mallard (*Anas platyrhynchos*)	7	1	0	0
Falconiformes				
Bald Eagle (*Haliaeetus leucocephalus*)	6	1	0	0
Sharp-shinned Hawk (*Accipiter striatus*)	14	3	1	0
Cooper's Hawk (*Accipiter cooperii*)	33	3	0	0
Northern Goshawk (*Accipiter gentilis*)	3	1	0	0
Broad-winged Hawk (*Buteo platypterus*)	1	1	1	NA
Red-tailed Hawk (*Buteo jamaicensis*)	36	11	0 (0)	0
American Kestrel (*Falco sparverius*)	12	4	3	0
Merlin (*Falco columbarius*)	4	1	0	0
Peregrine Falcon (*Falco peregrinus*)	7	1	0	0
Galliformes				
Impeyan Pheasant (*Lophophorus impeyanus*)	1	1	0	0
Charadriiformes				
Herring Gull (*Larus argentatus*)	3	1	0	0
Great Black-backed Gull (*Larus marinus*)	3	2	1	0
Columbiformes				
Mourning Dove (*Zenaida macroura*)	75	6	0	0
Strigiformes				
Great Horned Owl (*Bubo virginianus*)	25	12	0 (0)	1
Passeriformes				
Blue Jay (*Cyanocitta cristata*)	339	166	133 (80)	1 (1)
American Crow (*Corvus brachyrhynchos*)	1,076	702	608 (87)	7 (2)
Fish Crow (*Corvus ossifragus*)	22	10	2 (20)	0
Common Raven (*Corvus corax*)	2	1	0	0
American Robin (*Turdus migratorius*)	233	16	3 (19)	1 (1)
Gray Catbird (*Dumatella carolinensis*)	113	2	0	13 (12)
Brown Thrasher (*Toxostoma rufum*)	2	1	0	0
European Starling (*Sturnus vulgaris*)	85	1	1	1 (1)
Scarlet Tanager (*Piranga olivacea*)	5	1	0	0
Northern Cardinal (*Cardinalis cardinalis*)	21	6	4	0
Common Grackle (*Quiscalus quiscula*)	189	6	3	1 (1)
Brown-headed Cowbird (*Molothrus ater*)	8	1	0	1
House Finch (*Carpodacus mexicanus*)	17	7	7	1
House Sparrow (*Passer domesticus*)	222	41	31 (76)	1 (1)
Gouldian Finch (*Chloebia gouldiae*)	1	1	0	0
Society Finch (*Lonchura domestica*)	1	1	1	NA
Other species[f]	339	0	NA	8[g] (2)
Total all species	2,913	1,013	799 (79)	36 (2)

[a]RT-PCR, reverse transcriptase-polymerase chain reaction; NA, not available.
[b]Principally tests of kidney (brain or heart alternate when kidney missing).
[c]Percentages are provided for species where n $\geq$ 10.
[d]No. and percentage of RT-PCR–positive birds that were VecTest-positive.
[e]No. and percentage of RT-PCR–negative birds that were VecTest-positive.
[f]84 species representing 16 orders.
[g]Mute Swan (*Cygnus olor*) (1), Green Heron (*Butorides virescens*) (6), Northern Mockingbird (*Mimus polyglottos*) (1).

WNV in ≥50% of RT-PCR–positive birds of the following species: American Kestrels (*Falco sparverius*) (3/4), Northern Cardinals (*Cardinalis cardinalis*) (4/6), Common Grackles (3/6), and House Finches (*Carpodacus mexicanus*) (7/7) but was unable to detect WNV in RT-PCR–positive Red-tailed Hawks (*Buteo jamaicensis*) (0/10) and Great Horned Owls (*Bubo virginianus*) (0/12). Poor VecTest sensitivity was also recorded in small numbers of Mourning Doves (*Zenaida macroura*) (0/6), American Robins (*Turdus migratorius*) (3/16), and Fish Crows (*Corvus ossifragus*) (2/10).

VecTest sensitivity did not appear to be seriously compromised by extensive postmortem deterioration or freezing of the carcasses. RT-PCR–positive American Crows (n = 124) and Blue Jays (n = 30) that showed moderate or more severe autolysis of tissues, including extensive maggot activity in many, were positive by VecTest in 89% and 87% of cases, respectively. VecTest sensitivity in birds that had been frozen was 80% (49/61) in American Crows and 79% (49/62) in Blue Jays. In addition, the freezing (–20°C) of 18 VecTest-positive oral swab sample solutions for 2 days to 7 months had no effect on results. All repeat tests were positive at what appeared to be the same intensity.

Nineteen oral swab samples in buffer solution from American Crows and Blue Jays were tested with VecTest strips, and then refrigerated at 4°C for 3 to 7 days. Samples were then retested with VecTest strips, and all pre-, and postrefrigeration results were the same. Seventeen of the samples tested were positive, and 2 were negative in both phases of testing.

False-positive results in oral VecTests were observed in 36 (2%) of 1,900 RT-PCR–negative birds and rarely occurred in species, with the exception of Gray Catbirds (*Dumatella carolinensis*) (12%, 13/111) and Green Herons (*Butorides virescens*) (75%, 6/8) (Table 1). Thus, the overall specificity (identifying an RT-PCR-negative as negative) of the VecTest was high (98%, 1,864/1,900), as were the VecTest–positive predictive value (96%, of 835 VecTest-positive birds, 799 were also RT-PCR-positive) and –negative predictive value (90%, of 2,078 VecTest-negative birds, 1,864 were also RT-PCR-negative).

Most (24/36) of the false-positive results, including all those involving Gray Catbirds and Green Herons, consisted of very narrow lines at the lower border of the test region, unlike the full-width colored bands described in the manufacturer's instructions as positive results, and recorded in oral tests of RT-PCR–positive birds (Figure). These lines, in contrast with the VecTest-positive results, which usually developed to their full extent within 10 min, often continued to intensify beyond 15 min (sometimes only noticeable after >15 min had elapsed). Narrow-line results were not identified in oral tests of RT-PCR–positive birds, but such results could have been merged with true-positive

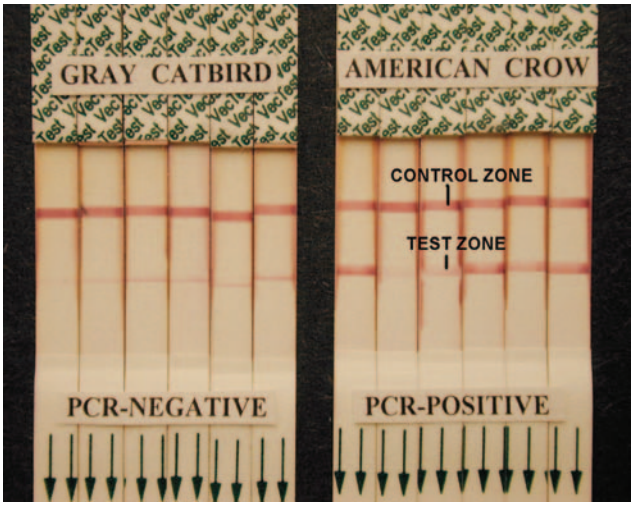

Figure. West Nile virus (WNV) VecTest results from oral swabs of Gray Catbirds showing narrow-line false-positive results compared with typical true-positive VecTest results from reverse transcriptase-polymerase chain reaction-positive American Crows. Note the near exclusive deposition of pigment at the lower margin of the test zone on the dipsticks of catbirds, and the distribution of pigment across the full width of the test zone in the WNV-positive crows, even in very weak positive tests.

wide-band results in tests where wide-band results developed. At least four of the other false-positive results were faint positive reactions in multiple test zones of the WNV/St. Louis encephalitis/eastern equine encephalitis version of the VecTest; two of the false-positive results appeared intermediate, between narrow-line and wide-band results. The other six false-positives also were obtained with the WNV/St. Louis encephalitis/eastern equine encephalitis test. No distinct wide-band–positive results were obtained in RT-PCR–negative birds.

VecTest results for swabs taken from the cloaca, heart blood, and kidney of RT-PCR–positive corvids and House Sparrows produced results generally similar to those obtained from oral swabs (Table 2). When oral tests were positive (n = 62), tests of the other three tissues were almost uniformly positive (cloacal [95%], heart blood [97%], kidney [98%]). When oral tests were negative (n = 22) in these RT-PCR–positive birds, however, positive VecTest results (often weak) were sometimes recorded in cloacal (27%), blood (14%), and kidney (55%) samples. VecTest of oral swabs, and swabs of alternate tissues, for 18 RT-PCR–positive raptors (Table 2) were negative, with the exception of one American Kestrel (oral) and one Great Horned Owl (kidney). Narrow-line results recorded in three heart blood samples and one kidney sample from these raptors were not included as positives in Table 2.

In RT-PCR–negative birds, VecTests of internal tissues (Table 3) produced narrow-line false-positive results (as described above and in the Figure) far more frequently

Table 2. Comparative sensitivity of VecTest with swabs from different sources in RT-PCR–positive birds[a,b]

Species	N	No. positive (% positive[c]) by VecTest			
		Oral	Cloacal	Heart blood	Kidney
Blue Jay	37	29 (78)	31 (84)	28 (76)	33 (89)
American Crow	36	24 (67)	25 (69)	25 (69)	30 (83)
House Sparrow	11	9 (82)	9 (82)	10 (91)	10 (91)
Raptors[d]	18[e]	1 (6)[f]	0	0	1 (6)[g]

[a]RT-PCR, reverse transcriptase–polymerase chain reaction.
[b]Four sources from each bird, with exceptions noted in footnote e.
[c]Wide band positive only (see text).
[d]Sharp-shinned Hawk (1), Cooper's Hawk (1), Red-tailed Hawk (7), American Kestrel (1), Merlin (1), Peregrine Falcon (1), Great Horned Owl (6).
[e]n = 14 for cloaca; n = 15 for heart blood.
[f]Positive VecTest in one American Kestrel.
[g]Positive VecTest in one Great Horned Owl.

than occurred in tests of oral swabs. These lines were most common in heart blood (30%, 62/208 RT-PCR-negative birds). As with their occurrence in oral testing, these lines were also far more prevalent in Gray Catbirds. Tests of cloacal swabs produced results similar to the oral testing (4% false-positives, 5/117; all were in catbirds).

VecTest of brain tissue in 49 oral-negative, RT-PCR–positive birds yielded eight wide-band–positive results (Table 4). Sensitivity in corvids (21%, 3/14) was similar to that in raptors (18%, 5/28). Five narrow-line results were also recorded in raptors (two Sharp-shinned Hawks (*Accipiter striatus*), three Red-tailed Hawks). VecTest results of brains from 17 RT-PCR–negative raptors (including eight Red-tailed Hawks) were negative.

VecTest of feather pulp for 43 RT-PCR–positive corvids (37 American Crows, 5 Blue Jays, 1 Fish Crow) identified WNV in 36 (84%) of the birds tested. VecTest of oral swabs identified WNV in 32 (74%) of the same birds. VecTest specificity for feather pulp was 99%; the test correctly identified 93 of 94 RT-PCR–negative corvids as negative.

Oral VecTest sensitivity in American Crows early in the WNV season (April – June) was poor 17% (1/6) in 2003 but was 82% (14/17) during the same period in 2004. No difference in sensitivity was found in tests of hatch year (86%, 162/188) and after hatch year (87%, 418/480) American Crows. Sensitivity of the oral VecTest in Blue Jays was somewhat higher in after hatch year birds (86%, 57/66) than hatch year birds (75%, 71/95), but the difference was not significant ($p < 0.10$).

The VecTest analyses showed low levels (2.6%; 26/1,013) of false-negatives in RT-PCR testing. Twenty-six birds with positive, broad-lined, VecTest results and initially negative RT-PCR results were positive on subsequent tests, which included RT-PCR of original or reextracted sample, indirect fluorescent-antibody assay of cell culture–isolated virus, or assay of an alternate tissue. This group consisted of 15 American Crows, 6 Blue Jays, 2 House Finches, 2 Northern Cardinals, and 1 House Sparrow.

Discussion and Conclusion

The sensitivity of oral VecTest reported here for American Crows and Blue Jays in New York State was similar to that reported in smaller scale evaluations (8,9) and appears acceptable for seasonal and geographic surveillance, provided an adequate supply of these corvids exists for testing. Our study further suggests that oral tests of House Sparrows, House Finches, and Northern Cardinals, three common urban or suburban species, might be efficiently used to survey for WNV in some areas where corvid populations have been diminished by WNV (11) or are uncommon for other reasons.

Komar et al. (7) detected high WNV titers in cloacal and oral swabs from experimentally infected corvids, a

Table 3. Frequency of false-positive VecTest results[a] in tests of swabs from cloacal and tissue sources in RT-PCR–negative birds

Species	No. false-positive/no. RT-PCR–negative (%)			
	Cloacal	Kidney	Liver	Blood
Cooper's Hawk	0/3	1/7	1/2	1/9
Mourning Dove	0/2	0/7	0/4	0/7
Blue Jay	0/3	3/26 (12)	0/8	0/8
American Crow	0/23 (0)	19/112 (17)	6/51 (12)	14/47 (30)
American Robin	0/9	3/30 (10)	0/13	4/14
Gray Catbird	5/15 (33)	16/28 (57)	4/15	16/18
European Starling	0/8	4/30 (13)	3/11	3/5
Common Grackle	0/9	0/55 (0)	1/25 (4)	4/12
House Sparrow	0/9	0/15	1/5	4/15
Other avian	0/36 (0)	9/87 (10)	1/29 (3)	16/73 (22)
All species	5/117 (4)	55/397 (14)	17/163 (10)	62/208 (30)

[a]Principally narrow lines at the lower margin of the test zone on the dipstick; RT-PCR, reverse transcriptase–polymerase chain reaction.

Table 4. VecTest results from brain swabs of RT-PCR–positive[a] birds with oral VecTest-negative results

Species	N	No. positive	No. narrow-line results[b]
Sharp-shinned Hawk	2	0	2
Cooper's Hawk	2	0	0
Northern Goshawk	1	1	0
Red-tailed Hawk	11	4	3
American Kestrel	1	0	0
Peregrine Falcon	1	0	0
Great Horned Owl	10	0	0
Blue Jay	4	0	0
American Crow	9	3	0
Common Raven	1	0	0
Other species[c]	7	0	0
All species	49	8	5

[a]RT-PCR, reverse transcriptase–polymerase chain reaction.
[b]Type of result most commonly seen in internal tissues of RT-PCR—negative birds (see text).
[c]Great Blue Heron (1), Mallard (1), Herring Gull (1), Great Black-backed Gull (1), Mourning Dove (2), Scarlet Tanager (1).

finding that indicates that both of these orifices may be useful for virus detection. In our comparison, the sensitivity of the VecTest for detecting WNV in both cloacal and oral swabs from American Crows was similar (69% and 67%, respectively). Lindsay et al. (9), however, found that VecTests of cloacal swabs were less sensitive than oral VecTests for detecting WNV in American Crows (58.3% and 92.8%, respectively). This discrepancy may be due to limited sample sizes in both studies and warrants further comparison of the two swabs. Lindsay et al. (9) demonstrated that swab solutions could be held up to 7 days at temperatures ranging from –20°C to 18°C. Our data also showed that neither freezing of the swab samples, at –20°C for 2 days to 7 months, nor refrigeration at 4°C for 3 to 7 days, had any effect on the sensitivity of the VecTest.

When testing will include or be limited to oral sampling, we suggest the following protocol to help standardize the technique and maximize the amount of tissue and fluid captured by the swab tip. After moving the swab tip against the lining of the mouth, compress the throat immediately behind the head and vigorously move the swab tip within the constricted entrance to the esophagus. This aggressive technique should be used only in dead birds.

Recent findings have shown vascular flight feather pulp of corvids to be a superior source for WNV isolation by culture (12). Our limited data showed feather pulp to be slightly more sensitive than oral swabs for detecting WNV with VecTest assays in corvids, and feather pulp specificity was excellent. Using feather pulp as an antigen source for VecTest assays may be advantageous, especially for testing live birds or where oral samples from dead birds may be compromised by autolysis or contamination. However, whether feather pulp or some other tissue would be useful in detecting WNV in species for which oral swabs appear ineffective requires further evaluation. The results obtained with other tissues from Great Horned Owls and Red-tailed Hawks were not encouraging. In experimentally exposed corvids, WNV has been shown to be present at roughly similar concentrations in a wide variety of tissues at death (13). A similar study of viral distribution and concentration in species, such as raptors and songbirds like robins, that showed poor VecTest results would be useful. A substantial fraction of birds from this group would likely have died from other causes (e.g., traumatic injury, poisoning) during periods when antigen levels were low. Low antigen levels and poor VecTest sensitivity may occur in WNV-susceptible species early in the incubation period or during recovery.

In this study, VecTest of oral swabs correctly identified RT-PCR–negative birds as negative in most cases (high specificity), which was consistent with results obtained in a similar study (9). Yaremych et al. (8), however, reported lower specificity with Illinois birds but, as mentioned by the authors, this finding may have been due to small sample size in their analysis. Also, the Illinois study tested a mixture of fecal, saliva, and tissue samples, which may not be directly comparable to tests of oral swabs alone. Lower specificity occurred in our study in oral tests of Gray Catbirds and Green Herons and in tests of internal tissues in a variety of species. VecTest results in these tests all involved the occurrence of the narrow-line false-positives mentioned earlier. The cause of these potentially misleading lines in the WNV test region of the dipstick was not determined. Although they can be readily distinguished from true-positive results in most cases, their elimination from this assay should be a high priority for the manufacturer of the VecTest. The number of species showing narrow-line results will likely increase as this test is used on a wider array of avian species. Also, in rare cases, results appear equivocal even to experienced test evaluators. In the interim, we recommend that the VecTest instruction sheet be modified to alert users to this phenomenon.

The VecTest has many attributes that make it a useful substitute for RT-PCR or other more complicated techniques. It is fast, easy to use, relatively inexpensive, and can be readily employed in the field. The VecTest has good sensitivity in key WNV–vulnerable species, can potentially be used with a variety of tissue sources, and has similar efficacy in fresh and decomposed carcasses. Clear wide-band–positive results have to date shown a 100% positive predictive value. The most serious disadvantages of the VecTest are its poor sensitivity in some species, and the narrow-line false-positive results. In situations in which improved sensitivity is desired, testing of kidney could reduce the number of false-negatives in American Crows, Blue Jays, and House Sparrows. However, the use of internal tissues requires opening the body cavity and increases human risk for WNV exposure**.** We recommend RT-PCR

or other backup for negative results in cases where detection is critical, for diagnostic work, and in assessing threats to humans or animals.

In addition to its use in surveillance activities, the VecTest could be used as a diagnostic tool in some veterinary practices (e.g., zoos, exotic birds), some wildlife rehabilitation operations, and by biologists studying illness and death in wildlife. Both surveillance and diagnostic applications would benefit greatly from new findings concerning the test's sensitivity relative to a much larger array of avian species. Similar evaluation of the VecTest relative to amphibians, reptiles, and mammals also would be useful.

Acknowledgments

We acknowledge the technical assistance of Kevin Hynes, Darci Dougherty, Bill Keuhnle, Ricky Woods, and Robert Benson (bird necropsy, swab and tissue collection, performing the VecTest), and Susan Jones, Mary Franke, and Joseph Maffei for RT-PCR and virus isolation assays. We thank the County Health Departments, New York City Department of Health, Yoichiro Hagiwara, Richard Chipman, Richard A. Watt and other Animal Control Officers, wildlife rehabilitators, and the concerned public for coordination of and participation in the dead bird reporting and submission. We also thank Kate Schmit and Yves Moliere Jean-Louis for initial analyses and Nicholas Komar, Kirti Dave, and Lawrence Skinner for editorial input.

The New York State Department of Environmental Conservation and the New York State Department of Health jointly supported this research. This research was also supported in part by the International Research Partnership for Infectious Diseases, through a grant from the National Aeronautics and Space Administration's SYNERGY program, by the Public Health and Response to Bioterrorism Cooperative Agreement Number U90/CCU216988 from the Centers for Disease Control and Prevention (CDC), and by a supplement to National Science Foundation grant #9983304, "Developing a National Infectious Disease Information Infrastructure: An Experiment in West Nile Virus and Botulism." Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC.

The authors declare no ownership, stock holdings, or other financial interest with the manufacturers or developers of VecTest.

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Experimental Everglades Virus Infection of Cotton Rats (*Sigmodon hispidus*)

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Everglades virus (EVEV), an alphavirus in the Venezuelan equine encephalitis (VEE) serocomplex, circulates among rodents and vector mosquitoes and infects humans, causing a febrile disease sometimes accompanied by neurologic manifestations. EVEV circulates near metropolitan Miami, which indicates the potential for substantial human disease, should outbreaks arise. We characterized EVEV infection of cotton rats in South Florida, USA to validate their role in enzootic transmission. To evaluate whether the viremia induced in cotton rat populations regulates EVEV distribution, we also infected rats from a non–EVEV-endemic area. Viremia levels developed in rats from both localities that exceeded the threshold for infection of the vector. Most animals survived infection with no signs of illness, despite virus invasion of the brain and the development of mild encephalitis. Understanding the mechanisms by which EVEV-infected cotton rats resist clinical disease may be useful in developing VEE therapeutics for equines and humans.

Everglades virus (EVEV; *Togaviridae*: *Alphavirus*) circulates among rodents and vector mosquitoes in South Florida and can tangentially infect humans, causing a febrile disease with occasional neurologic signs. The most closely related Venezuelan equine encephalitis (VEE) complex viruses, enzootic subtype ID strains, are the progenitors of subtype IAB and IC strains responsible for major epidemics and epizootics (1). This relationship raises the possibility of epidemic emergence in South Florida, involving mutations in the EVEV genome, with serious public health consequences for >2 million people in metropolitan Miami-Dade County.

EVEV was first recognized in South Florida in the 1960s, when Seminole persons living north of Everglades National Park were shown to have seroprevalence as high as 58% (2). Recorded EVEV activity has been limited to South-Central Florida from Everglades National Park, north to Indian River County (Figure 1) (3–10). Although EVEV circulation in South Florida has been documented repeatedly, little is known about the dynamics of its ecology and transmission. Strains isolated from mosquitoes, laboratory transmission experiments (11), and rodent host preferences (12) found that *Culex (Melanoconion) cedecei* was the primary vector. Field studies in the 1960s implicated cotton rats (*Sigmodon hispidus*, subspecies not reported) and cotton mice (*Peromyscus gossypinus*) as reservoirs on the basis of high seroprevalence for EVEV (4–6,8). Subsequent studies characterized EVEV and related VEEV infection in experimentally infected cotton rats and laboratory rodents (13–16). However, the duration and magnitude of viremia titers needed to infect mosquito vectors, the clinical outcome of infection, and the immune response were never defined in cotton rats from the enzootic region. To better understand the enzootic EVEV cycle, we experimentally infected cotton rats from South Florida.

Factors that regulate the geographic distributions of arboviruses are poorly understood. Animals of different genetic backgrounds can show differential susceptibility and responses to infection with mosquito- and rodentborne viruses (17–19). For example, rodents most closely related to reservoir species of hantaviruses are more susceptible to infection than more distantly related species are (20). Variation in the susceptibility of cotton rats to EVEV might explain why its distribution is restricted to South Florida. Twelve currently recognized subspecies of cotton rats native to the southern United States (21) differ by as much as 5% in their cytochrome b DNA sequences (22). If genetically distinct cotton rat populations living outside the EVEV-enzootic region do not sustain the magnitude or

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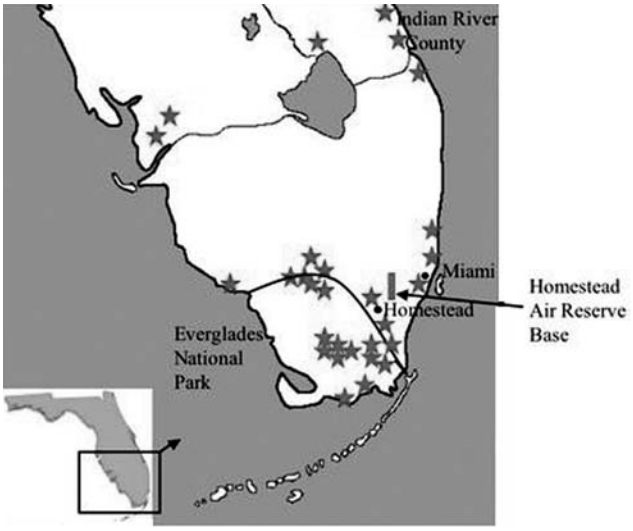

Figure 1. Map of South Florida, indicating locations of Everglades virus isolation, human cases or antibody detection (stars), and our cotton rat collection site (box). Dark line delineates national park boundary.

duration of viremia titers needed to infect sufficient numbers of vectors, they could be incapable of maintaining virus circulation. To test this hypothesis, we compared EVEV infection in a sympatric cotton rat subspecies to infection in a genetically divergent Texas subspecies outside the known EVEV and VEE complex alphavirus distribution (22).

Materials and Methods

Virus Strains

Two EVEV strains from ENP were used in the experimental infections: the prototype strain, FE3-7c, isolated in 1963 from *Culex* (*Melanoconion*) spp. mosquitoes, was passaged five times in suckling mouse brains (SMB) and twice in Vero cells (23), and FE4-71k (SMB1, Vero 1), a 1964 isolate from *Culex* spp. mosquitoes. Both isolates were used to assess strain variation and to determine any effects of the more extensive passage history of FE3-7c on infection or virulence. Virus stocks were prepared in Vero cells, and each animal was inoculated with approximately 1,000 PFU. All inocula were back-titered by plaque assay to determine the exact dose administered.

Cotton Rat Collection, Identification, and Colonization

Cotton rats were collected in baited live traps (Sherman Traps Inc., Tallahassee, FL) in April 2003 in Homestead Air Reserve Base (25.49°N, 80.38°W) within the EVEV-enzootic region of southern Florida. All procedures were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee and were performed in accordance with published guidelines (24). All rats were seronegative and virus-negative for several rodentborne pathogens enzootic in South Florida, including hantaviruses, arenaviruses, eastern equine encephalitis virus, and EVEV. First generation (F_1) offspring from mating pairs established in the laboratory were used for infections. To represent a cross-section of the natural population, rats of various ages (3, 6, 9–12 weeks) were infected. In most cases, infected animals and mock-infected controls were matched for age and sex. In addition to morphologic identification of the animals to the species level, DNA was extracted from the liver and purified by using the DNeasy extraction kit (Qiagen, Valencia, CA) or whole blood using the methods of Longmire et al. (25), and the cytochrome b gene was amplified and sequenced as described previously for cotton rat identification (22,26).

A second cohort of cotton rats, representing a different subspecies, was collected in Galveston Island State Park (29.27°N, 94.83° W) in June and August 2003 and used directly for experimental infections. Texas rats were chosen for the following reasons: 1) among U.S. subspecies, Texas cotton rats are the most divergent genetically from the Florida subspecies (26) and may exhibit a difference in susceptibility; 2) because the subspecies of cotton rats in which EVEV activity was detected previously is unknown, we wanted to test a subspecies unexposed to VEEV complex viruses; and 3) the use of local rats simplified animal use protocols**.** Although no VEE complex alphaviruses are known to circulate in Texas, all rats were tested and determined to be EVEV seronegative before infection. The ages of the field-collected rats were unknown, but their weights ranged from 50 g to 160 g, which represents the range of ages in natural populations of cotton rats because weight can be used to estimate life stage and age (27). The Texas rats were matched for sex and size, and the cytochrome b gene was sequenced.

Cotton Rat Infections

Cohorts of eight cotton rats from each location were injected subcutaneously (SC) in the left thigh with EVEV, and two rats per cohort were sham-injected with diluent. The virus dose (2.3–3.6 $\log_{10}$ PFU) and infection route is an appropriate simulation of the bite of alphavirus-infected mosquitoes (28,29). Individually housed animals were monitored daily for signs of illness typical of VEE complex virus infection and were bled from the retroorbitus at 1- to 2-day intervals, beginning 1 day postinfection.

In a subsequent experiment, 15 Florida rats (5–22 weeks of age) were administered 3.2 $\log_{10}$ PFU of strain FE4-71k SC and were serially killed at daily intervals (two rats/day) for histologic examination and virus assay of selected organs. Surviving animals were bled daily. Anesthetized rats were perfused with 20 mL to 50 mL of phosphate-buffered saline to eliminate viremic blood from

the organs, and organs were homogenized (MM300 homogenizer, Retsch Inc., Newton, PA) in Eagle's minimum essential medium (MEM) with 5% fetal bovine serum to yield a 10% weight/volume suspension. Each suspension was centrifuged at 5,760 x *g* for 6 min, and the supernatant was frozen at –80°C. Additional tissue samples were transferred to 10% formalin for 48 h and then stored in 70% ethanol before being embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were examined in a blinded manner for histopathologic lesions characteristic of VEEV infections of mice and hamsters (30,31).

Virus and Antibody Assays

Serum and organ samples were tested for EVEV by plaque assay on Vero cells (32). Log- transformed viremia levels were compared among cohorts by using the Mann-Whitney U test (33). The limit of detection of the assay was 80 PFU/mL (1.9 $\log_{10}$ PFU/mL). Antibody titers were measured by standard 80% plaque reduction neutralization tests (PRNT) (32).

Results

Identification of Cotton Rats.

Genetic distances among mitochondrial cytochrome b gene sequences of rats from Florida and Texas were obtained by using the Kimura 2-parameter model (34) and were used to construct a neighbor-joining tree (35) that reflected phylogenetic relationships (data not shown). Texas rats grouped closely with *S. hispidus berlandieri*, and Florida rats were identified as *S. hispidus spadicipygus*, another subspecies that differs by up to 5% in its sequence from *berlandieri*, which suggests that these populations represent the maximum level of divergence within the United States.

Infection Profile and Virus Replication Kinetics

A total of 46 of the 47 cotton rats from both localities injected with EVEV became viremic for 3 to 4 days (Figure 2). With the exception of a single death approximately 30 hours postinfection, all rats survived, and none exhibited detectible illness. The rat that died had viremia and organ titer levels comparable to levels in other rats 1 day postinfection. Rats from Texas did not experience viremia levels of shorter duration or lower magnitude ($p \geq 0.05$) than Florida animals, causing us to reject our hypothesis that Texas animals are less likely to exhibit EVEV viremia. Strain FE3-7c produced lower viremia titers than FE4-71k at 1 and 2 days postinfection; however, only differences in Florida rats were significant ($p = 0.02$ day 1, $p = 0.03$, day 2). Mean peak titers occurred 2–3 days postinfection and reached 4–4.5 $\log_{10}$ PFU/mL for all cohorts. By day 4, viremia levels were not detectable in most rats. Viremia profiles were independent of sex, age, or sibling relatedness among the colony Florida rats (data not shown).

Pathologic Manifestations and Viral Tropism

Although a single rat died approximately 30 hours postinfection, none of the other 46 infected rats exhibited signs of illness. The viremia profile for rats sacrificed daily (Figure 3A) showed no difference from that generated in the first experiment with animals from the same location infected with the same virus isolate (Figure 2A). Figure 3 shows the temporal course of organ infection in the heart, brain, salivary glands, and lungs (B) and spleen, kidney and liver (C). EVEV was detected in the heart (1–2 days

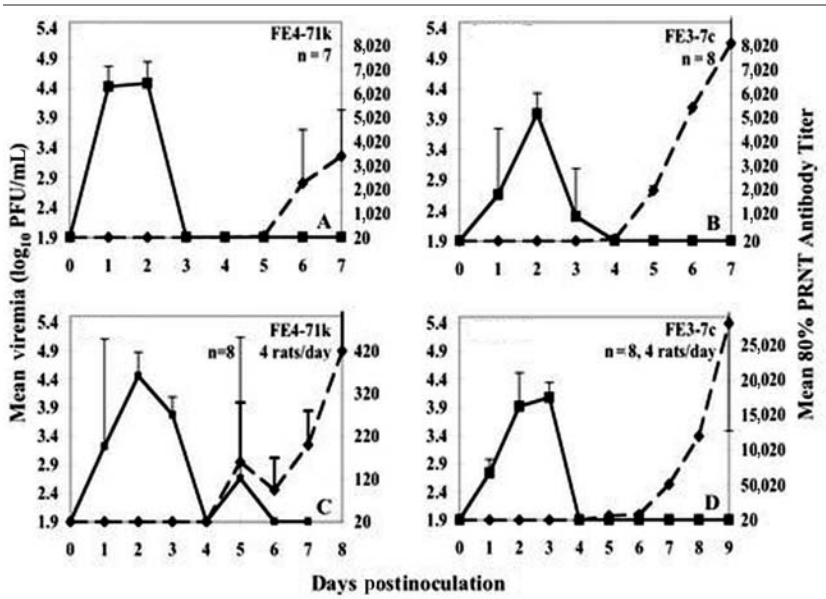

Figure 2. Viremia and neutralizing antibody profile in F1 Florida (panels A, B) or wild Texas (panels C, D) cotton rats injected with Everglades virus strains FE4-71k (A, C) and FE3-7c (B, D) administered subcutaneously in the left thigh. Inoculum doses were as follows: panels A and B: 2.9 $\log_{10}$ PFU/mL, panel C: 2.3 $\log_{10}$ PFU/mL, panel D: 3.6 $\log_{10}$ PFU/mL. Florida animals were bled daily; viremia or 80% plaque reduction neutralization test (PRNT) antibody titers represent geometric means of data from eight rats (strain FE3-7c) or seven rats (EVEV FE4-71k). Rats from Texas were each bled every 2 days; means (geometric) represent measurements from four animals. Bars denote standard deviations.

postinfection), salivary glands (3–4 days postinfection), lungs (1–4 days postinfection), brain (2–4 days postinfection) (Figure 3B), and in the spleen (1–3 days postinfection), and inconsistently in the liver (2–4 days postinfection) and kidney (1–6 days postinfection) (Figure 3C). Aside from virus in the kidney of one rat at day 6 postinfection (Figure 3C), virus was cleared from all organs by day 5, which coincided with the development of neutralizing antibodies (described below). We were unable to detect virus in urine or fecal samples collected 1–7 days postinfection.

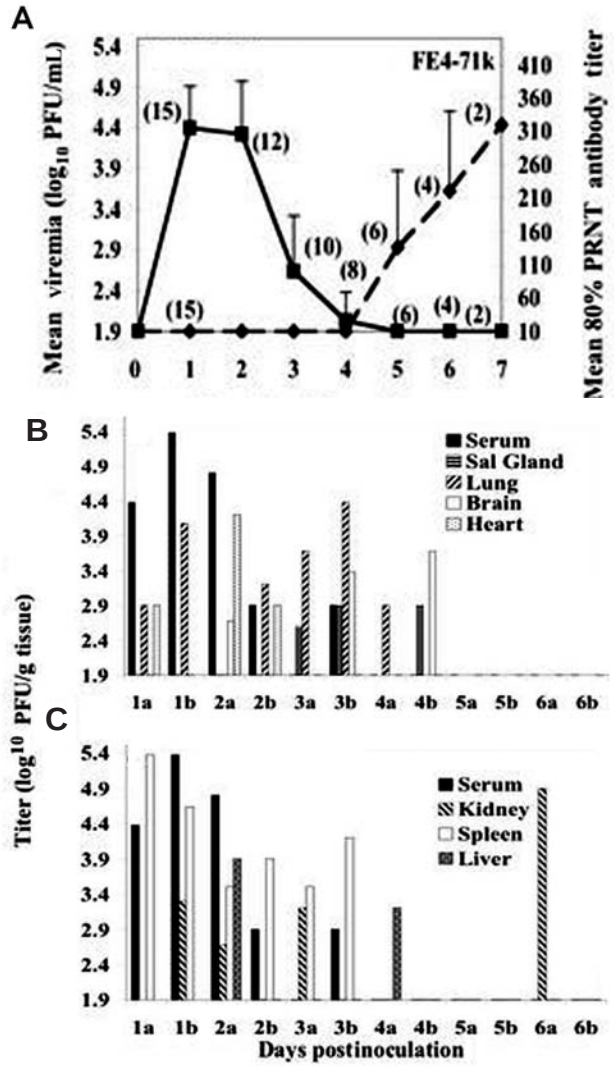

Figure 3. A) Viremia and neutralizing antibody profiles in F1 Florida cotton rats serially sacrificed at daily intervals after infection with 3.2 $\log_{10}$ PFU of Everglades virus strain FE4-71k administered subcutaneously in the left thigh. Lines on each graph represent the geometric mean viremia or mean 80% plaque reduction neutralization test (PRNT) antibody titers; the number of rats bled at each time point is denoted in parentheses above each point. Error bars denote standard deviations. Everglades virus organ titers from the brain, salivary glands, lung and heart (B) and liver, kidney, and spleen (C) of EVEV strain FE471k-infected F1 Florida cotton rats serially sacrificed at daily intervals. Two rats, denoted "a" and "b" were sacrificed daily from days 1–7 postinfection. No virus was detected in any organ on day 7.

Histopathologic examinations showed depletion of lymphoid cells in the spleen on day 2, followed by architectural reorganization and recovery 3 to 7 days postinfection (not shown). Brains of infected rats appeared similar to those of mock-infected rats until 4 days postinfection, coincident with virus clearance from the blood. After day 4, focal meningoencephalitis and associated perivascular mononuclear cell infiltration and neurophagia were observed. Figure 4 shows brain sections from sham-inoculated (A) and encephalitic rats infected with strain FE4-71k that were killed on day 7 postinfection (B), and approximately 5 weeks postinfection (C). The focal encephalitis observed in infected rats at 7 days postinfection was resolved by 5 weeks postinfection, without chronic inflammation or tissue reorganization.

Antibody Responses

All 46 surviving cotton rats seroconverted; neutralizing antibody was first detectable 5 days postinfection, concordant with or after the disappearance of viremia (Figures 2A–D, 3A). Levels of neutralizing antibody rose rapidly to maximum mean titers of 320 to 28,157 (Figures 2A–D, 3A), and some rats maintained high (>10,240) neutralizing antibody titers for 6 months.

Discussion

Infection Outcome

EVEV produced benign, systemic infection when delivered SC in relevant doses to cotton rats from EVEV-endemic and EVEV-nonendemic areas of the United States, and all surviving animals seroconverted. The appearance of antibody sometimes followed the disappearance of viremia, indicating that innate immune mechanisms may participate in virus clearance or that undetectable levels of neutralizing antibody may have preceded the disappearance of viremia. The lower levels of viremia generated by strain FE3-7c may reflect the more extensive cell culture passage history of this isolate. The nonfatal outcome of infection, combined with the high levels of viremia and observations from field studies (4–6,8), is consistent with the role of cotton rats as reservoir hosts for EVEV.

EVEV was neuroinvasive in cotton rats and caused transient, focal encephalitis as well as mild viscerotropic diseases, similar to those caused by other VEE complex alphaviruses. Although encephalitis developed in cotton rats, their ability to clear virus from the brain and the relatively minor inflammatory response they mounted

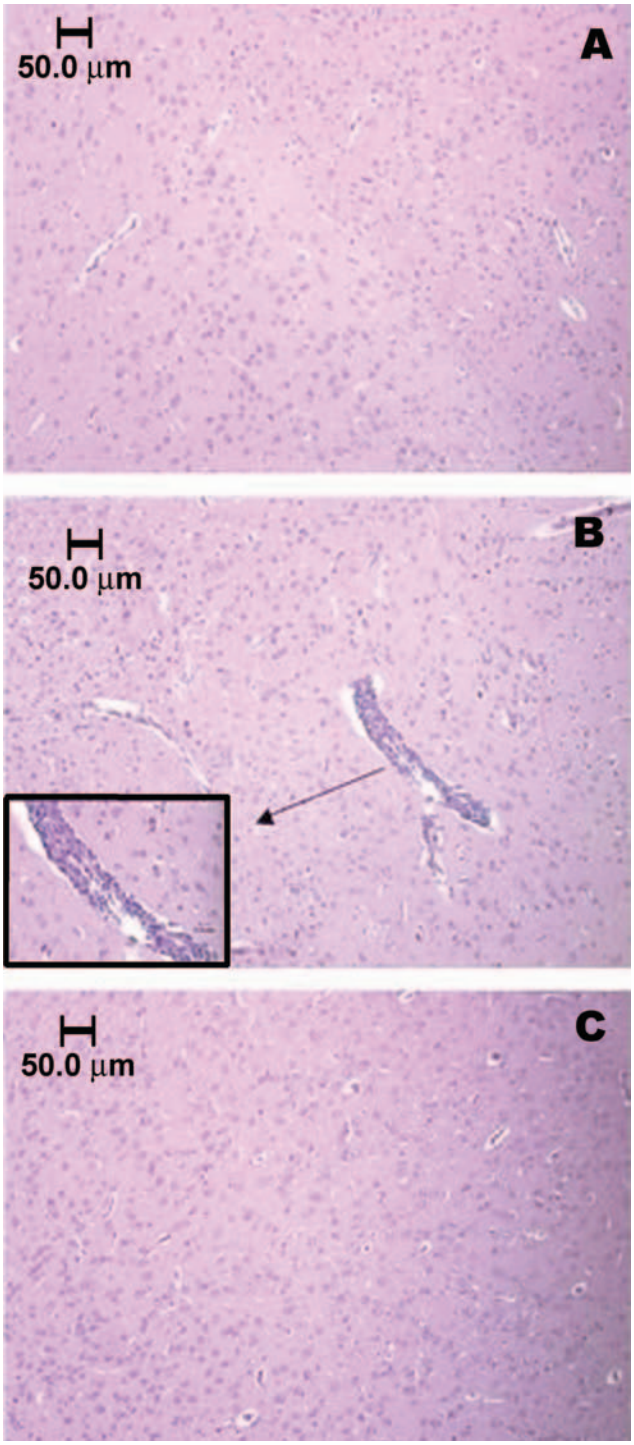

Figure 4: Brainstem section of sham-inoculated control rat, showing the absence of an inflammatory response (A). Vascular and perivascular infiltration of mononuclear cells within the brainstem of a Florida cotton rat 7 days after infection with 3.2 $\log_{10}$ PFU/mL EVEV strain FE4-71k; inset enlarged to show cell infiltration (B). Cortex of cotton rat 5 weeks after infection, showing absence of inflammatory response (C). Animals in panels A and B were anesthetized with pentobarbital and perfused with phosphate-buffered saline intracardially. The rat in panel C was not perfused.

contrasts dramatically with EVEV or VEEV infection of mice (36) and warrants further study.

In many respects, our results were similar to published cotton rat infection profiles of animals and VEE complex viruses from other localities. Wild-caught Panamanian cotton rats (probably *S. h. hirsutus*) (26) that had been injected with 2.8 $\log_{10}$ PFU of an enzootic VEEV subtype ID strain exhibited no virus-induced deaths, but viremia titers developed of 3.7 days mean duration with a peak median magnitude of 7.1 $\log_{10}$ Vero PFU/mL at day 2 postinfection (13), three orders of magnitude higher than the viremia levels we measured. Howard (16) reported that 9 (45%) of 20 cotton rats captured in central Florida near Tampa died after injection with 3.8 $\log_{10}$ suckling mouse intracerebral lethal dose 50% ($SCILD_{50}$) of a VEEV subtype IAB isolate, and a peak viremia level of 6.0 $\log_{10}$ PFU/mL developed in the surviving animals at day 2 postinfection. Possible explanations for the differences in VEE complex viremia levels in different rat populations include the following: 1) EVEV may generally replicate at lower levels in a variety of rodents, or 2) cotton rats from southern Florida are more resistant to the replication of VEE complex alphaviruses. Infection of cotton rats from southern Florida with other VEE complex strains is needed to test this hypothesis.

The only other reported experimental infections of North American cotton rats with EVEV involved seven animals from Homestead, Florida (C. Calisher, pers. comm.), which became viremic 2–4 days postinfection, with a peak of 6.4 $\log_{10}$ $SMICLD_{50}$/mL 3 days postinfection and no deaths (15). This peak viremia level is approximately equal to our 4.0 PFU/mL value measured by plaque assays, since the $SMICLD_{50}$:PFU ratio for EVEV is approximately 200:1 (L. L. Coffey, unpub. data).

Cotton Rats as Reservoirs of EVEV

The fact that high numbers of infected cotton rats in our study survived contrasts with results from EVEV infections of laboratory rodents and is consistent with their role as natural reservoirs. Golden Syrian hamsters and Swiss albino mice experience 75%–100% mortality with doses as low as 3 $\log_{10}$ Vero PFU, and pathologic lesions develop, consistent with VEE-like disease (14,30,37,38). Even though infection of laboratory rodents often causes death, EVEV infection is less virulent than most other VEE complex viruses, which generally cause 100% of infected animals to die (14,30,31,38).

For EVEV transmission by a vector, the reservoir must attain a threshold viremia level (minimum virus titer that infects approximately 1%–5% of vectors [39]). Susceptibility studies of *Culex* (*Mel.*) *cedecei* indicated that hamster blood meal titers as low as 0.9 $\log_{10}$ chicken embryo cell (CEC) PFU/mL (even lower than the viremia

detection limit in our study) infected 9% of mosquitoes, and infected *Cx. cedecei* transmitted EVEV to naïve animals after extrinsic incubation (11). With oral doses of 4.9 $\log_{10}$ CEC PFU/mL, slightly higher than the peak viremia levels observed in our cotton rats, 100% of *Cx. cedecei* became infected. One EVEV Vero cell PFU approximates one CEC PFU (L. L. Coffey, unpub. data), indicating that the infection threshold for *Cx. cedecei* is lower than the detection limits of our assays. Therefore, any viremia levels we observed should be sufficient to infect at least some *Cx. cedecei*.

The absence of virus in excreta from any of the infected animals indicates that EVEV is probably not transmitted horizontally between nest-mates through this route, despite the detection of virus in the kidney. However, the possibility of persistent infection should be addressed in further studies.

EVEV Distribution

Our data do not support the hypothesis that variation in the susceptibility of cotton rats explains the limited EVEV distribution. Another explanation supported by susceptibility testing (11) is that the mosquito vector limits EVEV distribution. The recorded distribution of *Cx. cedecei* is restricted to 13 counties in South Florida (40) and closely parallels the recorded distribution of EVEV activity.

Potential for EVEV Disease

Understanding arbovirus transmission cycles is important for delineating the epidemiology of human disease. Our data support the role of cotton rats as EVEV reservoirs in South Florida. Future work should focus on cotton rat ecology, with emphasis on population dynamics. Combined with quantitative information about vector-reservoir contact, mosquito population fluctuations, and virus circulation intensities, EVEV transmission dynamics can be elucidated.

Previous studies (1,41) indicate that epidemic VEEV emerges from enzootic subtype ID strains, the closest relatives of EVEV. Only a few mutations in enzootic VEEV can generate viruses with equine amplification phenotypes (42). If such epidemic EVEV strains arise, substantial human illness or deaths could occur. Reverse genetic studies under way in our laboratory are designed to assess this possibility.

Acknowledgments

The State of Florida Fish and Wildlife Conservation Commission granted a cotton rat trapping permit and Victoria Johnson and Humberto Rivera gave permission to collect rats in Homestead Air Reserve Base and access to collecting sites. The Texas Parks and Wildlife Commission granted permission to trap cotton rats in Texas. Christine Hice, Ivorlyne Greene, Jessica Tonry, Brad Schneider, and Darci Smith helped trap rats.

This research was funded by National Institutes of Health (NIH) grant AI48807. L.L.C. was supported by the James W. McLaughlin Fellowship Fund. S.P. received support from the NIH Emerging Tropical Diseases T32 Training Grant AI-107536 and K08 grant AI059491.

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Avian Influenza H5N1 in Tigers and Leopards

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Influenza virus is not known to affect wild felids. We demonstrate that avian influenza A (H5N1) virus caused severe pneumonia in tigers and leopards that fed on infected poultry carcasses. This finding extends the host range of influenza virus and has implications for influenza virus epidemiology and wildlife conservation.

The Study

The 2003–2004 avian influenza A (H5N1) virus outbreak in Southeast Asia resulted in 24 reports of fatal human cases (May 12, 2004) due to direct transmission of the virus from birds to humans. During the H5N1 virus outbreak in Thailand in December 2003 (1), two tigers (*Panthera tigris*) and two leopards (*P. pardus*) at a zoo in Suphanburi, Thailand, showed clinical signs, including high fever and respiratory distress, and they died unexpectedly. The animals had been fed fresh chicken carcasses from a local slaughterhouse. At that time many chickens around Suphanburi were dying with respiratory and neurologic symptoms of what was retrospectively identified as H5N1 virus infection (1). Postmortem examinations were performed on all four zoo felids, and samples were collected for histologic, immunohistochemical, and virologic analyses.

At necropsy, the primary gross lesions in all four animals were severe pulmonary consolidation and multifocal hemorrhage in several organs, including lung, heart, thymus, stomach, intestine, liver, and lymph nodes. Histologic examination was performed on formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin. Pulmonary lesions were characterized by loss of bronchiolar and alveolar epithelium; thickening of alveolar walls; and flooding of alveolar lumens with edema fluid mixed with fibrin, erythrocytes, neutrophils, and macrophages (Figures 1A and 1B). One tiger and one leopard had evidence of encephalitis, characterized by multifocal infiltration by neutrophils and macrophages. Tissues were examined for influenza A (H5N1) virus nucleic acid by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, with primer pairs specific for the hemagglutinin (HA) and neuraminidase (NA) genes (2). Lung samples from all four animals were positive for H5N1 with both primer pairs, and the identity of the PCR products was confirmed by nucleotide sequencing. Formalin-fixed, paraffin-embedded tissue sections from one of the leopards were examined for influenza virus antigen by an immunohistochemical technique (3). A monoclonal antibody against the nucleoprotein of influenza A virus was used as primary antibody. Alveolar and bronchiolar epithelial cells in affected lungs expressed influenza virus antigen (Figure 1C and 1D), confirming that influenza virus infection was the primary cause of the pneumonia.

Influenza A virus was isolated from lung samples of one of the tigers and one of the leopards by injecting into embryonated chicken eggs (3). The entire genomes of these two viruses were sequenced. RT-PCR specific for the conserved noncoding regions of influenza A virus was performed (4). PCR products were purified by using the QIAquick gel extraction kit (Qiagen, Leusden, the Netherlands) and sequenced with the Big Dye Terminator sequencing kit, version 3.0 (Amersham Biosciences,

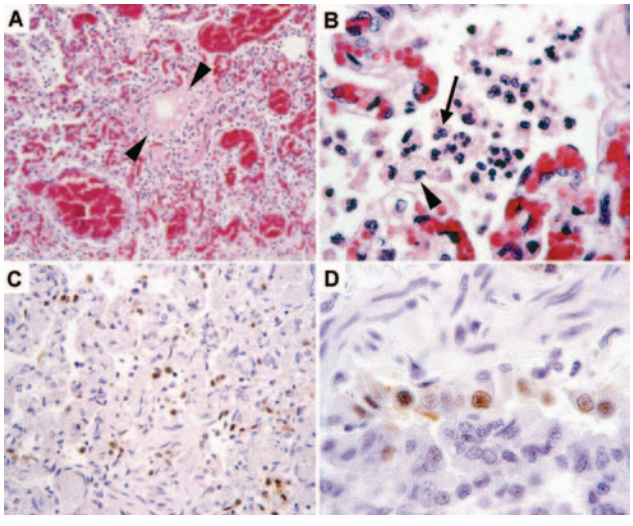

Figure 1. Histopathologic and immunohistochemical evidence of avian influenza A (H5N1) virus in leopard lung. A) Diffuse alveolar damage in the lung: alveoli and bronchioles (between arrowheads) are flooded with edema fluid and inflammatory cells. B) Inflammatory cells in alveolar lumen consist of alveolar macrophages (arrowhead) and neutrophils (arrow). C) Many cells in affected lung tissue express influenza virus antigen, visible as brown staining. D) Expression of influenza virus antigen in a bronchiole is visible mainly in nuclei of epithelial cells.

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Piscataway, NJ). Nucleotide sequences were aligned by using Clustal-W running under BIOEDIT 5.0.9 (Ibis Therapeutics, Carlsbad, CA) and maximum likelihood trees were generated with PHYLIP 3.6 (University of Washington, Seattle, WA) (5) with 100 bootstraps and three jumbles. The consensus tree was used as a user tree in DNAML to recalculate branch lengths. The trees had good bootstrap support (data not shown). Sequencing and phylogenetic analysis of the HA and NA genes of these two isolates showed that they were virtually identical to each other and to the H5N1 virus circulating in poultry at the time (Figure 2) (6). Therefore, the zoo felids were most probably directly infected with avian influenza A (H5N1) virus by feeding on infected poultry carcasses. Furthermore, phylogenetic analysis of the remaining six genome segments (data not shown; leopard accession no. AY646177–AY646182; tiger accession no. AY646169–AY646174) showed that they were of avian origin, which indicates that no reassortment with mammalian influenza viruses had occurred.

The virus isolates obtained from the tiger and the leopard contained a glutamine at position 222 (226 in H3) and a glycine at position 224 (228 in H3) in HA1, which were also found in other recent H5N1 isolates and which are related to preferential binding to avian cell-surface receptors (7). Both viruses contained a deletion of five amino acid residues in NS1, like other recent H5N1 isolates, and contained a glutamic acid at position 92 (6,8). The mutation glutamic acid to lysine at position 627 of PB2, which was responsible for the high virulence of A/Hong Kong/483/97 and was also found in fatal human cases of H7N7 infection in the Netherlands, was observed in the virus isolate obtained from the leopard, but not from the tiger (9,10). Thus, with the exception of position 627 at PB2 in the leopard isolate, the genomic sequences of these zoo felid isolates did not show substantial differences from other recent H5N1 isolates from Asia.

Lung samples from all four felids tested negative for canine distemper virus by RT-PCR (11), while those of three of four felids tested positive for a vaccine strain of feline panleukopenia virus (12), administered 2 weeks before death. Although absence of typical clinical signs and lesions ruled out feline panleukopenia as the primary cause of death, an immunosuppressive effect cannot be ruled out (13).

Conclusions

This report is the first of influenza virus infection causing disease or death in nondomestic felids. Generally, influenza virus is also not considered pathogenic for the domestic cat. Experimental infection of domestic cats in the 1970s and 1980s with influenza A viruses of subtypes H3N2 from humans, H7N3 from a turkey, and H7N7 from a harbor seal (*Phoc vitulina*) resulted in transient virus excretion and a temporary increase in body temperature but did not induce clinical signs of disease (14–16). However, anecdotes of fatal infection have been reported in this species during the 2003–2004 H5N1 virus outbreak

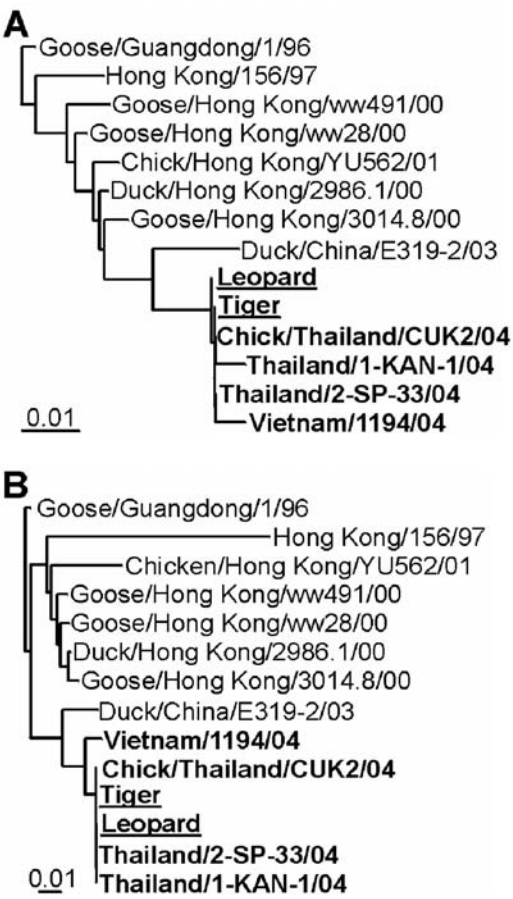

Figure 2. Phylogenetic comparison of zoo felid isolates with other H5N1 viruses. DNA maximum likelihood tree of hemagglutinin and neuraminidase sequences. Representative full-length Asian influenza A virus H5 (A) and N1 (B) sequences from 1996 to 2004 are shown with 2004 sequences in bold and leopard and tiger sequences underlined. Maximum likelihood trees were generated by using 100 bootstraps and three jumbles, and the resulting consensus trees were used as a user tree to recalculate branch lengths. The trees had good bootstrap support. Scale bars roughly indicate 1% nucleotide difference between related strains. Accession no. used: A/Goose/Guangdong/1/1996 (AF144305 and AF144304), A/Hong Kong/156/1997 (AF028709 and AF028708), A/Goose/Hong Kong/ww491/2000 (AY059480 and AY059489), A/Goose/Hong Kong/ww28/2000 (AY059475 and AY059484), A/Chicken/Hong Kong/YU562/2001 (AY221529 and AY221547), A/Duck/Hong Kong/2986.1/2000 (AY059481 and AY059490), A/Goose/Hong Kong/3014.8/2000 (AY059482 and AY059491), A/duck/China/E319-2/2003 (AY518362 and AY518363), A/Thailand/1-KAN-1/2004 (AY555150 and AY555151), A/Thailand/2-SP-33/2004 (AY555153 and AY555152), A/Chicken/Thailand/CU-K2/2004 (AY590568 and AY590567), A/Leopard/Thailand/2004 (AY646175 and AY646176), and A/Tiger/Thailand/2004 (AY646167 and AY646168).

(17), and these reports were recently confirmed experimentally (18).

Our findings in tigers and leopards extend the host range of this virus and, together with the findings in domestic cats (18), suggest that this H5N1 virus is more pathogenic for felids than other influenza viruses. This finding has important implications for wildlife conservation and influenza virus epidemiology. First, H5N1 virus infection may threaten the survival of endangered felids, as has been shown recently for other emerging viruses in susceptible wildlife (19,20). The severity of this threat is increased because H5N1 virus may be transmitted horizontally between domestic cats (18). Second, if the higher pathogenicity of H5N1 virus for felids also means longer excretion of more virus, the role of felids in avian influenza epidemiology, both in humans and in poultry, needs to be reevaluated. Finally, the confirmation of H5N1 virus infection as the probable cause of death in two other mammalian hosts besides humans implies that more species of mammals may be at risk for infection with this virus.

Acknowledgments

We thank Termsitthi Paphavasit and Somkid Kanda for technical support and Theo Bestebroer (virus isolation, PCR, and nucleotide sequencing), Pieter Derkx, Debby van Riel (immunohistochemistry), and Frank van der Panne (photography) for excellent technical assistance.

The study was supported by a grant from the Center for Excellence Research Fund, Emergency Fund of Chulalongkorn University, and the Thailand Research Fund, Senior Research Scholar and National Center of Genetic Engineering and Biotechnology.

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Novel Avian Influenza H7N3 Strain Outbreak, British Columbia

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Genome sequences of chicken (low pathogenic avian influenza [LPAI] and highly pathogenic avian influenza [HPAI]) and human isolates from a 2004 outbreak of H7N3 avian influenza in Canada showed a novel insertion in the HA0 cleavage site of the human and HPAI isolate. This insertion likely occurred by recombination between the hemagglutination and matrix genes in the LPAI virus.

Highly pathogenic avian influenza (HPAI) viruses cause systemic disease in poultry, which is associated with rapid death and a case-fatality ratio approaching 100%. To date, only H5 and H7 subtypes have shown this virulence, although not all of these subtypes are HPAI. HPAI viruses are not normally present in wild bird populations but arise from low pathogenic avian influenza (LPAI) viruses introduced into poultry flocks from wild birds (1,2).

The hemagglutinin gene plays a key role in defining virulence in avian influenza (AI). The hemagglutinin glycoprotein is produced as a precursor, HA0, which requires posttranslational cleavage by host proteases before infectious virus particles can be produced (3). Cleavage of the HA0 precursor in LPAI viruses is catalyzed only by trypsin and trypsinlike host proteases restricting virus replication to locations where these proteases are found, namely, respiratory and intestinal tracts. In contrast, HA0 cleavage in HPAI viruses is mediated by a poorly defined protease(s) that appears to be a proprotein-processing subtilisin-related endoproteases (4). The ubiquitous nature of these protease(s) enables the HPAI virus to replicate systemically, damaging vital organs and tissues, leading to disease and death (3).

All HPAI viruses encode a HA0 protein having a motif of multiple basic amino acids (R and K) flanking the cleavage site. In contrast, LPAI viruses have two basic amino acids at positions–1 and –4 from the cleavage site for H5 and at positions –1 and –3 for the H7 subtype. An increase in basic residues near the cleavage site, either as a result of nucleotide insertion or substitution, allows the HA0 precursor to be cleaved by ubiquitous host proteases (5).

Since 1996, several instances of AI viruses infecting humans have been reported; some of these cases have been fatal (6). Here we describe the sequence of avian and human LPAI and HPAI isolates obtained from the AI outbreak in the Fraser Valley of British Columbia in 2004. Avian HPAI and human virus isolates contain an insert that does not conform to the consensus sequence suggested to be the prerequisite for all HPAI viruses (7), and further analysis shows the insertion is the result of nonhomologous recombination between the hemagglutinin and matrix genes of the virus. Both human isolates have mutated since the original recombination event, and one of the two is likely not highly pathogenic in chickens. We also provide a homology model for HA0 of the HPAI human isolate and show that in addition to adding basic residues, the H1 insertion likely increases accessibility to the protease cleavage site.

The Study

All methods and materials, including supplementary data, are available online in the appendix (http://www.cdc.gov/ncidod/EID/vol10no12/04-0743_app.htm). At the index farm, two flocks were maintained in adjacent barns. Decrease in appetite and slightly increased death rate were noted in the older flock, followed by a dramatic increase in death rate (25% in 48 hours) in the younger flock. Influenza A virus was isolated from both flocks: A/Chicken/Canada/AVFV1/04 (AVFV1) from the older and A/Chicken/Canada/AVFV2/04 (AVFV2) from the younger. All birds on this farm were culled. However, the virus spread, resulting in a Canadian Food Inspection Agency order to kill all 19 million domestic birds in the Fraser Valley.

In two workers involved in the depopulation, symptoms developed, including conjunctivitis, headache, and coryza, 1–3 days after direct exposure of the eye to poultry tissue on infected farms. Influenza A (H7N3) was isolated from both persons (8). The genomes of the viral isolate were

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sequenced to determine whether genetic changes were associated with increased pathogenicity and to assess whether the virus had acquired human influenza A genes. Four isolates were sequenced: two poultry viruses, AVFV1 and AVFV2, and the two human isolates A/Canada/444/04 (human) (Hu444) and A/Canada/504/04 (human) (Hu504).

Consensus sequences for the eight genomic segments isolated from each of the four independent viral genomes are deposited in GenBank. Accession numbers are listed at Appendix Table (available at http://www.cdc.gov/ncidod/eid/vol10no12/04-0743_app.htm#table) and ClustalW lineups of the complete nucleotide and protein sequences are available (online Appendix Figures 1A and 1B, respectively; available at http://www.cdc.gov/ncidod/eid/vol10no12/04_0743-appG1.htm). Sequences for all 32 genes (and their encoded proteins) are highly related to previously determined sequences (BLASTN identities 93%–98% and BLASTP identities 98%–100%) (online Table) and do not suggest the presence of human influenza A genes. However, hemagglutinin (HA) genes in three of the Fraser Valley isolates (AVFV2, Hu444, and Hu504) have a 21-nucleotide (nt) (7 amino acid [aa]) insertion in the HA gene (protein) immediately upstream of the HA0 cleavage site relative to the most closely related H7N3 sequence. AVFV1 lacked this insertion. Although both human isolates contained the insertion, amino acid changes within the inserted sequence were observed compared with the avian AVFV2 sequence, indicating that sequence drift occurred after the initial insertion event. Figure 1 is an alignment of the HA0 cleavage region of all four isolates. An examination of the sequences indicates that the insertion of the 21 nt likely occurred once (QAYRKRM– AVFV2) and that the sequence has subsequently mutated to QAYQKRM and QAYQKQM in human isolates Hu504 and Hu444, respectively.

When the sequence of the AVFV2 virus from the FV outbreak was analyzed, the 21-base insert matched perfectly with a region from the influenza matrix (M) gene. For the two human isolates, the match is 20/21 nt (Hu504) and 19/21 nt (Hu444), indicating this sequence changed over a short period, which is consistent with a high rate of mutation in influenza viruses.

The two avian isolates were tested for pathogenicity by the National Centre for Foreign Animal Diseases by using standard in vivo testing in chicks (http://www.inspection.gc.ca/english/anima/heasan/disemala/avflu/avflufse.shtml). AVFV1 was not pathogenic, whereas AVFV2 is highly pathogenic. On the basis of the consensus sequence for highly pathogenic H7 viruses, we can predict that the Hu504 isolate is likely highly pathogenic in chickens while the Hu444 isolate is likely not pathogenic. These tests are currently under way in another laboratory.

To further characterize the hemagglutinin gene from the Fraser Valley HPAI isolate, a phylogenetic tree was generated by using an alignment of 65 full-length H7 HA sequences obtained from GenBank (Figure A2, available at http://www.cdc.gov/ncidod/eid/vol10no12/04-0743_appG2.htm). With the exception of a single isolate, A/duck/Hong Kong/293/78, the sequences clustered into two distinct sublineages based on location of origin; North American or European. Isolates within the North American sublineage were further divided on a 24-nt deletion (beginning at nt 710) not found in the Fraser Valley isolates. This deletion, first reported in LPAI H7N2 isolates originating from American live bird markets (9), lies within the receptor-binding site for influenza viruses and is thought to compensate for a concurrent NA stalk deletion (10). Consistent with the absence of this deletion in our isolates, no deletion was observed in the NA stalk region of the four Fraser Valley isolates.

The insertion in the HA protein sequence from the Fraser Valley isolates does not conform to the consensus motif (R-X-R/K-R*-G-L-F) for an HA1–HA2 connecting peptide in HPAI viruses because a threonine is at the -2 position. However, the AVFV2 and Hu504 insertions do conform to a minimum cleavage recognition sequence associated with HPAI viruses (R-X-X-R*G) (5). Thus, the insertion sequences for these two Fraser Valley isolates are expected to be cleaved by furinlike proteases. (They may also be susceptible to chymotrypsinlike enzymes because of the introduction of a tyrosine residue at –6 in the HPAI isolate and at –4 and –6 in the human isolates.) Hu444 is likely not pathogenic in chickens, which is consistent with previous observations that a basic amino acid at –4 is required for cleavage (5).

Since the insertion sequence we observed in the HA gene is novel, we used molecular modeling to visualize the effect this insertion has on the HA0 protein. The model for

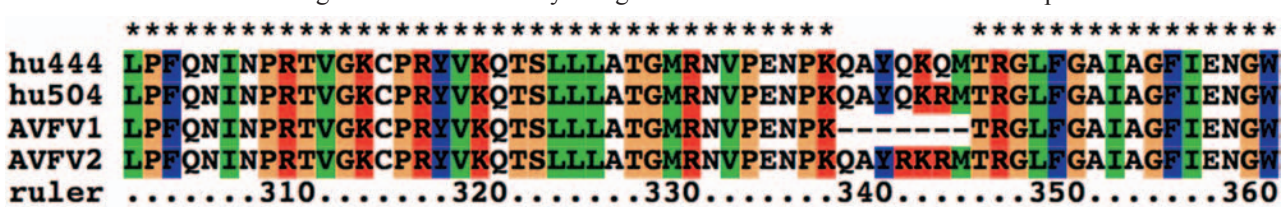

Figure 1. Alignment of the hemagglutinin cleavage region from four isolates of Fraser Valley H7N3 virus. A/Chicken/Canada/AVFV1/04 is designated AVFV1; A/Chicken/Canada/AVFV2/04 is designated AVFV2; A/Canada/444/04 (human) is Hu444, and A/Canada/504/04 (human) is Hu504). A 7–amino-acid (aa) insertion associated with the AVFV2 isolate and both human isolates is shown at aa 338.

the H7N3 hemagglutinin precursor is based on the 2.8 Å human H3 HA0 structure (11). From the structure-base sequence alignment (Figure 3, available at http://www.cdc.gov/ncidod/eid/vol10no12/04-0743_appG3.htm) and resulting homology model (Figure 2) the 7-residue insertion extends out in a loop. This loop formation likely increases the accessibility of the cleavage site, and the insertion of critical basic amino acids contributes to these viruses' marked increase in pathogenicity.

Conclusions

Cleavage of the HA0 protein of influenza viruses results in an essential conformational change in the HA protein, which enables the envelope of the endocytosed virus to fuse with the membrane of endosomes, releasing nucleocapsids into the cytoplasmic compartment (5,13). The 7-aa insertion described here generates an enlarged exposed loop, which contains multiple basic amino acids in the HA0 protein. Together these modifications to the HA0 protein likely result in an increased rate of cleavage by furinlike proteases, which increases pathogenicity. Analysis of the nucleotide sequences of HA genes from HPAI H5 and H7 isolates has shown that in many cases direct repeats of a purine-rich sequence (AAGAAA) occur. This sequence may arise because of the pausing of the transcriptase complex at a region of secondary structure, which results in slippage of the transcriptase complex and insertion of a short repeat sequence. Additionally, recombination events between the HA and NP genes (14) and the HA gene and host cell 28S ribosomal RNA have been documented (15). More recently, recombination between the NP and HA genes resulted in a 30-nt insertion near the HA0 cleavage site in HPAI viruses isolated in Chile in 2002 (16). The HA sequences from the Fraser Valley outbreak described here contain a novel insert derived from the M gene. Thus, in addition to transcriptase slippage, nonhomologous recombination represents an important mechanism in the acquisition of virulence in avian influenza viruses.

Acknowledgments

We gratefully acknowledge the support of the British Columbia Cancer Foundation.

This project was funded by Genome British Columbia. M. Marra, S. Jones, R. Holt, M. Griffith, and M. Paetzel are recipients of salary awards from the Michael Smith Foundation for Health Research.

Dr. Hirst is a research associate at the Michael Smith Genome Sciences Centre within the British Columbia Cancer Agency, Vancouver, Canada. He leads a group of researchers who are working on generating and sequencing full-length cDNAs for rare mammalian mRNAs as part of the Human Genome Project–Mammalian Gene Collection initiative.

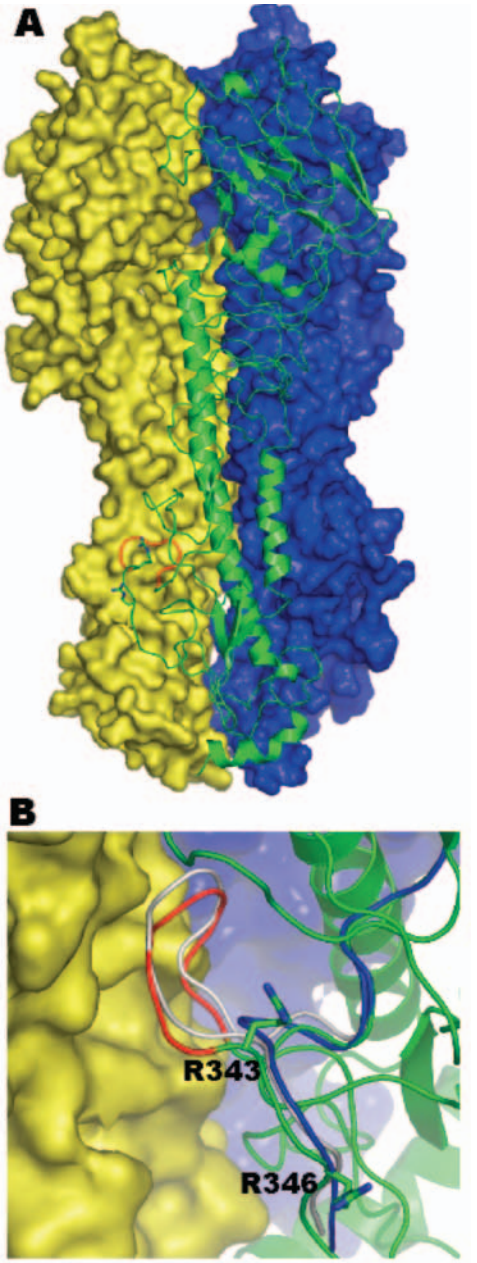

Figure 2. A homology model of the human A/Canada/504/04 (Hu504) hemagglutinin precursor (HA0) trimer based on the crystal structure of the human strain CV-1 HA0 (PDB: 1HA0) sequence identity 49.9%. A) Molecule A is shown as a green ribbon diagram; molecules B and C are shown in blue and yellow molecular surfaces, respectively. The 8–amino-acid (aa) sequence 335-342 (NPKQAYQK) is shown in red. B) A close up of this region located between molecules A (in green ribbon) and molecule C (in yellow surface). This 8-aa sequence forms a loop, which bumps into the adjoining molecule before energy minimization (gray). Shown in red is the loop after energy minimization, which results in the cleavage site's being pushed out slightly. Shown in blue is the corresponding region for the template structure (PDB code 1HA0). The side chains for arginine 343 and arginine 346 (–1 residue) are shown in stick form. (Since the preparation of this manuscript, the structure of an H7 HA protein has been reported [12])

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Human Illness from Avian Influenza H7N3, British Columbia

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Avian influenza that infects poultry in close proximity to humans is a concern because of its pandemic potential. In 2004, an outbreak of highly pathogenic avian influenza H7N3 occurred in poultry in British Columbia, Canada. Surveillance identified two persons with confirmed avian influenza infection. Symptoms included conjunctivitis and mild influenzalike illness.

Influenza is the most diversified in birds, particularly in wild waterfowl (1). Concern exists that outbreaks of avian influenza in domestic poultry could, through a process of genetic reassortment, mutation, or both, introduce new influenza subtypes into the human population. In the context of widespread susceptibility, such an event could be the precursor of a pandemic (2,3).

An outbreak of avian influenza emerged on a farm in the Fraser Valley of British Columbia on February 6, 2004. Slightly increased deaths (8–16 deaths/day) were noted among 9,200 chickens in one barn. Avian influenza infection was confirmed on February 16, 2004, and later genotypic and phenotypic intravenous pathogenicity index (IVPI) testing characterized the virus as low pathogenicity avian influenza (LPAI) H7N3. On the same farm, an adjacent barn that contained 9,030 chickens had a dramatic increased in deaths from February 17 through 19 (2,000 deaths in 2 days). Genotypic and IVPI testing confirmed highly pathogenic avian influenza (HPAI) H7N3 in this second flock.

The Canadian Food Inspection Agency ordered the culling of both flocks and initiated active avian influenza surveillance on all farms within 5 km, but the virus spread nonetheless. On April 5, the Canadian Food Inspection Agency ordered depopulation of all poultry in the Fraser Valley south of the Fraser River (19 million birds). In total, the Canadian Food Inspection Agency identified avian influenza in 42 of the ≈600 commercial poultry farms in the region and in 11 backyard flocks, which represented ≈1.3 million birds (4). The last infected farm was identified on May 21, 2004.

To mitigate the risk for human infection and the potential for genetic reassortment, federal workers involved in the depopulation were required to wear personal protective equipment, including N95/North 7700 masks, gloves, goggles, and biosafety suits and footwear. They were also required to take prophylactic oseltamivir at a dose of 75 mg per day for the duration of exposure plus 7 days and to receive the commercially available human influenza vaccine for the 2003-04 season, if they had not already done so (5). All protective measures were provided free of charge and were recommended also for exposed farm workers and their families. Following reports of human illness, these measures were more rigorously promoted and reinforced through worker screening, information letters prepared by the British Columbia Centre for Disease Control, and media bulletins.

We report the results of enhanced surveillance for human illness in association with this poultry outbreak of HPAI H7N3 in British Columbia.

The Study

After the first report to public health authorities of poultry outbreaks on February 18, 2004, enhanced surveillance for conjunctivitis and influenzalike illnesses was implemented for federal workers, farm workers and their household contacts, and any other potentially exposed persons. Illness was reported to the British Columbia Centre for Disease Control by using a standard questionnaire and report form. Respiratory specimens were tested at the British Columbia Centre for Disease Control by reverse transcription–polymerase chain reaction for influenza and by cell culture for all respiratory pathogens; influenza isolates were sequenced to determine the subtype (e.g., H7). Suspected human cases were defined as illness in persons presenting after February 6, 2004, with two or more new or worsening conjunctivitis or influenzalike symptoms, with onset from 1 day after first exposure (defined as direct contact or shared air space) to 7 days after last exposure to a potential source of avian influenza virus in the Fraser Valley. Confirmed cases had laboratory-confirmed

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influenza A (H7) virus in conjunctival, nasal, nasopharyngeal, or throat specimens by reverse transcription–polymerase chain reaction (6) or cell culture. Influenza hemagglutinin and neuraminidase subtyping was performed at the National Microbiology Laboratory. Serum samples were tested for antibody to influenza A (H7) by hemagglutination inhibition and microneutralization assays (7) at the National Microbiology Laboratory. Microneutralization assays were repeated at the U.S. Centers for Disease Control and Prevention on serum samples from two persons with confirmed infections and from eight persons with suspected cases.

Approximately 2,000 poultry farm workers are in the Fraser Valley. Approximately 650 federal workers assisted with outbreak management and control; not all had poultry exposure. From February 18 to June 1, 2004, a total of 77 symptomatic persons were reported to the British Columbia Centre for Disease Control. Fifty-seven had suspected (n = 55) or confirmed (n = 2) avian influenza infections.

Among the 20 reports that did not meet the suspected or confirmed case definitions, 9 had insufficient information to determine case status, 3 did not meet the symptom requirements, 3 did not have a relevant exposure history, 1 had onset before February 6, 3 had onset >7 days after exposure, and 1 had onset <1 day after exposure.

Baseline characteristics are shown in Table 1, and the epidemic curve is shown in the Figure. Respiratory symptoms predominated (Table 2) among the 55 patients with suspected cases. Symptom duration was 1–58 days. No patients were hospitalized. Twelve (22%) reported taking prophylactic oseltamivir at symptom onset, and 11 (20%) received oseltamivir for treatment. The remaining 22 patients with suspected cases were identified >48 hours after onset or refused treatment. All recovered fully.

Respiratory specimens (nasal, nasopharyngeal, throat, and conjunctival) were collected from 47 patients with suspected cases (86%) an average of 5 days after onset (range 0–27 days). Cell culture identified pathogens in two persons: adenovirus type 3 in one (conjunctival and nasal specimens) and HSV-1 in another (throat specimen). All other results were negative for respiratory viruses, including influenza. No antibody to influenza A H7 could be detected in paired acute- and convalescent-phase serum samples (n = 17), drawn an average of 9 days (range 0–33 days) and 31 days (range 18–88 days) after onset, respectively, or in convalescent-phase serum samples (n = 8) drawn an average of 28 days (range 8–56) after onset from patients with suspected cases.

Influenza A H7N3 infection was confirmed in two men (40 and 45 years of age) exposed on different farms March 13 and March 22–23. Both had direct conjunctival contact with infected poultry. One was not wearing eye protection, and the other was wearing glasses that were bypassed by a feather. Neither was taking oseltamivir prophylaxis. Neither was vaccinated against human influenza virus. Symptoms developed 1–3 days after exposure (March 16 and 24). Conjunctivitis and coryza developed in the first patient, and conjunctivitis and headache developed in the second. Both received oseltamivir treatment, and symptoms resolved fully. Active daily surveillance by the local health unit identified no secondary cases.

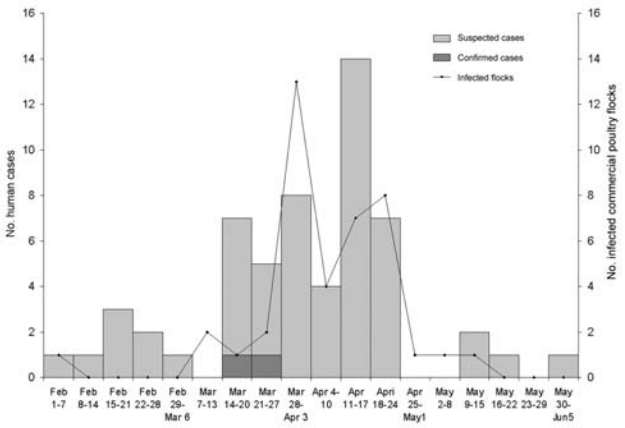

Figure. Onset of symptoms for suspected and confirmed cases in humans and identification of infected commercial poultry flocks, highly pathogenic avian influenza H7N3, British Columbia, 2004. Date for poultry flock is either the date the flock was suspected to be infected (because of clinical illness) or the date the sample was taken as part of surveillance.

Influenza A H7N3 virus was isolated from a nasal specimen from one man (A/Canada/444/04) and a conjunctival specimen from the other (A/Canada/504/04); both samples

Table 1. Characteristics of patients with suspected and confirmed A/H7N3 cases

Characteristic	Cases (%) (N = 57)
Male sex	32 (58)
Median age in y (range)	33 (1–68)
Received influenza vaccine	36 (65)
>2 wk before exposure	12 (22)
Occupation/relationship	
Farm owner	9 (16)
Family member	11 (19)
Farm employee	14 (25)
Farm manager	3
Egg collector	6
Chicken catcher	2
Miscellaneous worker	3
Federal worker	12 (22)
Veterinarian	4[a]
Inspector	3
General laborer	6[a]
Decomposition worker	1
Other	4 (7)
Unknown	5 (9)

[a]Includes confirmed A/H7N3 case.

Table 2: Clinical profile of suspected and confirmed infections of avian influenza H7N3 in humans, Canada

Symptoms	Cases (%) (N = 57)
Conjunctivitis	
Red eye	12 (21)[a]
Tearful eye	6 (11)[a]
Itching eye	13 (23)[a]
Painful eye	4 (7)
Burning eye	7 (12)
Discharge from eye	4 (7)[a]
Photophobia	5 (9)
ILI symptoms[b]	
Fever	17 (30)
Cough	42 (74)
Coryza	35 (61)[a]
Sore throat	39 (68)
Myalgia	26 (46)
Arthralgia	16 (28)
Fatigue	22 (39)
Diarrhea	11 (19)
Chills	16 (28)
Headache	28 (49)[a]
Other symptoms	12 (21)

[a]Includes confirmed A/H7N3 cases.
[b]ILI, Influenzalike illness.

were collected within 1 day of onset. No antibody to influenza A H7 could be detected by hemagglutination inhibition or microneutralization assays in serum samples collected 34 days and 8 and 22 days after onset, respectively.

Virus isolated from birds on the same source farm as the human isolate A/Canada/444/04 was confirmed as HPAI H7N3 by genotyping and IVPI. Virus from birds on the same source farm as A/Canada/504/04 showed insertion sequence match with HPAI H7N3, but IVPI was not performed (C. Kranendonk, National Centre for Foreign Animal Disease, pers. comm.). Both human isolates contained an insertion sequence similar to that seen only in the HPAI avian virus. These insertion sequences vary from the poultry virus by one and two amino acid differences, respectively. Based on the consensus sequence for HPAI H7 viruses, only A/Canada/504/04 is likely highly pathogenic in chickens (8). Phenotypic pathogenicity testing on the human isolates is ongoing.

Conclusions

We report the first known human avian influenza H7N3 infections. Although enhanced surveillance identified 57 persons meeting a suspected case definition, avian influenza infection was confirmed in only 2. The two patients had conjunctivitis and mild, influenzalike illnesses, similar to symptoms reported from the Netherlands in association with another H7 subtype (H7N7) (9). Neither confirmed case in British Columbia mounted a hemagglutination inhibition or serum neutralizing antibody response. This finding has been observed elsewhere in association with avian influenza infection (10,11). A possible explanation includes highly localized infection without induction of systemic antibody. Mechanical trauma, irritation due to dust or airborne particulate matter, or an allergic cause of symptoms associated with viral contamination, rather than infection, is less likely given the delay to symptom onset, consistent with the incubation period for influenza.

Among suspected cases, respiratory rather than conjunctival symptoms predominated. Other pathogens were also detected among suspected case reports, a finding consistent with the relatively nonspecific case definition applied.

From February 6 to May 21, 2004, routine influenza surveillance activities in the Fraser Valley also identified human influenza A from nine persons and two long-term care facility outbreaks. Although no coinfections were identified, this human influenza activity increased concerns about potential mixture of avian influenza with human influenza strains.

Avian influenza H7 has caused human illness previously, most notably 89 confirmed human infections, including one death in the Netherlands in 2003 (9). Based on the precedent set by the Netherlands in protecting exposed persons, British Columbia recommended comprehensive precautions for workers early in the outbreak. These precautions may have prevented further human infections. The strain circulating in British Columbia may have been more limited in its ability to cause human illness. The genomic sequence of the avian viruses from the source farms of the two human isolates was consistent with HPAI, whereas one of the human isolates was consistent with LPAI. The presence of an insertion sequence in the human LPAI isolate likely signifies that the virus in poultry mutated from HPAI to LPAI, and both were circulating among the birds on that source farm, the latter undetected. A less likely explanation is that mutation from HPAI to LPAI occurred in the human host.

To date, illness in humans from H7 subtypes differs markedly in severity from that of avian influenza H5N1 (12). Their lower virulence should not be inferred to indicate lower pandemic potential since subclinical or mild infections may have greater opportunity through surreptitious spread to reassort and through mutation to become more virulent. A compilation and detailed overview of the protective measures used in all avian influenza outbreaks would help to estimate the actual risk to persons and populations. Recommendations for precautions that are both necessary and reasonable during future poultry outbreaks could then be refined.

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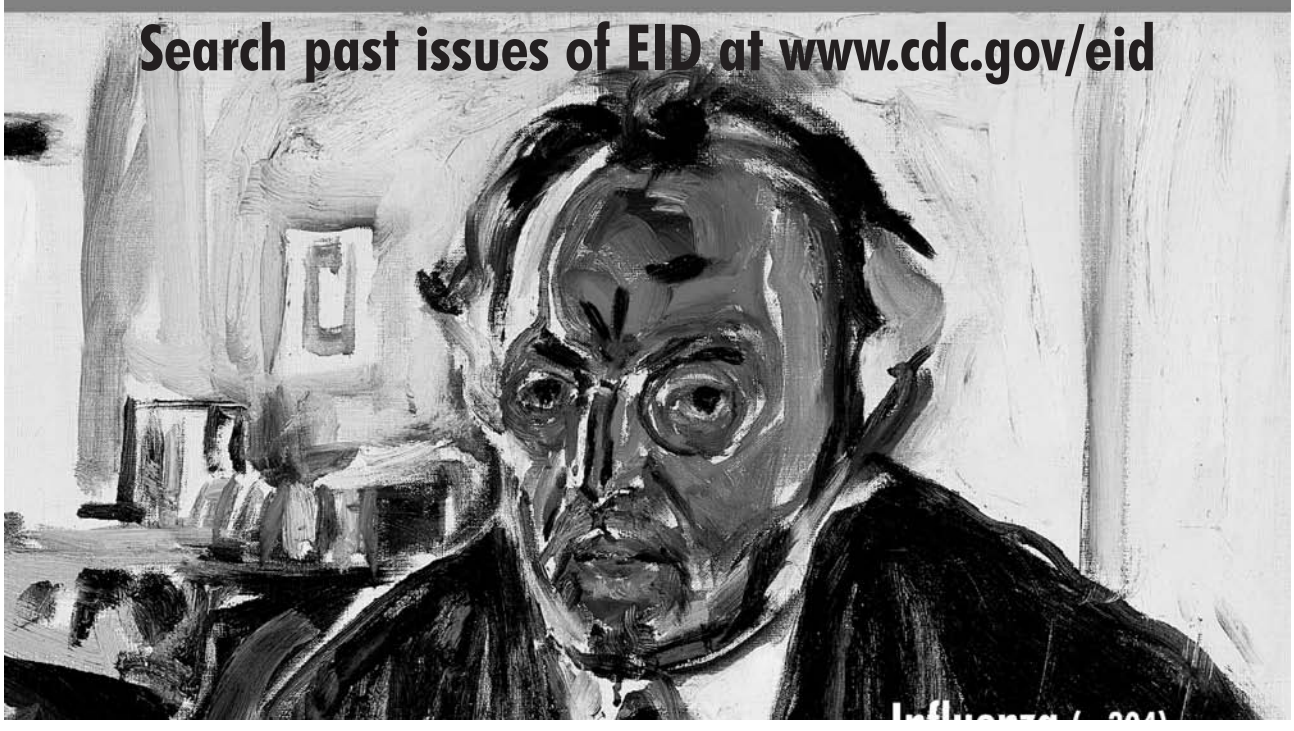

SARS Molecular Detection External Quality Assurance

Christian Drosten,* Hans Wilhelm Doerr,† Wilina Lim,‡ Klaus Stöhr,§ and Matthias Niedrig¶

Inactivated severe acute respiratory syndrome–associated coronavirus samples were used for an external quality assurance study within the World Health Organization SARS Reference and Verification Network and other reference institutions. Of 58 participants, 51 correctly detected virus in all samples ≥9,400 RNA copies per milliliter and none in negative samples. Commercial test kits significantly improved the outcome.

Severe acute respiratory syndrome (SARS) is an infectious interstitial pneumonia that causes death in a considerable portion of patients. The first epidemic of SARS began in November 2002 in southern China, spread to all five continents, and was interrupted in July 2003. It caused 774 deaths among the 8,098 cases. Two laboratory-associated infections and four new isolated cases have since occurred (1). SARS is caused by a novel coronavirus (SARS-CoV) that is shed in patients' respiratory secretions after infection (2–5). Immune response to SARS-CoV appears with a latency of up to 4 weeks from infection, and the concentration of virus particles varies greatly between patients or types of clinical samples. Thus achieving a reliable virologic diagnosis early after disease onset is difficult. Highly sensitive methods for virus detection, such as reverse transcription–polymerase chain reaction (RT-PCR) are required to confirm SARS in the acute phase and prevent transmission.

Molecular detection methods have been developed by several research laboratories, and the first commercial test kits have become available (6,7). The performance of such tests, however, has only been evaluated in pilot feasibility studies. Little data exist about the relative performance of different laboratories and methods. The World Health Organization (WHO) has made the comparing and standardizing of laboratory tests an issue of high priority in SARS research (8). Comparative testing of characterized samples is a direct way to identify weaknesses of single laboratories or certain methods.

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The Study

We present the results of the first external quality assurance study on SARS-CoV molecular detection. Ninety-three institutions involved in laboratory diagnostics of SARS were invited to participate in the study. Invitees were members of the international WHO SARS Reference and Verification Laboratory Network (9) or national and regional SARS reference laboratories. The study was announced as an external quality assurance study on diagnostic proficiency, which included certifying and publishing the results in a comparative and anonymous manner. Fifty-eight laboratories from 38 countries (21 European, 9 Austral-Asian, 7 North and South American, and 1 African) eventually enrolled in the study.[1] Four companies that produced commercial diagnostic test systems also participated but were evaluated separately because they do not fulfill public health duties.

Virus material was obtained from supernatants of Vero cell cultures collected one day after infection with SARS-CoV strains Frankfurt 1 and HKU-1. The supernatants were heated to 56°C for 1 h and γ irradiated with 30 kGy. Residual infectivity was excluded by Vero cell cultures (3 passages). Aliquots of the inactivated virus stock solutions were lyophilized and redissolved, and the virus RNA was quantified by two different noncommercial real-time RT-PCR assays (2,6). Virion integrity was confirmed by morphology by electron microscopy (data not shown). Test samples for the study were generated by diluting the inactivated virus stock solutions in human fresh-frozen plasma testing negative for HIV-1, hepatitis B virus, hepatitis C virus, and SARS-CoV by RT-PCR. Aliquots of 100 µL each were then lyophilized and shipped at ambient temperature to the participating laboratories. Each participant received a coded panel of seven positive and three negative samples. Virus-positive samples contained 94–940,000 RNA copies per milliliter after resuspending in 100 µL of water. The participants were asked to analyze the material

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with the molecular methods they routinely use in suspected cases in humans. Details about the methods were requested, such as the sources of RT-PCR primers and protocols, the type of extraction method used, and suppliers and types of commercial kits, if used. The following two criteria were chosen as minimum requirements for overall proficiency. First, laboratories had to correctly detect the four samples containing ≥9,400 copies of viral RNA per milliliter, a concentration well above the detection limit of published and commercial nucleic acid amplification tests (NAT) for SARS-CoV, (6,7,10–12). Second, no false-positive results were allowed with the negative samples. Indeterminate results in positive samples were treated as negative and in negative samples were treated as positive since the application of NAT usually does not involve indeterminate endpoints, and laboratories should be able to resolve unclear results by double testing with another amplification assay (13).

Before evaluating the performance of individual laboratories, we determined how many participants managed to detect virus in each sample (Table 1). The concentration-dependent, cumulative positivity rates per sample corresponded exactly with the response rates calculated by a probit regression analysis, which is equivalent to a dose-response model (Figure, $p \leq 0.0001$). The model could predict for the average laboratory that 50% of all test results could be expected to be correctly positive when 158 (95% confidence interval [CI] 76.55–269.15) copies of virus RNA per milliliter of sample were present, and 95% with more than 11,220 (95% CI 5,675–31,988) copies per milliliter. Good compliance with the model furthermore confirmed that all samples contained the expected concentration of RNA upon reception by the participants and that no RNA degradation had occurred even in samples containing low amounts of virus.

Applying the proficiency criteria, 51 (88%) of 58 laboratories passed the minimum requirements for successful participation. Failure in three laboratories was due to lack of sensitivity, in three due to false-positive results, and in one due to both. Thirteen of 51 successful laboratories (22.4% of all 58 participants) could also detect the virus in all three weakly positive samples (≤2,350 copies/mL), and another 17 missed only one positive sample. Ten of the 58 laboratories issued indeterminate results in one or more samples.

Whether common technical factors would influence the performance of laboratories was also assessed. We subjected cumulative results from low concentration samples (≤2,300 copies/mL) to analysis of variance (ANOVA) analysis. The overall positivity rate in these samples was 65.6% (95% CI 56.1%–75.0%). Seven technical factors (Table 2) were used to characterize the test procedures each laboratory was using. Only use of commercial RT-PCR test kits made a significant difference with regard to total sensitivity. This finding was in concordance with results of the four participating companies who manufacture these kits: all were 100% correct. Fourteen of 58 participants used commercial test kits. For noncommercial tests, whether laboratories developed primers themselves or adapted from other researchers did not make a difference. This finding might be due to availability of well-evaluated primers through a WHO internet resource during the outbreak (14). Forty-two of the 58 participants used at least one procedure listed on this site.

Table 1. Positive samples in test panel[a]

Sample code	SARS-CoV strain	Virus RNA concentration copies/mL	Fraction of laboratories with positive detection (%)
S-CV2	Frankfurt 1	940,000	100
S-CV9	Frankfurt 1	94,000	98.3
S-CV6	HKU-1	23,500	98.3
S-CV4	Frankfurt 1	9,400	94.8
S-CV10	HKU-1	2,350	87.9
S-CV1	Frankfurt 1	940	70.7
S-CV5	Frankfurt 1	94	43.1

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus.

We finally assessed whether laboratories belonging to the international WHO SARS Reference and Verification Network (9) were more proficient in SARS molecular detection than others. In the three samples containing ≤2,350 copies of SARS-CoV RNA per milliliter, the network laboratories achieved a cumulative fraction of correct positive results of 79.5% (95% CI 60.2%–98.9%) as opposed to 61.5% (95% CI 50.6%–72.4%) in the other

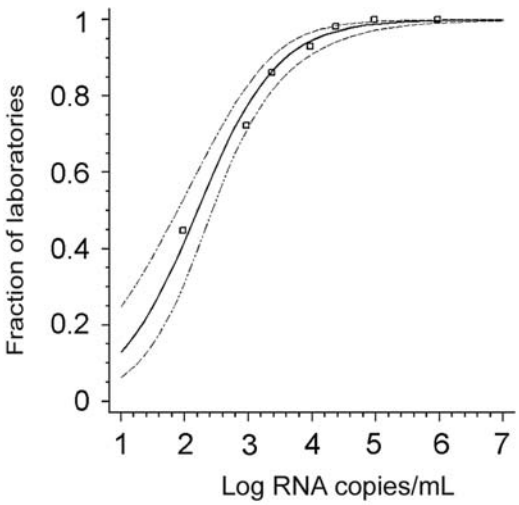

Figure. Probit analysis of the fractions of laboratories achieving a positive result (y-axis) in relation to the virus RNA concentration in a given positive sample (x-axis). Data points represent individual samples in proficiency test panel. The thick line is the regression line calculated on the basis of a probit model (dose-response curve); the thin lines are 95% confidence intervals. Data fit into the model with $p < 0.0001$.

Table 2. Factors influencing the performance of laboratories[a]

Possible technical influence factors	No. of laboratories	Positive influence on sensitivity p value
Qiagen viral RNA extraction kit	38	0.9
Roche MagnaPure/HighPure extraction kit	7	0.2
Silica particle-based extraction method (Boom)	9	0.9
Primers originally developed in own laboratory	16	0.5
Any nested PCR assay	25	0.9
Any real-time PCR assay	37	0.7
Any commercial test kit	14	0.03

[a]Analysis of Variance (ANOVA) by factor, eliminating the influence of other factors; PCR, polymerase chain reaction.

labs participating in the study. This difference was not significant (p value = 0.11, *t*-test).

Conclusions

The results of this first external quality assurance study on SARS-CoV molecular detection are assuring. Compared to an earlier study on molecular testing for filoviruses, Lassa virus, and orthopoxviruses, using very similar proficiency criteria (15), almost double the portion of participating laboratories completed the study successfully (88% vs. 45.8%). On the other hand, this study only examined paramount issues like sensitivity and control of contamination. Validation of other aspects, like cross-reactivity of primers or control of PCR inhibition, is the responsibility of each diagnostic laboratory.

Commercial tests clearly were the preferred way of achieving good diagnostic performance, possibly because SARS-CoV is a pathogen with which relatively few laboratories have had experience. However, developing and approving commercial tests is a lengthy process and high costs limit their application. Other approaches have to be adopted for efficiently providing good diagnostic tools in immediate response to an infectious disease outbreak. WHO's strategy of disseminating essential information through a public Internet resource before publication has proven successful. Laboratories have willingly shared protocols and positive control material with other institutions, enabling qualified diagnostics within weeks after the primary description of the new virus. The benefit is proven by good overall results in this study.

International strain collections should be complemented with noninfectious reference material of rare pathogens. Until now, such material has been available only for highly prevalent agents like HIV-1, herpes viruses, or hepatitis viruses. For SARS-CoV, reference material has been created in this study for the first time. All samples described can be obtained for a nonprofit charge through the WHO SARS Reference and Verification Laboratory Network.

This study was performed by the WHO and the European Network for Diagnostics of Imported Viral Diseases, funded by the European Community DG SANCO under the program AIDS and other communicable diseases, grant no. SI2.299717 (2000CVG4-26). Work of the Bernhard-Nocht Institute was funded by the German Ministry of Health under grant No. 325-4539-85/3.

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Detecting West Nile Virus in Owls and Raptors by an Antigen-capture Assay

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We evaluated a rapid antigen-capture assay (VecTest) for detection of West Nile virus in oropharyngeal and cloacal swabs, collected at necropsy from owls (N = 93) and raptors (N = 27). Sensitivity was 93.5%–95.2% for northern owl species but <42.9% for all other species. Specificity was 100% for owls and 85.7% for raptors.

The emergence of West Nile virus (WNV) in North America has created a demand for reliable, rapid, and economical tests for detecting this flavivirus (family *Flaviviridae*) in a variety of species and sample types. The VecTest (Medical Analysis Systems, Camarillo, CA), a rapid antigen-capture wicking assay, was previously reported to detect WNV in mosquitoes (1,2) and corvid birds (family *Corvidae)* (3).

Little is known about the ability of this test to detect WNV in avian species other than corvids. Low sensitivity of the test when applied to oropharyngeal swabs from dead raptors has been recently reported (3). Since the test is largely dependent on the concentration of viral antigen in the analyzed sample (2,3), the test is more likely to detect WNV in swabs collected from birds that shed large quantities of the virus. Timing of the sample collection with relation to the course of the infection (i.e., acute versus subacute or chronic) is also expected to play a key role, as virus shedding generally is short-lived (4).

The objective of this study was to evaluate the usefulness of the VecTest for detecting WNV in oropharyngeal and cloacal swabs from North American owls (family *Strigidae*) and raptors (families *Falconidae*, *Accipitridae*, *Pandionidae*). Based on an observed higher susceptibility of northern versus southern owl species to WNV (5), we hypothesized that patterns of virus excretion that influence the sensitivity of this test might differ. Owl species were classified as northern if most of their natural breeding range was north of latitude 48°N, or southern if it was otherwise (6).

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The Study

Oropharyngeal and cloacal swabs were collected at necropsy from 87 birds representing 14 species of North American owls, one Eurasian owl, and one falcon that died at the Owl Foundation, Vineland, Ontario (43°10' N, 79°20' W) from April 15 to December 25, 2002. This rehabilitation facility had a large-scale WNV outbreak from July to September 2002 (5). All birds were kept frozen at –20°C from shortly after the time of death until examination (8–12 months later). Before necropsy, carcasses were allowed to thaw for 24 to 48 h at 4°C.

Oropharyngeal swabs were also collected from 7 owls and 26 diurnal raptors submitted for necropsy to the Canadian Cooperative Wildlife Health Center diagnostic service at the Ontario Veterinary College. These birds were collected from a variety of localities in Ontario and died or were euthanized from August 10, 2002, to July 22, 2003. Most were originally presented to the college's wild bird clinic for veterinary care.

Swabs were collected by rubbing sterile cotton-tipped applicators (provided with the VecTest kit) against the oropharyngeal or cloacal mucosa for 10 s. They were then frozen at –80°C until analyzed (2–6 months for Owl Foundation birds) or tested immediately (wildlife center birds).

For each bird, a full diagnostic necropsy was performed, followed by collection of tissue samples. A pooled sample (about 50 μg in total) of brain, lung, kidney, liver and spleen (foundation birds), or kidney and brain (wildlife center birds), was collected from each bird and tested for WNV RNA by real-time reverse transcription–polymerase chain reaction (RT-PCR) as previously described (7). Reported mean C_T values (cycles required to reach a fluorescence threshold) are based on 2 to 3 runs per bird using the generic 3′NC primer set (6). C_T values were available only for Owl Foundation birds.

Before testing, swabs were soaked in 0.5 mL of the grinding solution (provided in the VecTest kit). If initially frozen, swabs were allowed to thaw in the solution for ≥30 min at room temperature. The test was performed according to the manufacturer's instructions, as previously described (2,3). All samples were centrifuged for 4 min at 5,200 x *g* before the test strip was inserted. Appearance of even the faintest red line on the test zone at 15 min was considered a positive result (Figure).

To assess the repeatability of the test, 15 samples were tested simultaneously in duplicates. In two cases, where results were inconclusive because of appearance of uniform red smearing in the test zone, samples were diluted 1:2 in grinding solution and retested. One of those tested

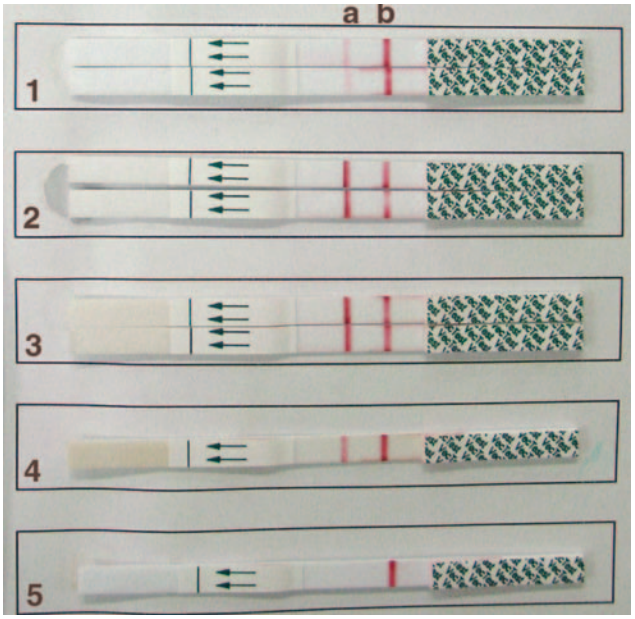

Figure. Results of testing by the VecTest assay. Each strip has a test zone (a) and a positive control zone (b). Samples 1–3 were run in duplicate. Note the difference in band intensity between sample 1 vs. samples 2 and 3 (all three are positive). Sample 4 was a positive control and sample 5 was a negative control.

positive; the other sample remained inconclusive. For each VecTest kit (50 tests) one positive control (brain and kidney homogenate from an American Crow confirmed to be WNV positive by RT-PCR) and one negative control (water) were tested.

The effect of geographic range and taxonomic group (e.g., owls vs. raptors) on the VecTest sensitivity and on C_T values was tested by the Fisher exact test and the Student *t* test, respectively, by using the SAS 8.2 software (SAS Institute Inc., Cary, NC). Based on natural breeding range, the following species were classified as northern: Snowy Owl, Great Gray Owl, Northern Hawk Owl, Boreal Owl, and Northern Saw-whet Owl (for scientific names see Table) These are also the species that had death rates >90% during the 2002 WNV outbreak at the Owl Foundation. All other owl species were considered southern and had death rates of up to 16.7%. These differences and the epidemiology of the outbreak at the Owl Foundation have been described elsewhere (5).

Of 120 birds tested by real-time RT-PCR, 89 (74.2 %) were positive, 30 (25.0 %) were negative, and one was inconclusive (and therefore excluded from further analysis) for WNV. All duplicates gave identical results for each pair.

Of the oropharyngeal swabs tested by VecTest, 71 (59.2%) of 120 were WNV positive (Owl Foundation and wildlife health center birds). When the RT-PCR results were used as the standard, the sensitivity of the VecTest was 77.5% for all birds. The test was significantly more sensitive for owls (85.5%) than for diurnal raptors (30.8%) ($p < 0.001$), and for northern owl species (95.2%)

Table. RT-PCR results and sensitivity of the VecTest assay in 19 species of North American owls and raptors tested for West Nile virus[a]

	RT-PCR		VecTest					
			OP swab		Cloacal swab		Combined[b]	
Species	N	No. positive	N	Sn (%)	N	Sn (%)	N	Sn (%)
Great Gray Owl (*Strix nebulosa*)[c]	19	17	19	100.0	19	100.0	19	100.0
Northern Hawk Owl (*Surnia ulula*)[c]	16	16	16	100.0	15	93.3	15	100.0
Boreal Owl (*Aegolius funereus*)c	13	10	13	80.0	13	90.0	13	90.0
Northern Saw-whet Owl (*Aegolius acadicus*)[c]	12	10	12	100.0	12	100.0	12	100.0
Snowy Owl (*Bubo scandiacus*)[c]	9	9	9	88.9	9	77.8	9	88.9
Great Horned Owl (*Bubo virginianus*)	10	8	10	62.5	4	50.0	4	50.0
Short-eared Owl (*Asio flammeus*)	3	2	3	50.0	3	50.0	3	50.0
Long-eared Owls (*Asio otus*)	3	1	3	100.0	3	100.0	3	100.0
Barred Owl (*Strix varia*)	2	0	2	–	2	–	2	–
Northern Pygmy-Owl (*Glaucidium gnoma*)	1	1	1	0.0	1	0.0	1	0.0
Flammulated Owl (*Otus flammeolus*)	1	1	1	0.0	1	0.0	1	0.0
Tawny Owl (*Strix aluco*)	1	1	1	0.0	1	100	1	100
Eastern Screech-Owl (*Megascops asio*)	2	0	2	–	2	–	2	–
Spotted Owl (*Strix occidentalis*)	1	0	1	–	1	–	1	–
American Kestrel (*Falco sparverius*)	1	0	1	–	1	–	1	–
Red-tailed Hawk (*Buteo jamaicensis*)	19	11	19	36.4	0	–-	0	–
Cooper's Hawk (*Accipiter cooperii*)	2	1	2	0.0	0	–	0	–
Sharp-shinned Hawk (*Accipiter striatus*)	4	1	4	100.0	0	–	0	–
Osprey (*Pandion haliaetus*)	1	0	1	–	0	–	0	–
Total	120	89	120	77.5	86	88.4	86	91.4

[a]RT-PCR, real-time reverse transcription–polymerase chain reaction; OP, oropharyngeal; N, sample size; Sn, sensitivity.
[b]Results of oropharyngeal and cloacal swab testing were considered in parallel (i.e., the bird was considered positive if one of the two tests was positive).
[c]Northern owl species.

than for southern owl species (42.9%) ($p < 0.001$). The difference between raptors and southern owl species was not significant.

The specificity of the VecTest when applied to oropharyngeal swabs was 93.3% for all birds, 100% for owls, and 85.7% for diurnal raptors. Both false-positive results involved Red-tailed Hawks (*Buteo jamaicensis*), one of which had nonsuppurative encephalitis and myocarditis consistent with WNV infection but was WNV negative on PCR and immunohistochemical tests. The positive predictive value (PPV) was 97.2% for all birds, 100% for owls, and 66.7% for diurnal raptors. The negative predictive value (NPV) was 58.3% for all birds, 59.3% for owls, and 57.2% for diurnal raptors.

Of the cloacal swabs tested by VecTest, 61 (71.8%) of 85 were WNV positive (Owl Foundation birds only). Based on the RT-PCR results, the sensitivity of the VecTest when applied to cloacal swabs from all Owl Foundation birds was 88.4%. The test was significantly more sensitive for northern owl species (93.5%) than for other species (42.9%) ($p < 0.001$). The specificity and PPV of the test were 100%, and the NPV was 66.73% for all Owl Foundation birds.

When the test results of both oropharyngeal and cloacal swabs were considered in parallel (Owl Foundation birds only), 64 (73.6 %) of 87 birds tested positive with an overall sensitivity of 91.4%, specificity and PPV of 100%, and NPV of 72.7%. The sensitivity was 96.8% and 50% for northern and southern owl species, respectively.

C_T values were significantly lower (mean 16.78 ± 0.32, $n = 62$) for northern owl species than for southern owl species (mean 24.56 ± 0.88, $n = 8$) ($p < 0.0001$). Birds that were misclassified as negative by the VecTest using either oropharyngeal or cloacal swabs had significantly higher C_T values (mean 26.13 ± 4.64, $n = 10$) compared to all other positive birds (mean 16.25 ± 1.53, $n = 60$) ($p < 0.0001$).

Conclusions

The VecTest proved to be highly sensitive for detecting WNV in oropharyngeal or cloacal swabs from northern owl species, but it showed low sensitivity for samples from southern owl species and raptors. Unlike results with corvids (3), cloacal swabs were slightly superior to oropharyngeal swabs. This finding may reflect greater virus shedding from the digestive or urinary systems in northern owl species; however, this hypothesis requires further investigation. Testing both swabs in parallel produced the highest sensitivity. The overall specificity of the VecTest was similar to that reported in corvids (3), but it was higher for owls than for raptors (100% vs. 85.7%). Both false-positive samples were from Red-tailed Hawks.

The difference between northern owl species and all other species may reflect higher titers of WNV in the carcasses of northern birds, as indicated also by lower C_T values. Northern owl species died significantly earlier during the outbreak period at the Owl Foundation and had high death rates (5). These findings suggest differences at the level of the host-virus interactions, possibly affecting virus replication, virus shedding, or the course of the disease (i.e., acute versus chronic). Again, this hypothesis requires further investigation.

The VecTest may be useful as a screening test in birds with suspected WNV infection. However, negative results should be interpreted with caution in light of the test's low sensitivity in some species.

Acknowledgments

We thank Katherine McKeever and the Owl Foundation staff for their assistance in sample and data collection, Chris Enright and Amanda Low for assistance in performing necropsies, Antonia Dibernardo and Heather White for technical assistance, and Dale A. Smith for critically reading the manuscript.

A stipend from the Rathlyn Foundation made this study possible.

Dr. Gancz is a veterinarian enrolled in the Doctor of Veterinary Science Program in Avian and Exotic Pet Medicine at the Ontario Veterinary College, University of Guelph, where he combines clinical training with West Nile virus research.

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Parastrongylus cantonensis in a Nonhuman Primate, Florida

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Parastrongylus (= *Angiostrongylus*) *cantonensis* is a parasitic nematode of Norway rats throughout tropical regions. This parasite is neurotropic and causes disease and death in humans and other mammals. We report the first identification of *P. cantonensis* as the cause of a debilitating neurologic disease in a captive primate in Florida.

The Case

On July 28, 2003, an acute and disabling neurologic disorder developed in a captive 49-year-old male white-handed gibbon (*Hylobates lar*) from the Miami Metrozoo in Florida. The gibbon was born in the wild but had been in captivity in Florida, USA since 1963. The onset of severe quadriparesis occurred overnight, without signs of prior illness. In addition to extreme weakness of all limbs and the inability to support his body, the gibbon had a slight lip droop but was able to swallow and had no other detectable cranial nerve signs. The gibbon was behaviorally depressed but responsive and aware of his surroundings. Blood analyses and thoracic radiographs did not show a cause for the clinical disease, and blood eosinophil count was not elevated. The animal did not show improvement after 48 hours and was euthanized by intravenous injection of Euthasol (Delmarva Laboratories Inc., Midlothian, VA). On postmortem examination, chronic renal disease and moderate cardiac fibrosis and endocardiosis were observed, but these conditions were considered to be age-related. Gross abnormalities were absent on external surfaces of the central nervous system (CNS). Tissues of the CNS were preserved in 10% formalin.

Tissue samples from regions of the CNS were embedded in paraffin by using standard procedures. Tissue sections were cut (6 µm) and stained with hematoxylin and eosin. In the transverse sections, the only gross lesion observed was a thin, white discolored area in the left dorsal funiculus in the cranial cervical spinal cord segments. Microscopic examination showed necrosis and inflammation located primarily in the dorsal gray columns throughout the cervical spinal cord. This lesion was bilateral with some involvement of the adjacent funiculi. The areas of inflammation included numerous eosinophils and transverse sections of one or more parasitic nematodes (Figure 1). A few lesions were found in the ventral gray columns. This destructive lesion extended into the caudal medulla. Rostral to this were a few scattered destructive lesions, and a few sections of the parasite were present in the leptomeninges. Wallerian degeneration occurred in the dorsal funiculus where the white discoloration was observed on gross examination.

Preserved tissues of the CNS were examined for nematodes at 4x magnification with a dissecting microscope (Zeiss, Micro-Med Instruments, Inc., Walden, NY). Four nematodes were recovered from the meninges of the brain and spinal cord. One intact male nematode and additional pieces of a male nematode were recovered from the

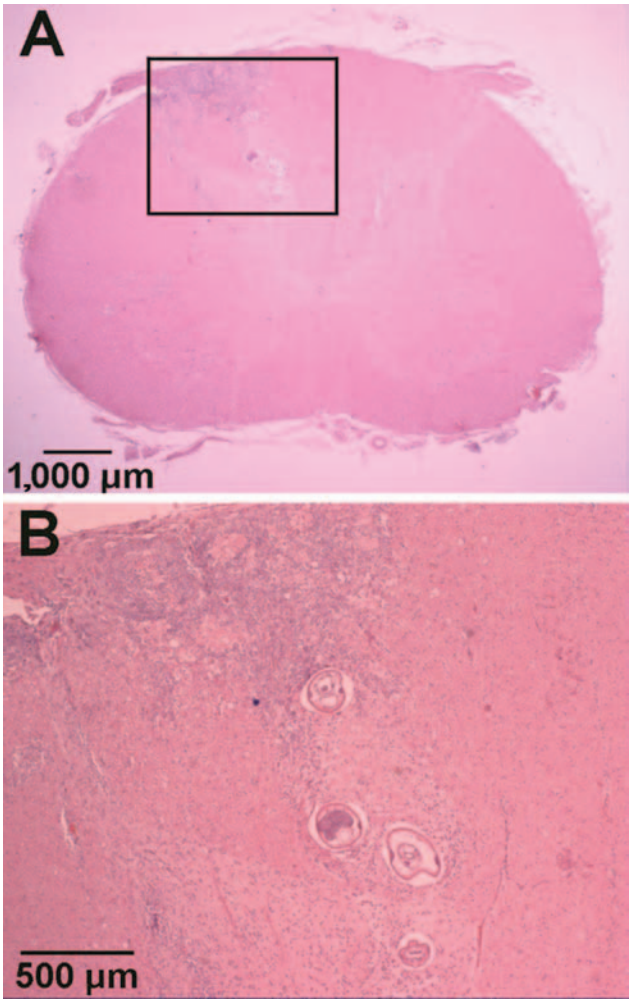

Figure 1. Hematoxylin and eosin–stained sections of *Parastrongylus cantonensis* in the parenchyma of the cervical spinal cord of a gibbon (*Hylobates lar*) from Florida (A). Enlarged image of inset from panel A (B).

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subarachnoid space of the cervical spinal cord. A second intact male nematode was recovered from the subarachnoid space of the cerebellum, and additional pieces of a nematode were recovered from the subarachnoid space of the cerebrum. The partial nematodes had damaged anterior and posterior ends. Nematodes were examined at 40–400x magnification, and digital images of specimens were captured (Olympus Model DP12, Olympus Optical Co., Ltd., Tokyo, Japan). Corresponding images of a stage micrometer allowed the sizes of morphologic characteristics to be determined. A key morphologic feature of the three male nematodes was the presence of a well-developed bursa (Figure 2). This feature indicated their classification within the order Strongylida. Within this group, members of the superfamilies Diaphanocephaloidea, Ancylostomatoidea, and Strongy-loidea were excluded based on morphologic features of the anterior end. Members of the Trichostrongyloidea were also excluded on the basis of the absence of a cephalic vesicle and longitudinal cuticular ridges. In addition, with the exception of the family Dictyocaulidae, all Trichostrongyloids are restricted to the intestinal tract. Furthermore, no strongylid nematodes from the aforementioned superfamilies have been reported from the CNS. The nematodes from the gibbon CNS were thus classified as members of the superfamily Metastrongyloidea.

Sprent (1) and Anderson (2) documented extensively the nematodes reported from the CNS of mammals. Within the Metastrongyloidea, these include *Parelaphostrongylus* spp., *Elaphostrongylus* spp., *Skrjabingylus* spp., *Gurltia paralysans*, and *Parastrongylus* spp. Of those nematodes, only *Parastrongylus* spp. have been reported previously from the CNS of primates. As such, the causative agent was presumed to be a species of the genus *Parastrongylus*. This genus comprises *P. cantonensis*, *P. malaysiensis*, *P. mackerrasae*, *P. sandarsae*, *P. siamensis*, *P. costaricensis*, *P. dujardini*, *P. schmidti*, *P. tateronae*, *P. ryjikovi*, *P. sciuri*, and *P. petrowi* (3). Of these species, *P. cantonensis*, *P. malaysiensis*, and *P. costaricensis* have been reported previously from primates.

Spicule measurements (1,140–1,180 μm) of nematodes from the gibbon (Figure 2) correlated well with measurements reported for *P. cantonensis*. With the exception of *P. malaysiensis*, *P. cantonensis* is distinguished from all other *Parastrongylus* spp. based on spicules ≥1,000 μm. Spicules from *P. malaysiensis* are on average 940 μm (800–1,200 μm). *P. cantonensis* and *P. malaysiensis* are distinguished on the basis of the morphology of bursal rays from male nematodes (4). The bursal rays of nematodes recovered from the gibbon were consistent with those of *P. cantonensis*. Based on a combination of morphologic features, spicule measurements, host species, and location within the host, we concluded that the infecting nematodes were *P. cantonensis*. The male specimens of *P. cantonensis* were deposited in the U.S. National Parasite Collection (Beltsville, MD) under accession number 94698.

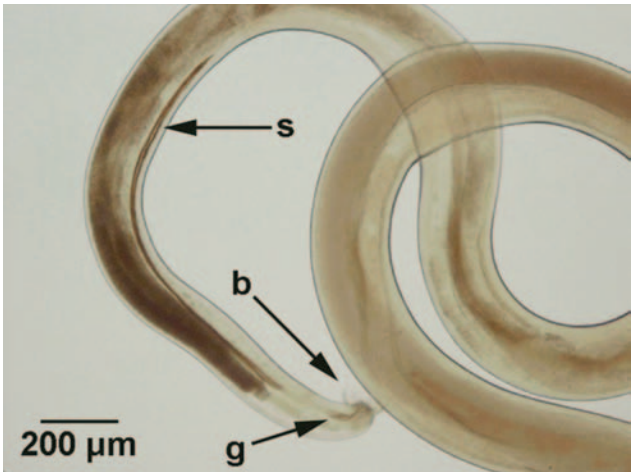

Figure 2. Morphologic features of a male nematode recovered from the central nervous system of a gibbon (*Hylobates lar*). The characteristics used for specific identification of *Parastrongylus cantonensis* were the presence of a bursa (b), a gubernaculum (g), and the size of spicules (s).

Conclusions

P. cantonensis are parasitic nematodes that reside in the cardiopulmonary system of their rat (*Rattus* spp.) definitive hosts. The parasite is common in Southeast Asia and the South Pacific and has been reported in Madagascar, Japan, Egypt, and India (5). Reports also document *P. cantonensis* from the Western Hemisphere in Cuba, Puerto Rico, Dominican Republic, Bahamas, Jamaica, and Haiti (6,7). From the mainland United States, *P. cantonensis* is known only in Louisiana (8) and Mississippi (9). Naturally acquired infections in these two areas resulted in neurologic disease in a horse (9), in wildlife species (10), and in both human (11) and nonhuman primates (10,12). Onset of neurologic signs in humans occur 1–45 days after infection (13,14). This feature suggests that the captive gibbon in this report likely acquired the infection shortly before onset of disease. The advanced age of the animal may have been a factor in the severity of the disease. Although deaths have been reported in human adults, infections typically result in transient neurologic debilitation (14). Conversely, death can occur in 5% of children (13). Comparative data are lacking for disease severity in older persons.

Transmission of *P. cantonensis* infection requires first-stage larvae (L1) from rat feces to infect and develop into the infective-stage (L3) within obligate gastropod intermediate hosts. Infection of rats and other mammalian hosts requires ingestion of L3, commonly through ingestion of

infected gastropods. However, the L3 of *P. cantonensis* can also emerge in gastropod mucus trails and thereby contaminate surrounding vegetation (15). This mode of transmission was reported in a human outbreak of eosinophilic meningitis in Jamaica, where disease was correlated with eating Caesar salad (16).

The present distribution of *P. cantonensis* within the United States is unknown outside of Louisiana (8) and Mississippi (9). The finding of *P. cantonensis* in the captive gibbon in this report suggests that the parasite may be established in Miami, Florida. Since neurologic disease may occur in human and nonhuman primates, dogs, horses, and numerous other species (9–12), documentation of the present distribution of *P. cantonensis* will prove valuable for monitoring the spread of this zoonotic parasite. *P. cantonensis* have a number of susceptible aquatic (17) and terrestrial gastropod intermediate hosts in the eastern United States (2,8). In combination with the ubiquitous distribution of rats, the probability for spread and establishment of *P. cantonensis* exists within eastern North America.

The dispersal of *P. cantonensis* has, in some instances, been linked to the introduction of the African giant land snail, *Achatina fulica* (5). Three specimens of *A. fulica* were introduced into Miami, Florida, from Hawaii in 1966 (18). These exotic snails became established but were considered eradicated from Florida by 1975 (18). A survey of *A. fulica* in Hawaii in the mid-1960s showed that *P. cantonensis* infection was both highly prevalent and intense (19), which suggests that the parasite may have been introduced to Miami with the translocated snails in 1966. *P. cantonensis* was believed not to establish successfully (18). However, this event or a similar translocation involving less conspicuous exotic gastropods may have resulted in the inadvertent introduction of *P. cantonensis*. Live *A. fulica* are confiscated routinely from tourists returning to mainland United States from Hawaii, and 75 other exotic gastropod species have been introduced to the United States accidentally, inadvertently, or intentionally (20). The introduction of *P. cantonensis* with infected rats from areas where the parasite is endemic would be equally conceivable, as was proposed for its introduction to New Orleans, Louisiana (8).

P. cantonensis may be established in rat and gastropod populations in Miami. However, given the role of emergent L3 in transmission of infections (15,16), the L3 involved in the present case may have been acquired from contaminated vegetation supplied commercially from another region. The gibbon in this report was fed a commercial monkey chow and a variety of produce both grown locally and imported from several regions of North, Central, and South America. *P. cantonensis* is not known to be in any of these supply regions. The source of *P. cantonensis* in the gibbon infection remains to be determined. However, *P. cantonensis* was definitely introduced to Miami through the translocation of either infected animals or contaminated plants.

Future investigations aim to conduct parasitologic surveys of rats and gastropods in Miami, Florida, and develop molecular tools for specific identification of *P. cantonensis*. The specific source of infection in the present case report remains unknown. However, our report indicates that infection with *P. cantonensis* should be included as a differential diagnosis for instances of neurologic disease in human and nonhuman primates, as well as in wildlife and veterinary species in the southeastern United States.

Acknowledgments

We thank Judith Appleton and Dwight Bowman for advice, assistance, and use of microscopes and imaging equipment and Paige Adams for helpful comments on the manuscript.

Dr. Duffy is a research associate at the James A. Baker Institute for Animal Health, Cornell University. His research interests include neurotropic parasitic nematodes and acquired immunity to these infections.

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Naturally Acquired *Plasmodium knowlesi* Malaria in Human, Thailand

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We describe a case of naturally acquired infection with *Plasmodium knowlesi* in Thailand. Diagnosis was confirmed by the small subunit ribosomal RNA and the mitochondrial cytochrome b sequences. The occurrence of simian malaria in human has signified the roles of wild primate populations in disease transmission in some malaria-endemic areas.

A number of emerging pathogens have been known to cross-transmit between humans and nonhuman hosts. Wild primate populations have the potential to serve as origins and reservoirs of certain human pathogens, ranging from virus to helminths (1). More than 26 species of *Plasmodium* circulate among primate populations (2). Several of the simian malaria species are closely related to the human ones, and some of these, e.g. *Plasmodium simium*, *P. brasilianum*, *P. cynomolgi*, *P. inui*, and *P. knowlesi*, have been implicated in symptomatic malaria in humans in experimental, accidental, or natural infections (2–7). Before the advent of molecular tools for diagnosing infectious diseases, identifying simian malaria in humans required expertise in the structure of these parasites, experimental studies in mosquito vectors, and tests for infectivity to primate hosts (6,7). In general, simian malaria is not included in the differential diagnosis of human infections, which could partly stem from lack of awareness about the zoonotic potential of these parasites. Furthermore, the current laboratory methods for species differentiation target only the four human plasmodia species. On the other hand, simian malaria species that display structural similarity to those species commonly found in humans may be unnoticed in routine examinations of blood smears. We describe a patient who acquired *P. knowlesi* infection while staying in a forest in southern Thailand where human malaria is endemic.

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The Study

In August 2000, a 38-year-old Thai man came to an outpatient department of King Chulalongkorn Memorial Hospital, Bangkok, with daily fever, headache, intermittent chill, sweating, and malaise for 4 days. His home was in a suburb of Bangkok, where no malaria transmission has been reported. During the past few months before the present illness, he spent several few weeks in a hilly forest area in Prachuap Khiri Khan Province in southern Thailand, ≈300 km from Bangkok near the Thai-Myanmar border. He reported having fever 1 week after returning home. He did not know of any underlying illness and had not experienced any previous malaria attacks. Although he stayed in a cottage and slept inside a mosquito net, he remembered being bitten frequently by mosquitoes, especially at dusk and dawn.

Upon examination, his temperature was 38.5°C, and pulse rate was 90 beats per minute. His hemoglobin was 14.0 g/dL, hematocrit was 0.4, and erythrocyte count was 4.2 x 10^6 cells/μL. The total leukocyte count was 5,500 cells/μL, with normal differential count. The platelet count was 90,000/μL. Levels of other laboratory investigations, including urinalysis, blood sugar, liver function test, blood urea nitrogen, and creatinine, were normal. Examination of Giemsa-stained thin blood films showed 10% young trophozoites, 45% growing trophozoites, 40% schizonts, and 5% gametocytes (n = 300). The parasite structure was compatible with that of *P. malariae*. The parasite density inferred from the number of malarial parasites per 500 leukocytes in thick blood smear yielded 1,155/μL or equivalent to parasitemia 0.03%. The patient was treated with 10 mg/kg of oral chloroquine initially, followed by 5 mg/kg, 6 hours later on the day 1, and 5 mg/kg/day for the next 2 days. On day 2, with a temperature of 37.5°C, he came to the hospital. Parasitemia decreased to 137/μL. Complete defervescence was observed on day 3, and parasitemia could not be detected. Two weeks and 2 months later, his blood smears were negative for malaria. Fever did not recur.

Meanwhile, we recently evaluated a DNA-based diagnostic method by the polymerase chain reaction (PCR) targeting the small subunit ribosomal RNA (SSU rRNA) genes of all four species of human malaria as reported (8). Ten isolates each for *P. falciparum*, *P. vivax*, and *P. malariae* and four isolates of *P. ovale* were used as positive controls. Results showed that all isolates gave concordant positive PCR products with those diagnosed by microscopy except an isolate from this patient (data not shown). Retrospective examination of blood smears has shown several developmental stages of malaria parasites similar to those typically seen in *P. malariae*. However, some erythrocytes that harbored mature asexual parasites possessed fimbriated margins. The cytoplasm of some young

trophozoites appeared spread out into the network of irregular pseudopodia, and the chromatin was distributed into fragments, conforming to the tenue forms. Pinkish dots varying from fine to large irregular masses called Sinton and Mulligan's stippling developed intracorpuscularly with the maturation of some parasites (Figure 1).

To elucidate the species of malaria infecting our patient, we determined the SSU rRNA gene by using similar methods as described by others (9), except that ExTaq DNA polymerase (Takara, Japan), pGEM-T vector (Promega, USA), and *Escherichia coli* strain JM109 were used. Results showed that the SSU rRNA sequence contained 97.8% to 99.6% homology with those of *P. knowlesi* transcribed during asexual stages or the type A gene (GenBank accession no. AY327549-AY327557, L07560, and U72542) (3,9). Nucleotide sequence data reported in this study are available in the EMBL, GenBank, and DDJB databases under the accession no. AY580317–8.

Phylogenetic tree showed that *P. knowlesi* in this study was closely related to the W1 and Nuri strains, although its divergence from Malaysian human isolates was not supported by bootstrap analysis (Figure 2). Consistently, the mitochondrial cytochrome b gene of this isolate, determined by the methods similar to previous report except the PCR primers (mtPk-F:5′-AGGTATTATATTCTTTATACAAATATTAAC-3′ and mtPk-R:5′-TCTTTTATAATGAACAAGTGTAAATAATC-3′), displayed perfect sequence identity with that of *P. knowlesi* strain H from monkey (AF069621) (4).

Conclusions

P. knowlesi is prevalent among crab-eating macaques, *Macaca fascicularis*, in the Malaysian peninsula and the Philippines (2,10). Other known natural hosts include pigtailed macaques, *M. nemestrina*, and leaf monkeys, *Presbytis melalophos* (2,10). Although in 1932, Knowles et al. (11) had shown that *P. knowlesi* isolated from monkey could be infectious to humans, the first naturally acquired human infection with *P. knowlesi* was not reported until 1965 (6); the patient was infected in a Malaysian forest. In 1971 the second case, albeit presumptive, occurred in a man who also acquired the infection in a forest in Malaysia (12). Recently, a large cluster of human infections caused by *P. knowlesi* has been identified from Malaysian Borneo (9). Our report has expanded the geographic range for natural transmission of *P. knowlesi* to a forest in Thailand near southern Myanmar border, where wild populations of crab-eating macaques, despite being considered endangered, are still substantial.

The prevalence of naturally acquired primate malaria in humans can be underestimated from examination of blood films. The reported abundance of ring stages of *P. knowlesi* found in the first naturally acquired human case led to the initial diagnosis of *P. falciparum*, while the mature parasites could masquerade as those of *P. malariae*, as we encountered in this patient (6). Although structural descriptions of young trophozoites of *P. knowlesi* have been delineated, we were unable to find the ring form with double chromatin dots (9). Conversely, a few young trophozoites resembled the tenue forms, proposed by Stephens in 1914 (13) to be a distinct species. However, the tenue form has recently been recognized to be a *P. malariae* variant found in Myanmar (8). The presence of the tenue form in the blood smears of our patient, despite the low number, rather suggests a shared structural feature among species of malaria. The possibility of coinfection

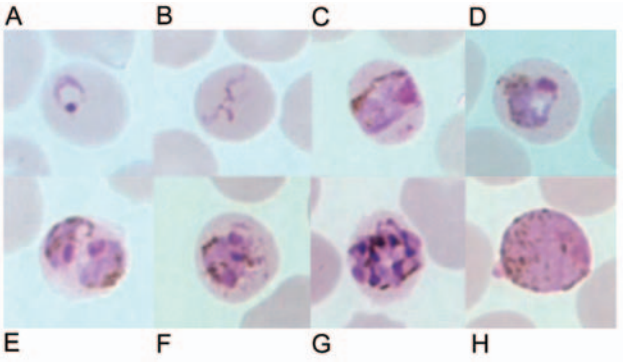

Figure 1. Giemsa-stained thin blood films depicting A) ring stage, B) tenue form of young trophozoite, C) band-shaped growing trophozoite, D) growing trophozoite with little or no amoeboid activity, E) double growing trophozoites, F) early schizont, G) late schizont in an erythrocyte with fimbriated margins, and H) mature macrogametocyte. Discernible Sinton and Mulligan stippling is in C, D, and F.

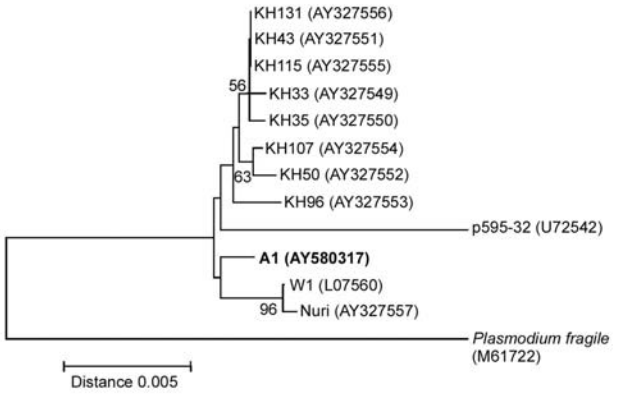

Figure 2. Neighbor-joining tree based on the asexually transcribed SSU rRNA sequences displaying the phylogenetic position of isolate A1 in this study in relation to other *Plasmodium knowlesi* isolates (AY327549-AY327556 from humans, and L07560, U72542, and AY327557 from monkeys) and *P. fragile* (M61722). The tree was constructed with Kimura's two-parameter distance. including transitions and transversions as implemented in the MEGA version 2.1 software. Bootstrap percentages more than 50% based on 1,000 replicates are shown on the branches.

between *P. knowlesi* with one or more of the four human malaria species was not supported by our PCR detection. The structure of *P. knowlesi* is highly dependent on the host erythrocytes, i.e., resembling *P. vivax* in *M. fascicularis*, *P. falciparum* in rhesus monkeys, and *P. malariae* in humans (2,9,11,12). Although stippling was not seen among *P. knowlesi*–infected blood smears of Sarawak's patients, the presence of Sinton-Mulligan stippling in infected erythrocytes in this study is in accord with the report by Fong et al., in which erythrocytic stippling served as one of the diagnostic feature (9,12). Such discrepancy could partly arise from differences in the condition for Giemsa staining, infecting parasite strains, or both.

The complete asexual erythrocytic cycle of *P. knowlesi* in human and its natural macaque host requires ≈24 hours, coinciding with a quotidian fever pattern. However, fever pattern per se may not be a precise indicator for differentiating malaria caused by *P. knowlesi* and *P. malariae*. Although the merogony cycle of *P. malariae* has been generally known to be 72 hours, fever patterns might not be strictly quartan (14). Meanwhile, the preexisting immunity to *P. vivax* has reportedly conferred partial resistance to induced infection during malariotherapy (2). Whether naturally acquired immunity against *P. vivax* can reduce symptoms in *P. knowlesi* infection requires further investigation.

To date, little is known about the extent of variation in the *P. knowlesi* population. Analysis of the SSU rRNA gene from the isolate in this study has shown minor difference from those of *P. knowlesi* from monkeys and patients in Malaysian Borneo (3,9). Evidence from malariotherapy showed that *P. knowlesi* could lose or increase its virulence on blood passage in humans, which suggests that strain difference could occur in wild populations and might effect humans differently (2). In conclusion, *P. knowlesi* could contribute to the reemergence of simian malaria in Thailand and southeast Asia, where its vectors, *Anopheles leucosphyrus* group, are abundant (15).

Acknowledgments

We thank the patient for participating in our investigation and S. Obama, M. Kinoshita, K. Komatsu, and M. Charoenkorn for assistance.

This study was funded by the Hitachi Scholarship Foundation and Research Grants from Ministry of Education, Culture, Sports, Science and Technology (Grant No. COE17301-F1) and from Ministry of Health, Labour and Welfare of Japan.

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Rabies in Endangered Ethiopian Wolves

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With rabies emerging as a particular threat to wild canids, we report on a rabies outbreak in a subpopulation of endangered Ethiopian wolves in the Bale Mountains, Ethiopia, in 2003 and 2004. Parenteral vaccination of wolves was used to manage the outbreak.

During the last decade, infectious diseases have posed a major risk to small populations of wild vertebrates. Highly pathogenic infectious agents have been implicated in the decline and extirpation of a considerable number of populations (1–3). Analysis of disease outbreaks suggests that carnivores appear to be particularly susceptible (2). Specifically, the susceptibility of wild canids may arise from a variety of intrinsic social and ecologic factors but is undoubtedly also due to their susceptibility to general pathogens carried by the most abundant carnivore, the domestic dog. Indeed, rabies has emerged as the most common cause of disease outbreaks in wild canids (1–3).

The Outbreak

We report on an outbreak caused by rabies in the world's rarest canid, the endangered Ethiopian wolf (*Canis simensis*) (Figure 1). The Ethiopian wolf (13–20 kg) is found in only seven Afroalpine highlands in Ethiopia. Wolves live in discrete and cohesive social packs of 2 to 18 adults that communally share and defend an exclusive territory but forage alone for small prey, primarily rodents. Breeding is usually monopolized by a dominant pair, although subordinate animals do attempt to breed, and all animals help raise young. With up to 300 of the global estimate of 500 wolves, the Bale Mountains in south-central Ethiopia are home to the largest and most important population of this species (4). Within these mountains, three areas of relatively high density of Ethiopian wolves can be identified (Figure 2), although wolves are found through the Afroalpine range. Until August 2003, one of these core areas, the Web Valley, harbored an estimated 95 wolves.

Wolf packs in the Bale Mountains are monitored by the Ethiopian Wolf Conservation Programme staff on foot or horseback, using binoculars, global positioning systems, and, following the rabies control intervention strategy, radio telemetry. Over the last year, the Ethiopian Wolf Conservation Programme monitored 47 packs containing 250–300 wolves, in seven areas in these mountains. During a 6-week period from mid-August to the end of September, the carcasses of four wolves were found in the Web Valley; 15 more dead wolves were found in the first half of October (Table). Through January 30, 2004, a total of 38 wolf carcasses were found in this core monitoring area. Two carcasses were also found in the Morebawa to the south and one in the Gaysay Valley to the north (Figure 2). In addition, 36 wolves disappeared from the Web Valley during this period. Extrapolating from background annual average probability of death (0.15 for adults and yearlings, 0.45 and 0.55 for juveniles males and females, respectively), we would normally have expected ≈12 of 95 animals in the Web Valley subpopulation to die during these 6 months, rather than the 74 that actually died or disappeared. Thus,

Figure l. Ethiopian wolves. Photo credit: Martin Harvey.

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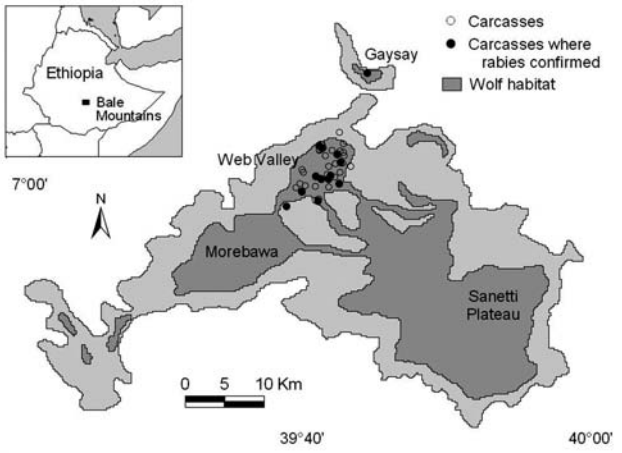

Figure 2. Ethiopian wolf subpopulations, habitat, and carcass locations during the reported rabies outbreak in the Bale Mountains, Ethiopia. Samples were not obtained from all carcasses, but those confirmed rabies positive are depicted with filled circles.

mortality clearly increased in this period. Clinical signs observed in 10 Ethiopian wolves (including 2–5 animals whose carcasses were not recovered) during this period were all consistent with rabies and included hind limb ataxia (8 wolves, including 3 that were repeatedly falling), depression (n = 6), severe weight loss (n = 3), restlessness or unusual ranging behavior (n = 4), aggression (n = 3), loss of fear of humans or other wolves (n = 2), excessive salivation at death (n = 2), and a poor coat (n = 1).

Rabies virus was diagnosed from 13 of 15 brain samples sent to the Centers for Disease Control and Prevention (CDC), USA. The negative samples most likely represent poor-quality samples, but these results are also consistent with background mortality. Diagnostics, RNA extraction from wolf brains, reverse transcription–polymerase chain reaction, N gene sequence generation, and phylogenetic analysis were performed as described in Kuzmin et al. (5). The nucleoprotein (N) gene sequences (strain ETH2003, GenBank accession no. AY500827) were identical for all 13 samples, which suggests a single point source of infection. Comparison with other N gene sequences available from GenBank (Figure 3) and limited sequences from the national CDC archival collection (to be submitted, GenBank accession nos. requested) demonstrated that the virus belonged to the Africa-1a group (6). The N gene of the virus ETH2003 was 98.3% identical to the N gene of the virus 8807ETH, isolated from an Ethiopian hyena (*Crocuta crocuta*) in 1987 but, in general, belongs to the overall group associated with domestic dogs in different regions throughout northern and central Africa (6). Overall identity in the group was 96.2% for the N gene sequences.

At least one previous rabies epidemic has occurred in the Bale Mountains: 77% of 53 known wolves died or disappeared in the Web Valley from 1991 to 1992 (7). Rabies was confirmed in samples from three animals (7,8). In addition, 52% of 23 known wolves died or disappeared in the Sanetti Plateau (Figure 2) from April to June 1990, but no samples were obtained for analysis (7). However, disease was identified as the prime candidate for this sudden reduction in numbers. Overall, the Bale Mountains wolf population was estimated to have declined from ≈450 to 120–160 animals in the early 1990s (9). There were, therefore, concerns that the recent outbreak would again spread throughout the whole Bale wolf population.

All available evidence suggests that domestic dogs are the reservoir for rabies both in the Bale Mountains and Ethiopia; the genetic analysis identified the virus to be of canid type and no wildlife reservoir has ever been identified in this country. Rabies is endemic in Ethiopia and remains both a public health and economic problem through livestock losses in these impoverished rural communities (10,11). More than 32 domestic dogs and 20 cattle exhibiting clinical signs consistent with rabies were reported in communities adjacent to the Bale Mountains National Park in the same period, and at least three people were bitten by suspected rabid dogs. Efforts have been made to reduce the threat of rabies to Ethiopian wolves in this area since 1996 through the vaccination of dogs. More than 70% of domestic dogs in core wolf areas within the national park have been vaccinated against rabies, and, where resources have allowed, the dog vaccination campaign has been extended to surrounding communities. Case traceback in this outbreak suggests rabies may have been brought into wolf habitat by an unvaccinated immigrant dog accompanying people and livestock searching for seasonal grazing.

Rabies was confirmed on October 28, 2003, and advice on its management was sought from a range of persons and institutions including the World Conservation Union/Species Survival Commission Canid Specialist

Table. Ethiopian wolf carcasses found from August 2003 through January 2004 in the Web Valley of the Bale Mountains, Ethiopia

Date interval	No. carcasses found	Cumulative total
Aug 11–24, 2003	1	1
Aug 25 to Sept 7, 2003	0	1
Sept 8–21, 2003	1	2
Sept 22–Oct 5, 2003	2	4
Oct 6–19, 2003	16	20
Oct 20–Nov 2, 2003	6	26
Nov 3–16, 2003	2	28
Nov 17–30, 2003	6	34
Dec 1–14, 2003	1	35
Dec 15–28, 2003	1	36
Dec 29, 2003, to Jan 11, 2004	1	37
Jan 12–25, 2004	0	37
Jan 26–Feb 8, 2004	1	38

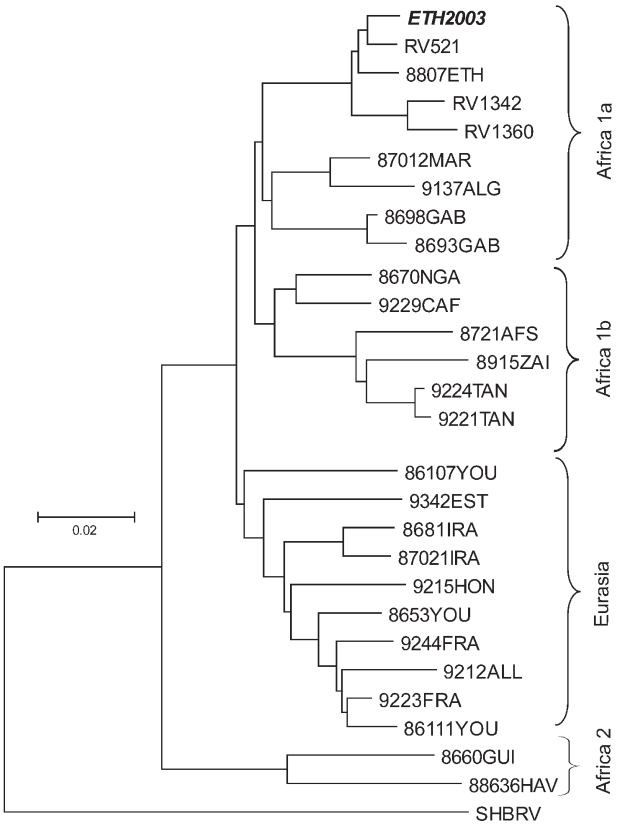

Figure 3. Neighbor-joining phylogenetic tree of African and Eurasian rabies virus samples, rooted with silver-haired bat rabies virus variant (SHBRV), based on a 400–bp region of the nucleoprotein gene. The sample names are given according to GenBank records.

Group and Veterinary Specialist Group. After recommendations were submitted, the Ethiopian Wildlife Conservation Organization decided to intervene with a trial emergency measure, on the grounds that that the species is rare and endangered and that rabies was apparently inadvertently introduced as a byproduct of human activities. The Ethiopian Wolf Conservation Programme implemented this trial intervention, which aimed to contain the disease within the area of the initial outbreak. Since oral rabies vaccines are not currently licensed for use in Ethiopia, parenteral vaccination was the only means to directly protect wolves. Wolf trapping and vaccination began in mid-November in packs adjacent to those already affected, and 69 wolves were trapped and vaccinated up to the middle of February in two high-density wolf areas, Sanetti (n = 33) and Morebawa (n = 36), outside of the core rabies-affected area. Wolves were injected intramuscularly with alternate 1-mL or 2-mL doses of inactivated rabies vaccine (Nobivac R, Intervet). A subsample of 19 wolves was recaptured 30 days later (±5 days) to determine the extent of antibody response to vaccination and to administer a booster dose of 1 mL of vaccine. Preliminary results have shown that all these 19 wolves seroconverted after vaccination. All but one vaccinated wolf were confirmed alive 1 week after vaccination, and all but two wolves were alive 2 months later; both figures are consistent with background death rates. A full analysis of this ongoing work will be reported when data are complete.

Conclusions

This outbreak has highlighted that rabies is a continued threat to endangered canids and that conservationists are often ill-equipped to manage infectious disease. First, lack of information often hinders management: few established models offer guidance. Indeed, some early and unsurprising failures have attracted damaging controversy (2). In addition, although a variety of theoretic approaches to disease management exist, relatively few may be feasible or effective (12). Finally, considerable funds are required to effectively prevent and control disease threats. Nevertheless, results obtained from this trial intervention will be invaluable in assessing the effectiveness of vaccination against rabies in Ethiopian wolves and of the approach in general. These findings will be particularly useful when considering disease management options for wolf populations in other areas of Ethiopia (12,13). With rabies-endemic dog populations around all Ethiopian wolf populations, further research and trials are required to ascertain the most cost-effective and feasible method to decrease the threat of disease for each population and to control any future outbreaks.

Acknowledgments

We thank all the wildlife disease, rabies, and canid experts who provided advice on the management of the rabies outbreak; members of the CDC Rabies Section, particularly Pam Yager, for their input and expertise; Jorgelina Marino for allowing us to use her base layer for the map; members of the Ethiopian Wolf Conservation Programme staff for their dedication and hard work, in harsh field conditions, during the rabies outbreak and vaccination; and the Ethiopian Wildlife Conservation Organisation and the Oromiya Rural Land and Natural Resources Administration Authority for permission to undertake conservation activities and research in Bale.

A consortium of funders, headed by Frankfurt Zoological Society and including the Born Free Foundation and Conservation International, supported this emergency intervention.

Ms. Randall is a doctoral student from the Wildlife Conservation Research Unit of the University of Oxford, United Kingdom. She conducts research on the determinants of genetic variation in Ethiopian wolf populations, including the impact of mating system, social structure, and disease outbreaks. The Bale Mountains are the principal field site for her research.

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Protective Effectiveness of Hantavirus Vaccine

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A case-control study in the Republic of Korea evaluated the protective effectiveness of the hantavirus vaccine. Point estimates showed increasing effectiveness with increasing numbers of doses received: 25% for one dose, 46% for two doses, and 75% for three doses. All 95% confidence intervals overlapped zero; therefore, the findings could be due to chance.

In 1990, the Republic of Korea (ROK) approved a vaccine against the Hantaan virus after accepting data that showed a high seroconversion rate as a surrogate for vaccine effectiveness (1). The recommended schedule for vaccination is two doses 1 month apart, as a primary vaccination, and one booster 12 months later. Although the hantavirus vaccine has been in use since approval, and millions of doses have been given, the effectiveness of the vaccine continues to be debated. However, protective effectiveness of the hantavirus vaccine has been measured mainly by serologic studies (2–4).

The Korean Army is one of the largest consumers of the hantavirus vaccine, second only to public health centers. Uncertainty about protective effectiveness of the vaccine has been enhanced by reports on military personnel in whom hemorrhagic fever with renal syndrome (HFRS) developed, even though they had received the vaccine.

The ROK Army defines a "high-risk area" for HFRS as an administrative district where HFRS cases have occurred during the previous 3 years. Vaccination programs focus on military units located in these high-risk areas, but vaccinating all personnel in those units is impossible because of budget limitations. Therefore, coverage is not 100%.

In a recent study (4), the authors noted that since a vaccination campaign began in 1991, the number of HFRS patients has decreased significantly (Figure 1). However, vaccination is likely not the only factor affecting secular trends in number of HFRS patients; climatic and environmental changes also likely play a role. The present case-control study was conducted to assess the protective effectiveness of the hantavirus vaccine.

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The Study

Cases were identified through the hospital-based active surveillance system of the HFRS maintained by the Korean Army. Cases occurring from January 1, 2002, to January 1, 2004, were enrolled prospectively. For early detection of HFRS, the Korean Army used the operational clinical criteria to identify cases. Patients with HFRS may have sudden onset of fever; experience pain in the head, abdomen, and lower back; and report bloodshot eyes and blurry vision. Petechiae may appear on the upper body and soft palate. The patient's face, chest, abdomen, and back often appear flushed and red, as if sunburned. A confirmed case of HFRS is defined as a positive result on the high-density particle agglutination test.

For each case, one control was selected from among the other patients at each hospital where the case-patient had been hospitalized. The control was matched with the case-patient according to unit, age at the time of hospitalization (±3 years), date of hospitalization (±3 months), and date of transfer to the present unit (±3 months). If no suitable control could be found, the intervals around the case-patient's unit, age, date of hospitalization and date of transference were progressively widened until one or more potential controls were found. For each case-patient, all eligible controls were listed, and one suitably matched control was identified at random. As with the case-patients, the final decisions about each control patient's eligibility for the study were made on the basis of a detailed review of hospital records. Decisions about the eligibility of potential controls were made without knowledge of their vaccination status.

History of vaccination was sought from vaccination records kept at each unit. Vaccine had to be received at least 3 weeks before hospitalization because of the time required for antibodies to develop and because the incubation period is ≈3 weeks on average (5). One patient vaccinated <3 weeks before hospitalization was excluded from the data analysis.

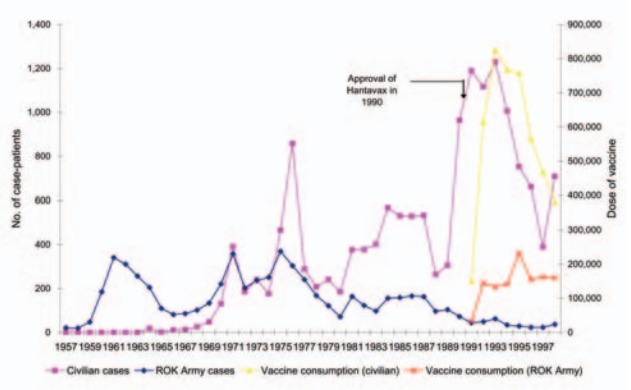

Figure 1. Secular trends in the numbers of hemorrhagic fever with renal syndrome cases, Republic of Korea (ROK), 1957-1998.

Estimates of the relative odds of HFRS associated with vaccination were estimated by using methods developed by Mantel and Haenszel (6), which are appropriate for matched designs. The protective effectiveness of the vaccine was estimated as 1 minus the relative odds associated with vaccine use, times 100. Ninety-five percent confidence limits for the effectiveness were derived from the 95% confidence interval (CI) of the relative odds. Data were analyzed with SPSS, version 10.0 (SPSS, Inc., Chicago, IL).

From January 1, 2002, to January 1, 2004, a total of 57 HFRS cases were identified among troops of the Korean Army. Of the 57 patients, 3 (5.3%) died. One of three deaths occurred in previously healthy, vaccinated (first and second doses) personnel. Twelve, 9, and 2 cases occurred in personnel who were vaccinated with one, two, and three doses, respectively. Most cases occurred in October (15.7%), November (35.7%), and December (17.1%), although disease also occurred during the spring and the summer (Figure 2).

Of the 54 persons identified with HFRS from January 2002 to January 2004, those who were vaccinated within 3 weeks of hospitalization were excluded from analysis. Because the effectiveness was calculated by comparing each dose (exactly one to three) with no vaccination, case-patients or controls not applicable to that comparison were excluded from each matched set. Finally, 41, 38, and 31 matched sets were formed for one, two, and three doses of the hantavirus vaccine, respectively (Table). Ages of the case-patients were similar to ages of controls. Estimates of vaccine effectiveness according to the number of doses received rose from 25% (95% CI –78% to 68%) for one dose to 46% (95% CI –35% to 78%) for two doses to 75 % (95% CI –18% to 95%) for three doses. When recipients for whom 1 year had passed since their second dose were excluded, effectiveness of two doses increased markedly to 70% (95% CI –9% to 92%).

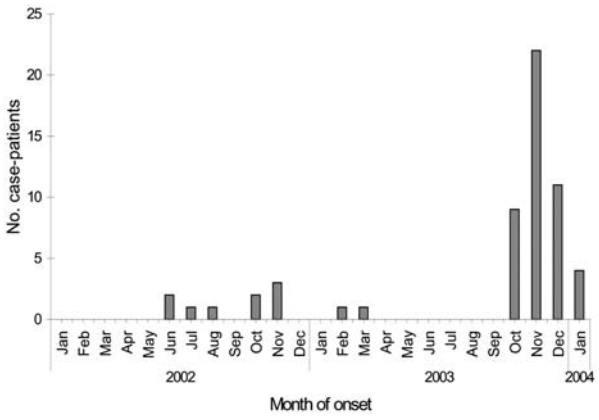

Figure 2. Hemorrhagic fever with renal syndrome cases among Republic of Korea military personnel, by month of onset, January 2002 to January 2004.

Conclusions

The results of this study suggest a trend toward protection for the hantavirus vaccine. The protective effectiveness of the vaccine strongly depends on the number of doses. In particular, effectiveness increased when persons for whom ≥1 year had passed since their second dose were excluded, which suggests that the protective effect of the second primary vaccination does not persist beyond the period recommended for having the booster dose. In addition, we do not know whether the recommended immunization schedule was optimal for military personnel and farmers, groups for whom hantavirus vaccination is recommended. The vaccination schedule should be epidemiologically relevant, immunologically effective, operationally feasible, and socially acceptable (7).

In a field study from the former republic of Yugoslavia conducted by Korean researchers, including the developers of the hantavirus vaccine (8), no case of HFRS was observed among 1,900 vaccinees, while 20 confirmed cases were observed among 2,000 nonvaccinated controls. Considering that our study showed low protective effectiveness for one or two doses, that no case of HFRS occurred in Yugoslavian vaccinees before they received the full three doses was surprising.

Because the case-control studies were not experimental, they may be subject to biases. The most important potential biases that might affect this kind of study are detection and selection bias. If all cases of HFRS were identified, no detection bias would occur. Because patients were identified prospectively by active surveillance, we believe that virtually all cases of HFRS diagnosed during the study period were identified. Selection bias may occur when controls do not represent the general population. In this study, controls were selected randomly from a list of

Table. Characteristics of patients and matched controls[a]

	No. doses (matched sets)					
	3 (31)		2 (38)		1 (41)	
Characteristic	Patients	Controls	Patients	Controls	Patients	Controls
Median age (y) (mean ± SD)	22.0 (23.2 ± 3.5)	22.0 (22.0 ± 3.6)	22.0 (23.2 ± 3.1)	21.0 (22.0 ± 2.1)	22.0 (23.4 ± 3.8)	21.0 (22.2 ± 0.7)
% vaccinated	6.5	32.3	23.7	39.5	29.3	36.6

[a]All participants were men.

potentially eligible controls by using a systemic algorithm. Confounding influences affect the results of a case-control study if controls differ from case-patients in characteristics related to risk of contracting the disease and likelihood of receiving the vaccine. Since the population of this study consisted of military personnel, bias due to sociodemographic differences may be negligible. Therefore, other candidate confounding factors determined by considering the military milieu were used as matching variables.

We could not show that vaccine effectiveness estimates were significant. All of our confidence intervals have lower bounds less than zero. Therefore, while point estimates show effectiveness, this finding could be due to chance. Of course, the range of point estimates in studies with relatively small samples can be wide, and wide confidence intervals that include zero are not uncommon in many studies on vaccine effectiveness (9). However, caution is appropriate in interpreting our estimates of vaccine effectiveness.

Finally, this study represents a short-term (7.3 months average) evaluation of protective effectiveness of three doses of the hantavirus vaccine. To assess the long-term effectiveness, protection must be monitored over a longer period.

The authors do not have commercial or other associations that might pose a conflict of interest. In addition, this work had no financial support.

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Genome Sequence and Attenuating Mutations in West Nile Virus Isolate from Mexico

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The complete genome sequence of a Mexican West Nile virus isolate, TM171-03, included 46 nucleotide (0.42%) and 4 amino acid (0.11%) differences from the NY99 prototype. Mouse virulence differences between plaque-purified variants of TM171-03 with mutations at the E protein glycosylation motif suggest the emergence of an attenuating mutation.

Since its introduction into North America in 1999, West Nile virus (WNV) has spread rapidly across the continent, and evidence for virus circulation has also been detected in the Caribbean and parts of Central America (1). In 2003, WNV was isolated from a dead raven in Villahermosa, in the state of Tabasco, Mexico (2). Nucleotide sequencing of the premembrane (prM) and envelope (E) structural protein genes of this strain, TM171-03, and comparison with sequences from other North American isolates indicated that this virus had accumulated several unique mutations from the New York 1999 strain 382-99 (NY99) prototype sequence. We describe the complete genomic sequence of TM171-03 and its relationship to other North American isolates, as well as the results of virulence phenotype comparisons.

The Study

The isolation and initial characterization of TM171-03 have been described elsewhere (2). For genomic sequencing, RNA was extracted from infected Vero cell culture supernatant (second Vero cell passage from the original brain material, designated V2) using the QiaAmp kit (Qiagen Inc., Valencia, CA), reverse transcribed with AMV reverse transcriptase (RT) (Roche, Indianapolis, IN) and amplified by polymerase chain reaction (PCR) as nine overlapping fragments by using Taq polymerase (Roche). The PCR products were purified from 1.5% TAE/agarose gels by using the QiaQuick kit (Qiagen) and directly sequenced on an ABI Prism model 3100 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Texas Medical Branch's Protein Chemistry Core Facility by using the amplifying primers and additional internal primers. The primers used for RT-PCR and sequencing were similar to those used by Lanciotti et al. (3) and Beasley et al. (4) (complete details are available on request). Sequence data were assembled into the complete genome sequence and analyzed as described elsewhere (2,4). In addition, Bayesian analyses were performed by using MRBAYES v3.0 (5) and 100,000 generations. A general time-reversible model was used with empirically estimated base frequencies and either a codon position-specific or a γ distribution of substitution rates.

The genomic sequence for TM171-03 (GenBank accession number AY660002) differed from the NY99 prototype sequence (GenBank AF196835) at 46 nucleotides (nt) (0.42%). As reported previously, sequencing the prM-E genes of TM171-03 (V1 passage) identified nonsynonymous mutations encoding substitutions at prM-141 Ile→Thr and E156 Ser→Pro (2). The E-156 mutation results in the loss of the E-154-156 "NYS" glycosylation motif. Complete genome sequencing identified only two other encoded amino acid changes from the NY99 sequence at NS4B-245 (Ile→Val) and NS5-898 (Thr→Ile). However, during the sequencing of the V2 passage material, a reversion from Pro to Ser encoded at E-156 was observed. Analysis of the sequence chromatograms from V1 and V2 passages, and for a PCR product obtained from the original brain material, indicated that this reversion was likely to be the result of a mixed virus population, with overlapping "T" and "C" peaks visible at residue 1432 in the sense or antisense sequences for each product. To confirm this finding, PCR products containing the E-156 region from each passage level of TM171-03 were cloned into pGEM-T(Easy) (Promega, Madison, WI), and five clones were sequenced for each. For the original brain tissue, four clones encoded Pro at E-156 and one clone encoded Ser. For products derived from either V1 or V2 passages, two or three clones encoded a Pro at E-156, and the remainder encoded a Ser. In addition, several variants of TM171-03 were purified through two rounds of plaque selection in Vero cells, and nucleotide sequencing of these variants also confirmed a mixed population. Sequences from approximately half of the plaques encoded a Pro at E-156, while the remainder encoded Ser. Western blotting of infected Vero cell lysate antigens for these variants with WNV E protein-specific monoclonal antibody 7H2 (6) showed differences in the electrophoretic mobility of the proteins consistent with the presence or absence of glycosylation (Figure 1).

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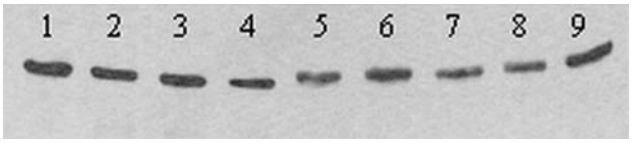

Figure 1. Western blot showing differing mobility of E proteins from nine plaque-purified variants of West Nile virus (WNV) strain TM-171 Mex03. Nucleotide sequencing of strains in lanes 5 to 9 indicated the presence of an "NYS" glycosylation motif at residues 154 to 156 of E, while strains in lanes 1 to 4 encoded "NYP." Antigens were separated in a nonreducing 5%/10% discontinuous sodium dodecyl sulfate-polyacrylamide gel, transferred to 0.2 μm nitrocellulose and detected with WNV-specific monoclonal antibody 7H2 (6).

Comparison of the TM171-03 nucleotide sequence with other genomic sequences of WNV strains showed that it was most closely related to strain NY00-grouse3282 (GenBank AF404755; Figure 2). The NY00-grouse3282 sequence differed from NY99 at only 13 nt (0.11%), and its relationship to TM171-03 was apparently based on 10 nonstructural protein region nucleotide differences from NY99 that were shared with TM171-03 (Table 1). None of these mutations encoded amino acid differences, and TM171-03 differed from NY00-grouse3282 at 39 other nucleotides. These were primarily additional mutations that had accumulated in the TM171-03 strain. Genomic sequence data from other East Coast U.S. isolates collected during 2000 and subsequent years are needed to attempt to establish a definitive relationship for TM171-03 with a particular North American isolate.

To assess the effects of the E-156 Ser→Pro mutation on the virulence of TM171-03, serial 10-fold doses from 1,000 to 0.1 PFU of TM171-03 and four plaque-purified (pp) substrains (TM171-03-pp1 and -pp2 encoding Pro at E-156; TM171-03-pp5 and -pp6 encoding Ser) were inoculated intraperitoneally (i.p.) and intracranially (i.c.) into groups of 3- to 4-week-old female NIH Swiss mice to determine mouse neuroinvasiveness and neurovirulence, as described elsewhere (7) and in accordance with guidelines of the University of Texas Medical Branch Institutional Animal Care and Use Committee.

TM171-03 was highly virulent following i.p. and i.c. inoculation, as were the plaque-purified variants, TM171-03-pp5 and -pp6, which encoded the E154-156 NYS glycosylation motif (i.p. and i.c. 50% lethal dose [LD_{50}] values for each ≤2.0 PFU; Table 2). The lethality of these strains was comparable to that of other North American isolates that have been evaluated by using the NIH Swiss mouse model (4,7). In contrast, the nonglycosylated variants TM171-03-pp1 and -pp2 were both attenuated, having i.p. LD_{50} values >1,000 PFU and i.c. LD_{50} values of 32 and 25 PFU, respectively.

To confirm that the mouse virulence differences between the plaque-purified variants could be primarily attributed to the mutation at E-156, regions that encoded the additional consensus amino acid mutations at prM-141, NS4B-245, and NS5-898 were sequenced. All four plaque-

Table 1. Summary of nucleotide and amino acid differences between West Nile virus strains NY99 (AF196835), NY00-grouse3282 (AF404755), and TM171-03[a]

Nucleotide (amino acid)	NY99	NY00	TM171-03
50	A	A	G
71	A	A	G
93	C	T	C
381	C	C	T
483	C	C	T
858	C	C	T
887 (prM-141)	T (Ile)	T (Ile)	C (Thr)
1137	C	C	T
1285	C	T	C
1432 (E-156)[b]	T (Ser)	T (Ser)	C (Pro)
1626	C	C	T
2328	C	C	T
2388	C	C	T
2466	C	C	T
2607	T	T	C
2832	T	T	C
2865	C	C	T
3111	G	G	A
4146	**A**	**G**	**G**
4212	T	T	A
4564	**T**	**C**	**C**
4749	C	C	T
6120	C	C	T
6138	**C**	**T**	**T**
6141	C	C	T
6279	G	G	A
6426	**C**	**T**	**T**
6495	G	G	A
6771	**A**	**G**	**G**
7015	**T**	**C**	**C**
7359	C	C	T
7648 (NS4B-245)	A (Ile)	A (Ile)	G (Val)
7672	C	C	T
7938	**T**	**C**	**C**
8067	A	G	A
8109	C	C	T
8676	A	A	G
8811	**T**	**C**	**C**
8838	T	T	C
8994	T	T	C
9378	T	T	C
9408	T	T	C
9453	C	C	T
10317	C	C	T
10373 (NS5-898)	C (Thr)	C (Thr)	T (Ile)
10393	**C**	**T**	**T**
10828	T	T	G
10851	**A**	**G**	**G**
10989	G	G	A

[a]**Bold** text indicates residues at which NY00-grouse 3282 and TM171-03 both differed from NY99.
[b]Strain TM171-03 had a mixed sequence at this residue. Consensus sequence of early passage material had "C" at nucleotide 1432.

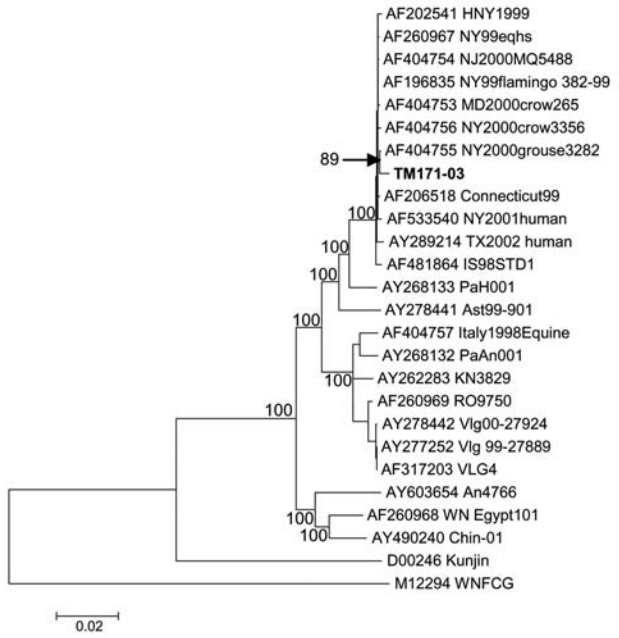

Figure 2. Neighbor-joining phylogenetic tree based on complete genome sequences of West Nile virus strains. Strain TM171-03 is indicated in bold text. The topology of maximum parsimony and maximum likelihood trees was essentially identical. Bayesian analysis also confirmed the close relationship between TM171-03 and NY00-grouse3282 sequences (data not shown). Bootstrap values are shown for major branches (500 replicates). GenBank accession numbers for sequences used to construct the tree are indicated on the branches.

purified variants encoded the three amino acid mutations that were present in the consensus sequence. No additional mutations encoding amino acid changes were identified in the regions that were sequenced for these strains (equivalent to ≈3,000 nt in total for each). Although the entire genome of each plaque-purified variant was not sequenced, we believe that it is highly unlikely that the mouse virulence differences observed between the variants would be attributable to other amino acid mutations in the unsequenced regions that were present in the two E-156 Pro variants but not the E-156 Ser variants or the parental TM171-03 strain.

Conclusions

These results are somewhat contrary to previously reported data that described attenuated variants with glycosylated E proteins that were derived from a virulent, nonglycosylated Israeli lineage 1 WNV strain (8). However, subsequent studies identified E glycosylated variants of the same strain that retained a virulent phenotype, which suggests that multiple determinants, most probably including mutations in the nonstructural protein genes, were responsible for the observed variations in virulence (9). Other comparisons of wild-type WNV strains suggested that absence of E protein glycosylation might be associated with attenuation of mouse neuroinvasiveness (7). Recently, some of us have shown that mutating the E protein gene of a WNV infectious clone derived from the NY99 prototype strain to prevent glycosylation resulted in a ≈200-fold attenuation of neuroinvasiveness, but not neurovirulence, in the NIH Swiss mouse model (D.W.C. Beasley, et al., unpub. data). Given the greater degree of attenuation of neuroinvasiveness and neurovirulence observed for the nonglycosylated TM171-03-pp1 and -pp2 variants described here, we hypothesize that one or more of the other mutations (at prM-141, NS4B-245, or NS5-898) also contributed to the phenotype, but this hypothesis remains to be determined experimentally. All of these mutations in the absence of the E-156 Ser→Pro mutation (as occurred in the TM171-03-pp4 and -pp5 variants) did not appear to significantly affect the mouse virulence phenotype.

E protein glycosylation appears to play an important role in flavivirus assembly in mammalian cell culture (10); the mechanism by which this particular mutation would emerge in a wild-type WNV population, as is the case with the TM171-03 isolate, is not clear. However, the posttranslational processing of glycoproteins differs between mosquito and mammalian cells (11), and adaptation of dengue virus to mosquito cells resulted in loss of the equivalent glycosylation motif (12), which suggests that the presence of carbohydrate on the E protein may be of lesser importance during virus replication in mosquito cells.

Recent data from the Mexican Department of Health indicate that no human cases of encephalitis attributable to

Table 2. Neuroinvasiveness and neurovirulence of TM-171 Mex03 parental isolate and plaque-purified variants after injection into 3- to 4-week-old female NIH Swiss mice[a]

Virus	E154-156 sequence	i.p. LD_{50} (PFU)	i.p. AST ± SD (d)[b]	i.c. LD_{50} (PFU)	i.c. AST ± SD (d)[b]
TM171-03	NYP/S	1.3	7.9 ± 0.7	0.8	6.2 ± 1.8
TM171-03-pp1	NYP	>1000	NA	32	6.0 ± 0.9
TM171-03-pp2	NYP	>1000	NA	25	6.7 ± 1.7
TM171-03-pp5	NYS	2.0	9.0 ± 1.4	2.0	7.4 ± 1.2
TM171-03-pp6	NYS	2.0	8.5 ± 1.7	1.3	7.4 ± 0.9

[a]NIH, National Institutes of Health; AST, average survival time; i.p., intraperitoneal; i.c., intracranial; LD_{50}, 50% lethal dose; SD, standard deviation; NA, not applicable.
[b]Average survival time ± SD was calculated for all animals that died following inoculation with 1,000 - 0.1 PFU doses of indicated virus.

local transmission of WNV have occurred during 2004 and, although many WNV-seropositive horses have been identified, few cases of overt clinical disease have been reported (http://www.cenave.gob.mx/von; accessed 26 Aug, 2004). The epidemiology of WNV disease in Mexico is likely to be complicated by preexisting immunity to other flaviviruses, but the emergence of an attenuated WNV strain would be important. WNV nucleotide sequences obtained from infected horses in Mexican states closer to the U.S. border suggest that they are closely related to recent isolates from Texas that do not encode mutations at the E glycosylation motif (13; J. Estrada-Franco, et al., unpub. data).We are unaware of any other WNV isolates from Tabasco or other southern regions of Mexico. Obtaining additional isolates from southern Mexico is important to determine if a nonglycosylated WNV population is emerging and to ascertain what impact this may have on the prevalence of severe WNV disease in Mexico.

Acknowledgments

We thank Igor Romero Sosa and Pedro Paz-Salazar for their contributions to this project.

This study was funded in part by the State of Texas Advanced Research Program and NIH contract NO1-AI25489. Resources for field collections were provided by the Office of Exotic Diseases of the Agricultural Ministry of Mexico. D.B. is the recipient of a fellowship from the James W. McLaughlin Fellowship Fund.

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Animal-to-Human Transmission of *Salmonella* Typhimurium DT104A Variant

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Salmonella enterica serovar Typhimurium was isolated from a pig, a calf, and a child on a farm in the Netherlands. The isolates were indistinguishable by phenotyping and genotyping methods, which suggests nonfoodborne animal-to-animal and animal-to-human transmission. Persons in close contact with farm animals should be aware of this risk.

Salmonellae are widespread in humans and animals worldwide. In industrialized countries, nontyphoid salmonellae are an important cause of bacterial gastroenteritis. In the Netherlands, the estimated incidence of salmonellosis is 3 cases per 1,000 inhabitants per year (1). In the United States, *Salmonella* is estimated to cause 1.4 million illnesses and 600 deaths annually (2). *Salmonella enterica* subspecies enterica serovar Typhimurium can cause infections in humans and animals. Most human cases are foodborne; however, nonfoodborne *Salmonella* infection may be transmitted during contact with animals, contaminated water, or the environment (3–9). We report apparent transmission of *S.* Typhimurium on a farm.

The Case

A farm in IJsselstein, the Netherlands, housed 80 dairy cows and 250 finishing pigs (for fattening) in separate sheds. The farmer took care of animals in different stables without changing clothes, and his children had access to all the stables.

In January 2001, the farmer consulted a veterinarian of the Pig Health Unit of Utrecht University regarding a problem with his pigs. In a compartment where 95 pigs (≈6 months of age) were housed, 1 of the pigs was very listless, had a rectal temperature of 41.2°C, and had yellowish diarrhea. Another pig had died suddenly that morning. At that time, the other pigs in the compartment were asymptomatic, but the farmer had noticed diarrhea in several pens a few days earlier. A fecal sample was taken from the ill pig for bacteriologic examination. Despite therapy with enrofloxacin, the pig died. Veterinarians of the Ruminant Department of Utrecht University were consulted 20 days later regarding five 3-week-old calves on the same farm. The calves had diarrhea and fever, and two of them had symptoms of pneumonia. A fecal sample was taken from one of the calves, and the calves were medicated intramuscularly with trimethoprim/sulfadiazine and polymyxin orally. All calves recovered after treatment.

Three weeks after the first veterinarian's visit, the farmer's 5-year-old son became ill with diarrhea and a fever. At that time, the pig was known to have had salmonellosis. Amoxicillin was prescribed for the boy, and a fecal sample was taken for bacteriologic examination. The farmer, his wife, and the other children were not tested because they were healthy.

All three samples (two from animals, one from the child) yielded *Salmonella* after direct plating without preenrichment (10). No other pathogens were found. Susceptibilities to 17 antimicrobial agents (Table) were assessed by using the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method (11). Breakpoints given by NCCLS and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (11,12) were used.

Additionally, serotyping based on O and H antigens, according to the Kauffmann-White scheme (13); phage typing in accordance with the methods of the Health Protection Agency, London (14 and L.R. Ward, pers. comm.); plasmid profiling (15); and pulsed-field gel electrophoresis (PFGE) after digestion with *Xba*I and *Spe*I (16) were performed.

Serotyping and phage typing all three samples identified *S.* Typhimurium DT104A variant, a subtype of DT104 that is similar but not identical to DT104A. Antimicrobial-drug susceptibility tests showed that the salmonellae had identical resistance patterns. They were sensitive to most of the antimicrobial agents tested, except for tetracycline, sulfamethoxazole, trimethoprim, and trimethoprim-sulfamethoxazole; MIC values for these agents were similar (Table). All isolates possessed a single plasmid of ≈7 MDa, and all isolates had the same PGFE pattern after digestion with each of the enzymes (Figure).

Conclusions

S. Typhimurium DT104A variant was isolated from a diseased pig, calf, and child on a Dutch farm. All three strains were typed by phenotypic and genotypic methods and appeared to be identical, which suggests an epidemiologic link. *S.* Typhimurium DT104A isolates are uncommon and show less resistance determinants in comparison to other DT104 isolates (17,18). The *S.* Typhimurium

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Table. Three *Salmonella enterica* serovar Typhimurium DT 104A isolates from a boy, a pig, and a calf on a Dutch farm, with MIC values for antimicrobial drugs

Antimicrobial drug	MIC (μg/mL)[a]		
	Porcine strain	Bovine strain	Human strain
Tetracycline	>32 R	>32 R	>32 R
Sulfamethoxazole	>512 R	>512 R	>512 R
Spectinomycin	32 S	32 S	32 S
Chloramphenicol	8 S	8 S	8 S
Florfenicol	8 S	8 S	8 S
Streptomycin	8 S	32 R	16 I
Ampicillin	2 S	2 S	2 S
Neomycin	≤2 S	≤2 S	≤2 S
Amoxycillin-clavulanic acid	≤2/1 S	≤2/1 S	≤2/1 S
Nalidixic acid	≤4 S	8 S	≤4 S
Gentamicin	≤1 S	≤1 S	≤1 S
Trimethoprim	>32 R	>32 R	>32 R
Colistin sulfate	≤4 S	≤4 S	≤4 S
Trimethoprim-sulfamethoxazole	>8/152 R	>8/152 R	>8/152 R
Ciprofloxacin	≤0.03 S	≤0.03 S	≤0.03 S
Ceftiofur	1 S	1 S	1 S
Kanamycin	≤4 S	8 S	≤4 S

[a]The categories susceptible (S), intermediate (I), or resistant (R) were assigned on the basis of breakpoints recommended by the National Committee for Clinical Laboratory Standards and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (11,12).

DT104A variant strain in the present study was resistant to sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole, which is a common resistance pattern of DT104A isolates. Unlike other *S.* Typhimurium DT104 isolates, resistances to ampicillin, chloramphenicol, and florfenicol are rare in *S.* Typhimurium DT104A, as is resistance to kanamycin, neomycin, and gentamicin (18).

Because the boy had free access to the stables, we assume that he was infected by direct or indirect contact with animals. The boy was not likely to have been infected with this particular DT104A variant by any other route because this is an uncommon phage type. Transmission of *Salmonella* spp. by direct contact with animals has been reported before (3–9). Close contact with farm animals is a risk factor for *S.* Typhimurium DT104 infections (8,9).

The primary source for human disease was difficult to identify, but it was most likely the pigs. Calves were 1 day old when the pig died and 3 weeks old when they became ill, and *Salmonellae* may have been transmitted from pigs to calves shortly after the calves were born. However, the incubation period of salmonellosis is short (1–3 days), and therefore the calves were probably infected when they were nearly 3 weeks old. The farmer, other members of the family, or visitors may have transmitted contaminated pig feces to the calves on dirty boots, clothes, or fomites. The pigs or the calves could have infected the boy. The calves are more likely because the boy's rabbits were housed in the calves' stable and therefore he had more intensive contact with the calves than with the pigs. Another possibility is that the farmer transmitted the infection to the boy as a result of inadequate handwashing, wearing inadequately disinfected footwear, or wearing working clothes indoors.

We advise those who are at high risk for *Salmonella* infection, e.g., farmers, veterinarians, and slaughterhouse workers, to follow general hygiene guidelines. The amount of bacteria shed by hosts is probably much larger in clinical salmonellosis than in the carrier state, and great care must be taken to clean and disinfect hands and tools to prevent spread of the bacteria after contact with clinically ill animals. Veterinarians must inform animal caretakers about the zoonotic aspects of disease when they diagnose a *Salmonella* infection.

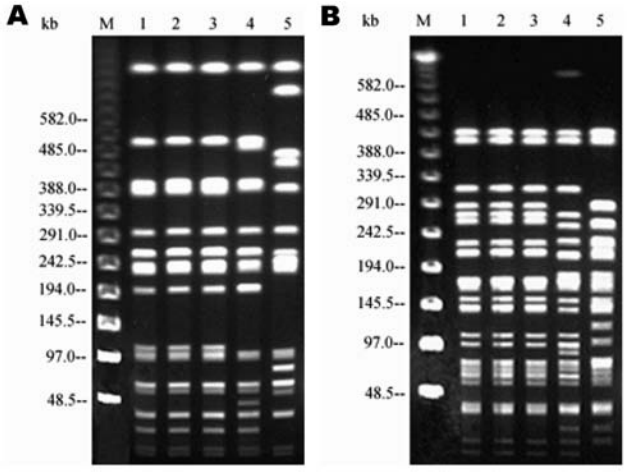

Figure. Pulsed-field gel electrophoresis profiles of the three strains after digestion with *Xba*I (A) and *Spe*I (B). Lane M, molecular size markers. Lane 1, porcine strain. Lane 2, bovine strain. Lane 3, human strain. Lane 4, comparison strain *Salmonella enterica* serovar Typhimurium DT104A with a different resistance pattern. Lane 5, *S.* Typhimurium DT104L strain with the common pentaresistance pattern.

Acknowledgments

We thank M. Nielen for writing assistance and critically reviewing the manuscript; R. Helmuth and A. Schroeter for their help with serotyping, phage typing, and antimicrobial susceptibility testing; and L. Ward for phage typing.

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Isolation and Molecular Identification of Nipah Virus from Pigs

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Nipah viruses from pigs from a Malaysian 1998 outbreak were isolated and sequenced. At least two different Nipah virus strains, including a previously unreported strain, were identified. The findings highlight the possibility that the Malaysia outbreaks had two origins of Nipah virus infections.

An outbreak of febrile encephalitis with high death rates (≈56%) occurred among pig farmers in a pig-farming community in Tambun, Perak, Malaysia, in 1998 (1). The disease spread southward within 4 to 6 months to several other pig-farming communities near or around Seremban, a city ≈300 km from Tambun (2). By April 1999, at least 85 deaths were reported in Seremban; 15 were recorded in Tambun (3). All the cases from Tambun were recorded before the outbreak in Seremban. During the outbreaks, pigs in Seremban and Tambun manifested acute respiratory distress syndrome and encephalitis and subsequently died (2,3). Since the incidence of the disease in humans paralleled the occurrence of the disease in pigs, infected pigs were presumed to be the main and perhaps only source of infections in humans (4,5). Culling almost one million suspected infected pigs effectively curtailed the spread of the disease, except for a cluster of infections, the last to be reported, which occurred in the south, in Sungai Buloh, in 1999 (3).

Nipah virus (NV) was eventually isolated from patients manifesting the typical pig-farming–associated fatal encephalitis, confirming the viral source of the infection (4,5). The whole genome sequence of the virus was determined, and its close phylogenetic relationship to Hendra virus (HV) was shown (6,7). The viruses were designated as members of a new genus, *Henipavirus*, of the *Paramyxoviridae* family (8,9). Several other human isolates were also sequenced and found to share a high degree of sequence similarity to that of the first isolate (10). More recently, an NV was isolated from flying foxes of Tioman Island, located east of peninsular Malaysia (11). The virus had such high sequence similarity to all the human NV isolates that it was suggested as the potential source of NV (12). It was proposed that pigs in the north contracted the infection through contact with NV of flying foxes (from flying fox urine or leftover fruit) and that humans then acquired the infection by direct handling infected pigs (12–14). Movement of infected pigs was responsible for subsequent foci of outbreaks in the south (2,3). Whether the index outbreak in Tambun was a result of a single event (transmission of a flying fox NV to pig), which resulted in clonal propagation and transmission of the virus to pigs and subsequently humans, was unclear. To date, no reports have indicated that NV found in humans shared similar genome sequences to those found in pigs, although NV has been detected and isolated from infected pig samples (15). We present results from analyses of the whole genome sequence of three representative NV isolates from pigs from the three outbreak clusters, one from the north, Tambun, and two from the south, Seremban and Sungai Buloh.

The Study

In this study, NV from pigs (NV/MY/99/VRI-0626, NV/MY/99/VRI-1413, and NV/MY/99/VRI-2794) were isolated by the Veterinary Research Institute, Malaysia. The isolate from a human patient sample from Seremban (NV/MY/99/UM-0128) was isolated at the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia. NV of pigs was isolated after infection of Vero (African green monkey kidney) cells with lung tissue samples of pigs that died from the acute respiratory distress syndrome and encephalitis. In Vero cell cultures, all the NV pig isolates manifested similar cytopathologic effects. RNA was extracted from NV-infected cells at passage three, and the genome of the four virus isolates was sequenced in its entirety and analyzed together with the genome sequences of all other NV isolates available in GenBank (Table). The genome sequence of the human NV isolate AF212302, designated as CDC, was used as the reference sequence. The sequences were aligned and manually edited. The phylogenetic trees were constructed and displayed as previously described (16). All new NV genome sequences were deposited in European Molecular Biology Laboratory (accession numbers AJ564621, AJ564622, AJ564623, and AJ627196).

A high sequence similarity (>99%) between NV sequences of pigs and humans was noted after aligning the whole genome sequences. A maximum likelihood phylogenetic tree, constructed by using pig NV genome

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Table. Nipah virus isolates used in the study

Isolate	Host	Accession no.
CDC	Human	AF212302
UMMC1	Human	AY029767
UMMC2	Human	AY029768
NV-Flying Fox	Flying foxes	AF376747
UM-0128 (NV/MY/99/UM-0128)[a]	Human	AJ564623
NV-Tambun (NV/MY/99/VRI-0626)[a]	Pig	AJ627196
NV-Seremban (NV/MY/99/VRI-1413)[a]	Pig	AJ564622
NV-Sungai Buloh (NV/MY/99/VRI-2794)[a]	Pig	AJ564621

[a]New isolates described in the study.

sequences, showed that NV isolates of pigs clustered tightly together with all known human and flying fox Nipah viruses (Figure A). As expected, all NV sequences clustered with HV to form a distinct group from other established genera within the *Paramyxovirinae* subfamily. The NV-Tambun showed the most divergent genome sequence from all other known Nipah viruses, in a phylogram constructed by using cumulative nucleotide sequence differences (Figure B). The overall divergence value was nonetheless small (<1%); hence, the isolate remained within the NV cluster.

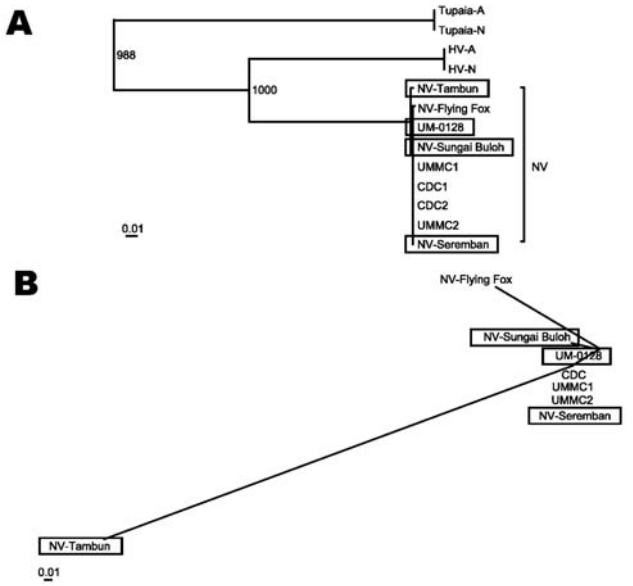

Figure. Phylogenetic trees illustrating the relationships of the pig Nipah virus isolates to all other known Nipah viruses and the related members of subfamily *Paramyxovirinae*. A) The maximum likelihood tree was drawn by using alignments of the full genome sequences. All the new isolates described in the study are shown in boxes. Abbreviations used and accession numbers not described elsewhere in the text are in parenthesis: Tupaia paramyxovirus (Tupaia-A) (AF079780); (Tupaia-N) (NC_002199), and Hendra virus (HV-A) (AF017149); (HV-N) (NC_001906). B) Unrooted maximum likelihood plot was constructed by using alignments of all the nucleotide differences in the Nipah virus gene coding regions (N, P, M, F, and G) shown in the online Appendix (http://www/cdc/gov/ncidod/EID/vol10no12/04-0452_app.htm) by artificially treating all the differences as a single stretch of nucleotide sequence.

Subsequent analysis of the deduced amino acid sequences of the NV-Seremban pig isolate showed that they were identical to the human NV isolates CDC and UMMC2 (online Appendix; available from http://www/cdc/gov/ncidod/EID/vol10no12/04-0452_app.htm). By contrast, the NV-Sungai Buloh pig isolate–deduced amino acid sequences were identical to the human NV isolates UMMC1 and UM-0128. NV-Seremban differed from NV-Sungai Buloh at only one amino acid position (1645) within the polymerase protein (L). Both isolates, however, differed from the flying fox NV isolate, NV-Flying Fox, at three amino acid positions, residues 30, 206, and 348 in the coding regions of nucleoprotein (N), phosphoprotein (P), and fusion protein (F), respectively. In contrast, the NV-Tambun pig isolate had a distinct signature sequence in comparison to all other NV. NV-Tambun differed from all known NV at 47 nucleotide positions; 28 of these differences occurred within the virus coding regions. The nucleotide differences were translated into amino acid changes at 11 positions; residues 274, 304, and 378 of the P protein, residues 147 and 250 of the matrix protein (M) and F protein, respectively, residues 20 and 272 of the glycoprotein (G), and residues 223, 1645, 1753, and 2039 of the L protein. Amino acid changes noted in the highly phosphorylated P protein at positions 274 and 304 resulted in residue changes from serine to arginine and threonine to alanine, respectively. These changes may reduce the potential phosphorylation sites in the P protein since serine, threonine, tyrosine, and histidine residues are the common targets for protein phosphorylation. Substitution of amino acids at positions 223 (threonine → asparagine), 1645 (serine → phenylalanine) and 2039 (histidine → asparagine) in L protein may also reduce the number of predicted potential phosphorylation sites on L. A substitution of amino acid isoleucine for asparagine at position 20 of the G protein added a potential glycosylation site apart from the eight identified N-linked glycosylation sites (6). However, the additional potential glycosylation site is located at the cytoplasmic tail of the protein and might make the addition of an additional glycan extremely unlikely, if not impossible. No nucleotide and consequently deduced amino acid variations, however, were observed in the N protein.

Conclusions

Findings reported here present for the first time molecular evidence that at least two major strains of NV of pigs were circulating during the 1998 NV outbreak in Malaysia, one strain from the initial outbreak in the north (NV-Tambun) and the other strain from the subsequent outbreak

approximately 4 months later in the south (NV-Seremban and NV-Sungai Buloh). The NV-Seremban and NV-Sungai Buloh pig isolates had identical sequences to those reported from human infections, which confirmed that the infections in humans during the southern outbreak originated from infected pigs. No record of isolation of the NV-Tambun is available from patients from the initial outbreak in Tambun or from the subsequent outbreaks. Isolation of NV-Seremban and NV-Sungai Buloh was not reported from the Tambun outbreak. Hence, ascertaining if the two major strains originated from the same initial focus of infection, Tambun, is not possible. Alternatively, the NV-Tambun could be the basal ancestral strain from which the later southern strain evolved. Two findings suggested this hypothesis: the Tambun outbreak occurred at least 4 months before the Seremban outbreak, and the sequence differences between NV-Seremban and NV-Sungai Buloh occurred as a result of genetic drift, a phenomenon not uncommon amongst RNA viruses. Then again, this occurrence is unlikely considering that the genome sequence of NV-Tambun diverges from the NV-Flying Fox, purportedly the initial source of NV infections. In addition, given that the NV sequences of both humans and pigs (UM-0128, NV-Seremban and NV-Sungai Buloh), sequenced independently in different laboratories, were practically identical, the sequence differences were not likely caused by inherent polymerase chain reaction errors or adaptation to tissue culture conditions. Therefore, the NV-Tambun strain is the more likely causal agent for the initial outbreak among pigs in Tambun, resulting from an infection acquired from a yet-to-be-identified source. By contrast, the subsequent outbreaks in the south were due to the pig NV isolates with higher sequence similarities to the NV-Flying Fox of Tioman Island. This finding implied that the 1998 Malaysia NV outbreak is unlikely to be due to a single transmission of NV from flying foxes of Tioman Island to pigs, but it points to the possibility of at least two different origins of NV infections.

This project was supported by the Ministry of Science, Technology and Innovation, Malaysia research grant #01-02-03-004BTK/ER/28. The Department of Veterinary Services, Veterinary Research Institute, Ipoh, Perak, Malaysia, and the Department of Medical Microbiology, Faculty of Medicine, University of Malaya permitted us to use their facilities and provided technical and laboratory assistance.

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Serologic Evidence of Lyssavirus Infection in Bats, Cambodia

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In Cambodia, 1,303 bats of 16 species were tested for lyssavirus. No lyssavirus nucleocapsid was detected in 1,283 brains tested by immunofluorescence assay. Antibodies against lyssaviruses were detected by enzyme-linked immunosorbent assay in 144 (14.7%) of 981 serum samples. Thirty of 187 serum samples contained neutralizing antibodies against different lyssaviruses.

The genus *Lyssavirus* belongs to the family *Rhabdoviridae* and includes seven species and one tentative species (1). Six of these seven species (or genotypes) have been isolated from bats. Rabies virus (RABV), responsible for most human rabies cases in the world, is associated with bats only in the Americas, and this association is currently responsible for most human rabies cases in North America. Lagos bat virus (LBV) and Duvenhage virus (DUVV) are found in Africa. European bat lyssavirus-1 (EBLV-1) and European bat lyssavirus-2 (EBLV-2) have been isolated in Europe. Australian bat lyssavirus (ABLV) has been detected in Australia. DUVV, EBLV-1, EBLV-2, and ABLV have been responsible for several fatal cases in humans (2–4).

In Asia, rabies infection of bats has rarely been reported. A human case of rabies with history of bat bite was first reported in 1954 in southern India. Two large surveys in the Philippines and in Malaysia failed to detect any rabid bats. Lyssavirus infection was detected in Thailand in a frugivorous bat, *Cynopterus brachyotis*, and in India in a frugivorous bat, *Pteropus poliocephalus* (2). Recently, new lyssaviruses (Aravan, Khujand, Irkut, and West Caucasian bat viruses) were isolated in southern Kyrghyzstan, northern Tajikistan, eastern Siberia, and the Caucasus from *Myotis blythi*, *M. mystacinus*, *Murina leucogaster*, and *Miniopterus schreibersi*, respectively (5). Furthermore, neutralizing antibodies against ABLV were detected in the Philippines in two frugivorous species and four insectivorous species, notably *M. schreibersi* (6).

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In Cambodia, rabies is endemic, transmitted mainly by dogs. Since 1997, the Institut Pasteur du Cambodge (IPC) has received heads of suspected rabid domestic dogs from 11 of the 23 Cambodian provinces. Dogs from nine provinces had laboratory-confirmed rabies infection. Since mid-1995, ≈9,000 people per year have received free post-exposure rabies treatment at IPC. No case of a human with rabies and a history of bat bite has ever been reported to IPC (7), but potential exposure to rabies from bats is often underappreciated (8).

Surveillance for lyssaviruses in bats in Southeast Asia has been very limited to date. No isolate has been identified, and no particular bat species has been implicated as a potential reservoir. We therefore conducted a survey to look for lyssavirus infection among bat populations in Cambodia.

The Study

A total of 1,303 bats were sampled from 35 locations in nine Cambodian provinces (Figure). Of these, 467 came from restaurants in Phnom Penh and belonged to the species *P. lylei*. The other 836 animals were captured in nine provinces and belonged to 16 species representing six of the seven bat families known in Cambodia (Table 1, Figure). Bats were captured at roosts by hand and with hand nets, or along flyways by night with mist nets or hard traps. Anesthetized captured animals were euthanized by cardiac blood puncture, and their organs were collected. Sampling bats from restaurants was restricted to collecting

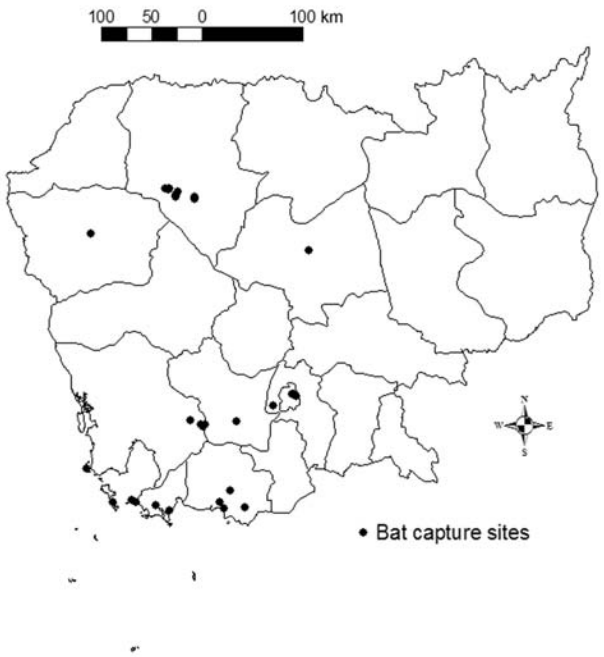

Figure. Location of bat capture sites during a survey on lyssavirus infection in bats in Cambodia, September 2000–May 2001.

Table 1. Lyssavirus enzyme-linked immunosorbent assay–reactive serum samples from bats, according to place of capture, Cambodia, September 2000–May 2001

Species (no. captured)	No. positive/no. tested (%)										
	Phnom Penh restaurants	Phnom Penh National Museum	Phnom Penh and vicinity	Siem Reap	Kirirom National Park	Kampot	Kompong Thom	Battam-bang	Kompong Som	Islands	Total (%)
Frugivorous											
Cynopterus brachyotis (1)					0/1						0/1 (0)
Cynopterus sphinx (83)			1/17	4/40	1/13					0/11	6/81 (7)
Macroglossus sobrinus (1)										0/1	0/1 (0)
Pteropus lylei (471)	25/224			0/4							25/228 (11)
Roussetus leschenaulti (16)					1/16						1/16 (6)
Insectivorous											
Hipposideros armiger (1)						0/1					0/1 (0)
H. larvatus (96)					0/16	2/40			1/36		3/92 (3)
H. pomona (6)									0/3		0/3 (0)
Murina cyclotis (1)					0/1						0/1 (0)
Rhinolophus acuminatus (2)					0/1				0/1		0/2 (0)
R. luctus (1)					0/1						0/1 (0)
R. malayanus (2)											
Scotophilus kuhlii (153)				21/110							21/110 (19)
Tadarida plicata (227)		26/104				23/78		8/33			57/215 (27)
Taphozous melanopogon (85)		0/6		1/1		1/27	3/6			1/32	6/72 (8)
T. theobaldi (157)		13/94				12/63					25/157 (16)
Total (1303)	25/224	39/204	1/17	26/155	2/49	38/209	3/6	8/33	1/40	1/44	144/981 (15)

blood and brain. All bats specimens were stored in 70% ethanol until species was identified.

Direct immunofluorescence assay (IFA) was performed on the brain of 1,283 (20 were not testable) bats to detect lyssavirus nucleocapsid (9). Rabbit antirabies nucleocapsid immunoglobulin G (Bio-Rad, Marnes-la-Coquette, France) was used at a concentration (2x) that reliably detects infection with the seven lyssavirus genotypes. None of the brains tested was positive. Attempts to isolate virus in newborn mice (9) from the brains of 24 bats that gave uncertain IFA results were unsuccessful.

Serum samples of bats were first screened for antibodies against lyssavirus by enzyme-linked immunosorbent assay (ELISA). Antigens were obtained from inactivated and titrated supernatants of BHK21 clone BSR cell cultures infected independently by four different genotypes circulating in bats, RABV (CVS strain), LBV, ABLV, and EBLV-1. These strains were chosen according to their ability to detect cross-neutralizing antibodies (10). Each supernatant was diluted to a titer of 6 x 10^4 focus-forming units (FFU)/mL in carbonate buffer. Polysorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 µL of RABV (CVS)/LBV mixture and 100 µL of ABLV/EBLV-1 mixture and incubated at 4°C overnight. The samples were diluted 1:50. Peroxidase-labeled protein A/G (Pierce, Rockford, IL) was used as conjugate. Three negative control serum samples and one positive control sample (equine rabies immunoglobulin 200 IU/mL) diluted 1:50 were included in each plate. Washing and diluent buffers, incubation, cutoff value, and positive definitions followed Rossi and Ksiazeck's technique (11). Of the 981 bat serum samples tested by this ELISA, 144 (14.7%) had a positive result (Table 1). Animals with ELISA-positive samples belonged to eight different species of both frugivorous and insectivorous bats.

ELISA test results were confirmed by using lyssavirus rapid fluorescent focus inhibition test (RFFIT) on 144 ELISA-positive and 43 ELISA-negative samples chosen at random. Each sample was tested independently against four different lyssaviruses: RABV, EBLV-1, ABLV, and LBV by an adaptation of RRFIT (12) and was considered positive for an average titer >42 after two independent assays.

Of the 146 samples with interpretable RFFIT results (76% were ELISA-positive), 10 (31%) of 32 samples from frugivorous bats and 20 (18%) of 114 samples from insectivorous bats were positive for neutralizing antibodies against at least one of the four genotypes (Table 2). In some cases when high titers against one virus were recorded, cross-neutralization occurred with other viruses. Geometric means for the 30 positive responses were 76.2 (n = 6), 71.9 (n = 13), 97.4 (n = 12), and 83.8 (n = 7) against CVS, EBLV-1, ABLV, and LBV, respectively. Eleven samples exhibited titers >100. Among the 10

RFFIT-positive frugivorous bats, neutralizing antibodies against ABLV were the most frequent (50%). Conversely, neutralizing antibodies against EBLV-1 were the most frequent (50%) among the 20 RFFIT-positive insectivorous bats. Positive responses were in the majority (83.3%) against these two viruses. The 30 bats with neutralizing antibodies belonged to frugivorous species, *Cynopterus sphinx* (n = 3) and *P. lylei* (n = 7), or insectivorous species, *Hipposideros larvatus* (n = 2), *Scotophilus kuhlii* (n = 5), *Taphozous theobaldi* (n = 2), *T. melanopogon* (n = 1), and *Tadarida plicata* (n = 10). No meaningful geographic trends were identified.

Conclusions

This study reports the first evidence of anti-lyssavirus neutralizing antibodies in serum samples from insectivorous and frugivorous bats in Cambodia. These serologic data support the likely occurrence of rabies, possibly from a previously undescribed lyssavirus, among bats in Cambodia.

A simple ELISA was developed to detect antibodies against lyssavirus in bat serum samples as a first screening. The sensitivity and specificity of this test can be estimated by comparing its results with those of the RFFIT, which is considered the most effective and reliable method of detecting anti-lyssavirus antibodies. This comparison gives us a relatively high sensitivity (83%, n = 30) and a low specificity (27%, n = 116); therefore, ELISA could be used to test large numbers of samples. RFFIT, a time-consuming technique, could be used to double-check ELISA-positive samples. However, prevalence results obtained with ELISA should be considered cautiously because RFFIT was performed on samples selected according to ELISA results (and not performed simultaneously with ELISA on randomly chosen samples).

The threshold for RFFIT positivity chosen in this study was slightly higher than that used in recent bat studies performed in Europe (3) and the Philippines (6). Although no accepted standard for bat sera exists, the titer of 42 obtained in RFFIT against CVS-11 corresponds in our hands to a titer of 0.8 IU/mL using the World Health Organization (WHO) human standard. The arbitrary cutoff chosen during this study is then slightly higher than the arbitrary value (0.5 IU/mL) established by WHO as evidence of neutralizing antibodies against rabies having been induced after vaccination (13). This cutoff was chosen to avoid problems of test specificity because of hemolysis present in some specimens. The samples considered to be positive in this study should then be considered as highly indicative of anti-lyssavirus–specific antibodies.

None of the brain samples showed evidence of lyssavirus antigen or infectious particles. Similar studies did not succeed in detecting lyssavirus antigen or RNA in bats (3,6,14,15). Because the 1,303 bats collected in Cambodia during this study were healthy and belonged to 16 different species, the expected number of positive reactions would not be very high. One positive bat among them would indicate a global prevalence of active infection of 8 per 10^4 bats, which would be high for randomly selected healthy bats.

Further investigation is needed to determine whether the circulation of lyssavirus in the Cambodian bat population poses a threat to human health. In the meantime, postexposure treatment should be considered in the event of a bat bite. The public, especially persons in close contact with bats (guano collectors, sugar palm tree collectors, persons with bats roosting in their houses), should be educated about the risk for rabies transmission from bats and should be encouraged to participate in surveillance by shipping specimens from sick bats for laboratory diagnosis of rabies.

Acknowledgments

We are grateful to the Cambodian authorities for authorizing bat captures and Y. Buisson and J.L. Sarthou for facilitating this study.

Dr. Molia was the recipient of a fellowship from the Fondation de France, Jeunesse Internationale.

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Table 2. Reactivity of serum samples from bats against four lyssaviruses with rapid fluorescent focus inhibition test, Cambodia, September 2000–May 2001

	Frugivorous bats (n = 32)		Insectivorous bats (n = 114)		All bats (N = 146)	
Virus[a]	Negative	Positive	Negative	Positive	Negative	Positive
RABV	30	2	110	4	140	6
EBLV-1	29	3	104	10	133	13
ABLV	27	5	107	7	134	12
LBV	28	4	111	3	139	7
Total[b]	22	10	94	20	116	30

[a]RABV, rabies virus; EBLV-1, European bat lyssavirus-1; ABLV, Australian bat lyssavirus; LBV, Lagos bat virus.
[b]Columns may add up to numbers higher than those mentioned in the total because of the reactivity of individual serum samples against more than one lyssavirus.

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Human-to-Dog Transmission of Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) was cultured from the nose of a healthy dog whose owner was colonized with MRSA while she worked in a Dutch nursing home. Pulsed-field gel electrophoresis and typing of the staphylococcal chromosome cassette *mec* (SCC*mec*) region showed that both MRSA strains were identical.

The Case

In 2000, a methicillin-resistant *Staphylococcal aureus* (MRSA) strain was isolated from a patient admitted to a multisite 1,100-bed tertiary-care teaching hospital in the Netherlands. This strain recurred during several outbreaks and spread to a 190-bed nursing home, which is part of the hospital. During a large outbreak in 2003 in the nursing home, 48 patients and 15 nurses were identified as carriers of MRSA, either in their nares, throat, perineum, or a combination of these sites. All MRSA isolates from the outbreaks were sent to the National Institute of Public Health and the Environment (RIVM) for identification and genotyping by pulsed-field gel electrophoresis (PFGE). Most MRSA had the same PFGE pattern, RIVM cluster 35.

In 2003, a 31-year-old female nurse who had psoriasis was identified as an MRSA carrier during the above-mentioned MRSA outbreak. The nurse was treated to eliminate MRSA carriage by applying mupirocin ointment in her nares and washing with a chlorhexidine in ethanol solution for 7 days. Initially she became MRSA-negative but later converted to a carrier state again: samples from her nose, throat, perineum, and skin lesions were taken, and MRSA could be isolated from all sites. She was treated for her psoriasis with topical application of triamcinolonacetonide/tetracycline to minimize the skin lesions and thereafter with oral doxycycline and rifampin to eliminate MRSA. However, after some weeks, she became colonized again at all previously mentioned sites. Screening her home environment showed that her 1-year-old daughter, who also had psoriasis, was colonized in the nose, throat, and skin lesions. The nares, but not the perineum, of their healthy pet dog were also colonized. The dog had not been treated with antimicrobial drugs in the past. Samples from nose, throat, and perineum from the patient's husband were MRSA negative, as were samples from nose, throat, perineum, and skin lesions of the baby's grandmother, who also had psoriasis and took care of the baby when the mother worked.

The staphylococci isolated from the nurse, her child, and the dog were all identified as *S. aureus* by conventional methods, Vitek 2 (BioMérieux, Marcy-l'Etoile, France) and Martineau polymerase chain reaction (PCR), which targets the *tuf* gene (1). Susceptibility testing was performed by using the Vitek 2, according to the manufacturer's instructions. The isolates were resistant to penicillin G, ofloxacin, trimethoprim-sulfamethoxazole, and fusidic acid and tested susceptible to aminoglycosides, tetracyclines, erythromycin, clindamycin, vancomycin, rifampin, and oxacillin. Disk diffusion testing demonstrated susceptibility to mupirocin. Because the *mecA*-positive MRSA isolates from previous outbreaks in the nursing home were also resistant to ofloxacin, trimethoprim-sulfamethoxazole, and fusidic acid, and tested either oxacillin resistant or oxacillin susceptible by Vitek 2, the oxacillin resistance of our isolates was also tested by several other methods. The oxacillin screening test (Mueller-Hinton agar supplemented with 4% NaCl containing oxacillin at a concentration of 6 μg/mL) showed no growth after 24 h and 48 h of incubation at 35°C. However, the Etest (AB Biodisk, Solna, Sweden) showed that the isolates of the dog and the baby had an oxacillin MIC of 6 mg/L and the isolate from the nurse had an oxacillin MIC of 4 mg/L and could therefore be classified as oxacillin resistant. In addition, a PBP2 slide latex agglutination test (Oxoid, Haarlem, Netherlands) was positive for all three isolates. The presence of the *mecA* gene (2) was demonstrated by a positive *mecA* PCR for all three isolates.

Characterization of the staphylococcal chromosome cassette *mec* (SCC*mec*) was performed by typing the *ccrA/B* gene complex, the *mec* complex, and by means of a PCR strategy, which detects structural variants of SCC*mec* covering the entire genetic element (2–6). Typing the SCC*mec* of the MRSA strains cultured from the dog and its owner showed that these seemed to be identical (*ccrA/B* gene type 4, *mec* complex class B, and no loci A-H were found).

PFGE was carried out as described by Schwarzkopf et al. (7). PFGE showed that all three MRSA isolates (from the dog, the child, and the nurse) had indistinguishable

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patterns and that they belonged to RIVM cluster 35, an epidemic human MRSA cluster. This cluster was the same one cultured previously from patients and contacts in the nursing home during several outbreaks since 2000. On the basis of these data, we assume that the dog became colonized with the same strain as its owner through contact, and that either the dog or the baby or an unknown source within the nursing home reinfected the nurse. We finally treated mother and child for the skin lesions simultaneously as mentioned before. Afterward, the mother and the dog received an oral course of doxycycline and rifampin; the baby was treated with clarithromycin and rifampin. This treatment finally eliminated MRSA from the mother, the child, and the dog. Topical application of antimicrobial drugs in dogs is impractical. Follow-up cultures from the dog and the child were taken for 2 months, the mother was monitored for 9 months, and all cultures remained negative.

Conclusions

MRSA is an important cause of human nosocomial and community-acquired infections worldwide. In contrast, few cases of MRSA infections in dogs have been reported (8–11). We report the first known case of human-to-animal transmission of MRSA in the Netherlands. In the United States, the transmission of MRSA between a pet dog and its owners has been reported (12): a patient with diabetes had recurrent infections of the leg with a mupirocin-resistant MRSA strain, and his wife had cellulitis. Culture from their dog's nose grew an MRSA isolate with the same antimicrobial-resistance pattern and an identical PFGE pattern as the isolate cultured from wounds and nares of the couple. Further recurrence of the MRSA infection of the couple was only prevented when the dog was no longer an MRSA carrier (12). Cefai et al. (13) isolated an MRSA strain with an identical phage type from the nose of a male nurse, his wife, and their pet dog. Transmission of MRSA between humans and horses has also been suspected in a veterinary teaching hospital in the United States (14).

SCC*mec* is a mobile genetic element that carries the *mecA* gene, which mediates methicillin resistance in staphylococci. To date, four SCC*mec* types have been described (2,3,6). Typing the SCC*mec* of the MRSA in our study showed that they were of an uncommon type, which could not be classified as one of the four SCC*mec* types. Oliveira et al. (2) defined SCC*mec* type IV as harboring *ccrA/B* type 4 in combination with characteristic loci, whereas Ito et al. (3) defined SCC*mec* type IV as the unique combination of the class B *mec* and type 2 *ccr* gene complex. Our MRSA had *ccrA/B* type 4 and class B *mec* but no loci. Therefore, this MRSA cannot be classified in the current system and may present a new SCC*mec* type. This new type may spread successfully, as has been seen with SCC*mec* types I–IV. During the 1960s, MRSA carrying SCC*mec* type I spread across the world, followed by a second wave of MRSA during the late 1970s that carried SCC*mec* II; strains with SCC*mec* III spread during the 1980s, and strains with SCC*mec* type IV have been isolated worldwide since the beginning of the 1990s. Recently, an isolate with a *ccr*5 type was described (15), which indicates that additional SCC*mec* types are present in *S. aureus*. Isolates with new SCC*mec* types may be the frontrunners of new waves of MRSA, posing an unknown health threat.

Automated systems such as Vitek 2 are generally reported to be reliable for testing methicillin or oxacillin susceptibility, but the Vitek 2 did not detect MRSA in our study. Misclassification of *mecA*-positive *S. aureus* as oxacillin susceptible by the Vitek 2 has been reported before by Felten et al. (16), especially in strains with relatively low oxacillin MICs. The oxacillin-screening test also did not detect our MRSA strains. This can be explained by their relatively low oxacillin MICs (4 mg/L and 6 mg/L, respectively). The most reliable procedure for detecting MRSA remains the PCR amplification of the *mecA* gene. MRSA in this study was resistant to fluoroquinolones, which is common in MRSA but not in methicillin-susceptible *S. aureus* (17). Therefore, fluoroquinolone-resistant *S. aureus* strains should always be suspected of being MRSA and should be tested for the presence of the *mecA* gene by PCR.

In conclusion, dogs and other pets living in close contact with human MRSA carriers can become colonized with MRSA. Failure to detect and treat these colonized pets can result in recurrent MRSA colonization or infection in humans. Therefore, the risk of pets being the source of unexplained carriage or relapse of infection in humans should be recognized. Antimicrobial therapy of healthcare workers and, at the same time, of MRSA carriers and any infected family members or pets, can eliminate recurrent MRSA carriage. Pets should be treated systemically, since topical application is impractical.

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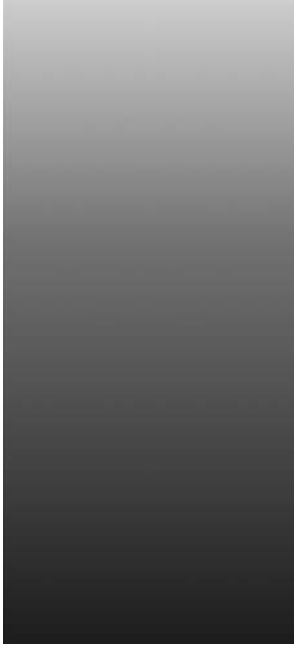

Francisella tularensis Peritonitis in Stomach Cancer Patient

Xiang Y. Han,* Linus X. Ho,* and Amar Safdar*

Tularemia with peritonitis developed in a 50-year-old man soon after diagnosis of stomach cancer with metastasis. The ascites grew *Francisella tularensis* subsp. *holarctica*, which was identified by sequencing analysis of the 16S rDNA. The infection resolved with antimicrobial treatment. Antibodies detected 4 weeks after onset disappeared after chemotherapy-associated lymphopenia.

Case Study

A 50-year-old man arrived at the emergency department in September 2003 with a 2-day history of high fever (temperature up to 40.8°C), rigors, dry cough, nausea, vomiting, lower abdominal pain, and melena. The patient had recently been diagnosed with signet-ring–cell carcinoma of the stomach with evidence of metastasis to the lung and peritoneum and multiple thoracic and abdominal lymph nodes. Chemotherapy had been planned to start soon.

Physical examination showed fever (temperature 39.7°C), hypotension (96/51 mm Hg), a systolic heart murmur with regular rhythm, and lower abdominal tenderness and rebound. Laboratory examination showed microcytic anemia (hemoglobin 87 g/L), relative neutrophilia (82% of 7.8 x 10^9/L total leukocytes), and relative and absolute lymphopenia (7% of leukocytes or 0.55 x 10^9/L). A chest x-ray was normal, as were liver function tests and pancreatic enzymes. A presumptive diagnosis of sepsis with peritonitis was made, and blood and urine were collected for cultures. Empiric cefepime (2 g every 8 h) and tobramycin (one dose 500 mg) therapy was started before hospital admission.

The following day, an esophagogastroduodenoscopy showed cancer ulceration as the source of melena. An echocardiography excluded endocarditis. An abdominal sonogram showed small pockets of ascites in the abdomen and pelvis, and the fluid showed many neutrophils, lymphocytes, and macrophages, consistent with peritonitis. The ascites (5 mL) was also cultured. Despite cefepime treatment, the patient's fever persisted for 36 hours, which prompted a change to imipenem (500 mg every 6 h) and vancomycin (1 g every 12 h). The fever subsided in 1 day, as did the abdominal manifestations. The patient was discharged the following day with further oral gatifloxacin (400 mg four times a day) and amoxicillin/clavulanate (875 mg twice a day) for 10 days.

Anticancer therapy that consisted of radiation to the stomach and daily capecitabine and weekly paclitaxel was begun 5 days after discharge. Two weeks later, at completion of these treatments and the oral antimicrobial drugs, the abdominal lymphadenopathy showed improvement on computed tomography. However, the tumor itself, as well as the lung nodules, remained stable. Additional chemotherapy with three cycles of paclitaxel and carboplatin was started soon afterwards.

Meanwhile, the ascites culture (Bactec Aerobic/F bottle with resins) became positive after 8 days of incubation, and a small gram-negative coccobacillus (strain MDA3270) was isolated. Its fastidious growth and unusual Gram stain features prompted sequencing analysis of the 16S rDNA for identification (1). A 586-base pair DNA fragment, amplified by polymerase chain reaction, demonstrated 100% sequence homology with *Francisella tularensis* subsp. *holarctica* (GenBank accession no. L26086, Wilson et al., unpub. data, and AF227312) (2). On review, the culture and stain features fit *F. tularensis*. The subspecies was confirmed by the Centers for Disease Control and Prevention (CDC) (Fort Collins, CO). The blood culture remained negative after 7 days of incubation.

The diagnosis of typhoidal tularemia (24 days after onset) led the patient to be further treated with intravenous gentamicin for 2 weeks (120 mg every 8 h), followed by 2 weeks of oral ciprofloxacin (750 mg twice a day). A query of exposure history was also made. The patient was a farmer from northeastern Mississippi and had cut hay in a field infested with rodents 3 weeks before onset. He had traveled from home to Houston for the cancer care. The patient had no history of camping, hunting, or bites by ticks or deerflies. After 6 weeks of anticancer therapy (7 weeks after tularemia), the patient's carcinoembryonic antigen decreased substantially. However, a predominant 6-cm mass in the gastrohepatic ligament region persisted, which raised the question of infection versus cancer. Thus, a percutaneous needle biopsy was performed, and cancerous mucin was demonstrated. Further chemotherapy continued.

A convalescent antibody against *F. tularensis* was detected 4 weeks after onset (titer 1:40, direct agglutination method); however, it disappeared at 3 months after chemotherapy-associated lymphopenia (Figure). Before chemotherapy started, the lymphocyte counts had been

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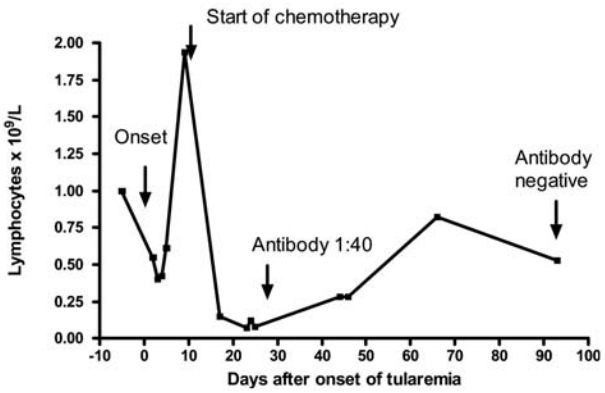

Figure. Effect of tularemia and anticancer chemotherapy on the lymphocyte counts and antibody response in a patient with gastric cancer.

1.0 x 10^9/L before infection, 0.55–0.40 x 10^9/L in early infection, and 1.94 x 10^9/L 8 days postonset. After chemotherapy, however, the counts dropped sharply to 0.07 x 10^9/L (96% reduction) in 2 weeks. During the remaining weeks of therapy, lymphopenia persisted despite improvement. In contrast, the patient's neutrophil counts were normal to slightly elevated during the entire course.

Conclusions

The interest in tularemia and its pathogen, *F. tularensis*, is renewed due to the high bioterrorism potential of the organism, i.e., listed as a category A by CDC (www.bt.cdc.gov). *F. tularensis*, a gram-negative coccobacillus, survives well in the environment and is facultative in infected host cells (macrophages). It has a high infectivity rate and is zoonotic. Human infection occurs mainly in animal handlers and those who are bitten by ticks, deerflies, or both. Airborne and waterborne outbreaks have also been reported (2–4). From 1990 to 2001, a total of 1,497 tularemia cases was reported to CDC (mean 125 cases per year), with 55% occurring in the states of Arkansas, Missouri, South Dakota, and Oklahoma (5,6). During those 12 years, however, Mississippi, the home state of our patient, had only one case. In view of the highest disease activity in neighboring Arkansas (324 cases), underreporting of the disease in Mississippi was a possibility in addition to other explanations, such as geographic differences and barriers (Mississippi River).

Tularemia manifests a few clinical forms and, before the antimicrobial era, carried a high fatality rate. The diagnosis of tularemia is often difficult to make, especially for the typhoidal and pneumonic forms. Most cases are diagnosed by serologic tests late in infection or afterwards. In an epidemiologic study of >1,000 cases (7), only 11% were diagnosed by isolation of *F. tularensis* from a body source, such as ulcer fluid, blood, lymph node aspirate, and pleural fluid. With improved blood culture methods in the past 2 decades, however, cases of *F. tularensis* bacteremia have been reported (3,8–14). These blood cultures became positive after an incubation period of 3 days to 3 weeks (median 7 days). Most cases were in patients with underlying conditions or diseases, such as old age, alcohol abuse, diabetes mellitus, transplantation, or AIDS. The immunocompromised patients tend to have prolonged infection or die. In a syngeneic bone marrow transplant patient (15), the infection presented as a 3-cm solitary pulmonary nodule, and after 6 weeks of antimicrobial treatment, the culture-positive nodule vanished.

Tularemia with associated peritonitis is extraordinary rare. Our patient's peritonitis was likely related to metastatic stomach cancer that had breached the integrity of peritoneum and regional blood vessels and lymph nodes, leading to peritoneal spill of the organism (free or intramacrophage ones). The ascites did contain many macrophages. To combat the infection, neutrophilia developed. Because the patient was severely anemic, absolute lymphopenia developed from normal baseline (Figure). Lymphopenia is generally absent in tularemia, and this patient's response was likely a compromise for neutrophilia. However, the lymphocyte count rebounded a few days later. The patient's response to cefepime therapy was suboptimal in view of the persistence of fever and concurrent isolation of the organism. Streptomycin or gentamicin, not a cephalosporin, is recommended to treat tularemia. Successful treatment with a fluoroquinolone has also been reported in at least 10 recent cases (11). The source of infection could not be determined definitively; however, living and working in a farm and the history of exposure to rodent-infested hay were probably important. Recently, landscaping occupation, such as lawn mowing and weed-whacking, is recognized as a risk for exposure (16).

The antibody response against tularemia is usually strong, peaking at 2–3 months after onset (2). In an outbreak caused by *F. tularensis* subsp. *holarctica*, the peak titer reached 1:256 to 1:8,192 (median 1:1,024) (2). In our patient, urgent initiation of the lymphotoxic anticancer chemotherapy blunted the initial response by ablating the antibody-producing lymphocytes. One of the agents, capecitabine, causes lymphopenia in >90% of patients following treatment (17). During the 11-week chemotherapy, existing antibodies (titer 1:40) were degraded in the circulation (3–4 half-lives) and became undetectable. Therefore, this case illustrates that, after anticancer chemotherapy, lack of antibody does not exclude an infection that usually elicits antibody response.

F. tularensis has four subspecies (biovars): *tularensis*, *holarctica*, *novicida*, and *mediasiatica*, and the first two subspecies are the main causes of tularemia in the United States. *F. tularensis* subsp. *holarctica*, also known as type B or biovar *palaearctica*, is generally less virulent than

F. tularensis subsp. *tularensis* (type A). Both typhoidal and cutaneous forms have been reported for *F. tularensis* subsp. *holarctica* (2,3,9,11,13,14). For the typhoidal cases, including ours, all nine reported patients recovered, and the median incubation of blood cultures was 9 days (4 days–3 weeks) (3,9,11,13,14), similar to the 8 days in our ascites culture.

Identifying *F. tularensis* may be difficult because of its rarity and fastidious growth, especially in areas where disease is nonendemic. Our laboratory has been using the 16S rDNA sequencing method to identify mycobacteria and other fastidious organisms. The method is considered to be the single best method to identify bacteria and will likely impact patient care in addition to microbiologic research.

Acknowledgments

The authors thank Jeannine Peterson, Martin Schriefer, and Paul Mead for confirming subspecies identification and Jeff Tarrand for review and discussion.

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First Human Cases of Tickborne Encephalitis, Norway

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The first reported case of tickborne encephalitis (TBE) in Norway occurred in 1997. From 1997 to 2003, from zero to two cases of human TBE have been diagnosed per year in Norway, for a total of eight cases. Clinical TBE cases in dogs are not reported in Norway.

In Scandinavia, tickborne encephalitis (TBE) is endemic in the coastal areas along the Baltic Sea. The first reports of TBE from Sweden and Finland date back to1954 and 1956, but the disease was not been found in Norway until 1997. Since then, eight cases of human TBE have been reported, and five cases have been published in a Norwegian journal (1,2).

In a study of serum samples from dogs in Aust-Agder County, immunoglobulin (Ig) G antibodies to TBE virus (TBEV) were detected in 16.4% of the samples (3). Clinical TBE cases in dogs are not reported in Norway, but the disease is probably underdiagnosed because antibody testing is not usually done. We present three new cases of human TBE and summarize the clinical characteristics and laboratory findings from all eight patients.

The Study

Patient 6 is a 62-year-old man from the town of Mandal who was bitten by a tick; onset of symptoms began 2 weeks later. At the end of May, the patient was dizzy and weak, had a headache, chills, and fever. He was hospitalized on June 11, 2002.

The antibody from serum sample profiles showed previous infection with herpes simplex and varicella zoster viruses. *Borrelia* antibodies could not be detected in serum samples taken at 5-week intervals. IgM antibodies against *Mycoplasma pneumoniae* were not detected, and virus cultures were negative. Nucleic acids from herpes simplex, varicella zoster, or enterovirus were not detected in cerebrospinal fluid (CSF).

TBEV IgM and IgG antibodies were detected in serum samples, with high levels of IgM (optical density [OD] 1.580 on June 13 to OD 0.899 on July 18) and high IgG levels (13.06, OD 1.235 on June 13 to OD 1.742 on July 18). Cut-off values were 0.250 for IgM and 0.263 for IgG on June 13 and 0.271 for IgM and 0.278 for IgG on June 18. Neutralization test antibodies in serum samples rose from <5 in samples taken on June 13 to 10 in samples from July 18. Symptoms gradually disappeared, and the patient completely recovered in 2 months.

Patient 7 is a 53-year-old man who was visiting a cabin in the coastal area near Mandal. Symptoms began at the end of June, with fever, increasing headache, nausea, and vomiting. He was hospitalized on July 20, 2002. His liver enzymes were slightly raised. Computed tomographic scan was normal. *Borrelia burgdorferi* antibodies were detected in serum, without intrathecal production of *Borrelia* antibodies. Nucleic acids from herpes simplex virus, varicella-zoster virus, or enterovirus were not detected in CSF.

TBEV IgM and IgG antibodies were detected in serum samples, with high levels of IgM (OD 2.064 on July 22; OD 1.916 on July 30; and OD 1.499 on August 8) and rising IgG levels (OD 0.597 on July 22; OD 0.876 on July 30; and OD 1.993 on August 8). Cut-off values were 0.277–0.280 for IgM and 0.266–0.275 for IgG). Neutralization test antibody levels rose from <5 in serum taken July 21 to 10 in serum from November 25. Borderline values of TBEV antibodies were found in spinal fluid. During the first several months after illness onset, the patient had cognitive dysfunction but gradually returned to work.

Patient 8 is a 74-year-old man, who lives in Kristiansand and has a camper in Søgne. Since August 2003, he had increasing headache and from October 3, 2003 the headache was intense and accompanied by nausea and vomiting. His personality was altered during these weeks, with reduced memory about recent events in particular, irritability, and verbal aggressiveness. He was admitted to the hospital on October 6, 2003. Results from computed tomography were normal, and electroencephalogram showed changes consistent with encephalitis. *Borrelia* antibody levels in serum samples were low. Intrathecal production of *Borrelia* antibodies could not be detected. Nucleic acids from herpes simplex or enterovirus were not detected in spinal fluid.

High levels of TBEV IgM (OD 1.461 on October 6 and OD 1.200 on November 5) were detected in sera together with rising IgG levels (OD 0.652 on October 6 and OD 1.475 on November 11). Cut-off values were 0.281–0.286 for IgM and 0.259–0.265 for IgG. In spinal fluid from October 3, intrathecal production of TBEV antibodies could not be detected, but one month later, intrathecal IgM was produced. During hospitalization, the patient recovered well. After 10 to 11 days, he was aware, and his mental situation improved considerably. He was also able to walk on stairs. After 4 to 5 months, he was fully recovered.

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The Agder counties have the highest incidence of *Borrelia* infections in Norway (33 cases/100,000 persons, 1997–2003). The incidence of neuroborreliosis is 10 cases per 100,000 persons (4). The first case of TBE in Norway was reported in 1997 (1). The previously published clinical signs and symptoms and results from these five patients (1,2) are summarized as case 1–5, while the three new patients are presented as patients 6–8 (Tables 1 and 2).

The eight patients included seven men and one woman from 42 years to 74 years of age. Biphasic courses were described in two patients. All patients had intense headache, seven had vertigo and nausea, and six had vomiting. Seven patients were hospitalized, three with reduced consciousness, two with mental disturbances; all seven had more or less severe neurologic abnormalities. Three had ataxia; one had diplopia; and one had speech difficulties, bilateral ptosis, paresis of eye and pharynx muscles, and paresis of muscles in the left shoulder. One had an epileptic seizure. All patients had fever, with temperatures from 38°C to 40°C. Serum samples were obtained from all eight patients and had signs of inflammation with C-reactive protein level of 10–105 mg/L and elevated leukocyte count of 8.3–15.4 x 10^9/L. Seven patients underwent lumbar puncture; CSF pleocytosis and elevated protein levels were found in all patients. Nucleic acids from herpes simplex virus, varicella-zoster virus, or enterovirus were not detected in the spinal fluid specimens, which excludes the most common differential diagnostic causes of encephalitis.

In all patients, high serum levels of TBEV IgM and IgG antibodies were detected with enzyme-linked immunosorbent assay methods. In neutralization tests, serum antibody titers increased from ≤5 to 10 in five of the patients, 10 to 20 in one, and 10 to 40 in one patient (1,2). Seven patients recovered during the first 6 months. Two had cognitive dysfunctions during the first several months. One person still had paresis and atrophy of the shoulder muscles 1 year later.

Although the diagnostic tests are not absolutely specific for TBE compared to the closely related Louping ill virus, no cases of Louping ill virus in livestock (Snorre Stuen, pers. comm.) or human infections have been reported in Norway since 1991; none of the eight patients lived close to or worked with sheep or goats. The clinical characteristics of the Norwegian patients are similar to those of Swedish patients (5). In Sweden, the disease is caused by TBEV subtype 1.

All eight patients with TBE in Norway became ill after being bitten by a tick in the coastal areas of the Agder counties. Four had been on Tromøy Island in Aust-Agder County before becoming ill, while one had been in Lyngdal and three in Mandal and Søgne in Vest-Agder County. None of the patients had been abroad in the 3 weeks before becoming ill.

TBE was assumed not to be present in Norway. Thus, all patients with suspicious cases of TBE may not have been tested for antibodies to TBEV. In Agder, we have tested for TBE since 1999, but the disease may still be underdiagnosed.

Some seroprevalence studies have been carried out. TBEV IgG antibodies were detected in 0.3% to 0.4% of the serum samples from persons in Agder counties. From persons on Tromøy Island, antibodies were found in 2.4% of serum samples, and in other coastal districts, the seroprevalence was 0%–11% (1,6). The number of human serum samples tested is limited, and the vaccination status is unknown. However, vaccination is unlikely because Norwegians are only vaccinated against flaviviruses on special travel indications.

In Sweden, the incidence of human cases of TBE has risen during the last few years, and new TBE foci have been reported (7). During the last 2 decades, an increased number of TBE cases have been reporting in most European countries. Changes in the distribution of TBEV

Table 1. Characteristics of patients with tickborne encephalitis, Norway, 1997–2003

Date	Patient no.	Age	Sex	Symptoms/neurologic disturbances	Disease duration
08/1997	1	42	Male	Biphasic course. Headache, nausea, vomiting, migrating myalgia/hyperreflexia.	1 mo
08/1998	2	72	Male	Fever, nausea, vomiting, confusion, speech disturbance/somnolence, mental disturbance, vertigo, bilateral ptosis, paresis of eye muscles, light throat paresis, paresis of the left shoulder	Sequela >1 y
10/1999	3	60	Male	Fever, headache/normal organ status	1 month
10/2000	4	67	Male	Fever, headache, nausea, vomiting/confusion, cognitive dysfunction.	Cognitive dysfunctions in 2–3 mo
10/2000	5	43	Female	Biphasic course. Fever, headache, nausea, vomiting/diplopia, ataxia.	1 mo
06/2002	6	62	Male	Fever, headache, nausea/ataxia.	2 mo
07/2002	7	53	Male	Fever, headache, nausea, vomiting/paresthesia, ataxia	Cognitive dysfunctions in months
10/2003	8	74	Male	Headache, nausea, vomiting, altered personality, irritability, verbal aggressiveness/confusion, and ataxia.	4–5 mo

Table 2. Laboratory findings in serum and spinal fluid specimens from patients with tickborne encephalitis, Norway, 1997–2003[a]

		Serum		Spinal fluid	
Patient no.	Temperature °C	CRP mg/L	Pleocytosis 10^9/L	Pleocytosis 10^6/L	Protein mg/L
1	38.4	10	ND	ND	ND
2	39.6	105	8,3	500	850
3	–	–	–	47	790
4	39.6	32	11,3	39	622
5	40	15	8,6	24	609
6	39.5	18	13	130	1,180
7	38	46	15,4	115	1,337
8	38.3	15	12,1	22	649

[a]ND, not done. CRP, C-reactive protein TBE was diagnosed retrospectively. The patient was not hospitalized; –, not published.

have been indicated, and the Norwegian cases are from areas where new foci have been predicted (8).

Conclusions

In Norway, 0–2 cases of TBE were diagnosed per year from 1997 to 2003. All patients have been bitten by a tick in the Agder counties in southern Norway. Of the first eight Norwegian patients, four had been on Tromøy Island in Aust-Agder County before becoming ill. The four most recent patients were bitten by ticks in Lyngdal, Mandal, and Søgne in the coastal areas of Vest-Agder County. The seroprevalence studies indicate that Tromøy and some spots along the coast in the southernmost part of Vest-Agder County may have a higher incidence of TBE than the rest of the Agder counties.

Our results confirm that TBE occurs in the coastal areas of southern Norway. Although TBE is a rare disease in Norway, the situation has to be monitored carefully. Further studies are required to establish guidelines for preventive measures such as vaccination.

Dr. Skarpaas is a medical microbiologist. Her research interests include infectious diseases and microbiology, especially tick-borne infections.

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Antibodies to SARS Coronavirus in Civets

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Using three different assays, we examined 103 serum samples collected from different civet farms and a market in China in June 2003 and January 2004. While civets on farms were largely free from SARS-CoV infection, ≈80% of the animals from one animal market in Guangzhou contained significant levels of antibody to SARS-CoV, which suggests no widespread infection among civets resident on farms, and the infection of civets in the market might be associated with trading activities under the conditions of overcrowding and mixing of various animal species.

Severe acute respiratory syndrome (SARS) first appeared in November 2002 in Guangdong Province, China (1). The outbreak was caused by a newly emerged virus now known as the SARS-associated coronavirus (SARS-CoV), which is believed to originate from animals. Most of the early index cases in Guangdong Province were concentrated in food handlers, and workers in live-animal markets had higher rates of antibodies to SARS-CoV than persons in other occupations (2,3). Studies have indicated that Chinese ferret-badgers (*Melogale moschata*), masked palm civets (*Paguma larvata*), and raccoon-dogs (*Nyctereutes procyonoides*) could be naturally infected by SARS-CoV or a closely related virus (4). Furthermore, experimental infection studies indicated that a variety of animals, including monkey, cat, ferret, mouse, and pig, are susceptible to SARS-CoV infection (5–9). These findings highlight the difficulties facing investigation into the origin of SARS-CoV.

Civets have been considered one of the most likely animals responsible for animal-to-human SARS-CoV transmission, and on this basis, more than a thousand civets in Guangdong were culled in January 2004. However, no conclusive evidence suggests that civets are the natural reservoir host of SARS-CoV or that civets in their natural habitat are infected with SARS-CoV. Lack of access to wild civets and regulatory issues involved make conducting detailed field studies of wild civets difficult, if not impossible, for the foreseeable future. Since most civets in markets are sourced from civet farms, we have conducted a preliminary serologic study on the prevalence of antibodies to SARS-CoV in civets from the market and farms.

The Study

After detecting SARS-CoV in civets from animal markets in Shenzen in late November 2003, the Guangdong government launched a campaign to cull all civets in the province to reduce the risk of SARS-CoV transmission to humans (10). To study the distribution of SARS-CoV and antibodies in these culled animals, intestine tissues and serum samples were taken from 56 animals: 38 civets from four farms in different regions of Guangdong Province (10 from Zhuhai, 10 from Shanwei, 9 from Shaoguan, and 9 from Qingyuan; Figure) and 18 civets from the Xinyuan Live Animal Market in Guangzhou.

Because of time constrains and regulatory issues, selection was conducted on the basis of convenience and personal contact with groups involved in the slaughter campaign. However, we tried to select civets from farms ≥100 km apart in the Guangdong Province. A total of 41 civet farms were in Guangdong Province at the time of the slaughter campaign, and most had <100 animals. No biosecurity measures were used in farms or markets, and no veterinary examination or accreditation was required for civet farming or trading. All of the farms tested had obtained their original seed stock from markets.

Also included in the study were 47 civet serum samples that had been previously collected in early June 2003 from two civet farms in Luoning City of Henan Province and Changsha City of Hunan Province. The farm conditions were similar to those in Guangdong, basically small-scale farms without biosecurity or animal health safeguards.

All serum samples were inactivated at 56°C for 30 min, transferred to the Australian Animal Health Laboratory, and inactivated by gamma irradiation before analysis. Anti–SARS-CoV antibody in serum was detected by using immunofluoresence antibody assay (IFA) and quantified in a microtiter virus neutralization test (VNT). The SARS-CoV (strain HKU-39849) used in both VNT and IFA was plaque purified three times in Vero cells, and stock virus (titer 5 x 10^7 50% tissue culture infective dose [$TCID_{50}$]) prepared by two low-multiplicity passes in Vero cells. In IFA, monolayers of Vero cells infected with SARS-CoV at a multiplicity of infection of 0.02 $TCID_{50}$/cell were methanol-fixed 24 h postinfection, exposed to a range of serum dilutions, and bound antibody detected by using

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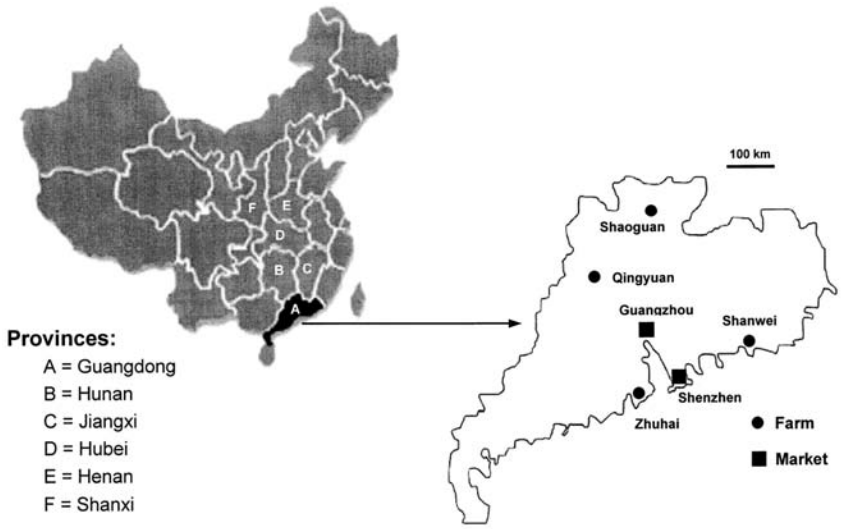

Figure. Geographic distribution of the farms and market examined in this study. The diagram on the left identifies the six provinces relevant to this study. The diagram on the right is an enlarged map of Guangdong Province showing the locations of the four farms and the capital city Guangzhou, where the live animal market was located. Also shown is Shenzhen, where civets from live animal markets were tested by Guan et al. in May 2003 (5).

fluorescein isothiocyanate–conjugated protein A (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Groups of samples that reacted positively in either VNT or IFA were also subjected to Western blot analysis with a recombinant SARS-CoV nucleocapsid (N) protein expressed in *Escherichia coli*. Bound antibodies were detected by using alkaline phosphatase–conjugated protein A/G (Pierce, Rockford, IL).

Intestine tissues collected from the 56 animals in January 2004 were also tested for SARS-CoV viral nucleic acid by using reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from these samples by using the Trizol method (Invitrogen, Carlsbad, CA), followed by first-strand cDNA synthesis using the Superscript II RNase H reverse transcriptase (New England Biolab, Beverly, MA) and random hexamer primers. PCR amplification was conducted by using Ex Taq polymerase (TaKaRa). Three pairs of SARS-CoV–specific primers were used to amplify regions in the N gene (forward, 5′-ATGTCTGATAATGGACCCCAAT; reverse, 5′-TTATGCCTGAGTTGAATCAG), the M gene (forward, 5′-ATGGCAGACAACGGTACTATT; reverse, 5′-CTTACTGTACTAGCAAAGCAAT) and the S gene (forward, 5′-ATGTTTATTTTCTTATTATTTC; reverse, 5′-GTCGACATGCTCAGCTCCTAT), respectively.

Of 103 civet serum samples tested, 18 were positive on at least one of the three assays used, for ≈17% overall seroprevalence. However, when seroprevalence among civets from farms and the market was compared, differences were observed. For samples taken in January 2004, 14 of 18 obtained from the Xinyuan Live Animal Market in Guangzhou tested positive by all three assays (Table), for a seroprevalence of 78%. In contrast, the prevalence on each farm was ≤40% (4 of 10 animals from the farm in Shanwei tested positive, and no positive animals were found on the other farms); the overall prevalence on farms was 4 (≈10%) of 38. SARS-CoV antibody levels in the four animals at the farm in Shanwei, which is located ≈240 km east of Guangzhou (Figure), were lower than those from the market, and two samples positive by VNT failed to react on IFA or Western blot (Table).

Intestinal tissues collected from the 56 civets were tested by RT-PCR using N-gene primers; none of the samples were positive. Negative results were confirmed by RT-PCR with M- and S-gene primers. Therefore, virus isolation from these tissues was abandoned. The other 47 serum samples taken in June 2003 from Henan and Hunan provinces were negative by VNT or IFA (Table). Western blot was not performed on this group of serum samples.

Discussion

While civet selection was derived from a convenience sample and limited because of time constraints imposed by the slaughter campaign, this study showed a marked difference in SARS-CoV antibody prevalence between animals from the market and those selected from the farms. Animals selected from one market in Guangzhou in January 2004 had a much higher prevalence of SARS-CoV antibodies than those selected from farms in the same period or from farms in two other provinces in June 2003. These results raise the possibility that civets, rather than being the natural animal reservoir of SARS-CoV, are infected mainly in markets or during other trade-related activities. Our results suggest that mass slaughter of civets on farms might not be necessary to control SARS-CoV spread. A more effective approach might be to implement testing in live animal markets and farms for susceptible animals and to apply quarantine regulation and targeted slaughter for markets or farms with infected animals.

While Guan et al. (4) were able to detect SARS-CoV infection by RT-PCR in six out of six palm civets collected in one particular live animal retail market in Shenzhen

Table. Summary of serologic analyses of civet serum samples[a]

	Farm													Market		
	Hunan		Henan		Guangdong									Guangdong		
	Changsha		Luoning		Qianguan		Shaoguan		Shanwei			Zhuhai		Guangzhou		
Sample no.	VNT[b]	IFA[c]	VNT[b]	IFA[c]	VNT[b]	IFA[c]	VNT[b]	IFA[c]	VNT[b]	IFA[c]	WB[d]	VNT[b]	IFA[c]	VNT[b]	IFA[c]	WB[d]
1	–	–	–	–	–	–	NA	NA	10	–	–	–	–	10	+++	++++
2	–	–	–	–	–	–	–	–	10	+	+	–	–	640	++	++++
3	–	–	–	–	NA	NA	–	–	–	–	–	–	–	CONT	+++	+++
4	–	–	–	–	–	–	–	–	–	–	–	–	–	20	+++	++++
5	–	–	–	–	–	–	–	–	40	+/–	+	–	–	–	–	–
6	–	–	–	–	–	–	–	–	20	–	–	–	–	30	++	+++
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	10	++	+++
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–										10	++	++++
12	–	–	–	–										NA	NA	NA
13	–	–	–	–										20	++	++++
14	–	–	–	–										30	++	++++
15	–	–	–	–										NA	NA	NA
16	–	–	–	–										10	++	++++
17	–	–	–	–										10	+++	++++
18	–	–												10	++	++++
19	–	–												240	+++	++++
20	–	–												60	+++	++++
21	–	–														
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25	–	–														
26	–	–														
27	–	–														
28	–	–														
29	–	–														
30	–	–														

[a] VNT, virus neutralization test; IFA, immunofluorescent antibody test; WB, Western blot test; NA, not assayed because of sample damage or loss during transportation or storage; CONT, contaminated samples that exhibited toxicity in VNT test.
[b] Titer of antibody that neutralized infectivity of 200 50% tissue culture infective dose of SARS-CoV.
[c] Immunofluorescent antibody test was performed by using a serum dilution of 1:50, and the intensity of staining in SARS-CoV–infected cells, but not in mock infected cells, was indicated by the sign +, with +++ representing the strongest signal observed.
[d] Western blot was conducted by using a serum dilution of 1:50, and the reactivity with the recombinant N protein expressed was indicated by the sign +, with ++++ representing the strongest signal observed.

in May 2003, a similar study conducted by us in the same period yielded different results. In our study, we collected civets from Xinyuan Live Animal Market in Guangzhou (n = 7), the Guangdong Centre for Rescue and Care of Wildlife Animals, also located in Guangzhou (n = 9), and a civet farm in neighboring Jiangxi Province (n = 15). While 2 civets from the market and 2 from the center were positive for SARS-CoV by RT-PCR, all 15 farmed animals from Jiangxi had negative results (C. Tu et al., unpub. data).

Results of these studies and those from our current study are similar. We observed a high percentage of infected civets in one particular market at a specific time. However, no indication of civet infection was seen on most farms during the same period. These results support the hypothesis that civets are highly susceptible to SARS-CoV, perhaps especially when they are stressed, and that most infections occurred in the market.

We observed a number of practices during our study. First, most animal traders deal with multiple species. Second, housing different animals in close proximity is common. Third, although civets are in high demand in Guangdong Province, they are expensive, so a batch of animals may remain in a storehouse for weeks. All of these factors facilitate interspecies transmission, which would be followed by rapid transmission among the civet population. Finally, civet farming and trading has been in practice in China for >10 years, but SARS has not been observed in workers until recently, which points to a recent introduction of SARS-CoV in the civet population in markets.

In a study conducted by the Guangdong Province Centre for Disease Control and Prevention and the World

Health Organization (3), epidemiologic data were analyzed from 1,454 clinically confirmed SARS cases (and 55 deaths) from November 2002 to April 30, 2003. One important observation from this study was that patients who became ill early in the epidemic were more likely than those who became ill later to report living near a produce market but not near a farm, which supports the notion that no widespread SARS-CoV infection occurred among farmed animals.

In the market study conducted by Guan et al. (4), all of the civets collected were positive for SARS-CoV. These animals were collected in the same market at the same time, but they originated in different regions of southern China; consequently, most, if not all, of these animals were likely infected in the market. In addition, SARS-CoV infection was also observed in at least one raccoon dog (*Nyctereutes procyonoides*) and one Chinese ferret-badger (*Melogale moschata*) from the same market at the same time, which demonstrates possible interspecies SARS-CoV transmission during trading. Sequence analysis of the S genes showed that one civet isolate (SZ16) was more closely related to the raccoon dog isolate (SZ13) than the other two civet isolates (SZ1 and SZ3), which further supports interspecies transmission in the market (4). Since that study, several experimental infection studies have shown most mammalian species tested to be susceptible to SARS-CoV infection (5–9), and animal-to-animal transmission can occur under experimental conditions as well (6). Caution should be taken in determining the origin of SARS-CoV; data collected from markets where a wide variety of species are housed in close proximity may be unreliable.

Out of the four farms in Guangdong Province, four animals from one farm in Shanwei had low levels of neutralizing antibodies to SARS-CoV, and two of the four samples did not react in IFA or Western blot. This farm in Shanwei is unique in that they farmed civets not for meat, but for the pet market in Southeast Asia. Most of their animals were obtained from various markets at various times from 2002 to 2003. These animals had possibly been exposed to SARS-CoV before arriving on the farm, and they still had low levels of convalescent antibodies in January 2004.

To assess the specificity of the serologic tests used in our study, we tested for cross-reactivity of SARS-CoV to four known coronaviruses from group 1 (porcine epidemic diarrhea virus and transmissible gastroenteritis virus), group 2 (porcine hemagglutinating encephalomyelitis virus), and group 3 (infectious bronchitis virus) and found no cross-reactivity (data not shown). We cannot rule out the possibility that an unknown coronavirus can infect civets, which may give low levels of cross-reactivity in the assays used in this study. However, such cross-reactive antibodies are not likely to positively react in all three of the assays used in this study.

The most basic limitation of our study was the nonrandom sampling, which limits the generalization of our results. However, this study is a first step in investigating the role of civets in transmitting SARS-CoV. Much remains to be done, including studies on the prevalence of infection with SARS-CoV and related coronaviruses that use more robust methods to sample susceptible animals in markets, farms, and the wild. Improved serologic tests should be developed that can detect SARS-CoV–specific antibodies from different animal species, without relying on live SARS-CoV. Other issues that remain to be resolved include the rate of new infections in susceptible animal species, the characteristics of the animals that become infected, and the nature of the exposures that lead to interspecies transmission.

Acknowledgments

We thank Zhaoan Xin, Liancheng Lei, Yuwei Gao, Yanhua Ao, Sandra Sapats, Chris Morrissy, Laurie Gleeson, Patrick Kluver, and Gary Anderson for providing either leadership support, project supervision, technical assistance, animal coronavirus antisera, critical reading of the manuscript, or help in statistical analysis.

This study was part of a project supported by Guangdong Provincial Program on SARS Prevention and Treatment to H.X. (project no. 2003FD02-06), the State High Technology Development Program (the 863 Program) on SARS origination research to X.K. (project no.2003AA208407). The investigation was organized and supported by the Chinese Ministry of Agriculture. The serologic study carried out at the Australian Animal Health Laboratory was supported in part by a grant from the Australian Biosecurity Co-operative Research Centre for Emerging Infectious Diseases (Project No. 1.007R) to L.F.W.

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Salmonella Typhimurium Outbreak Associated with Veterinary Clinic

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A *Salmonella enterica* serovar Typhimurium outbreak was associated with a veterinary clinic. Confirmed cases were in one cat, two veterinary technicians, four persons associated with clinic patients, and a nurse not linked to the clinic. This outbreak emphasizes the importance of strong public health ties to the animal health community.

Zoonotic transmission of *Salmonella enterica* has been associated with exposure to sick and healthy cattle on farms (1), sick cats at animal shelters (2), and cats at small animal veterinary clinics (2). Salmonellosis is a well-recognized nosocomial problem at large-animal veterinary hospitals (3,4), but it is associated with few, if any, human outbreaks. In small-animal medicine, salmonellosis is likely underrecognized because gastrointestinal illness is common and often self-limiting. As in human medicine, salmonellosis is rarely confirmed in a laboratory, which results in underreporting of cases. However, in 1999 two salmonellosis outbreaks at veterinary clinics were linked to cats with either confirmed or suspected salmonellosis (2). From September to October 2003, the New York State Department of Health and three local health departments identified seven human infections with *S. enterica* serovar Typhimurium, which exhibited an uncommon pulsed-field gel electrophoresis (PFGE) pattern. These cases had an apparent link to a veterinary clinic. This report describes the outbreak investigation and underscores the importance of integrating veterinary medicine into public health surveillance.

The Study

In September 2003, five culture-positive human cases of *S.* Typhimurium infection were identified in three adjacent counties in New York State. All five isolates were indistinguishable by PFGE and were resistant to ampicillin, chloramphenicol, sulfisoxazole, streptomycin, and tetracycline. Onset dates were July 22 to August 22, 2003 (Figure). A local veterinary hospital, clinic X, was the only exposure common to all patients (Table). Patients 1 and 2 were veterinary technicians at clinic X, and patients 3–5 were pet owners whose pets had visited clinic X from July 15 to July 22, 2003. Laboratory surveillance identified two additional cases (cases 6 and 7) with matching PFGE patterns (Table, Figure). Symptoms of the infection included diarrhea, cramps, fever, and nausea. The median duration of illness was 8 days. A full investigation was conducted, including a site visit, case-finding, and diagnostic testing of clients and staff of clinic X.

Interviews with clinic X staff and veterinary chart reviews determined that two cats and one dog owned by patients 3, 4, and 5 (pets A, B, and C, respectively) were admitted on two different dates in July for dental procedures (Table). All three procedures were performed by one veterinarian and one technician (patient 1). All three animals were held overnight, with evening treatments performed by patient 2, who works 1 evening per week. All procedures were performed in a designated room that was also used for other nonsterile procedures. The cats were held overnight in procedure room cages; the dog was held overnight either in the procedure room or in a dog run in a separate room.

All three animal patients were treated after the procedure with a prophylactic course of clindamycin. Pet B had a history of diabetes with chronic intermittent diarrhea attributed to diabetes-related dietary changes. The other pets had no prior illness. All three owners reported transient diarrhea in the pets after the dental procedure. Pet B developed severe mucoid diarrhea ≈5 days postsurgery and was treated with additional antibiotics (amoxicillin and enrofloxacin).

Patient 6 owns a dog but does not use clinic X. Patient 6 had occasional contact with his neighbor's dog (pet D),

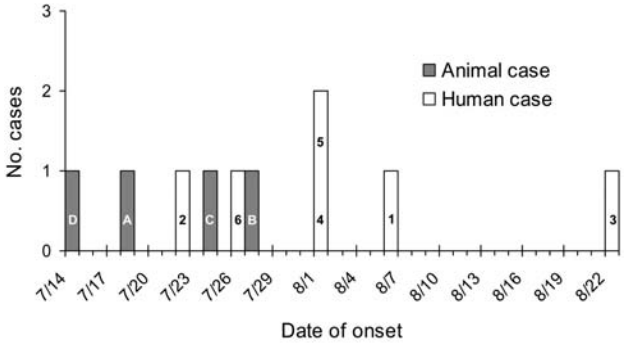

Figure. Epidemic curve of onset dates for human salmonellosis cases (white bars) and animal cases (gray bars). Numbers 1–6 refer to human cases, letters A–D refer to animal cases (see text).

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Table. Human and animal *Salmonella enterica* serovar Typhimurium cases associated with a veterinary clinic, New York, 2003

Case	Age, sex/species	Possible exposures	Clinic date(s)[a]	Estimated onset date	Status[b]
Patient 1	31, F	Clinic technician		8/6	Confirmed
Patient 2	27, F	Clinic technician		7/22	Confirmed
Patient 3	64, F	Owner of pet A		8/22	Confirmed
Patient 4	2, F	Owner of pet B		8/1	Confirmed
Patient 5	93, F	Owner of pet C		8/1	Confirmed
Patient 6	2, M	Neighbor owns pet D		7/26	Confirmed
Patient 7	44, F	Unknown		9/14	Confirmed
Pet A	Cat	Dental work by patient 1	7/15–7/16, 7/23–7/25, 8/1	7/18	Suspected
Pet B	Cat	Dental work by patient 1	7/22–7/23, 7/27, 8/4	7/27	Confirmed
Pet C	Dog	Dental work by patient 1	7/22–7/23	7/24	Suspected
Pet D	Dog	In clinic for vomiting/diarrhea	7/14–7/15, 7/16–7/21	7/14	Suspected

[a]Dates when each animal was at clinic X.
[b]Confirmed, pulsed-field gel electrophoresis match on stool culture; suspected, clinical signs of enteritis or gastroenteritis, stool culture negative.

which had been to clinic X for several overnight visits because of severe vomiting and diarrhea attributed to eating mulch. Pet D was last discharged from clinic X on July 21, 5 days before patient 6's onset date. Whether patient 6 had contact with pet D between July 21 and July 26 is unclear.

The seventh patient was an emergency room nurse at a hospital in the outbreak area. She has a dog but does not use clinic X; the dog had no recent illness and had not recently been to a veterinarian. No other exposures to clinic X or other patients and pets could be identified. Stool culture of the patient's dog was negative.

After identification of the outbreak in September 2003, stool cultures were collected from the pets of all patients, including healthy contact pets from the same households. Only pet B, the diabetic cat, had a positive stool specimen collected at the end of September with a PFGE match to the human isolates.

Clinic X is a large, multidoctor practice that primarily treats dogs and cats, although one clinician (not linked to this outbreak) sees exotic animals, including reptiles. In addition to exam rooms, procedure room, and sterile surgery suite, the practice has separate rooms for isolation, animal wards, dog runs, surgery preparation, laboratory, reception, and patient files. No animals had been placed in isolation during the outbreak. A break room and meeting room are on a separate floor of the practice.

Thirty-seven of 38 uninfected staff members completed questionnaires regarding exposure and illness history. Seven reported diarrhea, and two reported nausea only between June 1 and August 31, 2003. None of the staff members submitted stool cultures. Stool culture from the asymptomatic veterinarian for the case-pets was negative, but patient 1 had continued signs of illness and was still culture-positive in mid-September. She voluntarily excluded herself from direct patient care until illness resolved.

A review of infection control practices in mid-September 2003 did not identify significant lapses in handwashing; cleaning; or disinfecting instruments, floors, or surfaces. No food was visible in the work areas during a walk-through, but the owner reported that he frequently reminds staff to avoid eating in work areas. Twenty-three environmental swabs were taken from the procedure room, anesthesia machines, animal wards (including isolation), and the laboratory (including a microscope used for fecal parasitology exams). Samples were collected by using sterile gauze sponges dampened with sterile double-strength skim milk. All were negative for *Salmonella*.

Clinic X staff telephoned dental clients treated since June 1, 2003 with a questionnaire developed by health department staff. The script asked about illness in pets or people in the household. No additional human or animal illnesses were identified.

Conclusions

A likely source of *Salmonella* for this outbreak was not identified. The animal with the earliest illness onset (pet D) could have contaminated the clinic. However, pet D was not confirmed with *Salmonella* infection, was never in the dental room, and had no exposure to other case-pets. Another, unidentified animal patient may have been the source of contamination, or a person on the clinic staff may have been the source. If the clinic environment was the source of infection, cleaning apparently eliminated contamination by the time environmental specimens were collected in late September. Polymerase chain reaction (PCR) testing may have yielded different results; however, a positive result on a PCR test might represent nonviable bacterial DNA (3,4).

No epidemiologic link could be found to patient 7. However, out of 457 *Salmonella* isolates tested at Wadsworth Center Laboratories from January 2003 through July 2004, only the seven human patients and one cat reported here had this PFGE pattern. Patient 7 may have been exposed to undiagnosed cases through her work at the emergency room or through some other unidentified common exposure.

The outbreak described here was identified because the standard questionnaire used to interview patients included

animal exposure questions, which shows the importance of animal exposure history in detecting potentially zoonotic diseases. This outbreak also shows the importance of zoonotic disease education for pet owners and increased awareness of zoonotic diseases by veterinarians. As is often the case with gastroenteritis in pets, the potential for transmission to humans was not considered. Since pet owners are frequently unwilling or unable to pay for diagnostic testing, veterinarians often do not consider stool culture for animals with diarrhea, which might have prevented some human cases in this outbreak. However, even without a definitive diagnosis, veterinarians can emphasize infection control practices with staff and educate owners that, although pets are rarely confirmed as the source of human salmonellosis, zoonotic transmission of gastrointestinal illnesses from sick pets may occur. Veterinarians should emphasize handwashing and infection control in the home. This practice is particularly important for households with immunocompromised persons or young children, who could become a source of secondary infection to other children, especially in daycare settings.

Finally, the importance of a good working relationship between public health and the veterinary community is underscored by the strong, cooperative relationship between public health authorities and clinic X. The clinic owner and staff were enlisted early as public health partners who took an active role in preventing further cases. Increasing concern about emerging zoonotic diseases and zoonotic agents as bioweapons has raised awareness of the risk of zoonotic disease exposure for persons employed in animal health. Agriculture, veterinary, and public health agencies in many states are promoting zoonotic disease awareness among veterinary professionals. As we continue to integrate the veterinary community into public health, information from these types of outbreaks should be used to develop protocols for zoonotic disease response and education in the veterinary and pet-owning communities.

Acknowledgments

The authors thank Mike Murawski for assistance with the environmental investigation, Danielle Phillips for assistance with the site visit, Dianna Schoonmaker-Bopp and Tim Root for assistance with diagnostic testing at the Wadsworth Center, and the staff of clinic X for cooperation and assistance with the investigation.

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West Nile Virus Viremia in Wild Rock Pigeons

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Feral rock pigeons were screened for neutralizing antibodies to West Nile virus (WNV) during late winter/spring and summer of 2002 and 2003. Additionally, virus isolation from serum was attempted from 269 birds collected during peak transmission periods. The observed viremia levels and seroprevalence indicate that this species could be involved in amplifying WNV in urban settings.

The prototypical amplifying host for most bird-maintained arboviruses, such as West Nile virus (WNV) (*Flavivirus*; *Flaviviridae*), is a species that is locally abundant and readily accessible to arthropod vectors, develops a high level of viremia for an extended duration, and does not develop clinical disease (1). Therefore, both the bionomics of the bird species (e.g., population numbers, distribution, association with human habitation/mosquitoes) and its host competence (i.e., susceptibility to infection and ability to circulate virus at titers high enough to infect vectors) need to be evaluated when assessing whether it may be important in amplifying WNV (1). Historically, potential host competency for WNV has been determined through experimental infections (2–4) and, accordingly, supporting viremia levels from free-ranging birds to validate such laboratory-derived competence indices are usually unavailable.

Knowledge regarding the potential host competency of most North American bird species for WNV is limited. In a recent study, Komar et al. experimentally determined WNV viremia levels for 25 bird species encompassing 17 families, whereby an index for reservoir competence was calculated based on the susceptibility of each species to infection, the mean daily infectiousness, and the duration of infectious viremia (5). As all species tested were susceptible to infection, the calculated reservoir competence was therefore inherently dependent on the magnitude and duration of viremia. Species that had viremia levels of $<10^5$ PFU/mL were considered to be noninfectious for two enzootic vectors, *Culex pipiens* and *Cx. quinquefasciatus*, and hence deemed incompetent hosts. Rock pigeons (*Columba livia*) were included in this group.

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Rock pigeons are distributed throughout the entire continental United States and are a gregarious and abundant species, especially in urbanized areas. Field studies have demonstrated that this species has high seroprevalence rates (6–8) and therefore may be useful as a sentinel to monitor WNV transmission. Additionally, rock pigeons are nonmigratory, which allows for a more accurate determination of approximate sites of exposure than nonresident species. The objectives of this study were to assess the extent of natural infection in free-ranging rock pigeons from metropolitan Atlanta 1 and 2 years subsequent to the recognition of WNV in Georgia and to field-validate experimental results relating to potential levels of viremia in this species.

The Study

During February, March, and August 2002 and April, July, and September 2003, rock pigeons from northwest Atlanta rail yard, Fulton County, Georgia (33°48′40.1′N, 84°27′28.4′′W) (Figure), were collected by Wildlife Services personnel by using rocket nets as part of a cooperative nuisance wildlife removal project. Captured birds were identified as hatch-year or adult before being bled by brachial venipuncture for serum collection. A subset of these birds was transferred to captivity as part of an unrelated study. Serum samples collected during late winter/spring (February–April) were frozen at –70°C until screening for antibodies by a plaque-reduction neutralization test (PRNT). Serum samples collected during summer transmission periods (July–September) were tested for circulating virus before being frozen at –70°C until further processing (see below). From 8 separate collections, 499 pigeons were sampled during the 2-year period.

WNV antibody titers were determined by PRNT (6), with the following modifications. Infected Vero Middle America Research Unit (MARU) cell cultures were overlaid

Figure. Satellite image of northwest Atlanta rail yard, Fulton County, Georgia, which shows its close proximity to human habitation (courtesy of the United States Geological Survey).

with 1% gum tragacanth/1x minimum essential media (MEM) (supplemented with 2.2 g/L sodium bicarbonate, 3% heat-inactivated fetal bovine serum, 200 units/mL penicillin, 200 µg/mL streptomycin, and 500 ng/mL amphotericin B) rather than agarose, and cultures were inactivated on day 4 postadsorption with 10% buffered formalin and stained with 0.25% crystal violet for plaque visualization. Additionally, 100 pigeons from the August 2002 collection were also tested for antibodies to pigeon paramyxovirus-1 (PPMV-1) (*Avulavirus*; *Paramyxoviridae*) by a hemagglutination inhibition (HI) test (9).

For statistical analysis, seroprevalence between late winter/spring and summer collections were compared by using a Yates corrected chi-square test (Epi Info version 3.2.2) and 95% confidence intervals were determined according to Newcombe (10). Serum samples collected during summer months (July–September) were screened for circulating virus before being frozen at –70°C until titration (positive) or PRNT (negative). Briefly, 5 µL of serum was inoculated into a 2-mL culture of 2-day-old Vero MARU cells and monitored daily for cytopathic effects. WNV isolates were identified by reverse transcription–polymerase chain reaction by using degenerate WNV-specific primers (WN310F, sense primer: 5′-GTSAACAAAACAAACAGCRATGAA-3′; WN686R, antisense primer: 5′-ACWGMTGAYTTYGTGCACCA-3′) that amplify a 376-bp fragment spanning the nucleocapsid and premembrane genes. The Newcastle disease virus (NDV) isolate was identified by using primers directed against the fusion protein gene (sense primer, 5′-CCTTGGTTGAITCTATCCGIAG-3′; antisense primer, 5′-CTGCCACTGCTAGTTGIGATAATCC-3′) (11) and further classified as PPMV-1 by monoclonal antibody binding profiles (12).

Viral titers of WNV-positive serum samples collected during the summer were determined by plaque assay. Briefly, samples were rapidly thawed from –70°C, and 200 µL of each 10-fold dilution (10^{-1}–10^{-6}) of serum in MEM was added to duplicate wells of a six-well plate seeded with 4-day-old Vero MARU cells. Adsorption, overlay, and staining procedures were performed as in the PRNT protocol. Dilutions in which 20–100 plaques could be counted (when applicable) were used in determining WNV titers ($\log_{10}$ PFU/mL).

WNV-specific antibodies were detected in 128 (25.7%) of 499 rock pigeons tested (Table 1). Overall seroprevalence rates per collection for 2002 were 16%–45% and 11%–50% in 2003. Significant differences in seroprevalence rates were observed between late winter/spring collections (February–April, 37.4%) versus summer collections (July–September, 15.6%) ($p < 0.0000001$). Of the 133 samples with ≥90% plaque reduction on the initial screen, 128 were WNV-positive (96.2%), 4 were flavivirus-positive (3.0%), and 1 was St. Louis encephalitis virus (SLEV)–positive (0.8%). Of 269 birds tested for virus isolation, 11 (4.1%) were viremic (Table 2). Viremia levels were $10^{2.2}$ to $10^{7.2}$ PFU/mL (mean = $10^{4.0}$ PFU/mL).

Conclusions

In 2002–2003, we conducted a serologic study on WNV exposure rates of rock pigeons from a single locale adjacent to metropolitan Atlanta. Consistent with previous studies documenting high WNV exposure rates in this species (6–8), overall seroprevalence rates per collection for 2002 were 16%–45% and 11%–50% in 2003. The seasonal discrepancy in seroprevalence between late winter/spring collections (37.4%) versus summer collections (15.6%) may be partially ascribed to the influx of naïve juveniles into the population during months of quiescent or reduced virus activity before the onset of peak transmission in late summer.

Table 1. Flavivirus seroprevalence rates in free-ranging rock pigeons from northwest rail yard, Fulton County, Georgia, 2002–2003[a]

Collection date	No. tested	n (%) WNV+ [95% CI]	n (%) SLEV+ [95% CI]	n (%) FLAVI+ [95% CI]	n (%) viremic [95% CI]
2002					
Feb 28	56	25 (44.6) [32.4–57.6]	0	2 (3.6) [1.0–12.1]	NT
Mar 6–7	107	35 (32.7) [24.6–42.1]	0	1 (0.9) [0.2–5.1]	NT
Aug 15	58	9 (15.5) [8.4–26.9]	0	0	0
Aug 22	68	13 (19.1) [11.5–30.0]	0	0	7 (10.3) [5.1–19.8]
Total	289	82 (28.4) [23.5–33.8]	0	3 (1.0) [0.4–3.0]	7/126 (5.6) [2.7–11.0]
2003					
Apr 16	34	17 (50) [34.1–65.9]	0	0	NT
Apr 29	33	9 (27.3) [15.1–44.2]	0	0	NT
Jul 30	71	8 (11.3) [5.8–20.7]	0	0	2 (2.8) [0.8–9.7]
Sep 5	72	12 (16.7) [9.8–26.9]	1 (1.4) [0.3–7.4]	1 (1.4) [0.3–7.4]	2 (2.8) [0.8–9.6]
Total	210	46 (21.9) [16.8–28.0]	1 (0.5) [0.1–2.7]	1 (0.5) [0.1–2.7]	4/143 (2.8) [1.1–7.0]

[a]WNV, West Nile virus; SLEV, St. Louis encephalitis virus; FLAVI, flavivirus; WNV+, samples in which a fourfold difference in WNV antibody titer over SLEV could be demonstrated; SLEV+, samples in which a fourfold difference in SLEV antibody titer over WNV could be demonstrated; FLAVI+, samples in which a fourfold difference in titer between WNV and SLEV could not be demonstrated and therefore classified as flavivirus positive; CI, confidence interval; NT, not tested for viremia.

Table 2. West Nile virus (WNV) viremia titers of free-ranging rock pigeons from northwest rail yard, Fulton County, Georgia, 2002–2003

Bird ID no.	Collection date	Log_{10} PFU/mL
3309[a]	8/22/2002	2.3
3316	8/22/2002	5.3
3325	8/22/2002	4.4
3494	8/22/2002	3.4
3498	8/22/2002	3.5
3518	8/22/2002	3.3
3524	8/22/2002	2.2
4025	7/30/2003	4.4
4070	7/30/2003	4.4
4288	9/5/2003	3.6
5206[b]	9/5/2003	7.2

[a]Hatch-year bird; all other viremic pigeons were identified as adults.
[b]Died from a pigeon paramyxovirus-1 infection 11 days postcapture; virus identification of serum isolate as WNV was confirmed by reverse transcription–polymerase chain reaction of extracted RNA from serum and neutralization assays using Newcastle disease virus and WNV-specific antisera.

Of 269 birds tested for virus isolation, 11 (4.1%) were viremic. Since viremic birds were provisionally identified by cell culture, the lag time from serum collection to virus isolation did not afford daily screening for subsequent serum titers. Thus, we cannot delineate the daily mean titer, maximum titer, or duration of viremia for any of these birds. With an overall average WNV viremia titer of $10^{4.0}$ PFU/mL, our findings are similar to the daily mean titers ($10^{2.9}$–$10^{4.3}$ PFU/mL) of rock pigeons reported in experimental infections (5). However, while the maximum titer seen experimentally ($10^{4.8}$ PFU/mL) was below the inferred threshold necessary to infect *Cx. pipiens* and *Cx. quinquefasciatus* ($10^{5.0}$ PFU/mL), 2 of 11 (18%) naturally infected birds had titers in excess of this threshold.

Of note, the rock pigeon with the highest WNV viremia titer ($10^{7.2}$ PFU/mL) became ill 8 days postcapture and died within 72 hours of the onset of clinical signs. PPMV-1, an antigenic variant of NDV, was subsequently isolated from brain and heart tissue. PPMV-1 was not detected in serum. Whether the high-level WNV titer in this viremic pigeon was influenced by coinfection with PPMV-1 (or an undetected pathogen) or whether the level is normal and may occur under natural conditions cannot be determined. Although the effects of WNV coinfection with most microbes and parasites remains unknown, antibodies to PPMV-1 were detected in 68% (n = 100) of the birds tested, and numerous additional pathogenic viruses, bacteria, protozoa, fungi, and helminths have been isolated from free-ranging rock pigeons (13). These findings suggest that multiple concomitant infections may occur with regularity in feral populations.

Rock pigeons are intimately associated with urbanization, such that stable populations do not exist outside of human development. Although accurate U.S. population numbers are not available, censuses from various North American cities have estimated urban densities to be in range of 11.4 to 30.8 birds/km^2 (14). This number would equate to a rock pigeon population of 1.7–4.6 million for a city the size of Atlanta. As rock pigeons are ubiquitous in all cities and towns throughout the United States, they could potentially provide an abundant host for enzootic/epizootic vectors such as *Cx. tarsalis* and *Cx. quinquefasciatus*, both of which have been shown to preferentially bloodfeed on columbiforms (15,16).

Apart from the study by Komar et al., which, because of its extensive scope, only included six pigeons for viremia determinations, detailed species-specific experimental and field studies assessing the competency of common, urbanized bird species for North American strains of WNV are lacking (5). Although the overall viremia titers obtained from naturally infected birds corroborate previous experimental reports that rock pigeons generally maintain low-level viremia titers in relation to passerine species (2–5), there were outliers that exhibited titers sufficient to infect engorging mosquitoes. This finding exemplifies the need, as duly noted by Komar et al., that experimentally derived competence indices should be consolidated with field data to better estimate host potential (5). To our knowledge, this is the first report of viremia levels from wild birds naturally infected with WNV.

Acknowledgments

We thank the Southeastern Cooperative Wildlife Disease Study personnel who assisted in blood collection and serum separation, Daniel J. King and Phillip Curry for characterization of the NDV isolate as PPMV-1, Robert Lanciotti for SLEV strain TBH-28, and the United States Geological Survey for permission to use the digital orthophoto quadrangle of the northwest Atlanta rail yard.

Funding for this research was provided by Centers for Disease Control and Prevention Grant U50/CCU420515-02. Additional supplemental support was provided through the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and through sponsorship of the Southeastern Cooperative Wildlife Disease Study from fish and wildlife agencies in Alabama, Arkansas, Florida, Georgia, Kansas, Kentucky, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Oklahoma, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia.

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Cryptosporidium felis and *C. meleagridis* in Persons with HIV, Portugal

To the Editor: *Cryptosporidium*, a pathogenic protozoan parasite with a worldwide distribution, causes diarrheal illness in humans and animals. The parasite can be transmitted from human to human through fecal-oral contact (household contact and nosocomial transmission), sexual contact, ingestion of contaminated food or water, and contact with infected animals. Molecular diagnostic methods indicate that *Cryptosporidium parvum* and *C. hominis* are the major causes of cryptosporidiosis in humans, and other *Cryptosporidium* species can be associated with human infection (1–5).

In Portugal, patients with AIDS have an 8% prevalence rate of cryptosporidiosis (6) with *C. parvum* and *C. hominis* as the etiologic agents, even though other *Cryptosporidium* species were found in these patients (3,7,8). This study characterizes clinical manifestations of infections with unusual two different *Cryptosporidium* species isolated from seven patients and demonstrates that these species can cause life-threatening disease.

Cryptosporidiosis was diagnosed in 40 patients from 1994 through 2002. All patients were serologically positive for HIV-1 and had diarrhea (at least two loose stools per day) when diagnosed with cryptosporidiosis. Demographic, clinical, and immunologic data were obtained from each patient's records. *Cryptosporidium* oocysts were identified by light microscopy after concentration from fecal material by a modified water-ether sedimentation method followed by the modified Ziehl-Neelsen staining (7). The intensity of infection was quantified before molecular analysis by scoring the number of oocysts counted per microscopic field (under a 20x objective) of 50-µL volume of concentrated stool sample as + (1–5 oocysts), ++ (6–10 oocysts), +++ (11–15 oocysts), or ++++ (>15 oocysts). Genetic characterization of the isolates was based on polymerase chain reaction–restriction fragment length polymorphism analysis of the small subunit rRNA gene (2,3).

The molecular analysis showed that 22 patients (55%) were infected with *C. parvum*, 11 (27.5%) were infected with *C. hominis*, 4 (10%) were infected with *C. felis*, and 3 (7.5%) were infected with *C. meleagridis*. Of the four patients infected with *C. felis*, three (75%) showed low (+) and one (25%) showed moderate (++) oocyst loads. All three (100%) patients infected with *C. meleagridis* showed low oocyst loads (+). In contrast, of the 22 patients infected with *C. parvum*, 9 (41%) showed low oocyst loads (+), 3 (14%) showed moderate oocyst loads (++), 3 (14%) showed high oocyst loads (+++), and 7 (32%) showed very high (++++) oocyst loads. Similarly, of the 11 patients infected with *C. hominis*, 2 (18%) had low oocyst loads (+), and 3 (27%) each had moderate (++), high (+++), or very high (++++) oocyst loads.

Five of the seven patients infected with *C. felis* and *C. meleagridis* were men and two were women; the median age of patients was 31 years (7–44 years). In this group of HIV-positive patients, three were heterosexual persons, two were homosexual persons, one was an intravenous drug user, and one acquired HIV infection through vertical transmission. Of the seven patients, all showed a range of clinical manifestations of infection, including transient diarrhea, chronic diarrhea, dehydration, and cachexia. Five (71%) of the patients spontaneously recovered, and two (29%) of the patients died. The median $CD4^+$ count/mm^3 was 20 (range 18–213).

All of the seven patients were prescribed antiretroviral therapy, but one of the patients did not adhere to the treatment. Two of the three patients infected with *C. meleagridis* died of cryptosporidiosis. Information on the risk factors for acquiring *Cryptosporidium* infection was available for one patient, the child infected with *C. felis*, who had contact with cats at home. No other potential intestinal pathogens were detected in the feces of these patients at the time of the cryptosporidiosis diagnosis.

Twenty-two of the 33 patients infected with *C. parvum* and *C. hominis* were men and 11 were women; the median age of patients was 32 years (7–58 years). Sixteen patients were intravenous drug users, 5 patients were heterosexual persons, 1 patient was a homosexual person, and 2 patients acquired HIV infection through vertical transmission; the remaining 9 patients had no HIV-exposure history information. Eighteen of the 22 patients infected with *C. parvum* showed a range of clinical manifestations of illness with transient diarrhea, chronic diarrhea, dehydration, and cachexia. Twelve (67%) of the patients spontaneously recovered, and 6 (33%) of the patients died. Information on $CD4^+$ count/mm^3 was available for 13 of the 22 patients with a median count of 20 (range 3–250). Information on the outcome of the patients infected with *C. hominis* was available for 10 of the 11 patients. All of the 10 patients showed a range of clinical manifestations of infection, including transient diarrhea, chronic diarrhea, dehydration, and cachexia. Seven (70%) of the patients spontaneously recovered, and 3 (30%) of the patients died. The median $CD4^+$ count/mm^3 was 20 (range 6–40).

Most reports on infections with unusual *Cryptosporidium* species in humans give a brief description of the genotyping results, leaving the clinical importance of these species uncertain. Unusual *Cryptosporidium*

species can cause disease (symptomatic infection) and death. *C. felis* and *C. meleagridis* infections showed low oocyst shedding (all seven patients had low to moderate oocyst loads in samples). On the contrary, *C. parvum* produced similar clinical manifestations but showed higher oocyst shedding; 46% had high to very high parasite loads. *C. hominis* infections had parasite loads even higher than *C. parvum* infections; 54% of patients had high to very high parasite loads. In immunocompetent persons, *C. hominis* infections produce higher oocyst loads in feces than infections caused by *C. parvum* or zoonotic species (2,9).

The transmission route for the unusual *Cryptosporidium* species is unclear. Because human infection by unusual *Cryptosporidium* species is less common, the principal transmission route for these parasites is likely through direct contact with infected animals. In our study, one of the four immunocompromised patients with *C. felis* was a child who had been in close contact with cats at home. No data on animal contact were available for other patients infected with unusual *Cryptosporidium* species. Cats are found in many homes with no evidence of cryptosporidiosis; therefore, it is difficult to attribute the occasional human *C. felis* infection to contamination by cats. Careful epidemiologic studies are needed to elucidate the transmission route of human infections with unusual *Cryptosporidium* species.

This work was supported by Fundação para a Ciência e Tecnologia / European Union/Fonds Social Européen/ Fonds Européen de Développement Régional.

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Bartonella henselae in African Lion, South Africa

To the Editor: Four members of the bacterial genus *Bartonella*, *Bartonella henselae*, *B. clarridgeiae*, *B. koehlerae*, and *B. bovis*, have been isolated from felids, mostly domestic cats (1,2). Of these four species, *B. henselae* and *B. clarridgeiae* are recognized human pathogens, which cause many illnesses, including endocarditis, prolonged fever, various ocular infections and, most commonly, cat scratch disease (1).

In 1994, domestic cats (*Felis domesticus*) were found to be a reservoir for *B. henselae*; subsequent surveys have shown that a large proportion of the domestic cat population worldwide has been exposed to, or infected with, bartonellae (1). The epidemiologic features of *Bartonella* infection in other felid species has been explored; a high prevalence of seropositivity has been found in free-ranging and captive wild cats from California and Florida (3), as well as panthers from Florida (4). *B. henselae* has been isolated from a captive cheetah in Zimbabwe (5).

During 2002, blood samples were collected from 65 African lions that inhabited three ranches in the Free State Province of South Africa. These ranches breed and rear lions specifically for game. Although the lions are contained within vast (several km^2) enclosures, they are free to move about and interact with one another. The lions have minimal contact with humans or other animals, except carcasses of horses and donkeys that are provided as food. The lions do not receive any other food, food supplements, growth enhancers, or antiparasite prophylaxis. All three ranches are deep in the veld, at least 20 km from any settlements. Blood samples were drawn from the lions as part of an ongoing health surveillance program

conducted by the African Large Predator Research Unit, University of Bloemfontein. Whole blood samples were drawn aseptically from each lion into EDTA tubes, stored at 4°C before being returned to the laboratory, and then frozen at –70°C in the laboratory. Subsequently, blood samples were thawed, and an aliquot was plated onto 10% sheep blood–enriched agar and incubated at 37°C in a 5% CO_2 atmosphere for a maximum of 45 days. One culture yielded putative bartonellae (small, smooth, white-gray colonies) after 14 days' incubation. A crude DNA extract was prepared from this isolate and used as a template in previously described polymerase chain reaction–based assays to detect and identify *Bartonella* species which targeted fragments of the 16S rRNA encoding gene and 16S/23S intergenic spacer region (6). Amplification products of the expected size were obtained from the DNA extract. The nucleotide base sequence of each product showed that each shared 100% similarity with sequences of other *B. henselae* isolates held in GenBank. The 16S rRNA gene sequence was identical to that of type II variants.

Antisera from 62 of the 65 samples were tested for the presence of anti-*Bartonella* immunoglobulin G antibodies using an enzyme-linked immunosorbent assay previously evaluated to detect antibodies in domestic cats (7). Eighteen of the samples had matrix scores above the upper limit of the normal range of values observed in uninfected cats, thus indicating past exposure to *Bartonella* species. No serum from the *B. henselae* culture-positive animal was available for testing.

Our findings confirm that lions are susceptible to infection by *B. henselae*, but their role as reservoirs for this species remain unclear. The observed prevalence of infection (1.5%) and exposure rate (29%) in our study are lower than those typically observed in domestic cats, particularly in warmer regions of the world. Nonetheless, our serologic data do suggest that a substantial proportion of the lions are exposed to bartonellae. Although limited, our assessment of the lion *B. henselae* isolate suggests that it is within the genetic spectrum of strains associated with domestic cats, and lions may serve as an extension to this reservoir. The extent of contact between domestic cats, or their ectoparasites, and the farmed lions we studied is likely to be minimal, given the remoteness of the enclosures (the infected lion lived on a cat-free ranch). However, the lions may have contact with other wild-living felids such as the African wild cat (*Felis silvestris lybica*), small spotted cat (*Felis nigripes*), and the caracal (*Caracal caracal*) which are endemic to the region.

Acknowledgement

We thank the African Large Predator Research Unit for providing biologic material from the lions.

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Mycobacterium tuberculosis Transmission from Human to Canine

To the Editor: This report is the first known of a case of epidemiologically associated tuberculosis (TB) in a human and a canine caused by the same strain, confirmed by genotyping. In Tennessee, a 71-year-old woman with a 3-week history of a productive, nonbloody cough was evaluated. She lived alone, and standard epidemiologic investigation of family members and other close contacts showed no apparent TB exposure. A TB skin test 20 years earlier had been negative. Chest radiograph showed infiltrates and atelectasis in the upper lobe of the right lung. A TB skin test resulted in a 14-mm area of induration. Sputum stained positive for acid-fast bacilli (AFB) and was positive for *Mycobacterium tuberculosis* by DNA probe and culture. The organism was sensitive to standard antitubercular medications.

Treatment was initiated with isoniazid, rifampin, and pyrazinamide. After 14 days of daily, directly observed therapy, the patient complained of nausea, vomiting, and diarrhea. Treatment adjustments were made, and therapy was completed 11 months later with a complete recovery.

Six months after the patient's TB diagnosis, she took her 3-1/2-year-old male Yorkshire Terrier to a veterinary clinic with cough, weight loss, and vomiting of several months' duration. The dog lived indoors and had been a constant companion to the patient for 3 years. Because of the owner's diagnosis, TB was suspected. The dog's initial sputum sample was negative on AFB staining and *M. tuberculosis* nucleic acid amplification assay.

Eight days after discharge from a referral veterinary teaching hospital with a presumptive diagnosis of TB, the dog was euthanized because of urethral obstruction. Liver and tracheobronchial lymph node specimens collected at necropsy were positive by AFB stain and positive for *M. tuberculosis* complex by polymerase chain reaction. Cultures of liver, lung, and kidney specimens were positive for *M. tuberculosis.* The *M. tuberculosis* isolates from the dog and its owner had an indistinguishable 10-band pattern by IS*6110*-based restriction fragment length polymorphism genotyping (Figure) (1).

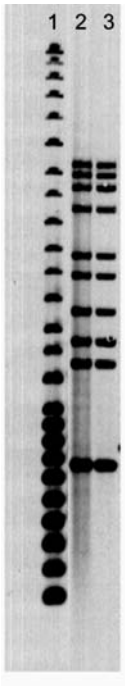

Figure. Ten-band *Mycobacterium tuberculosis* restriction fragment length polymorphism pattern. Lane 1, 25-band Centers for Disease Control and Prevention standard; lane 2, human case; lane 3, canine case.

This report is the first known of epidemiologically associated TB in human and canine cases to be confirmed by *M. tuberculosis* genotyping. The weight of historic data on human-canine TB supports our conclusion that the human owner was the likely source of the canine TB in this instance (2–5). In a review of 48 dogs and cats with known exposure to human TB, 7 (14.6%) were culture-positive for *M. tuberculosis* (2). Another series of eight canine TB infections documented by necropsy showed that seven of the dogs had a close association with humans with active TB disease (5). Other forms of mycobacterial infections, most notably *M. bovis*, have also been epidemiologically linked in humans and dogs (4,6). Cases in which dogs and cats infected with *M. bovis* or *M. tuberculosis* have infected humans have also been reported (4).

Genotyping has become a powerful tool for confirming epidemiologically linked transmission of *M. tuberculosis*. Two previous reports showed genotype matches between human and elephant TB cases and between human and monkey TB cases (7,8).

In our case, signs and symptoms that likely represented onset of TB appeared first in the woman and followed several weeks to months later in her pet dog. The owner often kept the dog in her lap, and the dog was allowed to lick the owner's face. A thorough, standard epidemiologic investigation did not identify any other infected contacts of the dog or owner; however, because the patient had limited social contacts, nonstandard investigation such as social network analysis was not formally conducted.

Cross-contamination of specimens was unlikely to have occurred at the laboratory at which cultures for *M. tuberculosis* were confirmed; several months passed between the times the two isolates were identified and subsequently sent to the Centers for Disease Control and Prevention for genotyping. This pattern has never been identified in a national database of >10,000 unique patterns, so this match is not likely to be due to anything other than transmission between the dog and its owner. Because systematic genotyping is not performed routinely in Tennessee, we are unable to determine more definitively whether this pattern has ever occurred in the state.

Although the true risk for TB transmission from humans to dogs, and vice versa, is not known, pet owners, physicians, and veterinarians should be aware of this potential. While standard tests, such as culture for *M. tuberculosis*, may be helpful in understanding the dynamics of TB between humans and other animals, genotyping has become the standard for confirming the association.

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Taura Syndrome Virus and Mammalian Cell Lines

To the Editor: Audelo-del-Valle et al. concluded that human and monkey cell lines (rhabdomyosarcoma [RD], human larynx carcinoma [Hep-2C], and Buffalo green monkey kidney [BGM]) could be infected by a penaeid shrimp virus, Taura syndrome virus (TSV) (1). They also concluded that *Penaeus* spp. could likely be a reservoir of a virus that might become pathogenic to humans and other mammals (1).

Though researchers have tried to develop continuous marine crustacean cell lines for >30 years, their efforts have not been successful. The lack of continuous marine crustacean cell lines has become an obstacle to conducting research on viral disease in shrimp (2,3). During the last 20 years, many researchers searched for substitute cell lines on which to study shrimp viruses (4,5). Audelo-del-Valle et al. likely chose RD, Hep-2C, and BGM cell lines because TSV was "recently reported to be genomically related to the cricket paralysis virus of the *Cripavirus* genus, family *Dicistrovirdae* of the 'picornavirus superfamily'" (1), and these cell lines were susceptible to some picornaviruses. If their findings are correct, they may have found substitute cell lines for isolating and studying TSV. To confirm their findings, we selected two mammalian cell lines, Hep-2 and Vero, which are highly sensitive to some picornaviruses (6,7), and tested them to determine their susceptibility to TSV.

The TSV extract was prepared from frozen cephalothoraxes of shrimp, *Litopenaeus vananmei*, that were infected with TSV (confirmed by standard reverse transcriptase–polymerase chain reaction [RT-PCR]) (8). To verify the TSV extract's validity, 50 µL of diluted TSV extract (approximately 0.8% volume of shrimp body weight) was injected into each of eight healthy shrimp, *L. vannamei.* Another eight healthy shrimp (control group) were injected with a diluted extract prepared from frozen cephalothoraxes of healthy shrimp. All of the TSV-injected shrimp died within 6 days and were TSV-positive; control shrimp did not die and were TSV-negative, which showed that our TSV extract was active and viable. The TSV extract was transferred into cell culture flasks according to a method previously reported (9). The cell monolayers were exposed to 100 µL of diluted and filtered TSV extracts for 1 hour; the extracts were then removed from the flasks, 2 mL of maintenance medium was added to each flask, and the flasks were incubated in three separate rooms at 37°C, 35°C, and 33°C, respectively. If a cytopathic effect (CPE) was not evident within 7 days, cell monolayers were washed with Hank's balanced salt solution (HBSS) six times to eliminate viral particles from the primary extract or from infected cells. Then cells were lysed in 2 mL HBSS, the lysate was clarified, and a portion of it was used for the first passage. This procedure was repeated three times. The control cell lines were injected with diluted extract from healthy shrimp, and passage was conducted as described earlier. RNA samples were extracted and purified from 150-µL lysates of primary cells and four passage cells and used as templates for RT-PCR analysis to determine the presence of TSV.

No CPE was observed in either the Hep-2 or Vero cell line that had been injected with TSV after 7 days of culture at any of the three temperatures tested, and CPE was not found after the fourth passage. The RT-PCR analysis resulted in weak amplification (positive) from the first lysate, but no amplification was found in lysates of four passage cells. Had TSV replicated (productive infection) in either of the two cell lines, RT-PCR would have shown a strong amplification from each lysate. Such a weak amplification may have been the result of residual extracellular viruses that remained in the cell culture flask after washing. However, after first passage and repeated washing with HBSS, any remnants of the original medium were not likely to have been present. Therefore, our result showed that TSV was incapable of infecting Hep-2 and Vero cell lines.

Generally, aquatic viruses replicate in cells of aquatic animals at 20°C–35°C, their natural environmental temperature. We incubated cultures as noted earlier, as we did not know which temperature was most conducive for viral replication; all attempts were unsuccessful. Hep-2

and Hep-2C derive from the same tissue (human Caucasian larynx carcinoma), while Vero and BGM derive from another organ (Africa green monkey kidney). Thus, Hep-2 and Vero cell lines that we used are likely susceptible to TSV if the virus can infect Hep-2C and BGM as reported by Audelo-del-Valle et al.

The difference between our methods and those used by Audelo-del-Valle et al. may explain the discrepant result. If CPE occurred "usually from 19–23 hours" and "cells were then harvested and lysed" for next injection, TSV most likely persisted in the lysate after the third passage because the cell monolayers had not been washed as they were in our method. According to the time the CPE was observed and the methods of Audelo-del-Valle et al., we assumed that, in their study, cells might be passaged at least three times within 1 week, whereas TSV might remain viable and infective for 1 week. Additionally, the Office International des Epizooties recommended an injection volume of 1% of shrimp body weight (8); Audelo-del-Valle et al. used 10%. With such a large dose, shrimp could be infected easily with TSV from the initial medium and die suddenly. Moreover, the evidence of successful infection from photos of CPE only is not sufficient; Audelo-del-Valle et al. should offer more convincing evidence from images of viral particles in cells by electron microscope or in situ hybridization. Therefore, we think the CPE that Audelo-del-Valle et al. reported was not caused by TSV but by a virus contaminant or some harmful component from shrimp extract.

The structure of the TSV genome is similar to that of small insect–infecting RNA viruses (10), which belong to a renamed virus genus, *Cripavirus* (11). No published reports have shown that other viruses in this genus are able to infect mammalian cells or cell lines. Moreover, TSV is prevalent in shrimp farming areas in the world, and *L. vannamei* (principal host for TSV) are eaten by people worldwide (8). In China, some persons eat fresh shrimp without disinfecting them; however, no evidence shows that TSV can infect humans. The results of our study show that TSV cannot infect mammalian cell lines or cells.

This work was supported by innovation-projects funds provided by The Chinese Academy of Sciences (Projects No. ZKCX2-211).

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Bartonella clarridgeiae and *B. henselae* in Dogs, Gabon

To the Editor: The genus *Bartonella* contains several recently described species, many of which are emerging human pathogens. Human infections are mostly due to *Bartonella henselae* and *B. quintana*. Like many vectorborne disease agents, *Bartonella* species have a natural cycle. This cycle contains a reservoir host, in which *Bartonella* species cause an intraerythrocytic bacteremia, and a vector, which transmits the bacteria from the reservoir host to a new susceptible host (usually the uninfected reservoir host) (1). In the case of *B. quintana* and *B. bacilliformis*, the natural host is human. In *Bartonella* diseases, humans act as accidental hosts. Among the nonhuman *Bartonella* species that infect humans, *B. henselae* is most commonly encountered and usually causes cat-scratch disease. However, several cases of infections in humans attributable to other *Bartonella* species, including *B. elizabethae*, *B. grahamii*, *B. vinsonii arupensis*, *B. vinsonii berkhoffii*, and possibly *B. clarridgeiae*, have been reported (1).

Isolation of *Bartonella* species in animals that have contact with humans can help identify new human pathogens or new diseases. We report results of isolation of *Bartonella* spp. from the blood of 258 dogs in Gabon.

The study was performed in the Ogooué-Ivindo province of Gabon, a country of Central Africa with an equatorial climate. Blood samples were taken from dogs in the town of Mékambo and in all villages connecting Mékambo and Mazingo (nine villages) and Mékambo and Ekata (seven villages) during July and August 2003. Each dog brought by its owner for the study was weighed and sedated by injection with 50 μg/kg of medetomidine (Pfizer Santé Animal, Orsay, France). After the dog was examined, a blood sample was drawn from the jugular vein by Vacutainer (Becton Dickinson, Meylan, France). Each dog was tattooed with an identification number and given both antihelminthic and external antiparasitic treatments. During the examination, the dogs were treated with care; upon completion of the examination, the dogs were given 250 μg/kg of the reversal agent atipamezola (Pfizer Santé Animal) intramuscularly. A physical examination form and a questionnaire were completed for each test participant by its owner. A total of 258 dogs (155 males and 103 females) were examined and had blood samples drawn during the study. All animals were of mixed breeds and were 6 months to 14 years old (average 3 years 1 month). The Vacutainer tubes were kept on ice until blood samples were dispensed into cryotubes and frozen in liquid nitrogen. Samples were stored at –80°C until isolation attempts were made on Columbia agar (Biomérieux, Marcy l'étoile, France) as described previously (2). In this study, six *Bartonella* isolates were obtained and identified as *B. clarridgeiae* (five isolates) and *B. henselae* (one isolate), by internal transcribed spacer amplification and sequencing (3).

B. vinsonii subsp. *berkhoffii* was the first *Bartonella* species found in dogs (1). Isolation of *B. clarridgeiae* (4,5) and *B. washoensis* (6) in dogs was recently reported. Infection of dogs by other *Bartonella* species was also detected in the DNA of *B. henselae* (7,8), *B. clarridgeiae* (7), and *B. elizabethae* (8). The presence of these *Bartonella* species is not surprising, since *Ctenocephalides felis*, the vector of *B. henselae* in cats, has a wide range of hosts, including the domestic dog. However, attempts to isolate this species in samples collected from 211 dogs in the United Kingdom failed (9). *Bartonella* species are supposedly difficult to isolate in dogs because of a low concentration of bacteria in the blood (1). This supposition was apparent in our study; we identified approximately 100 bacterial colonies per milliliter of blood from three of the six dogs in our study. From the other three dogs in our study, including the dog infected with *B. henselae*, we identified two to four bacterial colonies per milliliter of blood.

Most of the data pertaining to *Bartonella* have been obtained in the United States and Europe. Increasingly, *Bartonella* infections are being reported in Africa, especially in southern Africa (10). We report here the first isolation of *B. henselae* from a dog and the first isolation of *B. clarridgeiae* in Central Africa. That dogs also act as reservoirs of *B. henselae* likely has implications in Africa where HIV infections are prevalent.

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Serologic Evidence of Hantavirus Infection in Humans, Colombia

To the Editor: Several New World hantaviruses cause hantavirus pulmonary syndrome (HPS) in the Americas. All hantaviruses that cause HPS are hosted by the rodent family *Muridae*, subfamily *Sigmodontinae* (New World rats and mice). Since the Sin Nombre virus (SNV) was documented in 1993 (1), ≈25 sigmodontine hantavirus genotypes from the Americas have been described; each is associated with a different rodent species or subspecies. Hantaviruses have been documented in South America from Argentina, Chile, Paraguay, Uruguay, Bolivia, Brazil, Peru, and Venezuela (2), and in Central America from Costa Rica and Panama (3,4).

Although documented in four bordering countries, hantaviruses have not been documented in Colombia. We assessed hantavirus antibody prevalence in humans by screening blood samples from rural volunteers within Córdoba and Sucre provinces in Colombia. Workers 16–65 years-of-age from 12 communities were enrolled. The research committee of the University of Córdoba approved the protocol, and informed consent was obtained from all participants. Participants were of low socioeconomic status and lived in homes with no running water and frequently no electricity. These workers had lived in the same general area all of their lives; none had traveled outside of Colombia. From January to October 2003, 88 blood samples were collected in 12 localities. Samples were screened for immunoglobulin (Ig) G antibody reactive with SNV antigen by using enzyme immunoassay (5). This assay detects, but does not distinguish among, all known sigmodontine hantaviruses. SNV antibody was detected in 12 samples (13.5%) representing 10 of 12 sites. Site-specific prevalences (Figure) ranged from 5% (1 of 19) to 50% (1 of 2). Except for one category with no positive samples, prevalence of anti-SNV IgG was similar across occupations (chi square = 0.03, df = 3, p = 0.998). All 12 antibody-positive samples were from male workers. We divided the study population into five age categories (18–24 years, n = 19; 25–34 years, n = 24; 35–44 years, n = 20; 45–54 years, n = 15; 55–70 years, n = 10) and found significant differences among the proportions of antibody-positive persons (chi square = 9.8, df = 4, p = 0.04). The higher prevalences were in the two youngest age groups (16%–17%) and the oldest age group (40%); one antibody-positive sample was found in the 35- to 54-year-old group (3%).

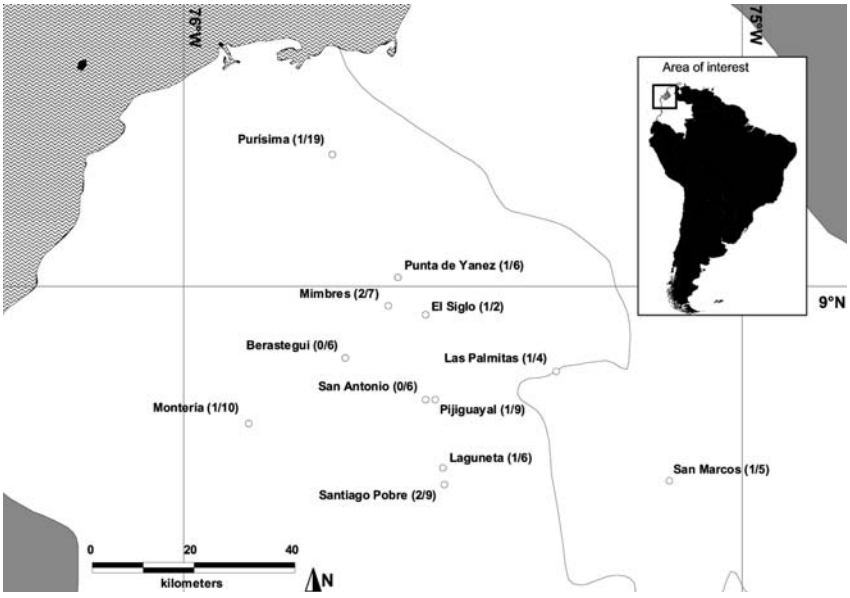

Figure. Locations of 12 towns in Córdoba and Sucre departments, Colombia, where rural workers were screened for antibody to Sin Nombre hantavirus. Numbers in parentheses represent number of antibody-positive persons and number of persons tested.

The prevalence of SNV-reactive antibody in rural workers indicates that at least one hantavirus is endemic in rodents in northern Colombia and is frequently transmitted to rural residents. This finding further supports mounting evidence that hantaviruses and HPS are a Pan-American problem (2,6). The bimodal infection distribution among age groups suggests human exposure might be episodic with an extended periodicity. Preliminary and limited rodent sampling has not produced any hantavirus antibody–positive samples. Although their distributions are poorly studied, both *Zygodontomys brevicauda* (reservoir of Calabazo virus in Panama) and *Oligoryzomys fulvescens* (reservoir of Choclo virus, a known agent of HPS in Panama [4]) are believed to be found in northern Colombia (7).

Despite the prevalence of antibodies to a hantavirus in humans in northern Colombia, we did not find any human illnesses. None of the SNV antibody–positive volunteers reported illness compatible with HPS; however, some hantaviruses may cause mild or no illness. Surveys have shown hantavirus antibodies (13%–40% prevalence in Paraguay and northern Argentina [8], Bolivia [J. Montgomery et al., unpub. data], northern Brazil [P.F.C. Vasconcelos et al., unpub. data], and Panama [6]) in some populations with little evidence of illness.

Infection in rural workers and the likely presence of *O. fulvescens* in northern Colombia underscore the importance of physician awareness and surveillance for HPS. A systematic survey of sigmodontine rodent populations that will identify the hantaviruses and their hosts is imperative.

Acknowledgments

We thank W. Ariz, A. Iguaran, A. Aleman, S. Najera, S. Florez, and A. Miranda for field work and collection of samples; T. Ksiazek and A. Comer for performing enzyme-linked immunosorbent assay; and J. Mills for consultation, assistance, and encouragement.

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Notice to Readers and Contributing Authors

Beginning in January 2005, summaries of emerging infectious disease conferences will be published online only.

Summaries submitted for online publication may include illustrations and relevant links. For more information on conference summary requirements, please refer to author guidelines at http://www.cdc.gov/ncidod/eid/instruct.htm.

Submit conference summaries at http://www.eid.manuscriptcentral.com

Instructions for Infectious Disease Authors

Letters

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

Emergence and Control of Zoonotic Viral Encephalitides

C.H. Calisher and D.E. Griffin, Editors

Springer-Verlag, Wien, New York
ISBN: 3-211-20455-5 (hardcover)

Pages: 244, Price: US $189; Euros 147.85

The viral encephalitides of Eastern, Western, and Venezuelan equine encephalitis viruses have been of public health concern for years. Over the last decade, several outbreaks caused by emerging zoonotic viral encephalitides, such as West Nile virus in North America and Nipah virus in Malaysia and Singapore in 1999, resulted in serious illnesses and deaths in persons, domesticated food animals, and wildlife. The Institute of Medicine has cited a number of factors that have led to these and other emerging disease outbreaks: 1) a growing human population that is moving into habitats of wildlife and domesticated livestock and poultry; 2) global climate changes that have caused changes in arthropod vector and rodent reservoir populations; 3) rapid travel and movement of people and animals worldwide; and 4) changing human behaviors (1). Emergence and Control of Zoonotic Viral Encephalitides is a timely book that gives an overview of agent, host, environmental, and other factors that have led to the emergence and transmission of several zoonotic viral encephalitides, including flaviviruses, alphaviruses, and rabies virus. The book also details important avenues for their control.

This book is a special issue of the Archives of Virology, and its 244 pages comprise 21 presentations that were made at a symposium on "Emergence and Control of Zoonotic Viral Encephalitides." The symposium was held April 6–8, 2003, in Les Pensieres, Veyrier du Lac, France. The first presentation gives an overview of the emergence of zoonotic viruses maintained by wildlife reservoir hosts and describes a conceptual model of processes that would account for the transmission of viruses among species. The second presentation describes the role of disease surveillance in polio eradication and the identification of emerging viral encephalitides. The third presentation gives an overview of the mechanisms of genetic changes and neurovirulence of encephalitogenic arboviruses.

The following 13 presentations include overviews of molecular determinants of virulence of West Nile virus in North America, genetic determinants of Venezuelan equine encephalitis virus, evolution and dispersal of encephalitic flaviviruses, and West Nile and other zoonotic viruses in Russia. Presentations that follow address lyssaviruses and henipaviruses transmitted by frugivorous bats, host-management strategies of novel viral encephalitides associated with bats, regulation of transcription and the nature of the cell receptor with regard to henipaviruses, and entry machinery of flaviviruses. Also included are presentations on persistent infection and suppression of host response by alphaviruses, subversive neuroinvasive strategy of rabies virus, neurovirulence and host factors in flavivirus encephalitis, regulation of apoptosis by viruses infecting insects, and Semliki Forest virus infection of laboratory mice as models to study the pathogenesis of viral encephalitis.

The book finishes with presentations on a novel principle of attenuation for developing new generation live flavivirus vaccines, on tick-borne encephalitis, and on a recombinant vaccine developed from a canarypox virus carrying the prM/E genes of West Nile virus that will protect horses against a West Nile virus–mosquito challenge, and on diagnosis of zoonotic viral encephalitis.

The book will be worthwhile to virologists and other infectious disease researchers and practitioners interested in the biology, virulence, and genetic evolution of viral encephalitides, and the factors involved in their emergence.

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Prions and Prion Diseases: Current Perspectives

Glenn C. Telling, Editor

Horizon Bioscience, Norfolk, England
ISBN: 0-9545232-6-1

Pages: 307, Price: U.S. $180

Prion diseases, also known as transmissible spongiform encephalopathies, are rapidly progressive, uniformly fatal brain diseases that can infect humans and animals, including cattle, sheep, goats, mink, deer, elk, cats, and zoo ungulates. In humans, prion diseases can occur as a sporadic

or inherited disease, or as a result of iatrogenic transmission. Prion diseases generated great public concern after an outbreak of bovine spongiform encephalopathy occurred in many European countries and scientific evidence indicated its transmission to humans.

Research in prion diseases is hampered by certain unconventional properties of the presumed etiologic agent and the long incubation period associated with these diseases. Most conventional laboratory methods used to study viruses and bacteria may not be applicable. In the past, the etiologic agent of transmissible spongiform encephalopathies was believed to be a slow virus, primarily because of its transmissibility, ability to retain infectivity after filtration, and long incubation period. The successful transmission of scrapie, a centuries-old prion disease of sheep, to mice in 1961 greatly facilitated identification and characterization of the scrapie agent. Several characteristics of the scrapie agent suggest that the agent is not a virus but is likely composed primarily of a protein. The agent's characteristics include the absence of disease-specific nucleic acids; resistance to radiation, nucleases, and standard sterilization and disinfection methods; and inactivation by protein-modifying procedures. These observations and purification of the scrapie prion in the early 1980s led to widespread acceptance of the prion hypothesis.

Since the 1980s, both the scope and nature of prion disease research has progressed rapidly. The economic and human cost associated with the bovine spongiform encephalopathy outbreak fueled the need to better understand the etiologic agent of prion diseases and their basic transmission mechanism. Prions and Prion Diseases: Current Perspectives summarizes the advances in prion disease research. It expands on a previous volume edited by David Harris that was published in 1999 under the title Prions: Molecular and Cellular Biology. The book's 10 chapters describe the biochemical and molecular features of prions and the normal prion protein, various laboratory methods for studying prions, and advances in the pathogenesis and immunology of prion diseases.

Chapters 2 through 6 detail laboratory methods developed to study the unconventional agent of prion diseases. Chapter 2 describes a cell-free conversion reaction system to study how pathogenic prions associated with different species interact with host cellular prion protein. Such systems have been used to study the biochemical mechanisms of prion diseases and can potentially be used to screen new therapies for their effectiveness against prion diseases. Chapter 3 describes the mechanisms underlying the biosynthesis and cell biology of the cellular prion protein by using cell culture systems. Understanding the detailed biochemical properties of the cellular prion protein will help show the molecular basis of its interaction with, and conversion to, the pathogenic prions. Subsequent chapters in the book describe other laboratory methods, including transgenic mouse models, which can be used to investigate the transmissibility of prions among different species, the extent and degree of the "species barrier," the mechanism of prion propagation, and prion disease pathogenesis.

Overall, the book provides a wealth of information on the progress made in understanding the molecular, immunologic, and genetic aspects of prion diseases and the laboratory methods used to study them. This book will be valuable to prion disease researchers, to scientists who want to gain more knowledge about the progress made in understanding the mechanisms of prion propagation, and to persons just beginning to study these unconventional, fatal brain diseases.

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Veterinary Institutions in the Developing World: Current Status and Future Needs

Cees de Haan, Editor

World Organisation for Animal Health Scientific and Technical Review

ISBN 92-9044-605-6

Pages: 397, Price: US $62.50; 50 Euros (including airmail postage)

Veterinary institutions help improve animal health by providing training that will enhance livestock production and trade and protect public health. The increasing role of animals in emerging infectious diseases has emphasized the need to improve veterinary services and integrate them with public health services more effectively.

The World Organisation for Animal Health devoted its Scientific and Technical Review (Volume 23, No. 1, April, 2004) to addressing these weaknesses and the rapidly changing environment of veterinary services in developing countries. The issue consisted of an introduction and summary by the coordinator and contributions from 28 persons organized into six sections. The first section

examines the relative roles of the public and private sectors and included two papers on economic frameworks and two papers on technical experiences in providing public services by the private sector. A second section emphasizes changing international sanitary and phytosanitary regulations and how veterinary services could help meet these new requirements. The third, fourth, and fifth sections detail experiences in providing services across the developing world. The most common experiences include using paraprofessional staff, providing public services by the private sector, conducting surveillance, and monitoring and controlling infectious diseases. A final section explores anticipated financial and institutional capacity, research, and professional training needs.

Examples provided in the book detail many innovations relevant to delivering health services, particularly for providing access for poor and marginalized persons. However, the examples do not provide an analysis of different experiences across countries and systems. Veterinary services have evolved and should respond to social, economic, and political realities. Guidance should be provided to the decision makers who want an analysis of what works under different circumstances.

From an infectious disease perspective, the focus is on promoting livestock production and trade, with less emphasis on public health and food safety issues. This emphasis is consistent with current veterinary policy and practice in most developing countries, where economic development is more relevant than public health concerns. The World Organisation for Animal Health, which is responsible for international animal and animal product trade standards under the World Trade Organisation, also focuses on this perspective.

The book reflects the constraints and broad mandates of veterinary services in developing countries and the potential short-term conflicts of interests between livestock commerce and public health. However, the book does not address links between veterinary and public health services, including common surveillance, information, and disease control systems. Because the book highlights the veterinary components of disease control systems, it provides an important resource to develop such systems in the future.

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Conference Summary

Human Health Safety of Animal Feeds Workshop

Approximately 150 scientists attended the "Human Health Safety of Animal Feeds" workshop at the Centers for Disease Control and Prevention (CDC) on January 23, 2004, to discuss issues pertaining to *Salmonella*-contaminated animal feed and their impact on public health. The workshop followed an article published in Clinical Infectious Diseases, which provided three recommendations to reduce human foodborne disease caused by *Salmonella*-contaminated animal feed (1). The first recommendation stressed the need for microbial contamination surveillance to determine how feed contaminants, particularly *Salmonella*, pass through the food chain. The second recommendation was to establish hazard analysis and critical control point programs to minimize *Salmonella* contamination by identifying and controlling sources of feed contamination. The third recommendation was to implement the *Salmonella*-negative standard in the feed industry. The purpose of the workshop was to elicit discussion on these and other recommendations concerning the human health safety of animal feed.

A variety of organizations were represented at the workshop, including international government agencies, the United States Department of Agriculture (USDA), the United States Food and Drug Administration (FDA), and consumer groups. Speakers offered perspectives on bacterial contamination of animal feed, including examples of human illnesses traced to *Salmonella*-contaminated feed, and data showing how contaminated animal feed contributes to human foodborne illness.

The opening plenary session focused on international experiences in controlling *Salmonella* in animal feed. Officials from the National Veterinary Institute of Sweden and the Norwegian Agriculture Inspection Service gave an overview of the control measures implemented in Sweden and Norway to ensure *Salmonella*-negative animal feed. Norway and Sweden have extensive surveillance programs for *Salmonella* control in animal feed. The measures implemented in Norway and Sweden are important contributing factors to the virtual absence of *Salmonella* in the food supply in their countries.

Several U.S. government agencies, including CDC; USDA; and the National Institutes of Health, National Institute of Allergy and Infectious Diseases, presented research findings at the workshop. Presentations included results from animal feed commodity studies that look at the factors contributing to microbial pathogens, mycotoxins, and chemical residues in animal feed. Researchers from FDA and Washington State University also provided data indicating that contaminated animal feed continues to be a source of *Salmonella* in food animals.

Further studies are necessary to document the precise contribution of contaminated animal feed to human illness. Nevertheless, some presentations suggest that practical interventions are available to reduce the prevalence of *Salmonella*-contaminated animal feed. Collaboration among all groups was stressed as a useful measure in controlling contaminated animal feed in the future.

A compact disk, including all of the presentations, agenda, and list of participants from the workshop, is available from Heather Bair (hhb9@cdc.gov). The contents of the compact disk are also available online at http://www.cdc.gov/narms/mce/animalfeeds.htm.

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Edward Hicks (1780–1849). Peaceable Kingdom (c. 1833)
Oil on canvas (44.5 cm x 60.2 cm). Worcester Art Museum

"One Medicine" for Animal and Human Health

Polyxeni Potter*

"The wolf also shall dwell with the lamb, and the leopard shall lie down with the kid, the calf, and the young lion, and the fatling together, and a little child shall lead them." These biblical lines (Isaiah 11:6–9) provided Edward Hicks the allegorical framework in which to approach a theme of special fascination to him: the peaceable kingdom, an idyllic world in which all creatures live in harmony. He painted more than 100 versions of this theme, 62 of which have survived (1).

A native of Bucks County, Pennsylvania, Hicks was orphaned in infancy and raised by a Quaker family. At 13 years of age, he was apprenticed to a carriage maker and learned to decorate coaches. He showed natural talent for decorative painting and, even without academic training, became very successful. "I am now employing four hands, besides myself, in coach, sign and ornamental painting, and still more in repairing and finishing carriages," he wrote reflecting on his experiences, "and I think I should find no difficulty in doubling my business" (2).

As a young man, Hicks set out to explore the wild side of life but soon returned to the Quakers and became a popular itinerant preacher. This Religious Society of Friends espoused the principles of equality and nonviolence but frowned upon artistic ventures as too worldly. Hicks abandoned his ornamental painting business to become a farmer only to relent, reluctantly, in middle age and move from commercial decoration to easel painting. Inspiration came mostly from his religious faith and missionary work among Native American tribes (3).

Born into a new nation, just 4 years after the Declaration of Independence, Hicks became part of an 18th- and 19th-century American tradition that produced provincial or folk art (portraits, landscapes, religious, and historical themes) characterized by a naïve style. This style, the hallmark of self-taught artists who arrived at their art through a journey of discovery and realization of innate talent, often flourished in rural areas and small towns (4). Outside traditional rules of perspective and proportion, naïve style relied on intuitive organization and structure, and was imaginative, creative, and direct.

Even though in his day he gained notoriety as an impassioned preacher, Hicks is now remembered for his art. His paintings, a continuation of his religious beliefs, explored ethical and spiritual dilemmas and commemorated historical events. Human figures, animals, and landscapes were created to embody Quaker ideals. Color, size, proportion, placement, and other elements were used as symbols to compose a moral message, which was often inscribed on the frame.

In a world marred by strife—between nations, between animals and humans, between animals themselves—Hicks

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tapped into the universal wish for harmony and peace. Well ahead of his time, he invited to his kingdoms not only leading human figures and innocent children but also a consortium of animals whose presence he found indispensable. Domesticated animals, part of his life as a farmer, appeared in realistic detail, but wild beasts were more idealized and decorative.

In The Peaceable Kingdom on this month's cover of Emerging Infectious Diseases, Hicks once more assembled the world's creatures for an idyllic group portrait. Against a lighted backdrop of trees and river banks, animals and children gathered in the foreground. In mid-panel, leading Quaker William Penn concluded a peace treaty with the Lenni Lenape tribe. The colors were solid, the light well focused, and the curves of animal frames and horns gracefully outlined. Yet, Hicks was not denying tensions in the universe.

The animals, whose anthropomorphic features betrayed human emotions, seemed puzzled and apprehensive. Even as the bull offered the lion hay, the king of beasts seemed stiff and uneasy. Even as the lamb cuddled up, the wolf wore a noncommittal glare. The world's creatures may have been tamed, but peace in the scene seems precarious.

The connectivity that Hicks sensed between humans, animals, and the universe was greater than the artist could have imagined. The intensity in the animals' eyes was not the only troubling element in the picture. In the dander and under their breath, in the soil and in the water, on the leaves and the clothing of the dignitaries, lay creatures unknown to Hicks, microorganisms, insidiously moving from animals to humans, eating, multiplying, sharing, spreading, connecting. Even if Hicks could have arranged a perfectly peaceable kingdom, strife would have continued beneath the surface through the transmission of disease.

Not long after Hicks' death in 1849, German pathologist Rudolf Virchow (1821–1902) coined the term zoonosis, verifying the essential link between animal and human health (5). This link, further complicated by the emerging nature of disease and the ethical, ecologic, social, and economic values placed on the relation between humans and their pets, livestock, or fellow inhabitants of nature, has not been uniformly acknowledged or exploited—even in the face of AIDS, Ebola, West Nile virus, avian influenza, bovine spongiform encephalopathy, and SARS.

In the 1980s, American epidemiologist Calvin Schwabe proposed a unified human and veterinary approach against zoonotic diseases. This approach, "one medicine" (6), upholds Virchow's principles and affirms Hicks' wish for the control of subversive elements, whether they interfere with harmonious animal and human interaction or they disrupt animal and human health.

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends
Vol. 11, No. 1, January 2005

Upcoming Issue

Look in the January issue for the following topics:

Border Screening for SARS

Capacity of State and Territorial Health Agencies to Prevent Foodborne Illness

Hepatitis C Virus, Linxian, China

Hypersensitivity to Tisks and Lyme Disease Risk

Demand for Prophylaxis after Bioterrorism-related Anthrax Cases, 2001

Human Parechovirus-3 and Neonatal Infections

Multidrug-resistant A*cinetobacter baumanii*

Hybrid *Vibrio vulnificus*

Novel Paramyxovirus?

Mosquitoborne Viruses, Czech Republic, 2002

Emergent Strain of Human Adenovirus Endemic in Iowa

Vibrio parahemolyticus Diarrhea, Chile, 1998 and 2004

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

Upcoming Infectious Disease Activities

December 3–4, 2004
Focus on Hospital Infections
Trump International Sonesta Resort
Miami, Florida, USA
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

January 27–29, 2005
7th International Symposium on Febrile Neutropenia
Barcelo Gran Hotel Renacimiento
Seville, Spain
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

March 16–18, 2005
Focus on Fungal Infections 15
Sheraton Bal Harbour
Miami, Florida, USA
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

April 9–12, 2005
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Los Angeles, California, USA
Contact: (703) 684-1006
Web site: http://www.shea-online.org

May 1, 2005
International Society of Travel Medicine (ISTM) Offers Certificate of Knowledge in Travel Medicine Exam (Given prior to the opening of 9th Conference of the ISTM)
Contact: exam@istm.org
http://www.ISTM.org/

May 1–5, 2005
9th Conference of the International Society of Travel Medicine (CISTM9)
Lisbon, Portugal
Contact: +49 89 2180 3830
http://www.ISTM.org/

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.

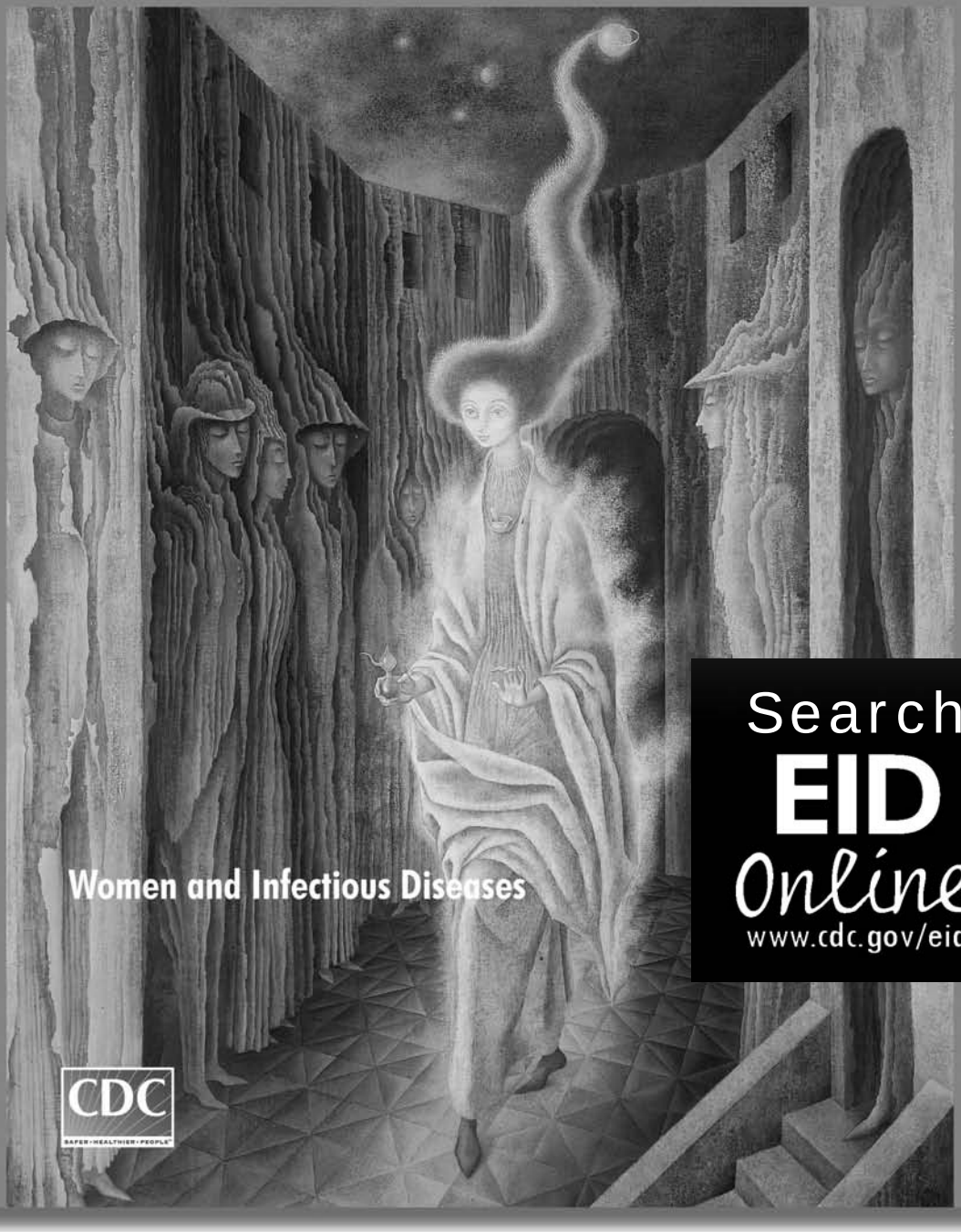
2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - 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.

EMERGING INFECTIOUS DISEASES

EID Online www.cdc.gov/eid

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.10, No.11, November 2004



Women and Infectious Diseases

CDC SAFER·HEALTHIER·PEOPLE

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

Editorial Policy and Call for Articles

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

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

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

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

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

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

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

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 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), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 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 no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

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

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

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

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.