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August 2009

Rabies

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On the Cover

Torii Kiyomasu I
(active 1696–1722)
Kintoki Wrestling with a Black Bear (c. 1700) (detail)
Color woodblock print (55.2 cm × 32.1 cm)
Courtesy of the Honolulu Academy of Arts, Hawaii, USA. Gift of James A. Michener, 1975 (16,576)

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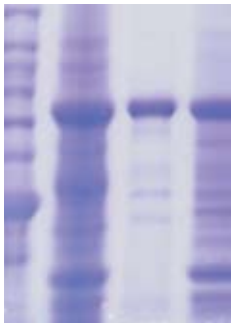

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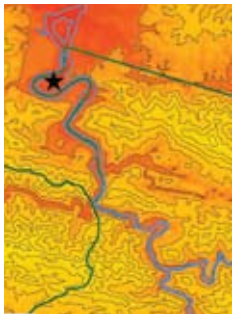

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Reemerging Rabies and Lack of Systemic Surveillance in People's Republic of China

Xianfu Wu, Rongliang Hu, Yongzhen Zhang, Guanmu Dong, and Charles E. Rupprecht

Rabies is a reemerging disease in China. The high incidence of rabies leads to numerous concerns: a potential carrier-dog phenomenon, undocumented transmission of rabies virus from wildlife to dogs, counterfeit vaccines, vaccine mismatching, and seroconversion testing in patients after their completion of postexposure prophylaxis (PEP). These concerns are all scientifically arguable given a modern understanding of rabies. Rabies reemerges periodically in China because of high dog population density and low vaccination coverage in dogs. Mass vaccination campaigns rather than depopulation of dogs should be a long-term goal for rabies control. Seroconversion testing after vaccination is not necessary in either humans or animals. Human PEP should be initiated on the basis of diagnosis of biting animals. Reliable national systemic surveillance of rabies-related human deaths and of animal rabies prevalence is urgently needed. A laboratory diagnosis–based epidemiologic surveillance system can provide substantial information about disease transmission and effective prevention strategies.

The record of rabies in Chinese history dates back to 556 BC in Master Zuo's tradition of the Spring and Autumn annals. He wrote, "In the eleventh month, people in the capital of Song were chasing a rabid dog. It entered the house of Hua Chen" (*1*). Sporadic descriptions of overt clinical signs of rabies can be found in records of various ancient civilizations (*2*). However, robust scientific investigation of the disease began only after 1885, with Louis Pasteur's discovery of postexposure vaccination against rabies. In the 1930s, a rabies virus (RABV) 3aG strain was isolated in Beijing and was eventually developed into a vaccine for human immunization. In the 1950s, another RABV strain (CTN) was isolated in Shandong Province and was characterized and attenuated as a vaccine for humans. However, to date, no dog RABV isolates in China have been developed into animal vaccines. Few domestically licensed vaccines for animal rabies exist, according to the Regulations for Veterinary Biologics in China (www.ivdc.gov.cn). The disconnection between human and dog rabies in China reflects a lack of awareness of the concept of one medicine, or health without regard to species, in approaches to rabies control in the public health system.

Although great progress has been made internationally in rabies control and prevention, >55,000 persons still die of rabies annually worldwide. In China, at least 108,412 persons died of rabies from 1950 through 2004 (*3*). A rabies epidemic occurs every 10 years in China (*4*). Despite high human mortality rates, only ≈30 rabies virus isolates have been recorded and partially characterized by sequencing (*3,5,6*). Therefore, human rabies is mainly reported without confirmatory laboratory diagnosis in most of China. Few statistics are available for dog rabies, indicating that a diagnosis and surveillance system for animal rabies is not fully functional. Obvious inconsistencies exist in published results of human rabies diagnosis (*6*). China is now facing another wave of rabies outbreaks resulting from the combined consequences of rapid economic development, a profitable domestic pet industry, and continuing family planning, resulting in increased numbers of family pets. Reemerging rabies in China has led to a carrier-dog myth, strict pet population control policies, counterfeit vaccines (low antigen, generating <0.5 IU of virus-neutralizing

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antibodies after administration), vaccine matching, seroconversion testing with an ELISA after completion of postexposure prophylaxis (PEP) in humans, virus–neutralizing antibody titration in vaccinated animals because of inferior vaccines, and other related issues. We discuss these issues and suggest a new approach to prevention and control of rabies when the disease reemerges in an unprepared country like China.

Carrier or Asymptomatic Rabies

Typically, rabies is fatal once clinical signs develop. Although persistent infections occur regularly for other virus infections, they have not been documented unquestionably in rabies, mainly because of the added complexity of the disease's relatively long incubation period. The carrier or asymptomatic rabies state was once considered to be important for public health, despite lack of adequate evidence that the phenomenon actually exists. This concern has been raised repeatedly from the early 1930s until recently (*7*). Reported carrier hosts have included vampire bats (*8,9*), cats (*10*), dogs (*11–15*), and hyenas (*7*). Because rabid dog bites are responsible for ≈99% of all human rabies cases in the world (*16*), the possibility of a carrier state or asymptomatic form of canine rabies deserves serious evaluation. Unfortunately, this possibility remains highly speculative. Although some investigators have questioned reports of a carrier state in dogs (*17*), an author reported RABV isolation from brains of healthy dogs (*6*). Carrier dog RABV isolates were even characterized at the molecular level in 1996 (*15*).

Similarly, reports of healthy-dog carriers were consecutively published in China in 1982 (*18*), 1999 (*19*), 2006 (*20*), and 2007 (*21*). Tang found that 5 (1.76%) of 283 healthy-looking dogs in Guangxi province were positive for RABV by reverse transcription–PCR (RT-PCR) and virus isolation (*5*). Another study showed that 24 of 42 RABV isolates were taken from dogs or cats classified as clinically normal (*3*). All animals from these studies were from areas experiencing rabies epidemics.

All cumulative convincing data for more than half a century from various parts of the world call into question either the concept of a rabies carrier state or the quality of research indicating such a state. Other persistent virus infections routinely occur in lymphocytes, monocytes, macrophages, and dendritic cells through the viruses' curtailment of the host's antivirus immune responses. Experimental RABV replication in murine bone marrow macrophages and in human macrophage–like cell lines suggests a mechanism of virus persistence (*22*). However, wild-type RABV is highly neurotropic. If RABV persistent infection occurs in neurons, these findings contradict the current understanding of RABV pathogenesis. Clearly, RABV infection causes minor morphologic changes in neurons but may result in neurophysiologic dysfunction. Also, virus persistence generally is life-long in infected hosts. The longest surviving presumed carrier dog lived for only 16 months; in this dog, the tonsil was found to be the only organ from which the virus was isolated (*14*). No data are available concerning how long such carrier dogs survive. In healthy vampire bats, the duration of salivary excretion of RABV was reported to be 690 days after infection by an extremely high dose of RABV (*8*). Generally, persistent infections are characterized by an excess of viruses or virus antigens; free antibodies, which circulate without binding to antigens, are difficult to detect. However, experimental carrier dogs and vampire bats presented high rates of virus-neutralizing antibodies in serum in these reports (*8,14*). From an evolutionary perspective, a carrier dog with normal behavior does not pose an advantage for virus survival because biting when an animal is controlled by an aggressive brain is the only major route for RABV to spread. Animal behavior change is fundamental for RABV survival/transmission. Therefore, if carrier dogs exist, they are inferior to rabid dogs for disease transmission.

In a report by Zhang (*17*), 15 dogs that were diagnosed as positive by ELISA were confirmed to be negative by the standard direct fluorescent antibody (DFA) method. Explanations for these contradictory findings include misidentification of infected dogs, detection of RABV early in the prodromal course once it has reached the central nervous system after infection and incubation, and inadequate diagnostics. These phenomena, rather than the existence of carrier hosts, may explain historical reports of asymptomatic rabies in enzootic areas.

Rabies Diagnosis in China

A well-established surveillance system for infectious diseases depends on reliable, laboratory-based diagnostic methods. Human and animal rabies cases in China have been reported mainly on the basis of clinical presentations and retrospective epidemiologic surveys. Animal rabies is rarely diagnosed in China. Human rabies diagnosis based solely on clinical symptoms is unreliable because human rabies can be confused with Guillain-Barré syndrome, poliomyelitis, and other types of encephalitis (*23*). Similarly, rabies in animals is difficult to distinguish from canine distemper and other encephalitic conditions. Postmortem rabies diagnosis should be routinely performed on rabid animals, animals that have bitten victims, and human patients who die after an animal bite. Antemortem diagnosis of rabies in humans is challenging because of the disease's long and variable incubation period. Also, distribution of virus antigens, virus nucleic acids, and antibodies is unpredictable at this stage (*24*). Consequently, all countries should establish standardized national rabies diagnostic protocols for postmortem examinations.

The DFA method, first introduced in the 1950s, is the global standard procedure for rabies diagnosis. It is simple, economical, and reliable (*25*). This method is approved by the World Health Organization (WHO) and the World Organisation for Animal Health (OIE) and has served as a cornerstone for rabies diagnosis for the past half century (*26*). All rabies diagnostic laboratories should follow a single standard protocol (*27*). Whether such a standard exists in China is unclear, and diagnostic reagents, equipment, and qualified diagnosticians are in short supply. The ELISA for RABV antigen detection is carried out in a few laboratories, but this method requires performance evaluation using the standard DFA method to assess specificity, sensitivity, and reproducibility. An ELISA-based rapid rabies enzyme immunodiagnosis (RREID) method that uses monoclonal antibodies against nucleocapsid and glycoprotein for rabies diagnosis has been developed at the Wuhan Institute of Biological Products (*3*). One analysis used the RREID method to detect potential virus antigens in dog saliva; when verified by the DFA method, all 15 samples showed false-negative results (*17*).

Much inconsistency exists when different methods are used for rabies diagnosis (*6*). Overall, of 76 positive samples examined by the DFA method (*6*, cited as an indirect immunofluorescent antibody method), only 36 were confirmed by RT-PCR. In a certified rabies reference laboratory, DFA-positive samples should be positive when confirmed by a sensitive RT-PCR method. Therefore, in China, either the DFA or the RT-PCR, or perhaps both protocols, have questionable validity. The high prevalence of rabies in China necessitates establishment of a standardized national DFA protocol in provincial Centers for Disease Controls and veterinary stations. Other methods should be compared against the established DFA standard. A direct rapid immunohistochemical test (dRIT) using low-cost light microscopy has been extensively investigated and shown to have excellent agreement with the DFA (*28*). The dRIT can be completed within 1 hour, and this method is a feasible alternative at the county level for confirmatory rabies diagnosis or enhanced field surveillance. The urgent need to establish an improved national standard DFA protocol in China should take precedence over current efforts to develop ELISA and RT-PCR methods.

Rabies Vaccines and Seroconversion Testing in China

From 1885, when the first human rabies vaccination occurred, to 1994, when the RV Street–Alabama-Dufferin (SAD) B19 strain was engineered with reverse genetics (*29*), methods for RABV manipulation have changed fundamentally from random attenuation to defined modifications. However, the basic concept for rabies vaccine development has not changed for more than a century. Several major modern human rabies vaccines include duck embryo vaccine, commercialized in 1957; human diploid cell vaccine, introduced in 1978; purified chicken embryo cell vaccine, developed in 1984; and a purified Vero cell rabies vaccine (PVRV), developed in the late 1980s.

Human Rabies Vaccines in China

Before the 1980s, nerve tissue-derived Semple vaccine was manufactured using the fixed RABV Beijing strain 3aG, which was isolated in 1931. After the 1980s, primary hamster kidney cells (PHKC) rabies vaccine using the same 3aG strain was investigated as a substitute for nerve tissue vaccines (NTVs) (*30*). In recent years, purified and concentrated Vero cell rabies vaccines using the 3aG and CTN-1 strains have been developed. The PVRV, using a RABV purified Vero (PV) strain imported from the US Centers for Disease Control and Prevention, is also being developed to meet the increasing demand for human rabies vaccine in China. In 2001, WHO issued a resolution for the complete replacement of NTVs by 2006 with cell-culture rabies vaccines. NTVs were gradually replaced by the PHKC vaccine during the 1980s in China.

Animal Rabies Vaccines in China

In contrast to human rabies vaccine development, animal rabies vaccine development in China has not progressed. In the United States alone, 11 different rabies vaccines are licensed for dogs, 12 for cats, 1 for ferrets, 3 for horses, 4 for cattle, and 5 for sheep (*31*). However, in China, only 1 pentavalent vaccine is licensed, and 1 Flury-low egg passage (LEP) vaccine for dogs has been tentatively approved. No regional RABV isolates were characterized for animal vaccine development. The LEP, Evelyn–Rokitnicki-Abelseth (ERA), PV, and challenge virus standard (CVS) strains being developed as vaccine candidates originate from other countries and have an unclear biological background. The inferior quality of the domestically manufactured dog vaccine in China has been documented (*32*). Consequently, development of animal rabies vaccines using carefully characterized RABV strains should be prioritized as a fundamental task.

Some believe that vaccine production should reflect the design of matched field isolates for regional control. However, vaccine-matching investigations to address concerns about mismatch between vaccine strains and epidemic RABV isolates are redundant (*21*). All fixed RABV strains recommended by WHO, such as PV, CVS, LEP, high egg passage, ERA, and SAD variants, have been successfully used in industrialized countries, where rabies is well controlled. Vaccine quality control and mass production, rather than matching, are urgently needed and most important for addressing the current rabies problem in China. Any potent rabies vaccine will protect against rabies.

Seroconversion Testing After Vaccination or PEP in China

Because human rabies vaccines in China are produced in cell culture using modern technology, the vaccine quality should follow the standard recommended by WHO. The minimum potency for all cell-culture and purified embryonated egg rabies vaccines is 2.5 IU per intramuscular dose using the National Institutes of Health test (*16*). After rabies exposure in humans, PEP is initiated or withheld based on the postmortem diagnosis of animals that were the source of exposure. Modern PEP is a combination of active and passive immunization, with 100% efficacy in rabies prevention if the process strictly adheres to WHO recommended guidelines (*33,34*). Seroconversion testing is not needed for patients completing preexposure or PEP unless the person is immunosuppressed or protocol is not followed (*33*). However, the seroconversion test is routinely performed at the provincial and central levels in China, mainly because of fear of rabies. In animal studies, virus-neutralizing antibody titer has been shown to be an imperfect marker for protection because it varies with time and the expertise of technicians performing the assay. For titration of rabies antibodies, the fluorescent antibody virus neutralization test and the rapid fluorescent focus inhibition test are the only methods approved by WHO and OIE (*35*). However, indirect ELISA or ELISA-based methods are routinely used and are still being developed in China for seroconversion testing after vaccination. Without a standard method, efforts to develop novel assays are a poor use of limited resources for rabies control in China. Potent rabies vaccines do not require routine serologic analysis.

Any failure of vaccination and PEP should be investigated thoroughly and independently to trace potential errors in the protocol. A national vaccine adverse-event reporting system should be established to track suspected problems for safety and efficacy.

Hosts and Virus Phylogeny

In theory, all mammals are susceptible to RABV infection. In China, dogs play the dominant role in rabies transmission (*4,5*). Statistically, >95% of human rabies cases in China are due to rabid dog bites (*16*). In isolated serologic studies of rabies in wildlife such as badgers, raccoon dogs, rodents, and bats, no RABV was successfully isolated from these animals. Rodents do not serve as reservoirs for rabies. In China, all RABV isolates characterized by phylogeny using the N and G gene sequences are categorized into classic RABV genotype 1 (*3,6*). Homology among isolates is >90% at the amino acid level. Although some subgroups are suggested, the differences would be minor. In addition, RABV isolates from dogs on all continents are grouped into genotype 1. Phylogeny analyses reinforce the perspective that vaccine matching in China is redundant. In a rabies-epidemic region such as China, rabies in wildlife may result from spillover from dogs. Without proper investigation of animal population density and characterization of the RABV isolates, wildlife rabies in China can be elucidated only after dog rabies is well controlled.

Interpretation of Rabies in China

The recent reemergence and severe incidence of rabies have attracted the attention of scientists and administrative authorities in China. However, efforts are distracted by and concerns misleadingly focused on healthy dog carriers, possible rabies in wildlife, vaccine matching, inferior or counterfeit vaccines, and seroconversion testing after vaccination. In the 1920s, long before the recognition of bat and other wildlife rabies and the availability of modern vaccines, rabies in Japan was successfully controlled through mass vaccination of dogs. At present, in large cities such as Beijing and Shanghai, although animal bites are fairly common, rabies cases are rare. In 2006, ≈140,000 animal bites were recorded in Beijing, but few human rabies cases were reported (*16*), mainly because of adequate dog vaccination. However, in rural areas of China, dog rabies vaccination coverage is <3% (*4,32*). WHO has determined that vaccination coverage ≥70% is needed to sufficiently control canine rabies, but the exact level of sufficient coverage varies according to demographic, behavioral, and spatial characteristics of dog populations. Historically, the 4 rabies epidemic waves in China reflect discontinuous efforts of dog control and vaccination. The periodic recess of rabies at 10-year intervals resulted from strict depopulation of dogs rather than mass vaccination. The density of 4.5 dogs/km^2 can lead to endemic rabies in vulnerable populations (*36*). The current estimated number of dogs in China is 80–200 million (*5*). If dogs are distributed evenly, the average density of dogs in China is 8–20/km^2. The estimated population density would be much higher in rabies-endemic provinces. With very low vaccination coverage in dog populations, rabies outbreaks are not surprising. The phenomenon of 4 rabies epidemic waves during the last half century in China corroborates our interpretation and shows that depopulation of dogs alone cannot efficiently control rabies. Mass vaccination of dogs has been demonstrated to be the most efficient way to control the disease (*37,38*).

Decisions to initiate or withhold PEP are based on postmortem diagnosis of the biting animal and use of a standardized method (*27*). Patients exposed to rabies in China were estimated to be between 1% and 10% of the population, according to disease prevalence in different areas. However, because of China's weak national animal rabies diagnosis network, the number of PEP patients in China is probably arbitrary or only roughly estimated. To maximize efficiency of the limited resource of human vaccines and rabies immunoglobulin for patients at real risk,

establishing a systemic rabies diagnosis network is imperative. At a minimum, several million persons are expected to require PEP each year.

RABV is highly neurotropic and functionally conservative. It is transmitted mainly through animal bites, although rare nonbite exposure routes have been reported. Because of this characteristic, the dissemination of rabies is relatively slow. The spread of the disease needs a minimum threshold support of host population density (*36*). Therefore, the frontline of waves of rabies infections garner little public attention. Once the density of dogs reaches a critical threshold, rabies spreads rapidly, and the spread can be accelerated through animal translocation. However, the animal population itself does not pose a rabies threat. An immunized dog population can be a solid barrier to prevent rabies from spreading to humans (*37,38*). The immediate challenge for rabies control in China is to stockpile enough vaccines for mass dog vaccination campaigns. A reliable systemic diagnostic network can support effective epidemiologic investigation, vaccine campaigns, and initiation of PEP by providing multiple opportunities for collaborations that work toward practical, humane, and economical rabies elimination in China.

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Dr Wu is a molecular biologist and associate service fellow in the Rabies Program at CDC. His main research interests are rabies virology, immunology, vaccines, pathogenesis, and disease control.

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Use of Revised International Health Regulations during Influenza A (H1N1) Epidemic, 2009

Rebecca Katz

Strong international health agreements and good planning created a structure and common procedure for nations involved in detection and evaluation of the emergence of influenza A (H1N1). This report describes a timeline of events that led to the determination of the epidemic as a public health emergency of international concern, following the agreed-upon procedures of the International Health Regulations. These events illustrate the need for sound international health agreements and should be a call to action for all nations to implement these agreements to the best of their abilities.

In March 2009, human cases of infection with a novel strain of influenza A virus (H1N1) emerged in Mexico, the United States, and Canada. As of May 26, this contagious virus has spread to 46 countries, accounting for ≈13,000 cases. To date, >90 deaths caused by this virus have occurred, most of which have been in Mexico (*1*). Suspected cases are even more widespread, and the number of cases will inevitably continue to increase and the virus will spread to more countries in the coming weeks and months.

Predicting the course of the epidemic is difficult, but one can state with certainty that good multilateral plans and agreements facilitated the initial notification of the disease. Good planning has also enabled communication and action around the emerging epidemic in a manner that has been rational, predictable, and productive. These plans, which only came into being in the past 5 years, enabled an unprecedented level of timely cooperation and communication for assessing and responding to the novel influenza A virus (H1N1).

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Some have argued that the initial detection of the outbreaks was delayed (*2*), and others have opined that the international disease surveillance and reporting system is severely crippled by a lack of resources (*3*). Although these debates will no doubt continue, it is crucial to document how, starting with initial notification by Mexico, the systems for communication and disease mitigation worked essentially as they were designed.

Planning

The International Health Regulations (2005)

A major international agreement, a regional agreement, and a multitude of pandemic plans put into place since 2005 have set the stage for the events of the past few weeks. In response to the threat of emerging infectious diseases, and pushed into action by the events related to the emergence of severe acute respiratory syndrome (SARS), the World Health Assembly agreed to accept the revised International Health Regulations in May 2005. These regulations, known as IHR (2005), are binding to all member states of the World Health Organization (WHO) and include several major provisions aimed at facilitating global communication and cooperation for early detection and containment of events termed public health emergencies of international concern (PHEIC). Although many international efforts in health have been disease specific, IHR (2005) focuses on the larger issues of ensuring competent surveillance and detection systems in every part of the world and a global commitment to work together to mitigate the consequences of a public health emergency.

Included in the regulations are provisions that member states are required to 1) establish a National IHR Focal Point for communication with WHO, 2) meet core capacity requirements for disease surveillance, 3) inform WHO in a

timely fashion of any incident that might be considered a PHEIC, and 4) respond to additional requests for information by WHO (*4*). The revised regulations broadened the type of events that needed to be evaluated and reported to WHO to include a list of always notifiable diseases and an algorithm for determining a potential public health emergency, regardless of source or origin (*5*). In addition, the regulations clearly articulate that the purpose is to "prevent, protect against, control and provide a public health response to the international spread of disease" in a manner that "avoids unnecessary interference with international traffic and trade" (*6*).

The IHR (2005) were implemented in the summer of 2007. Two nations submitted reservations; the United States cited federalism concerns, and India clarified how it would regard regions infected with yellow fever (*7*). By the terms of the regulations, all member states should currently have in place a National IHR Focal Point for communication, should complete assessments of their disease surveillance capacity by the summer of 2009, and should develop and maintain their core surveillance and response capacities by the summer of 2012.

Security and Prosperity Partnership of North America

In March 2005, the United States, Canada, and Mexico launched a trilateral agreement called the Security and Prosperity Partnership of North America (SPP). The purpose of this agreement was to enhance regional cooperation and information sharing around business competitiveness, energy, emergency management, securing of borders, and health (*8*). The health focus within SPP was to enhance public health cross-border coordination in infectious disease surveillance, prevention, and control. In particular, leaders of the 3 nations agreed to a set of principles that would guide collaboration in the detection and response to avian and pandemic influenza. These principles led to the formulation of the North American Plan for Avian and Pandemic Influenza (NAPAPI). This plan stresses the need for communication between nations and coordination in responding to the threat of a novel strain of influenza; it also lays out a set of actions for each nation relative to emergency coordination and communications, avian influenza, pandemic influenza, border monitoring and control measures, and critical infrastructure protection (*9*). A senior level coordinating body was established to facilitate planning and preparedness as well as to serve as a contact in the event of a human outbreak caused by a novel strain of influenza (*10*).

Pandemic Plans

Spurred by fears of avian influenza (H5N1), the United States embarked on an aggressive policy to put into place a series of plans at the federal, state, and local levels. These pandemic plans address continuity of operations, social distancing strategies, vaccine and antiviral production and distribution, hospital surge capacity, and special considerations for vulnerable populations. In addition to plans, there were accompanying implementation schedules for implementing necessary infrastructure in place to ensure the plans would be useful should a pandemic emerge (*11,12*).

WHO has had a pandemic planning and guidance document available since 1999. In 2005, WHO revised the document in response to the threat of avian influenza. This document was revised and rereleased in April 2009, in part to reflect advances in global pandemic planning, the IHR (2005) entry into force, and scientific advances in the development and stockpiling of countermeasures (*13*).

Events and IHR (2005)

I have outlined a series of events, beginning with the reporting by Mexico of an outbreak of acute respiratory illness. This event and subsequent events were linked with the corresponding article or provision in the IHR (2005), the SPP NAPAPI, or the WHO Pandemic Influenza Preparedness and Response guidance document. The events were organized according to the major goals of the IHR (2005): improving notification procedures, identifying public health emergencies of international concern, facilitating ongoing global communication during an emergency, and mitigating the consequences of the event through a coordinated response. In addition, the determination of pandemic phases as part of the IHR (2005) procedures, yet specific to this particular type of public health emergency, is discussed.

Notification

On March 18, 2009, surveillance systems in Mexico alerted authorities to an unusual number of cases of influenza-like illness (*2,14*). After a few days of discussion starting on April 11 between the Pan American Health Organization (PAHO) and Mexican authorities regarding unusual numbers of acute respiratory infections, the authorities notified PAHO according to recommendations in IHR Focal Points of a potential PHEIC. The event was an outbreak of acute respiratory illness in the states of Veracruz and Oaxaca, Mexico (*15,16*).

On April 18, the United States, through the National IHR Focal Point, notified PAHO of 2 cases of human influenza A (H1N1) in children in San Diego County and Imperial County, California. The United States assessed that these cases could be a potential PHEIC (*17*).

The initial notification by Mexico and the United States of a potential PHEIC within their borders aligns with the following articles of the IHR (2005):

- IHR (2005) Article 4 (Responsible Authorities). Each state is responsible for designating a National IHR Focal Point for 24 × 7 × 365 communication with WHO, including for dissemination of information from WHO to relevant sectors of the state. These National IHR Focal Points were used to officially communicate the potential PHEICs to the regional WHO office (PAHO).
- IHR (2005) Annex 2 (Decision Instrument). The decision instrument in Annex 2 helps nations determine which events should be reported to the WHO as potential PHEICs. Mexico and the United States presumably used this decision instrument to determine if the events constituted a potential PHEIC.
- IHR (2005) Article 6 (Notification). State Parties shall notify WHO (through their WHO Regional Office–PAHO in this case) by way of the National IHR Focal Point of all events that may constitute a PHEIC. This notification must occur within 24 hours of assessment of the public health information by the national authority. After a notification, the State Party and WHO shall continue to communicate in a timely fashion about the notified event.

Determination of a PHEIC

On April 25, the Director-General of WHO, after convening a meeting of the Emergency Committee, determined that the outbreak of novel influenza A (H1N1) constituted a PHEIC and made a public announcement. This was the first declaration of a PHEIC after the entry into force of the IHR (2005) (*18,19*). The IHR Emergency Committee, which was convened by the Director-General on April 25, and which provides advice regarding the determination of the PHEIC, proposed that nations increase their active surveillance for unusual outbreaks of influenza-like illness (*20*).

The formation of the Emergency Committee and the process of declaring a PHEIC proceeded according to the following provisions of the IHR (2005):

- IHR (2005) Article 12 (Determination of a PHEIC). The Director-General determines on the basis of information received from the affected states whether an event constitutes a PHEIC. If the Director-General assesses the event to be a PHEIC, she then consults the affected states regarding her determination. Subsequently, the Director-General seeks the views of an Emergency Committee.
- IHR (2005) Article 48 (Emergency Committee: Terms of Reference and Composition) and Article 49 (Emergency Committee: Procedures). The Director-General establishes an Emergency Committee to provide views on whether an event constitutes a PHEIC; the termination of a PHEIC; and proposes issuance, modification, extension, or termination of temporary recommendations for mitigating the consequences of the PHEIC. The Emergency Committee may meet by teleconference, videoconference, or electronic communications.

Ongoing Communication

After initial notification of the potential PHEICs by the United States and Mexico, WHO continued to maintain constant contact with the National IHR Focal Points. PAHO coordinated communication between the United States, Mexico, and Canada to better understand the emerging events (*14,21*).

National IHR Focal Points around the world continue to supply daily reporting of confirmed and suspected cases to WHO (*22,23*). WHO communicated with all member states through the National IHR Focal Points and the WHO public website to inform them of recommendations for actions to mitigate the consequences of the epidemic (*24*). On April 28, PAHO hosted a teleconference with health officials and ministers from 26 countries to exchange information on the influenza A (H1N1) epidemic (*25*).

The continued communication between the WHO and Member States, as well as between Mexico, Canada, and the United States, followed the regulations and provisions in the IHR (2005) and the SPP NAPAPI:

- IHR (2005) Article 6 (Notification). Following the initial notification, the State Party and WHO shall continue to communicate in a timely fashion about the notified event, including sharing updated detailed public health information on the notified event. This information includes case definitions, laboratory results, source and type of risk, and number of cases and deaths.
- IHR (2005) Article 11 (Provision of Information by WHO). WHO, in the most timely fashion possible, shall send information to all States Parties that enable the States to respond to the public health risk.
- IHR (2005). As part of IHR (2005), WHO developed a secure Event Information website to share timely information about public health events and emergencies among State Parties and WHO. This password-protected site is accessible to National IHR Focal Points.
- SPP NAPAPI. Chapter 2: Emergency Coordination and Communications. Mexico, Canada, and the United States agreed to share accurate and timely information before and during an outbreak. The 3

countries committed to working together so that all 3 nations use the same information to inform decision making and action.

Coordinated Response

On April 25, a team of experts from PAHO arrived in Mexico to assist with the outbreak. The team comprised WHO experts from Geneva and Washington, DC, and experts from the US Centers for Disease Control and Prevention. The team supported the efforts of Mexico in the epidemiologic investigation, laboratory diagnosis, clinical management, communication, and outbreak management, and reported daily to WHO and PAHO (*14,26*).

WHO and PAHO arranged to have 489,000 treatments (treatment for an adult was 75-mg capsules, twice a day for 15 days) of oseltamivir shipped to Mexico and other countries in the Americas. Approximately 220,000 treatments of oseltamivir were shipped to 21 countries in the Americas from the United Nations Humanitarian Response Depot in Panama (*27,28*).

The Director-General of WHO, after receiving advice from the Emergency Committee, made temporary recommendations to support the mitigation of the epidemic. WHO did not recommend travel or trade restrictions related to the virus but did recommend that persons who were ill delay international travel and that persons in whom symptoms developed after international travel seek medical attention (*18,29*).

The United States and other countries with confirmed cases shared isolates and sequences of the influenza A virus (H1N1) with the international community in a timely fashion. Samples of the virus were shared for the purpose of risk assessment, analysis, and for making seed vaccine (*30,31*).

The role and responsibilities of WHO for coordinating and assisting in the global response to a public health emergency are outlined in the following provisions of the IHR (2005):

- IHR (2005) Article 15 (Temporary Recommendations). If a PHEIC has been declared, the Director-General shall issue temporary recommendations according to the procedure set out in Article 49 (Procedures for the Emergency Committee).
- IHR (2005) Article 13 (Public Health Response). At the request of a State Party, WHO will assist in the response to a public health emergency by providing technical guidance, assessing the effectiveness of control measures, and mobilizing international teams of experts to send to the affected area.

Pandemic Phases

At the initial meeting of the Emergency Committee on April 25, members decided to maintain the current WHO-designated pandemic phase at a level 3 (no sustained human-to-human transmission sufficient to sustain community-level outbreaks) (*13,19*). The Emergency Committee met again on April 27, and on the basis of the developing epidemic, recommended changing from pandemic phase 3 to pandemic phase 4 (human-to-human transmission is verified). Following this recommendation, the Director-General upgraded the classification to pandemic phase 4 (*20*).

The epidemic continued to expand globally, and the Emergency Committee met again and determined that the pandemic classification should be changed from phase 4 to phase 5 (the same identified virus is causing sustained community-level outbreaks in multiple countries). The Director-General announced on April 29 that the world was at phase 5 on the WHO pandemic scale (*32*).

The meetings of the Emergency Committee followed the protocol of the IHR (2005) discussed above. As part of their recommendations for action, the committee cited the following excerpt from the WHO preparedness document:

- WHO Pandemic Influenza Preparedness and Response Section 3.2.2 (The Designation of the Global Pandemic Phase). Per the pandemic plan of WHO, the Director-General designated the global pandemic phase, consistent with the applicable provisions of the IHR (2005) and in consultation with affected Member States. Phase 4 signals sustained human-to-human transmission of the virus. Phase 5 indicates the virus is causing sustained outbreaks in $\geq$2 countries. Phase 5 suggests that a pandemic is imminent, although not a forgone conclusion.

Actions Outside the Regulations

Although the global community generally adhered to the IHR (2005), supported WHO recommendations, and participated in unprecedented levels of information sharing, there are still areas in which nations may be withholding information or make unilateral decisions that do not support the language or spirit of the revised IHR. For example, certain countries recommended against travel to North America, although WHO did not issue such recommendations. Other nations interrupted trade of pork products from the United States, disregarding the determination by WHO and global scientists that cooked pork does not transmit the virus. In addition, some countries quarantined North American citizens, regardless of potential exposure to influenza A virus (H1N1). One of these countries defended its decision to quarantine persons from North America by citing what it believed is the failure of the United States and Mexico to implement entry and exit screening to detect cases of infection with influenza virus (H1N1) (*33*). However, the US government referred to WHO advisory

and IHR Emergency Committee recommendations, which to date are not advising entry and exit screenings because WHO believes it would not help to reduce the spread of the disease (*29,34*).

In the past few weeks, WHO received reports of infection with influenza virus (H1N1) from many nations outside North America, several of which involved sizable numbers of cases. However, these countries claimed that their cases were linked to importation, with no sustained human-to-human transmission within their borders. Recent evidence in some nations of substantial increases in case counts makes sustained human-to-human transmission almost a certainty (which may lead WHO to raising the pandemic level to 6, per the WHO definition of pandemic phases). Further examination will be required to determine if nations were hesitant to admit such transmission or if previous cases were caused by importation (*35*).

Discussion

The rapid succession of events in this timeline describing the first weeks of international communication and collaboration around the outbreak of a novel influenza A virus (H1N1) demonstrate the value of good planning and agreements for addressing public health emergencies. Creating solid structures and procedures for dealing with emergencies has been shown to be essential for an appropriate response and mitigation effort. Although it is impossible to predict the exact nature of an emergency, thoughtful planning enables all affected parties to know their responsibilities and to know with whom they need to work. Time is not wasted on developing procedures and contacts during an emergency. Instead, responders can focus on mitigating the consequences of the event. Planning does not guarantee that everything will run smoothly, or that all nations will adhere to agreed-upon regulations. However, the current situation suggests that mitigation outcomes and response efforts will be more successful than an outcome if plans and agreements did not exist.

Many of the provisions included in IHR (2005) came about as a result of WHO and global experience during the 2003 SARS epidemic. Comparing the experience of SARS with the current influenza (H1N1) event can serve as a means of measuring the usefulness of the regulations. An obvious comparison is the global communication mechanisms around an emerging epidemic. When WHO needed to reach the global community to alert it to the emergence of SARS, it needed to hold a press conference on a Saturday morning. No mechanism was in place for communicating with member states in a timely fashion. The creation of National IHR Focal Points enabled rapid communication between WHO and the entire global community, and guaranteed that proper authorities were notified and that information was shared with appropriate policy makers and responders. If only for this reason, the IHR (2005) can be deemed a success.

In this public health emergency, the revised IHR were used accurately and appropriately. The regulations were established in part to facilitate communication and formulate action in the identification of a PHEIC, and that is what happened. The SPP agreement was put into place to ensure regional cooperation in the event of a health emergency. Mexico, Canada, and the United States followed the SPP agreement and shared timely information. These events should serve as a call to action for each nation to do its best to fully implement IHR (2005) and engage in regional cooperation concerning disease surveillance and data sharing.

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Response to Imported Case of Marburg Hemorrhagic Fever, the Netherlands

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On July 10, 2008, Marburg hemorrhagic fever was confirmed in a Dutch patient who had vacationed recently in Uganda. Exposure most likely occurred in the Python Cave (Maramagambo Forest), which harbors bat species that elsewhere in Africa have been found positive for Marburg virus. A multidisciplinary response team was convened to perform a structured risk assessment, perform risk classification of contacts, issue guidelines for follow-up, provide information, and monitor the crisis response. In total, 130 contacts were identified (66 classified as high risk and 64 as low risk) and monitored for 21 days after their last possible exposure. The case raised questions specific to international travel, postexposure prophylaxis for Marburg virus, and laboratory testing of contacts with fever. We present lessons learned and results of the follow-up serosurvey of contacts and focus on factors that prevented overreaction during an event with a high public health impact.

In Western countries, Marburg hemorrhagic fever (MHF) is an imported disease with a low risk of occurrence, but it has a high profile in the public mind (*1*) because it can be transmitted from person to person, the course is fatal in up to 80% of cases, and the reservoir is uncertain (*2*,*3*). The infection is caused by the Marburg virus (MARV), an enveloped, nonsegmented, negative-stranded RNA virus belonging, with the Ebola virus, to the family *Filoviridae*. Although the main transmission route is direct contact with blood or other infected body fluids, transmission by droplets and aerosols cannot be ruled out and has been demonstrated in animal models (*4*).

MARV was identified in 1967 in Marburg, Germany, during a laboratory outbreak caused by handling tissues of African green monkeys (*5*,*6*). From 1975 through 1987, sporadic cases occurred in South Africa (1975, when the index case, a person exposed in Zimbabwe, was diagnosed in South Africa) (*7*) and in Kenya (1980, 1987) (*8*–*10*). Outbreaks were reported from the Democratic Republic of Congo in 1998–2000 (*11*,*12*), Angola in 2004–2005 (*2*) and Uganda in 2007 (*13*). Nonhuman primates and bats are suspected as sources of infection, but their role in the natural reservoir for MARV and transmission to humans is unclear (*14*).

In July 2008, an imported case of MHF was diagnosed in the Netherlands. We describe the public health response involving the management of 130 contacts at risk of acquiring the disease.

The Case

On July 5, 2008, a 41-year-old woman was referred by her general practitioner to the Elkerliek Hospital because of fever (39°C) and chills of 3 days' duration after returning from a June 5–28 holiday in Uganda. She was placed in a hospital room with 3 other patients. Malaria was ruled out by 3 negative blood films. Routine bacteriologic tests were performed, and empiric treatment with ceftriaxone, 2 g/day,

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[1]On behalf of the national response team.

was started. On July 7, hemorrhagic fever was included among other infectious causes in the differential diagnosis because of rapid clinical deterioration and impending liver failure. An ambulance stripped of all unnecessary devices and equipped in accordance with strict isolation protocols transferred the patient to a single room with negative air pressure ventilation and anteroom in the Leiden University Medical Centre (LUMC).

After admission, rash, conjunctivitis, diarrhea, liver and kidney failure, and finally, hemorrhaging developed in the patient. Extensive bacteriologic and virologic analyses were conducted, and plasma samples were sent to Dutch national laboratories and to the Bernhard-Nocht-Institute for Tropical Medicine (BNI) in Hamburg, Germany, for testing to detect antibodies to and RNA from filoviruses. Initial laboratory results from the Dutch national reference laboratory were ambiguous for hemorrhagic fever. On July 10, BNI reported a positive reverse transcription–PCR result for MARV (*15*), which was confirmed by sequence analysis of the polymerase gene. The strain was related to, but distinct from, known isolates. MARV was confirmed by PCR by the Department of Virology at Erasmus Medical College (Rotterdam, the Netherlands). On July 11, the patient died of consequences of cerebral edema.

Travel History and Hypotheses for the Source of Infection

The patient's travel group consisted of 7 Dutch tourists and 2 guides. Three of the tourists, including the patient, and 1 guide visited an empty cave on June 16 in Fort Portal and the Python Cave in the Maramagambo Forest on June 19. The patient's partner recalled bats flying around in the latter cave, bumping against the visitors, and large amounts of droppings on the ground. She incurred no bite wounds, and no preexisting wounds were exposed to bats. On July 23, the travel group came within 5 m of gorillas in the wild and visited a village inhabited by pygmies, where they saw an elderly sick woman lying under a blanket.

We postulated that the most probable source of MARV infection was the visit to the Python Cave, known for its colony of Egyptian fruit-eating bats (*Rousettus aegyptiacus*). The party had photographed these bats, and this species of bat has been shown to carry filoviruses, including MARV (*16*,*17*) in other sub-Saharan locations. We estimated the incubation period of the infection to be 13 days.

Organization of Public Health Response

On July 8, the attending physician at the LUMC notified the Dutch public health authorities about the case. A national outbreak response team was formed of clinicians, medical microbiologists and virologists, public health specialists, staff members from the national response unit, and a press officer. This team convened a nearly daily teleconference to 1) to perform a structured assessment of the public health risks in the 2 hospitals and in the community, 2) perform risk classification of contacts, 3) issue guidelines for follow-up, 4) provide information to professionals and media, and 5) monitor progression of crisis response.

Immediately after the diagnosis was confirmed, on July 10, a press conference was held. Various press statements emphasizing the control measures designed to prevent secondary transmission followed the press conference. The World Health Organization was notified according to the International Health Regulations by the National Focal Point, and international warnings were issued through the Early Warning and Response System and through ProMED.

Management of Contacts

Although MARV infectivity is highest in the last stage of the disease, when severe bleeding coincides with high viral load, we considered the onset of fever (July 2) as the starting point for contact monitoring. Follow-up measures tailored to the risk group were undertaken during the 21 days after last possible exposure (*14*,*18*,*19*). The high-risk group comprised anyone with unprotected exposure of skin or mucosa to blood or other body fluids of the index patient. It included the other 3 patients in the patient's room at Elkerliek and personnel who handled her specimens without protection. The low-risk contacts were LUMC and ambulance personnel who had employed the appropriate personal protective measures while caring for the patient or diagnostic samples. Persons who had been near the patient during her holiday, the return flight, and stay in the Netherlands until Elkerliek admission but who were not exposed to her body fluids during her febrile illness and personnel from reference laboratories who worked under BioSafety Level 3 conditions were categorized as casual contacts.

A total of 130 at-risk contacts were identified, 64 at high risk and 66 at low risk (Table). High-risk contacts were required to record their temperature 2×/day, report to the local health authorities 1×/day, and postpone any travel abroad. The low-risk contacts were asked to record their temperature 2×/day and to report to local health authorities if it was ≥38°C. No limits were imposed on the casual contacts.

Because asymptomatic MARV infection is rare (*20*,*21*) and thus unlikely to play a role in spreading the infection, we restricted further clinical and laboratory evaluation to persons with a temperature ≥38°C, measured at 2 points 12 hours apart. Every case of fever was to be assessed on an individual basis by the response team. Three academic hospitals provided stand-by isolation facilities for admission of contacts.

On August 1, the temperature monitoring of contacts ended. Fever of at least 12 hours' duration or clinical signs

Table. Control measures targeting contacts with risk for exposure to Marburg virus, the Netherlands, 2008*

Type of contact	Date of exposure, Jul	No. persons	Risk for exposure	Measures		
				Temperature monitoring 2×/day	Daily temperature reporting to health authorities	Asked to limit travel and to not leave the country
Household/family contacts	2–8	4	High	Yes	Yes	Yes
Persons exposed in hospital ward	5–7	6	High	Yes	Yes	Yes
GP of the index case-patient	5	1	High	Yes	Yes	Yes
Healthcare workers, Elkerliek Hospital	7	33	High	Yes	Yes	Yes
Local laboratory workers	5–7	18	High	Yes	Yes	Yes
Ambulance staff	7	2	Low	Yes	No	No
Health care workers, LUMC	7–11	66	Low	Yes	No	No

*GP, general practitioner; LUMC, Leiden University Medical Centre.

of MHF did not develop in any of the contacts. Fever within 21 days did not develop in any of the travel companions and local guide who joined the patient in the bat cave. Because sustained fever did not develop in any of the high-risk or low-risk contacts during the surveillance period, no clinical or laboratory follow-up for MARV was needed. The online Technical Appendix (available from www.cdc.gov/EID/content/15/8/1171-Techapp.pdf) summarizes other findings during the monitoring period, dilemmas encountered with respect to travel restrictions, postexposure options in case of a high-risk accident, and laboratory diagnosis in the early stage of infection. The online Technical Appendix also describes laboratory procedures used.

Serologic Follow-up

To identify asymptomatic seroconversion, a serosurvey was undertaken of 85/130 (65%) contact persons who participated in the study. They represented 78% (50/64) of high-risk contacts and 53% (35/66) of low-risk contacts and included the Dutch visitors to the bat cave. Blood samples were collected from December 2008 through February 2009, 5–7 months after possible exposure. The laboratory testing was performed at the BNI in Hamburg by using an immunofluorescent antibody (IFA) assay.

The IFA slides were prepared using the MARV strain of the index patient. Details about the laboratory testing are given in the online Technical Appendix. In 2 initial evaluations, all but 2 samples were negative for antibodies against MARV. Additional screening found that all serum samples tested negative for immunoglobulin (Ig) G and IgM to MARV.

Discussion

We have described the public health response to the case of MHF in a Dutch woman returning from travel abroad, who was most likely exposed to MARV by visiting a bat cave. Outbreaks caused by filoviruses constitute a serious public health threat in sub-Saharan countries and have disruptive consequences at the individual and societal level. In countries in which these viruses are not endemic, imported cases occur only sporadically and are associated with little or no secondary transmission (*22*). Our patient represents a rare case of MARV infection imported to a Western country, and her case is unusual in that her only likely exposure was visiting a bat cave while traveling in Uganda. Insectivorous bats may have been the source of sporadic cases in Zimbabwe in 1975 (*23*) and Kenya in 1980 and 1987 (*8*,*9*). Furthermore, epidemiologic evidence linked a large outbreak of MHF in Durba (Democratic Republic of Congo) to a mine containing a large population of fruit-eating bats (*24*). Although the source of infection in our case is not certain, circumstantial evidence points to transmission in the Python Cave. Ecological surveys to assess the presence of infected bats in that cave are ongoing (P. Rollin, pers. comm.).

Our case shows that unnoticed exposure to an unknown reservoir in a country with no apparent cases of MHF can lead to infection. In countries with previous cases of MHF, entry into bat caves should certainly be avoided until we know the role of bats as reservoir for MARV. The importance of MHF for western countries may be increasing, with more persons traveling to high-risk regions and incurring exposure by intrusion into unaccustomed ecological niches. Hospital staff in low-risk countries must be alert to this possibility. In most travelers returning from tropical destinations, fevers are caused by common pathogens or by malaria. However, fever together with rapid clinical deterioration and hemorrhaging in a patient returned from a suspect region should suggest viral hemorrhagic fevers, especially if exposure to a possible reservoir could have occurred.

Inclusion of MHF in the differential diagnosis of a patient triggers an immediate public health response. This response aims primarily at reducing the chance of secondary transmission by identifying contact persons at risk. Person-to-person transmission occurs in countries to which MARV is endemic (*22*) but only once has been reported elsewhere (*23*). In this case we identified 130 contacts with possible risk. Two hospitals, 2 public health departments, and 3 laboratories were involved. We decided to trace all people who were in contact with the index patient after her fever developed and to assess their risk for exposure on a

case-by-case basis. All contacts complied with temperature monitoring and daily reporting. All but 2 high-risk contacts postponed further travel until the theoretical incubation period of 21 days had elapsed.

In the Netherlands, statutory power to prevent a healthy person from traveling abroad is limited, but the Public Health Law is being revised, and emergency legal provisions are being considered. Despite various recommendations (*14,18,25–27*), no evidence-based, widely accepted international protocol is available to guide contact classification and monitoring in the case of MHF. Legislation on containment of dangerous pathogens (*1*) and measures applied to contacts differ among countries, sometimes with extreme consequences. These differences, together with privacy issues, make international exchange of information difficult.

The serosurvey of the contacts of this patient confirm that no secondary transmission took place between her and any contact who provided a blood sample. Our results are consistent with those of Borchert et al. (*21*), who found no serologic evidence for asymptomatic or mild MARV infection in a serosurvey of household contacts.

The present case was an exceptional situation in which visiting a tourist attraction led to MHF, a disease with a high potential for overreaction. Given this potential, a rational response must be built on a thorough and evidence-based risk assessment (*1*). The response in the Netherlands was low profile and did not lead to overreacting or public alarm. Its key factors were a coordinated risk assessment and contact monitoring, together with factual updates for health professionals and the public. MHF may be more often encountered in industrialized countries in the future due to adventure travel to regions endemic for MHF.

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Tactics and Economics of Wildlife Oral Rabies Vaccination, Canada and the United States

Ray T. Sterner, Martin I. Meltzer, Stephanie A. Shwiff, and Dennis Slate

Progressive elimination of rabies in wildlife has been a general strategy in Canada and the United States; common campaign tactics are trap–vaccinate–release (TVR), point infection control (PIC), and oral rabies vaccination (ORV). TVR and PIC are labor intensive and the most expensive tactics per unit area (≈$616/km^2 [in 2008 Can$, converted from the reported $450/km^2 in 1991 Can$] and ≈$612/km^2 [$500/km^2 in 1999 Can$], respectively), but these tactics have proven crucial to elimination of raccoon rabies in Canada and to maintenance of ORV zones for preventing the spread of raccoon rabies in the United States. Economic assessments have shown that during rabies epizootics, costs of human postexposure prophylaxis, pet vaccination, public health, and animal control spike. Modeling studies, involving diverse assumptions, have shown that ORV programs can be cost-efficient and yield benefit:cost ratios >1.0.

Rabies continues to pose major public health concerns in Canada and the United States (*1*–*5*). Effective pet vaccination programs have controlled rabies in domestic dogs (*Canis familiaris*) in both countries, but rabies persists in wildlife reservoirs. In 2007, a total of 6,776 cases in wildlife were reported for the contiguous United States (*1*).

Oral rabies vaccination (ORV) is an evolving rabies control technology for use in wildlife (*6*). It involves distribution of baits containing orally immunogenic vaccines onto the landscape, thereby targeting wildlife to establish population immunity and prevent spread or eliminate specific rabies variants (*6*).

We reviewed the literature on ORV programs and economics in Canada and the United States. The first use of ORV sought to control rabies in red foxes (*Vulpes vulpes*) in Switzerland; subsequent programs were reported throughout much of western Europe (*7*,*8*). Switzerland, France, Belgium, and Luxembourg were deemed free of the red fox variant by 2001 (*8*).

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ORV in Ontario, Canada

Arctic Fox–Variant Rabies in Red Foxes

During 1989–1995, ORV was used in Ontario to progressively eliminate arctic fox (*Alopex lagopus*)–variant rabies that had spilled into (i.e., had been transmitted to another species) red foxes and spread southward (*9*). Each year ORV baits were distributed in southern Ontario (≈20 baits/km^2, from aircraft or by hand, over 8,850–29,590 km^2). The strategy was termed progressive elimination and resembled an expanding ORV wedge, which started near the center of the outbreak and expanded during successive years (Figure 1).

Within 5 years of program initiation, reported cases of rabid foxes declined from 203 cases/year to 4 cases/year in the baited areas (*9*,*10*). Spillover cases from red foxes to striped skunks (*Mephitis mephitis*) and livestock dropped from preepizootic (30-year) means of >36 and >42, respectively, to 0 by 1997 (*9*). Since 2003, only 13 cases of the variant in red foxes have been reported; these continue to be addressed by using focused control and enhanced surveillance (i.e., increased public health monitoring, examination of road-killed target animals, and rabies analyses of samples from trappers) (D. Donovan, Ontario Ministry of Natural Resources, pers. comm.).

Rabies in Raccoons and Skunks

During 1987–1991, to reduce spillover of rabies from red foxes to urban raccoons (*Procyon lotor*) and skunks, trap–vaccinate–release (TVR; capture live, vaccinate parenterally, and release on site) was integrated into ORV

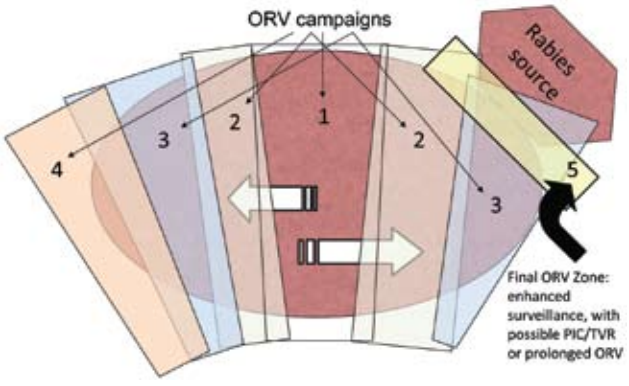

Figure 1. Expanding-wedge tactic with progressive elimination (*9*). Numbers represent successive oral rabies vaccination (ORV) zones. Potential savings are assumed for the area of progressive elimination, southern Ontario Province. The rectangle bordering the rabies source (i.e., 5) highlights an area of enhanced surveillance, possible point infection control (PIC) activities, trap–vaccinate–release (TVR) activities, or an ORV zone intended to deter future reemergence of the virus.

campaigns in the Toronto area (*10*). TVR was part of the red fox ORV program because Evelyn-Rokitnicki-Abelseth oral rabies vaccine is not immunogenic in skunks and raccoons (*6*,*9*). Live traps were set (20–75 traps/km^2) in a 60-km^2 portion of the city, and 66,168 ORV baits were distributed by hand in natural areas (20–40 baits/km^2). Of sampled foxes, 46%–80% had biomarkers from baits, and only 1 rabid fox was found during 1987–1992 (*10*). A recent update of ORV baiting in Toronto stated that 332,257 baits had been distributed during 1989–1999, and only 5 rabid foxes were found during 1990–2006 (*11*).

During 1999–2000, the raccoon variant of rabies was confirmed near Brockville, Ontario (*12*). To eliminate raccoon-variant rabies from the province, a point infection control (PIC) tactic, which integrated population reduction (PR; sometimes referred to as culling or depopulation), TVR, and ORV, was implemented (*12*). The initial PIC operation included concentric zones, each consisting of 1) an inner 5-km PR zone, 2) a middle 5-km TVR zone, and 3) an outer 8–15-km ORV zone (Figure 2). Additional PIC or modified PIC (no PR) operations were centered on newly discovered rabid raccoons (≈40).

Mean raccoon densities in PR zones dropped from 5.1–7.1/km^2 before to 0.6–1.1/km^2 after PIC operations. However, within 1 year, >37 more cases of raccoon-variant rabies occurred in the PIC regions (*12*). Intensive PIC was begun again and eliminated the variant from Ontario. Subsequently, to reduce the chances of raccoon-variant rabies recurring in southern Ontario, enhanced surveillance and annual ORV was conducted along the border of Ontario and New York (D. Donovan, pers. comm.). Elimination of raccoon rabies from Wolfe Island at the mouth of the St. Lawrence River using similar tactics was recently reported (*13*).

ORV in the United States

Canine-Variant Rabies in Coyotes in Southern Texas

During 1988–1994, a canine-variant of rabies described in Mexico was confirmed in 163 domestic dogs and 296 coyotes from 18 counties in southern Texas (*14–16*). In 1995, to prevent the northward spread of this variant, ORV baits (9–27 baits/km^2) were distributed in an arc-shaped band over a 24-county area (39,850 km^2) ≈200 km north of Laredo (*16*). During 1996–2003, annual baiting continued; ≈9.35 million baits were distributed onto ≈741,766 km^2 (*17*). Gradually, baits were distributed farther south, toward the Rio Grande River, in subsequent years, thereby collapsing the rabies-infected area (Figure 3). To protect livestock, coyotes were also removed from portions of the ORV zone during these years, but the effect of PR relative to ORV was not assessed (*18*,*19*). PR is considered an important component of many rabies-control models (*20*).

After 1 year of baiting, the mean rate of canine-variant cases at the leading edge of the epizootic area was 2.8/10,000 km^2. This rate was similar to that of the preepizootic period and suggestive that the northward spread of the epizootic had ceased (*16*). Subsequent surveillance showed a gradual decline in cases from 122 in 1995 to 0 in 2004 (*17*). Currently, to maintain an immune buffer and prevent canine rabies from reemerging in southern Texas, this program baits an ORV zone 30–65 km wide along the international border each year (E. Oertli, Texas Department of State Health Services, pers. comm.).

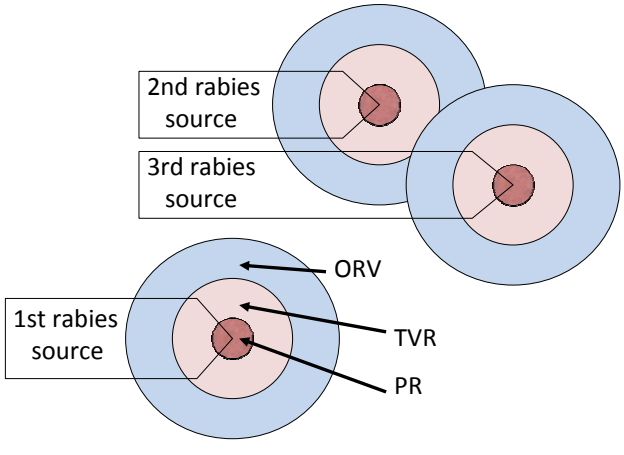

Figure 2. Point infection control (PIC) tactic. Concentric rings around the location of a rabid animal represent vector population reduction (PR), trap–vaccinate–release (TVR), and ORV zones (*12*). Each new source leads to repeated, overlapping ORV, TVR, and PR rings. Potential savings are assumed within the zones and for assumed distances beyond the zones.

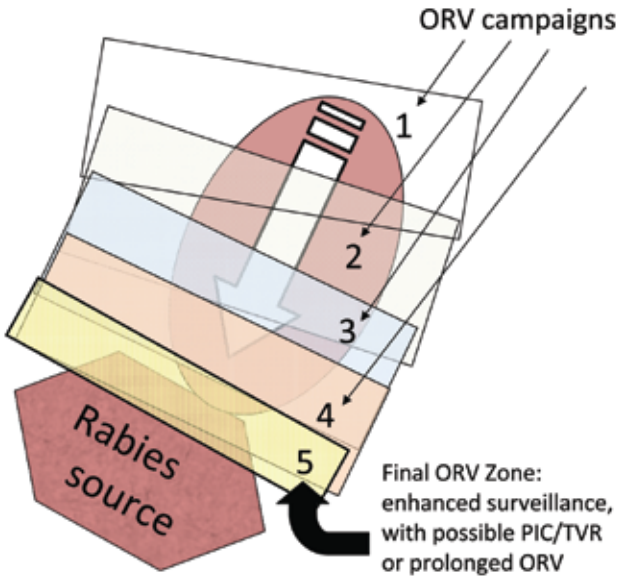

Figure 3. Collapsed-bands tactic with progressive elimination (*17*). Numbers represent successive oral rabies vaccination (ORV) zones that attempt to collapse the baited area, exclude virus incursion outside, and lead to a maintenance zone that prevents reintroduction of the disease after the current population matures and vaccination effects are lost. Potential savings are assumed to occur within the ORV areas and for assumed distances beyond the zone. The rectangle bordering the rabies source (i.e., 5) high-lights an area of enhanced surveillance, possible point infection control (PIC) activities, trap–vaccinate–release (TVR) activities, or an ORV zone intended to deter future reemergence of the virus.

Gray Fox–Variant Rabies in West-Central Texas

During 1988–1994, a total of 283 gray foxes (*Urocyon cinereoargenteus*) and 241 other domestic and wild animals in west-central Texas were confirmed positive for a unique rabies variant typically found in gray foxes (*17*). This outbreak was spatially distinct from the outbreak of canine rabies in southern Texas. To control this epizootic, during 1995–2009 (and ongoing), ORV (29–39 baits/km^2) was conducted annually by encircling the epizootic area using ≈32-km–wide ORV strips; an added 16- to 24-km vaccination buffer of ORV baits was created along the northern and eastern edges of the rabies-variant area; this tactic has been referred to as a purse string–like tactic (i.e., encircle and shrink) (*17*; Figure 4). An area of ≈350,000 km^2 was baited annually. Evidence of bait biomarkers and positive rabies virus neutralizing antibody titers was found for 39% and 62% of foxes, respectively, sampled from the ORV zone, confirming that numerous foxes had been vaccinated.

In 2007, new cases of gray fox rabies occurred northwestward along the Pecos River and in west-central Texas. To prevent further spread of this variant, ORV was used (E. Oertli, pers. comm.). The rabies-control goal has not changed from one of containment and elimination of the gray fox variant from Texas. However, in light of recent surveillance, the anticipated strategy of establishing and maintaining an ORV zone along the Rio Grande River to prevent potential reemergence from Mexico has been delayed and is being refined to include prolonged enhanced surveillance as a key factor in allocating resources and gauging success (E. Oertli, pers. comm.).

Raccoon-Variant Rabies in the Eastern United States

The National Rabies Management Program began in 1997 and coordinates ORV and related wildlife rabies–control activities in the United States (*21*,*22*). One of its priorities is to prevent the spread of raccoon-variant rabies into uninfected areas, particularly west of its current distribution along the Appalachian Ridge (*22*). The Program integrates natural terrain features (e.g., rivers, lakes, and poor habitat along mountain ridges) with ORV zones (baited at 50–75 baits/km^2) to create a 40–50 km zone of vaccinated raccoons to help prevent the spread of the virus (Figure 5).

During 1997–2007, the ORV zone was expanded from parts of Ohio to encompass parts of 8 states (i.e., Ohio, Pennsylvania, West Virginia, Virginia, Tennessee, North Carolina, Georgia, and Alabama) along the Appalachian Ridge. A total of 58 bait distributions (usually 1/year) total-

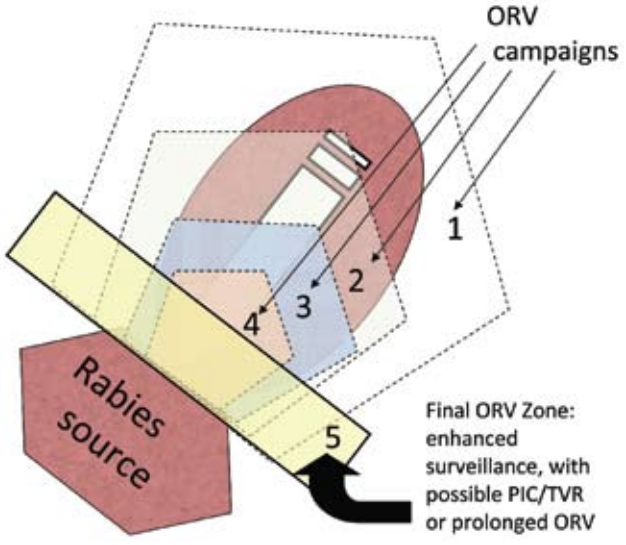

Figure 4. Purse string–like tactic with progressive elimination (*17*). Numbers represent successive oral rabies vaccination (ORV) zones that attempt to roughly encircle and shrink the baited area, exclude virus incursion from outside, and lead to a maintenance zone that prevents reintroduction of the disease after the current population matures and vaccination effects are lost. Potential savings are assumed to occur within the ORV areas and for assumed distances beyond the outer zone. The rectangle bordering the rabies source (i.e., 5) highlights an area of enhanced surveillance, possible point infection control (PIC)/trap–vaccinate–release (TVR) activities, or an ORV zone intended to deter future reemergence of the virus.

ing ≈41,018,800 baits and covering ≈530,825 km² (range of 28,660 km² to 84,225 km²/distribution) have characterized this effort as of 2007 (R. Hale, US Department of Agriculture, pers. comm.). On the basis of rates of spread of 30–60 km/year in the Mid-Atlantic states before 1997 (*22–24*), ORV is viewed as having slowed movement of the virus and, with contingency actions to eliminate some dispersed cases, prevented westward spread of rabies among raccoons. Relatively low and variable vaccination rates have been found, despite the use of relatively high bait densities (50–100/km²). Estimated raccoon vaccination rates, based solely on the index of rabies virus neutralizing antibody response, range from 10% to 55% (*22*). The need to vaccinate annually is dictated mainly by high death rates for juveniles and a relatively young age structure for raccoons in North America; juveniles often account for 50% of raccoon populations (*22*). Still, enhanced and public health surveillance indicate that areas west of the Appalachian Ridge remain free of raccoon-variant rabies (*1*,*22*,*23*).

To maintain the integrity of the Appalachian Ridge ORV zone, contingency actions have been needed. In 2004, emergency ORV baiting and TVR were used in northeast Ohio between the established ORV zone and the eastern suburbs of Cleveland (*25*). TVR of >300 raccoons and multiple ORV distributions occurred in this contingency action. This ORV zone had been widened earlier because of encroachment of rabid raccoons from Pennsylvania (*26*).

Other contingency actions unrelated to the westward spread of raccoon rabies have also been implemented. In 2004, an ORV zone created near the Cape Cod Canal to prevent spread of raccoon-variant rabies onto Cape Cod, Massachusetts, was breached, and raccoon-variant rabies spread rapidly throughout the peninsula (T. Algeo, US Department of Agriculture, pers. comm.). Currently, ORV is used twice a year (spring and fall) in the eastern half of the Cape, and baiting is moved gradually westward until an ORV zone can be reestablished along the Cape Cod Canal (J.C. Martin, Tufts Cummings School of Veterinary Medicine, pers. comm.). Additionally, to prevent raccoon rabies from reemerging in southern Ontario, ORV baiting for raccoon-variant rabies continues in northern New York. Confirmed positive raccoon-variant cases in southern Quebec have led to extensive PIC and ORV campaigns to prevent the disease from reaching Montreal. Together, these events and contingency actions illustrate the challenges posed by raccoon rabies, the importance of enhanced surveillance, plus the need to anticipate unexpected contingency actions and their related costs as a component of ORV campaigns.

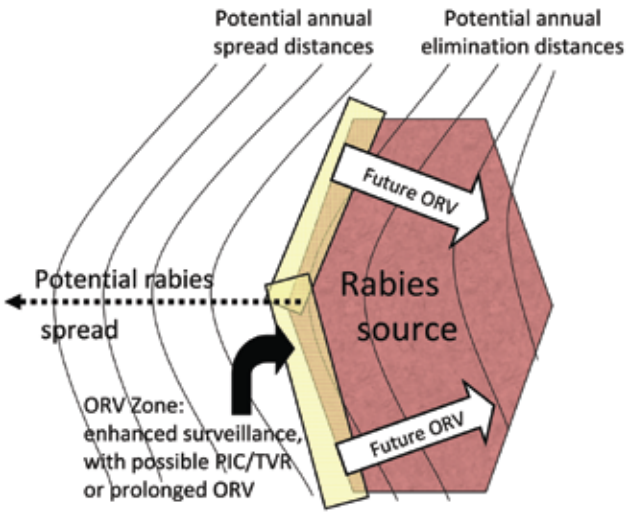

Figure 5. Oral rabies vaccination (ORV) preventive spread or elimination tactic with eventual progressive elimination (*22*). The ORV zone of vaccinated animals is intended to prevent spread of the disease beyond the ORV zone; potential elimination is assumed to result from successive baiting campaigns into the infected area. Potential savings are assumed beyond the ORV zone (or within the zone, if elimination is possible); disease spread rates, final distances of infectious impacts, and durations of ORV bait distributions ultimately determine the magnitude of potential savings. PIC, point infection control activities; TVR, trap–vaccinate–release activities.

Rabies-related Costs

Several studies have documented the costs associated with wildlife-rabies epizootics (*27–31*; see online Technical Appendix 1, available from www.cdc.gov/EID/content/15/8/1176-Techapp1.pdf). Costs have been adjusted for inflation to 2008 US$ or Can$. A raccoon-variant rabies epizootic in the early 1990s in Hunterdon and Warren Counties, New Jersey, more than doubled rabies-related control costs from $6.67/county resident at $591/km² ($4.05/county resident and $359/km², US$ in 1990) to $16.13/county resident at $1,503/km² ($9.79/county resident at $913/km², US$ in 1990) (*27*).

In Massachusetts, a multiyear study found that the median cost of postexposure prophylaxis (PEP) was $3,356/patient ($2,376/patient; range $1,038–$4,447, US$ in 1995); 69% of the cost was for biologics (*28*). Numbers of PEP administrations increased 26-fold, from 1.7/100,000 residents in 1991 to 45/100,000 residents in 1995 (*28*). Estimates for Connecticut were similar (*29*).

A raccoon-variant epizootic in New York State began in 1991, and the resultant rate of PEP administrations ranged from the equivalent of 24 to 34/100,000 residents (no preepizootic estimates of PEP given) (*30*). During 1998–1999, the mean PEP cost was $1,501/person treated ($1,136/person, US$ in 1998; biologics and administration), equivalent to between $36,024 and $51,034/100,000 residents ($27,264 and $38,624/100,000, US$ in 1998); New York City's population is excluded from these estimates. This lower cost compared with that for Massachusetts (*28*) and Connecticut (*29*) may be the result of local

public health department coordination of PEP administrations in New York State (*30*).

Recently (1998–2002), rabies exposure costs were estimated at $4,066/patient ($3,688/patient, US$ in 2005) in southern California (*31*). Average direct (biologics, medical costs) and indirect costs (travel to physicians, day care for medical appointments) were estimated at $2,827/patient and $1,239/patient, respectively ($2,564/patient and $1,124/patient, US$ in 2005).

ORV Program Costs

Bait costs and detailed descriptions of the areas baited, which allowed computations of unit area expenses, are available in Table 1 and online Technical Appendix 2 (available from www.cdc.gov/EID/content/15/8/1176-Techapp2.pdf). ORV programs in Canada and the United States have lasted from >1 for some to >11 years for others and have often required integration of contingency actions (Table 1). The most expensive tactics have been labor-intensive PIC and TVR, but their effectiveness is crucial to maintaining the overall integrity of certain ORV campaigns (*10–13*,*22*,*25*). PIC programs have been reported to cost $612/km^2 ($500/km^2, Can$ in 1999); costs reported for 3 PIC operations for raccoons totaled $469,247 ($363,100, Can$ in 1999; *12*). TVR costs have ranged from $616/km^2 to $1,573/km^2 ($450/km^2 to $1,150/km^2, Can$ in 1991; Table 1).

The target species of ORV greatly affects costs, mainly because of species-specific, bait-density requirements. Bait densities for foxes and coyotes have been less than half those for raccoons (Table 1). Thus, gray fox and coyote ORV programs in Texas averaged $48/km^2 ($42/km^2, US$ in 2004; Table 1), and raccoon programs in the eastern United States averaged between $111/km^2 ($108/km^2, US$ in 2007) and $198/km^2 ($153/km^2, US$ in 1999). Cumulative cost of the Appalachian Ridge ORV program has totaled ≈$57 million since its inception in 1997; baits accounted for 72% of the funds expended (Tables 1, 2).

Annual costs vary as changes in ORV zones occur, as contingency actions occur, and as ORV programs shift from preventing spread to eliminating variants in given geographic areas. Individual bait prices in the United States range from $1.00 to $1.25 (US$ in 2008, depending on bait type). Because of improved production efficiency, bait prices have decreased slightly during the past 5 years.

Table 1. Major oral rabies vaccination campaigns, Canada and the United States*

Country and reference	Strategy or tactic	Duration, y	Target species	Unit bait cost	Target bait density, no./km^2	ORV, TVR, or PIC area, km^2/y†	Cost/km^2‡
Canada							
(*9*)	ORV progressive elimination	>7§	Red fox	Not reported	18–20	8,850–31,460	No estimate
(*10*)	TVR	5§	Skunk, raccoon, red fox	>$2.00 (Can$ 1991)	20/den fox only	60	$450–$1,150 (Can$ in 1991)
(*12*)	PIC	>1§	Raccoon	>$2.00 (Can$)	70	225 PR, 485 TVR, 1,200 ORV	$500 (Can$ in 1999)
United States§							
D. Slate, unpub. data (2007)	ORV zone (Appalachian Ridge)	>1§	Raccoon	$1.22 (US$)	50–75	28,659–84,225	$108 (US$ in 2007)
(*26*)	ORV zone (Ohio–Pennsylvania border)	4§	Raccoon	$1.37–$1.52 (US$)	75	3,872–6,497	$153; range $102–$262 (US$ in 1999)¶
(*17*)	ORV progressive elimination	>9§	Coyote	Not reported	19–27	38,850	$42 (US$ in 2004)#
(*17*)	ORV progressive elimination	>8§	Gray fox	Not reported	27–39	56,202	$42 (US$ in 2004)#

*Unless otherwise noted, costs are in Can$ or US$. No discounting for inflation was used; this article and online Technical Appendix 2 (www.cdc.gov/EID/content/15/8/1176-Techapp2.pdf) provide inflation-corrected costs in 2008 Can$ or US$.
†Components of reported areas differ according to tactic and strategy. Oral rabies vaccination (ORV) entails topographic areas at which baits are distributed at target densities over landscape. Trap–vaccinate–release (TVR) involves relatively limited topographic areas of intense live trapping and parenteral vaccination of captured animals. Point infection control (PIC) involves successive concentric rings of population reduction, TVR and ORV; the concentric rings become distorted if subsequent rabid animals are caught within these rings. New concentric rings are now formed according to these occurrences. Additionally, depending on habitat or location of urban centers, ORV may be used in a larger strip to create an added ORV preventive zone.
‡Most cost estimates include purchase of baits, aircraft, fuel, and equipment but often omit accurate labor charges.
§Surveillance, TVR, PIC, or ORV bait distributions continue at present; therefore, current duration and areas baited differ from those reported. According to Foroutan et al. (*26*), ORV baitings continue as part of the National ORV Program (Slate et al. [*22*]).
¶According to Foroutan et al. (*26*), areas were baited twice each year. In 1997, the first baiting was conducted over a smaller area (1,780 km^2), and in May 1997 (initial) and June 1999, 2 smaller emergency baitings (in response to a breach in the ORV zone) were conducted, covering ≤1,701 km^2. Average costs include a baiting in April 1999, when several tests of bait densities (high) were conducted.
#According to Sidwa et al. (*17*), the area baited had shrunk over time because of progressive coyote-variant rabies elimination, but the purse string (gray fox) tactic and ORV-baited area were expanded in 2007 as the gray fox variant spread north along the Pecos River and into southern Texas. The area cost estimate was derived as the quotient of a reported $3.8 million/year program cost and average annual 33,669 km^2 (dog and coyote) and 56,202 km^2 (gray fox) bait zones (sum 89,871 km^2) cited in online Technical Appendix 2.

Table 2. Approximate, undiscounted total costs of largest oral rabies vaccine programs, North America, 1989–2004*

Location, target species	Years	Total undiscounted costs, million $	Average undiscounted annual costs, million US$	Reference
Ontario, red foxes	1989–2000	Can$43†	3.5	S.A. Shwiff, unpub. data*
Texas, coyotes and gray foxes	1995–2003	US$34	3.8	(*17*)‡
Appalachian Ridge, raccoons	1997–2007	US$57	5.2	D. Slate, unpub. data§

*Costs are estimates in Can$ or US$ as reported in original publication or as cited by unpublished source.
†S.A. Shwiff et al. (unpub. data) based their calculations on certain data presented in *9*,*32*.
‡Sidwa et al. (*17*) stated that (for both programs combined) average annual costs were $3.8 million. We computed this value as follows: 9 years × $3.8 million = $34 million total (i.e., Sidwa et al. did not clarify what was included in their cost estimate).
§D. Slate (2007, unpub. data) provided air, bait, fuel, and staff costs, although some staff hours and fuel costs were omitted for initial campaigns during 1997–2001; a total of 9,394 staff hours, $5,868,262 aircraft costs, $923,481 fuel costs, and $50,187,380 bait costs were reported for 58 campaigns involving the dispensing of 41,018,811 baits over 530,825 km^2. The software used to determine bait distribution costs was prepared by staff of the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Rabies Management Program. After deselecting the bait zones, flight lines were drawn by using the topography (e.g., avoiding water and residential areas) to determine the flight lines and transects. After that had been established, the bait zones were populated with the lines and measured to determine the total length (km). Flight lines determine total flight hours: ([km × 0.539958 nautical miles]/flight speed [knots] = flight hours). Fuel usage is computed as follows: (flight hours × consumption rate [91 gallons/h] × fuel price/gallon = total fuel cost). Costs were also influenced by air transect width, distance to airports for refueling, and end-of-transect turning distance.

Potential ORV-Induced Savings

One ex post study (actual returns, after the fact) provided detailed estimates of PEP administrations in Ontario during 1956–2000 (*32*). Annual PEP administrations increased from ≈1,000/year during the 1960s and 1970s to >2,000/year during 1982–1993, then decreased to ≈1,000/year again after large-scale ORV campaigns targeting red foxes began in 1989 (*9*,*10*,*32*). Many factors could account for these changes, including revisions of PEP administration guidelines. The initial increase in PEP administrations possibly occurred as a result of fewer adverse effects from use of the new human diploid cell vaccine and stability in numbers of rabies cases (*32*). The latter decrease in PEP administrations was coincident with ORV-caused elimination of arctic fox–variant rabies from southern Ontario (*9*).

Modeling the Benefits and Costs of ORV

Measured costs of an epizootic of raccoon rabies in New Jersey were used to model the costs and benefits of a hypothetical ORV program (*27*). The model projected net savings for ORV (Table 3) based on the assumptions that the ORV program would require a 2-year campaign and that expenditures to protect human health would remain constant. The model did not allow for reintroduction of rabies or for the potential reemergence of rabies. Benefit:cost ratios (BCRs) related to this hypothetical use of ORV were reported as ≥2.2 (*27*; online Technical Appendix 3, available from www.cdc.gov/EID/content/15/8/1176-Techapp3.pdf) summarizes key principles of benefit:cost modeling).

Use of ORV to eliminate raccoon rabies from a hypothetical area of 34,447 km^2 was modeled under 2 scenarios (*33*). Scenario 1 assumed that concentric ORV zones (rings) would expand outward from a center over a 20-year period and that the ORV zone would be maintained for 10 more years to prevent reintroduction. Scenario 2 assumed that the entire area would be baited in the first 2 years and that a ring-shaped ORV zone would be maintained for 28 more years. In the first scenario, inclusion of an expected 20% increase in pet vaccinations (*27*) as a benefit resulted in $3.1 million net savings from ORV; removing pet vaccinations as a savings yielded a net cost of $7.7 million ($6.2 million, US$ in 2000; Table 3). The second scenario yielded no net savings unless the cost of maintaining a containment zone was removed from the model (*33*).

The economics of a large-scale ORV program to prevent the westward spread of raccoon-variant rabies in the eastern United States was modeled and used in planning the current Appalachian Ridge program (*34*). Scenarios assumed that a raccoon-variant rabies epizootic would advance in 40 or 127 km/year (fixed rates) bands to the west of the current leading edge of raccoon-variant rabies along the Appalachian Ridge (*22*). Input variables were as follows: 7% discount rate, 102,650 km^2 ORV zone, 75 baits/km^2, $1.63/bait ($1.30/bait, US$ in 2005), $10.78/km^2 ($8.62/km^2, US$ in 2000) aerial distribution, and $18.75/km^2 ($15.00/km^2, US$ in 2000) post-ORV evaluation. The effect of an epizootic was calculated in terms of unit human population within bands. Results showed that all 8 scenarios, except the 40 km/year spread rate with 20-year fixed baiting costs, yielded BCRs ≥1.1 and that total estimated net present values of the program were $48–496 million with >$96 million in discounted program costs (*34*). Because of natural geographic features, raccoon population dynamics, and other factors that affect the spatial and temporal spread of rabies, an assumed variable spread of the virus westward would have been more realistic (*25*,*26*). As in previous models (*27*,*33*), estimates of net savings (>50%) for scenarios were enhanced by inclusion of potential pet vaccination costs.

Another model examined specific costs of baiting campaigns for raccoon rabies along the Ohio–Pennsylvania border (*26*). This model incorporated movement and life-cycle data for rabid and nonrabid raccoons. An area of 400 km^2 with a 10-km ORV zone was assumed to be baited. Benefits were predicted to accrue mainly in a 5-km strip on the west side of the ORV zone. Assumptions about rac-

coon carrying capacity and percentage ORV vaccination efficiency influenced the rate of rabies spread. This model predicted a net cost for ORV; however, a simple extrapolation implied that net savings would have occurred if the benefits were projected for a 100-km strip west of the ORV zone (*26*).

Ex post modeling was conducted for the ORV campaigns that eliminated red fox–vectored rabies in Ontario. Estimated ORV benefits (PEP + animal rabies tests + livestock indemnity) ranged from $35.4 million to $99.3 million ($35 million to $98 million, Can$ in 2007; total program costs were $78.0 million ($77 million, Can$ in 2007) (S.A. Shwiff, unpub. data). BCRs ranged from 0.49 to 1.36, and outputs implied a lag effect for savings; BCRs were <1.0 during 1990–1992 and ≥1.0 during 1993–2000.

Recently, an ex post modeling analysis was performed for the 1995–2006 ORV program that eliminated canine-variant rabies from southern Texas (*16*,*17*,*35*). Total expen-

Table 3. Comparison of selected modeling studies that examined the economics of oral rabies vaccination programs*

Reference	Locale, tactics, target species	Type of study, model	Duration modeled, y	Cost and density of vaccine baits; distribution costs†	Results	Comments
(*27*)	2 counties in New Jersey, ORV, raccoon	Benefit:cost, cost data collected from field with hypothetical baiting program	5	$1–$2/bait; 62–200 baits/km^2; distribution $100/km^2	Net savings $13.34–$20.78/ county resident (1990 US$); $1,244/km^2 – $1,939/km^2	Probably unrealistic: assumed only 2 baitings; no contingency costs; main economic benefit = reduced pet vaccinations
(*33*)‡	Hypothetical 34,447 km^2-area, expanding circle then maintained barrier zone, raccoon	Benefit:cost of hypothetical baiting program, extensive sensitivity analyses	30	$1.50/ bait; 100 baits/km^2 (range 40–115); distribution $39/km^2 (maximim $100/ km^2)	Net savings of $3.1 million if reduced pet vaccinations included as benefit. Net cost ($6.2 million) if pet vaccinations excluded.	Lack of data required many assumptions; bait density, cost/ bait, and value of pet vaccinations were the most critical elements
(*34*)§¶	Appalachian Ridge area, ORV, raccoon	Benefit:cost model of program to deter westward spread of raccoon rabies	20	$1.30/bait, 75 baits/km^2 ; aerial distribution $8.62/ km^2; evaluation $15/km^2	Net savings $100–$500 million (2000 US$)	Assumed that without ORV, rabies would move 42 or 125 km/y west; distribution costs are low; animal vaccinations are critical component
(*26*)#	Ohio–Pennsylvania, ORV zone (400 km^2), raccoon	Simulation of individual raccoons + benefit:cost model to prevent westward spread of raccoon rabies	40	$1.47/bait; 3 scenarios of 70, 100, 175 baits/km^2. Distribution $23.23/km^2	Net costs (1999 US$; savings recouped 5 km band west of zone)	Complex model showing importance of many biological factors determining potential for success and net savings
(*35*)	Texas, progressive elimination, collapsed bands, coyote	Retrospective benefit:cost model; projected population-based PEP and animal test costs for 20 southern to 232-county expansion area	12	$26.3 million total cost (2006 US$; Texas Department of State Health Services accumulated value)	Net savings $98–$354 million; BCRs of 3.7–13.4; range of savings for 100%, 50% and 25% of PEP and rabies tests in epizootic area.	Simple model showing wide-area expansion. ORV proved cost-efficient if projections were reduced to 7% of the PEP and tests for epizootic counties
S.A. Shwiff, unpub. data	Ontario, progressive elimination, expanded wedge, arctic-fox variant, red fox	Benefit:cost measured costs but had to model savings	12	$77.4 million (2006 Can$) for total ORV	Net savings in 3 of 4 scenarios: reductions in animal rabies testing accounted for most net savings.	Assumed multiple estimates of future rabies-related costs

*No inflation corrections used. ORV, oral rabies vaccination; PEP, postexposure prophylaxis; BCRs, benefit:cost ratios.
†Distribution costs exclude cost of bait purchases. US$ except as indicated.
‡For example, Meltzer (*33*) posited a baseline assumption with a distribution cost of $39/km^2.
§Kemere et al. (*34*) assumed that the "… effectiveness of vaccination programs would be validated through surveillance and testing of raccoon populations in the ORV zones … [evaluation cost] also includes educational, promotional, and overhead expenses."
¶Although Kemere et al. (*34*) did not explicitly allow for contingency costs (to allow for breaches of ORV zones, etc.), they did sensitivity analyses assuming "… the full program costs are used for the entire period instead of dropping to 40% after 5 years."
#Foroutan et al. (*26*) only considered benefits extending up to 5 km west of the ORV zone. A simple extrapolation would suggest that net savings would occur if the calculated benefits were to extend some 100–150 km west of the ORV zone.

ditures for the ORV program were compared with benefits accrued from likely PEP administrations and animal rabies tests estimated for the 20-county epizootic area and projected to an area involving most of the state. Estimated benefits ranged from $95 million to $369 million ($89 million to $346 million, US$ in 2006); total ORV program costs were reported as $28 million ($26,358,221 US$ in 1995–2006). BCRs ranged from 3.4 to 13.1, depending on assumed incidence of PEP administrations and animal tests (*35*). This study confirmed that 56/100,000 residents received PEP during the epizootic, a high rate for the sparsely populated area of southern Texas where the disease occurred (*35*).

Conclusions

ORV of wildlife has had positive public health effects. Multiyear campaigns have led to progressive elimination of arctic fox–variant and canine-variant rabies in Ontario and Texas, respectively. PIC, TVR, and ORV zones have prevented raccoon-variant rabies from becoming established in Ontario. Campaigns to contain and eliminate rabies in gray foxes of west-central Texas continue, and spillover of gray fox–variant rabies into coyotes may pose new challenges for preventing the spread of this variant. The ORV zones and contingency actions along the Appalachian Ridge have, thus far, prevented westward spread of raccoon rabies. Habitat alterations to reduce potential carrying capacities of raccoons through local no-feeding regulations and improved refuse management would aid rabies control efforts, but these measures are difficult to implement and enforce. Improved bait-vaccine technology, potentially integrating reproductive inhibitors into TVR campaigns for specific urban raccoon and skunk populations, may improve wildlife rabies elimination.

Rabies campaigns have been relatively expensive. We estimate that ≥$130 million (combined Can$ and US$) has been spent on ORV programs in North America during the past 10 years. Programs have proved lengthy (typically >5 years), have required enhanced surveillance, and have often required contingency actions to ensure rabies elimination without reintroduction.

Most economic assessments and modeling studies indicate that ORV programs can yield cost savings (*32*–*35*). Regional increases in PEP administrations (and associated public health costs) from 2–4/100,000 before to 24/100,000 (*30*), 45/100,000 (*28*), or 66/100,000 (*27*) residents during or after have been documented for nonbat rabies epizootics. Reduced PEP, epizootic-related pet vaccinations, animal diagnostic tests, public education activities, and other factors represent costs avoided by ORV programs. Direct estimates of wild mammal populations and the relationship of these to numbers of PEP administrations are difficult to obtain; this topic was beyond the scope of our review but needs research.

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etymologia

Lyssavirus

[lis′ə-vi″rəs]

From the Greek *lyssa* (frenzy or madness) and Latin *virus* (poison). In Greek mythology, Lyssa was the goddess of rage, fury, and rabies, known for driving mad the dogs of the hunter Acteon and causing them to kill their master. Aristotle (4th century BCE) said, "Dogs suffer from the madness. This causes them to become irritable and all animals they bite to become diseased." The disease in humans was characterized by hydrophobia, in which the sick person was simultaneously tormented with thirst and with fear of water. Hippocrates is believed to refer to rabies when he said that persons in a frenzy drink very little, are disturbed and frightened, tremble at the least noise, or are seized with convulsions.

Lyssavirus is a genus of the family Rhabdoviridae, which includes rabies virus and other related viruses that infect mammals and arthropods (e.g., Australian bat lyssavirus, Duvenhage virus, European bat lyssaviruses 1 and 2, Lagos bat virus).

Source: Steele JH, Fernandez PJ. History of rabies and global aspects. In: Baer GM. The natural history of rabies, 2nd ed. New York; CRC Press; 1991. Philadelphia: Saunders; 2007; Mahy B. The dictionary of virology, 4th edition. London: Academic Press; 2009. Dorland's illustrated medical dictionary, 31st edition.

Spread of *Cryptococcus gattii* into Pacific Northwest Region of the United States

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Cryptococcus gattii has emerged as a human and animal pathogen in the Pacific Northwest. First recognized on Vancouver Island, British Columbia, Canada, it now involves mainland British Columbia, and Washington and Oregon in the United States. In Canada, the incidence of disease has been one of the highest worldwide. In the United States, lack of cryptococcal species identification and case surveillance limit our knowledge of *C. gattii* epidemiology. Infections in the Pacific Northwest are caused by multiple genotypes, but the major strain is genetically novel and may have emerged recently in association with unique mating or environmental changes. *C. gattii* disease affects immunocompromised and immunocompetent persons, causing substantial illness and death. Successful management requires an aggressive medical and surgical approach and consideration of potentially variable antifungal drug susceptibilities. We summarize the study results of a group of investigators and review current knowledge with the goal of increasing awareness and highlighting areas where further knowledge is required.

Cryptococcus gattii (formerly *C. neoformans* var. *gattii*) (*1*) is a basidiomycotic yeast acquired by inhalation. In a susceptible host, the disease (cryptococcosis), caused by *C. gattii* and the congeneric pathogen *C. neoformans*, usually results in pneumonia or dissemination to distant tissues, especially to the central nervous system. Some studies have shown that *C. gattii* appears to differ from other cryptococcal pathogens in phenotypic characteristics, natural habitat, epidemiology, clinical disease manifestations and response to antifungal drugs. We briefly summarize these features before discussing the emergence of *C. gattii* in the Pacific Northwest region of Canada and the United States.

Based on the distribution of specific antigenic determinants on the polysaccharide capsule, pathogenic cryptococci have been divided into capsular serotypes B and C (both *C. gattii*), A (*C. neoformans* var. *grubii*), D (*C. neoformans* var. *neoformans*), and the hybrid diploid AD (*1*). Clinical and environmental *C. gattii* isolates obtained from most parts of the world belong to serotype B (*2*), the serotype also responsible for cryptococcal disease in the Pacific Northwest. The global distribution of *C.*

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gattii serotype C appears relatively more geographically restricted (*2*–*4*).

C. gattii causes disease in healthy, immunocompetent persons and in persons with immunosuppressive conditions, including those with HIV infection, organ transplant recipients, and patients with hematologic malignancies (*2*,*3*,*5*,*6*). However, because clinical cryptococcal isolates are not routinely subtyped to the serotype or species level, the actual incidence of *C. gattii* infection in HIV-negative persons is not known. In a US population-based surveillance study, 2 of 27 cryptococcal isolates obtained from HIV-negative persons were *C. gattii* (*7*). Although *C. neoformans* serotype A has been the most common cause of human disease worldwide since the HIV pandemic began, *C. gattii* may have previously caused proportionally more disease. For example, in Thailand in the pre-AIDS era, *C. gattii* comprised 66% of cryptococcal isolates, which decreased to ≈4% during the AIDS epidemic (*8*).

In general, cryptococcal disease can occur in 1 of 2 ways: a primary disease progression in the context of immunosuppression, or reactivation of a subclinical, latent infection. Progression from infection or colonization to disease likely depends upon a complex interplay of factors associated with the host and the organism.

The natural history of *C. gattii* infection is not well understood, but some studies suggest subtle differences in disease caused by *C. gattii* and *C. neoformans*. *C. gattii* causes cryptococcomas in the lung and brain (often large, multifocal lesions) more commonly than *C. neoformans*, and *C. gattii* disease is more often associated with neurologic sequelae, frequently requiring aggressive neurosurgical management (*9*,*10*). Differences in clinical manifestations and outcome suggested by some studies may in part be explained by differences in host immune status (*9*), although not all studies have demonstrated these differences (*4*).

Some reports have observed variable-to-decreased in vitro antifungal drug susceptibilities in clinical and environmental *C. gattii* isolates (*11*,*12*); other studies have shown no differences (*4*). Clinical significance of in vitro susceptibility of cryptococcal isolates is unclear because testing methods are not standardized and MIC breakpoints have not been defined.

C. gattii is found typically in the tropics and subtropics (*2*). Accordingly, although detected in many regions, *C. gattii* has been found to be endemic to Australia and New Zealand, Papua New Guinea, South and Southeast Asia (Cambodia, Malaysia, Thailand, Vietnam, People's Republic of China, Taiwan, Singapore, Nepal, and the Indian subcontinent), parts of Latin America (Argentina, Brazil, Colombia, Uruguay, Paraguay, Peru, and Venezuela), southern California, Mexico, Hawaii, Central and South Africa, and certain parts of Europe (Austria, Germany, France, Italy, Greece, and Spain) (*2,13*).

Our understanding of the epidemiology of cryptococcosis is limited by our currently used diagnostic methods. Diagnosis of cryptococcosis frequently relies on direct microscopy, culture of clinical samples, or detection of cryptococcal antigen in body fluids. However, the organism is not identified to species level in many clinical microbiology laboratories. One biochemical characteristic that distinguishes *C. gattii* from *C. neoformans* is its ability to produce blue coloration on L-canavanine-glycine-bromothymol blue (CGB) medium (*14*), although, occasionally, some isolates identified as *C. gattii* by molecular typing may be CGB negative (L. Hoang, unpub. data).

Molecular typing studies, which are essential in tracking *C. gattii* epidemiology, have used the techniques of PCR fingerprinting (*15*), restriction fragment length polymorphism (RFLP) analysis (*16*), intergenic spacer sequencing (*17*), amplified fragment length polymorphism analysis (*18*), and more recently, multilocus sequence typing (MLST) (*19*,*20*). Molecular typing has identified distinct haploid *C. gattii* lineages among clinical, veterinary, and environmental isolates, such as VGI, VGII, VGIII, and VGIV (*19*,*21*,*22*), which appear to have distinct biogeoclimatic distribution zones. For example, most clinical isolates from Australia and eucalyptus-associated *C. gattii* isolates belong to molecular type VGI; VGII isolates have been found in domestic animals in Australia and in Aboriginal persons in the Northern Territory (*2*). On Vancouver Island and mainland British Columbia, most human, veterinary, and environmental *C. gattii* isolates belong to the genotype VGII, with its 2 molecular subtypes VGIIa (predominant genotype, ≈90% of VGII) and VGIIb (≈10%), representing 2 independent clonal populations (*20*,*22*,*23*).

Emergence on Vancouver Island and in Mainland British Columbia

Isolated cases of *C. gattii* disease were identified in the animal population of British Columbia in 2000 (*24*). *C. gattii* disease was recognized as an epidemic on Vancouver Island and lower mainland British Columbia (*23*,*25*) and retrospectively found to have been affecting residents and travelers to the island, as well as the domestic and wild animal population, since 1999 (*24*).

In 2003, cryptococcal infection became a reportable disease in British Columbia, which strengthened surveillance efforts. The British Columbia Centre for Disease Control reported a cumulative incidence of 218 *C. gattii* cases in humans during 1999–2007, with an average of 24.2 cases per year (*26*). The average annual incidence rate of *C. gattii* infection was 5.8 cases per million on mainland British Columbia and 25.1 cases per million on Vancouver Island; this rate is higher than those reported in other *C. gattii*–endemic areas of the world (*27*). During this same period, 19 deaths from *C. gattii* disease were docu-

mented, for a case-fatality rate of 8.7% (*26*). Of these 218 cases, 55% were in men; the mean age at diagnosis was 58.7 years. Possible risk factors included smoking, cancer, and HIV infection (*26*). Annual incidence, after reaching a plateau during 2000–2005, increased in 2006. Although most *C. gattii* cases have occurred among residents of Vancouver Island the numbers of cases have steadily increased among residents of lower mainland British Columbia, likely caused by an active expansion of the epidemic zone since 2004 from Vancouver Island to mainland British Columbia and the northwestern United States (Washington and Oregon) (*27*). The exact mode of transmission of *C. gattii* from Vancouver Island to lower mainland British Columbia is not known. However, studies of possible dispersal mechanisms have indicated an association of *C. gattii* cases with high-traffic locations, and evidence of anthropogenic dispersal through vehicle wheel wells, footwear, construction and forestry activity (leading to aerial dispersal), and water (*23*). A few domestic and international travelers to the disease-endemic zone on Vancouver Island have subsequently contracted cryptococcal disease caused by *C. gattii* VGIIa, the molecular subtype most commonly associated with the Vancouver Island emergence (*28*,*29*).

Emergence in the Northwestern United States

Use of MLST has documented that a *C. gattii* strain genetically similar to the Vancouver Island VGIIa strain caused disease in a patient in Seattle in 1971 (strain NIH444, also known as ATCC32609 and CBS6956). Another similar isolate was found in the environment in San Francisco, California, in 1990 (strain CBS7750) (*20*,*22*). These findings indicate that this subtype of *C. gattii* was present in the environment (and may have been a cause of human disease) in this part of the United States for many years before recognition of the Vancouver Island emergence (*20*). In recent years, several cases of *C. gattii* disease have been recognized in Washington and Oregon.

In early 2006, the first US case of human infection by a *C. gattii* strain identical to the Vancouver Island VGIIa (as confirmed by MLST) was diagnosed in a resident of Orcas Island, Washington (*30*). From 2006 through July 2008, a total of 9 additional culture-confirmed cases were reported in Washington in residents of Whatcom, Island, King, and San Juan counties (R. Baer, unpub. data). All *C. gattii* isolates from these patients in Washington belong to the VGIIa genotype, as indicated by RFLP (M. Morshed and L. Hoang, unpub. data) and MLST (*31*). Three of these 10 patients had no travel history to Vancouver Island or other *C. gattii* disease–endemic areas. In addition, 1 Washington resident with sarcoidosis received a diagnosis of *C. gattii* disease in Oregon; the isolate from this patient belonged to a novel VGII genotype, VGIIc (*31*). Washington State now considers *C. gattii* infection notifiable as a rare disease of public health significance (www.doh.wa.gov/notify/nc/cryptococcus.htm).

Cases of *C. gattii* disease have been identified in Oregon since 2004. During 2004–2005, two cases of culture-confirmed *C. gattii* infection were recorded in the United States. These case-patients did not report exposure to Vancouver Island or other *C. gattii*–endemic areas. Isolates from these case-patients (both Oregon residents) were VGII, but they were genetically distinct from Vancouver Island VGII isolates and their relationship to the outbreak is unknown (*27*). The current count is 19 confirmed cases, as of May 2009 (S. West and K.A. Marr, unpub. data). As with case-patients seen in British Columbia and Washington, many of these patients had pulmonary manifestations. A review of the travel history of these patients suggests in-state acquisition, although with the currently considered incubation period of 6 weeks to 11 months for *C. gattii* disease (*27*,*28*), out-of-state acquisition cannot be ruled out. Additional cases are suspected because of increased reports of cryptococcal disease documented in otherwise healthy persons (K.A. Marr, unpub. data). However, the current epidemiologic understanding of the extent and spread of *C. gattii* disease in Washington and Oregon is incomplete because of the lack of culture isolation and strain identification in clinical microbiology laboratories.

Veterinary Cases in the Pacific Northwest

Cryptococcal disease in animals is often characterized by upper respiratory symptoms, subcutaneous nodules, pneumonia, central nervous system or ocular disorders, lymphadenopathy, or subcutaneous nodules (*2*,*24*,*32*). *C. gattii* infection was recognized in wild and domestic animals in British Columbia and Washington before human cases were detected, which reiterated the value of sentinel animal surveillance for emerging disease. To date, many species of animals, including dogs, cats, ferrets, porpoises, llamas, alpacas, birds, and a horse, have been confirmed to have *C. gattii* infections (*23*). During 2003–2005, *C. gattii* VGIIa infections were detected on the lower mainland of British Columbia (in 1 ferret, 1 llama, and 6 cats) (*27*) and in northern Washington (3 cats and 4 porpoises) (*27*; E. Byrnes, pers. comm.). From August 2006 through July 2008, *C. gattii* VGIIa was reported in 5 cats, 2 dogs, and 1 parrot in Whatcom, Island, Yakima, and Snohomish counties of Washington (R. Wohrle, pers. comm.). In Oregon, VGIIa *C. gattii* infection was found in 2007 in 1 cat in Lebanon, 1 dog in Salem, and 2 alpacas in McMinnville and Sherwood (*33*).

Identified risk factors for animals include disturbance of soil or vegetation caused by hiking, digging, logging, and construction. These activities can potentially increase aerial dispersal of the infectious particles and contact with soil and tree cuttings (*23*). The distribution of isolates re-

covered from human and animal sources and from the environment is shown in the Figure.

Ecologic Considerations

A possible association between *C. gattii* disease distribution and eucalyptus trees was observed in the early 1990s in Australia (*2*), and sporadically elsewhere (*34*,*35*). However, this association is not uniform (*11*), which indicates additional environmental sources. In British Columbia, *C. gattii* has been isolated from surfaces of >10 noneucalyptus tree species, soil, air, freshwater, and seawater within the Coastal Douglas Fir and Coastal Western Hemlock biogeoclimatic zones. These zones are characterized by warm, dry summers and mild, wet winters, a climate different from that traditionally associated with *C. gattii* (*23*). This finding suggests either a change in the ecology and distribution of this organism, or identification of a previously unrecognized niche (*23*,*27*).

From the beginning of the *C. gattii* epidemic on Vancouver Island and adjoining areas, all humans and animals with cryptococcal infection either lived within or traveled to the areas where *C. gattii* has been repeatedly and consistently isolated in subsequent studies (*23*,*27*). The Coastal Douglas Fir and Coastal Western Hemlock biogeoclimatic zones are located along the eastern edge of Vancouver Island, and extend into the southern Gulf Islands and lower mainland of British Columbia. Ecologic modeling of *C. gattii* in British Columbia has identified niche areas that are characterized by low-lying elevations (<770 m, average 100 m above sea level) and above freezing daily winter average temperatures (S. Mak, unpub. data). Similar climates with comparable temperature and rainfall extend further south into Washington and Oregon. Additionally, the San Juan Islands, Puget Sound, and the Willamette Valley contain plant diversity ecologically similar to that on Vancouver Island and in mainland British Columbia, lending support to the idea that *C. gattii* may have niche areas suitable for colonization in the broader Pacific Northwest (*27*).

Large-scale environmental sampling conducted from October 2001 through December 2005 in mainland British Columbia, the Gulf Islands, and Washington showed that 60 (3%) of 2,033 non-Vancouver Island environmental samples (air, water, soil, swabs of trees and other structures) were positive for *C. gattii* serotype B (58 VGIIa, 2 VGI) (*27*). *C. gattii* was consistently isolated from some areas. However, it was not found in the environment in other areas, such as the San Juan Islands (*36*), which suggests that environmental hot spots (zones of high concentration) of *C. gattii* may be found within the same broad ecologic niches. Some areas may have transient colonization, with initially positive sites yielding subsequent negative results (*23*).

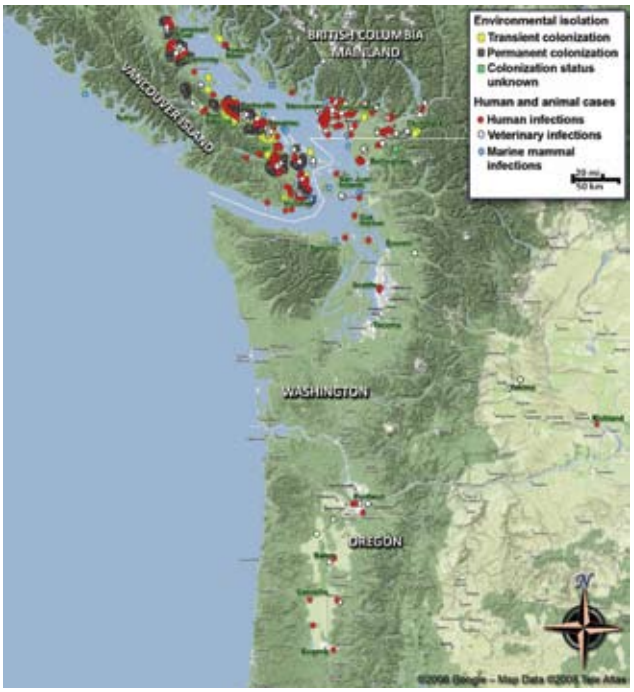

Figure. Map of the Pacific Northwest, comprising parts of British Columbia, Canada, and the states of Washington and Oregon in the United States, showing human and veterinary *Cryptococcus gattii* cases (including marine mammals) by place of residence or detection, and locations of environmental isolation of *C. gattii* during 1999–2008 (strain NIH444 [Seattle] or CBS7750 [San Francisco] not included). Data were collected from various state health departments and published reports referenced in the text. The map and icons have been used at a scale that shows gross geographic areas, effectively masking any personally identifiable patient locality information. Use of the map is courtesy of exclusive permission from Google Maps: ©2008 Google, map data ©2008 NAVTEQ.

Complicating the interpretation of epidemiologic data is the fact that the nature of the infectious propagule is currently unknown. Air sampling on Vancouver Island and in northern Washington has detected particles that are small enough to be either desiccated yeast cells or spores (*23*). *Cryptococcus* species are haploid yeasts that predominantly reproduce asexually (through mitosis and budding). However, they also possess a bipolar mating system, with mating types a or α. These mating types are capable of completing a sexual (meiotic) cycle that is either heterosexual (between a and α) or unisexual (nonclassical union between α and α or a and a; also known as monokaryotic fruiting) (*21*). This cycle can produce spores that can be up to 100-fold more infectious than yeast cells and thus might represent the infectious source (*37*). In tree hollows, *C. gattii* populations can exist equally as only α-mating type isolates or as a- and α-mating type isolates; both undergo recombination (*38*). This observation, taken together with

the isolation of a diploid, homozygous α-mating type *C. gattii* strain from Vancouver Island, indicates that unique monokaryotic fruiting between α-mating type strains may be producing the infectious spores on Vancouver Island (*20*). Moreover, recent studies have shown that *C. gattii* is stimulated to complete its sexual cycle during an association with plants (*39*).

The origin or introduction of *C. gattii* strains into the Pacific Northwest and factors encouraging their emergence as disease agents remain a mystery. Accurate determination of origin from any particular locale is difficult because of global dispersal and isolate recombination (*19*). Several hypothesized disease movement models have been inconclusive. As discussed above, *C. gattii* VGIIa strains may have existed for the past 35 years in the Pacific Northwest, and changing conditions of climate, land use, or host susceptibility may have caused it to emerge at this time. An alternative hypothesis is that the VGIIa genotype is well adapted to the local biogeoclimatic conditions and enhanced with regards to virulence by virtue of the same-sex mating of the parents (*20*). Genetic studies putatively show that the dominant strain on Vancouver Island (VGIIa) originated in Australia, South America, or the Pacific Northwest. Conversely, the Vancouver Island minor genotype (VGIIb) is identical to fertile isolates from Australia and may well have originated on that continent (*20*,*40*).

Issues and Questions

We will now attempt to identify several major unmet needs regarding *C. gattii* disease in the areas of public health, human and veterinary medicine, environmental science, and microbiologic or basic research. Although enhancing a multidisciplinary awareness of *C. gattii* infection will encourage prompt, accurate diagnosis of patients with compatible clinical symptoms, many clinical microbiology laboratories in the United States are not currently equipped to identify the organism to the species level. Most laboratories use antigen testing or histopathologic analysis and report all isolates simply as *C. neoformans*; few laboratories use biochemical tests that provide species level differentiation. This practice should be reconsidered, especially in laboratories servicing disease-endemic regions.

Although formal *C. gattii* surveillance has been ongoing in British Columbia since 2003, no routine surveillance program currently exists in the United States. Therefore, identified cases almost certainly represent an underestimate of the actual incidence of disease. Washington State has recently initiated a surveillance system for human cryptococcosis cases (www.doh.wa.gov/notify/nc/cryptococcus.htm). However, to gather sufficient information about the true incidence of this disease and to perform necessary clinical studies, a coordinated, systematic, regional approach to human and veterinary surveillance is needed.

Currently, the University of British Columbia and the British Columbia Centre for Disease Control hold a large collection of *C. gattii* isolates isolated from environmental, animal, and human sources. Additional central repositories of *C. gattii* strains and clinical databases would be useful for investigators in the field.

Animal models of *C gattii* disease are necessary for studying the pathogenicity of genetically diverse isolates and host immunity, as well as to answer more fundamental questions about the disease. Laboratory-based efforts should also focus on development of better diagnostic aids, such as a reliable system to isolate DNA from fixed tissue. Development of species-specific serologic tests would enable seroprevalence studies for a better understanding of epidemiology and disease mechanisms. Studies of soil persistence and propagation of the organism and those that address the nature and factors controlling the infectious propagule would be informative (*23*).

Antifungal drug susceptibility testing represents an area in need of improvement. Current susceptibility testing methods do not consider the unique in vitro growth conditions of cryptococci, and interpretation of results is difficult in the absence of clinical breakpoints.

Some standardization in the performance of molecular techniques for genomic research is required. Different genotyping methods have their own particular advantages and disadvantages, and the method of choice varies considerably among different laboratories. Random amplified polymorphic DNA analysis, RFLP, and amplified fragment length polymorphism analysis provide a lower resolution starting point, but may not be sufficient to rigorously establish strain identities, for which MLST approaches are necessary. The appropriate method is obviously dependent on the desired level of discrimination of the strains. However, because the organism designations may vary with different methods, common nomenclature should be standardized across molecular methods. Finally, further genome efforts should be encouraged; information comparing the genetic makeup of VG (I–IV) lineages might hold the key to understanding the differences in niches, virulence, mating, and the ability to cause an outbreak.

From a long-term public health perspective, it is important to gain an understanding of how *C. gattii* disease was introduced and spread within the Pacific Northwest, including the possible role of global climate change, deforestation and increased land use, as well as factors related to the biology of the organism and changing host susceptibility. This knowledge, acquired through environmental and genomic research studies, would help identify high-risk groups or regions, and provide information on risk-reduction and improvement of diagnosis and treatment.

Conclusions

C. gattii has become endemic to the Pacific Northwest. Efforts to understand the emergence on Vancouver Island and mainland British Columbia have generated valuable information. However, many questions remain unanswered. The Centers for Disease Control and Prevention (Atlanta, GA, USA) is currently working in conjunction with state public health departments to enable clinical (human and veterinary) surveillance. Additional organized national and international efforts are needed. As the first step, *C. gattii* should be recognized as an emerging infection in the United States and declared a priority pathogen in the eyes of funding agencies.

Dr Datta is a postdoctoral fellow at Johns Hopkins University School of Medicine. His research interests include pathogenic fungi and evaluation of host immune responses to these pathogens.

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Molecular Epidemiology of Rabies in Southern People's Republic of China

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In recent years, the number of human rabies cases in the People's Republic of China has increased during severe epidemics in 3 southern provinces (Guizhou, Guangxi, and Hunan). To analyze the causes of the high incidence of human rabies in this region, during 2005–2007, we collected 2,887 brain specimens from apparently healthy domestic dogs used for meat consumption in restaurants, 4 specimens from suspected rabid dogs, and 3 from humans with rabies in the 3 provinces. Partial nucleoprotein gene sequences were obtained from rabies-positive specimens. Phylogenetic relationships and distribution of viruses were determined. We infer that the spread of rabies viruses from high-incidence regions, particularly by long-distance movement or transprovincial translocation of dogs caused by human-related activities, may be 1 cause of the recent massive human rabies epidemics in southern China.

Rabies has been endemic to the People's Republic of China for many years; domestic dogs act as the main reservoir (*1*–*3*). After efforts to stabilize the dog population, the lowest incidence of human rabies (159 cases) was recorded in 1996. Subsequent annual increases followed: 505 cases were reported in 2000, 1,191 cases in 2002, and 2,651 cases in 2004 (*2*,*4*). In 2005, three southern provinces (Guizhou, Guangxi, and Hunan) recorded most of the human rabies cases throughout China (*5*). During the next 2 years, this region had ≈50% (6,595) of the total number of cases in China and contributed to one of the most serious human rabies epidemics in this country (*5*,*6*).

Various studies have been conducted to determine the causes and characteristics of rabies epidemics in China. Descriptive epidemiologic analysis showed that the increase in domestic dog populations and low vaccination coverage have contributed to rabies epidemics in the 3 provinces in southern China (*7*–*9*). Other authors have suggested that this trend may be caused by a carrier state in healthy dogs that remains undetected (*10*). Molecular epidemiologic analysis of dog specimens collected from 5 provinces (Guangxi, Hunan, Guizhou, Jiangsu, and Henan) demonstrated that rabies viruses (RABVs) in China are similar to those reported from previous epidemics (*11*). However, no studies have investigated the phylogenetic relationships among viruses circulating in different provinces during peaks of rabies incidence.

RABV is the prototype member of the family *Rhabdoviridae* and the genus *Lyssavirus*. It encodes 5 structural proteins: nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA-dependent RNA polymerase (*12*). The nucleoprotein (N) gene has been extensively used for genetic typing and evolutionary studies because of its relatively conserved variation among reservoir-associated variants and geographic lineages (*13*–*16*). Since the onset of a human rabies epidemic in China in 2005, we have collected brain specimens from dogs and human patients. Partial N gene sequences were obtained in an attempt to understand the sustained increase in rabies cases in China by analysis of RABVs circulating in the 3 southern provinces with the highest rabies incidence.

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Methods

Specimen Collection

From 2005 through 2007, we randomly collected 2,887 brain specimens from apparently healthy domestic dogs used as meat in restaurants of 15 cities in Guizhou, Guangxi, and Hunan provinces (Table 1, Figure 1). In addition, we included 4 brain specimens of suspected rabid dogs that had bitten other animals or humans and 3 brain specimens from patients who had died of rabies.

Detection and Sequencing of RABV

All specimens were examined by using a direct immunofluorescence assay (DFA) with fluorescent-labeled monoclonal antibody against RABV N protein (Rabies DFA Reagent; Chemicon Europe Ltd., Chandlers Ford, UK). For all identified RABV specimens, RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and used as template for cDNA synthesis with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Bioscience, Chalfont St. Giles, UK). The 720-nt sequence of the N gene, encoding regions corresponding to nt 704–nt 1423 of the total genetic sequence of the Pasteur strain of RABV, was amplified with primers N644F (5′-AAGATGTGYGCYAAYTGGAG-3′, nt 644–nt 663) and N1537R (5′-GGATTGACRAAGATCTTGCTCAT-3′, nt 1515–nt 1537). Locations of primer sequences were referred to the full genome sequence of the Pasteur strain of RABV (M13215). PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN Ltd., Crawley, UK) and sequenced with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1. Diagnostic results for rabies virus in southern People's Republic of China, 2005–2007*

Location	No. specimens	No. (%) positive
Guizhou Province		
Qianxinan	427	13 (3)
Qiannan	54	1 (1.9)
Anshun	173	8 (4.6)
Liupanshui	200	0
Guangxi Province		
Laibin	213	4 (1.9)
Guigang	304	8 (2.6)
Hechi	206	1 (0.4)
Yulin	302	4 (1.3)
Nanning	222	7 (3.2)
Liuzhou	105	2 (1.9)
Hunan Province		
Shaoyang	151	6 (4)
Yongzhou	162	2 (1.2)
Xiangtan	114	3 (2.6)
Changde	102	4 (3.9)
Xiangxi	152	3 (1.9)
Total	2,887	66 (2.3)

*Samples were obtained from apparently healthy domestic dogs.

Sequence Analysis

Complete alignment of nucleotide sequences was performed by using the ClustalX program, version 1.8 (*17*). MegAlign software version 5 (DNAStar, Inc., Madison, WI, USA) was used to analyze nucleotide and deduced amino acid sequence homologies. The neighbor-joining method in MEGA3 version 3.1 (*18*) was used with 1,000 bootstrap replications (*19*). All taxa used for the comparative analyses were obtained from GenBank (Table 2).

Results

Specimen Detection

Of 2,887 specimens randomly collected from dogs, 66 were positive for RABV (positivity rate = 2.3%) (Table 1). The 7 additional specimens obtained from 4 dogs suspected of having rabies and 3 patients who died of rabies all were positive for RABV.

Sequencing

A 720-bp region of the N gene of the RABV-positive specimens was obtained from 60 specimens (53 sequences were from 66 specimens obtained from healthy dogs and 7 sequences were from the 4 rabid dogs and 3 patients who

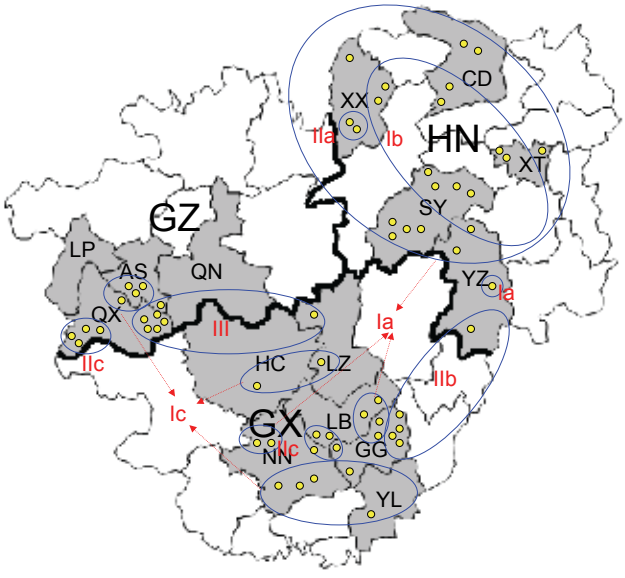

Figure 1. Locations of 15 cities selected for specimen collection in Guizhou (GZ), Hunan (HN), and Guangxi (GX) provinces, in southern People's Republic of China, 2005–2007, and genetic groups and subgroups of 60 samples analyzed for rabies virus. Roman numerals and letters indicate genotypes, gray areas indicate regions selected for specimen collection, yellow circles indicate specimens collected, ovals indicate regions with the same genotype, and arrows indicate specimens with the same genotype. LP, Liupanshiu; AS, Anshun; QN, Qiannan; QX, Qianxinan; XX, Xiangxi; CD, Changde; XT, Xiangtan; SY, Shaoyang; YZ, Yongzhou; HC, Hechi; LZ, Liuzhou; LB, Laibin; GG, Guigang; NN, Nanning; YL, Yulin.

died of rabies). The 13 other positive specimens were not sequenced. All 60 sequences were submitted to GenBank (accession nos. EF990564–EF990623).

Similarity Analysis

A sequence comparison of 60 N gene fragments showed 87.6%–100% nucleotide similarity. Some specimens from the same province were identical in their entire nucleotide sequence: 6 specimens from Guangxi Province (CGX0602D, CGX0604D, CGX0605D, CGX0 513D, CGX0620D, and CGX0622D); 3 specimens from Hunan Province (CHN0505D, CHN0506D, and CHN0507D); and 2 specimens from Guizhou Province (CGZ0506D and CGZ0514D). Identical sequences were also observed in different provinces: 7 specimens from Hunan Province (CHN0614D, CHN0516D, CHN0529D, CHN0609D, CHN0615D, CHN0528D, and CHN0525D) and 2 specimens from Guizhou Province (CGX0612D and CGX0606D), 2 specimens from Guizhou Province (CGZ0512D and CGZ0516D) and 3 specimens from Guangxi Province (CGX0614D, CGX0523D, and CGX0625D), and 4 specimens from Guizhou Province (CGZ0501D, CGZ0505D, CGZ0513D, and CGZ0518D) and 1 specimen from Guangxi Province (CGX0617D). Identity levels of deduced amino acid sequences of the 60 specimens ranged from 95.4% to 99.6%.

Phylogenetic Analysis

Results of a phylogenetic analysis of 60 sequences obtained in this study are shown in Figure 2; the Pasteur strain of RABV was used as an outgroup. The 60 virus specimens clustered into 3 statistically supported branches (bootstrap support 99%) and were designated as groups I, II, and III. Groups I and II can be further divided into 3 subgroups. Subgroup Ia contained specimens from Hunan Province (n = 9) and Guangxi Province (n = 6). All specimens in subgroup Ib were from Hunan Province. Most (7/11) specimens in subgroup Ic were from Guangxi Province, with 4 from Guizhou Province. The 3 specimens in subgroup IIa were from Hunan Province. Six of 7 specimens in subgroup IIb were from Guangxi Province; the other specimen was from Hunan Province. Subgroup IIc contained 4 speci-

Table 2. Lyssavirus strains used in study of molecular epidemiology of rabies virus in southern People's Republic of China, 2005–2007

Genotype	Strain	Year	Host species or type of strain	Origin	GenBank accession no.
GT1	SC02-90	2002	Dog	Indonesia	AB154243
	D664_45	–	Dog	Thailand	DQ267925
	CTN	1956	Vaccine	China	AF367863
	483a	–	Arctic fox	Russia	AY352487
	Ontario type 2/4	1991	Fox	Canada	U11734
	–	–	–	South Africa	AF467949
	FluryLEP	1939	Vaccine	USA	DQ099524
	PM1503	–	Vaccine	–	DQ099525
	SRV9	–	Vaccine	China	AF499686
	SAD B19	1935	Vaccine	USA	M31046
	CVS	–	Laboratory strain	USA	AF406696
	ERA	1935	Vaccine	USA	AF406695
	PV	1965	Vaccine	France	M13215
	92RABL00867	–	Skunk	Canada	AF344306
	3789	1998	Skunk	USA	AF461045
	Eth2003	–	Wolf	Ethiopia	AY500827
	RC-HL	–	Vaccine	Japan	D16331
	3aG	1931	Vaccine	China	AF155039
	DRV	2002	–	China	DQ875051
	ISR-40	2000	Fox	Israel	DQ837421
	–	–	Fox	Europe	AF045664
	3502f	–	Red fox	Russia	AY352455
	–	–	Dog	India	AF374721
	1294	1986	Dog	Sri Lanka	AY138549
	2054	2001	Bat	USA	AF394888
	V920	1993	Bat	Mexico	AY877435
	BR-BAT2	–	Bat	Brazil	AB201820
GT7	Ballina	1996	Bat	Australia	AF006497
GT5	8615POL	1985	Bat	Poland	U22844
GT4	86132AS	1986	Human	South Africa	U22848
GT6	9007FIN	1986	Human	Finland	U22846
GT3	MOK	1981	Cat	Zimbabwe	U22843
GT2	8619NGA	1958	Bat	Nigeria	U22842

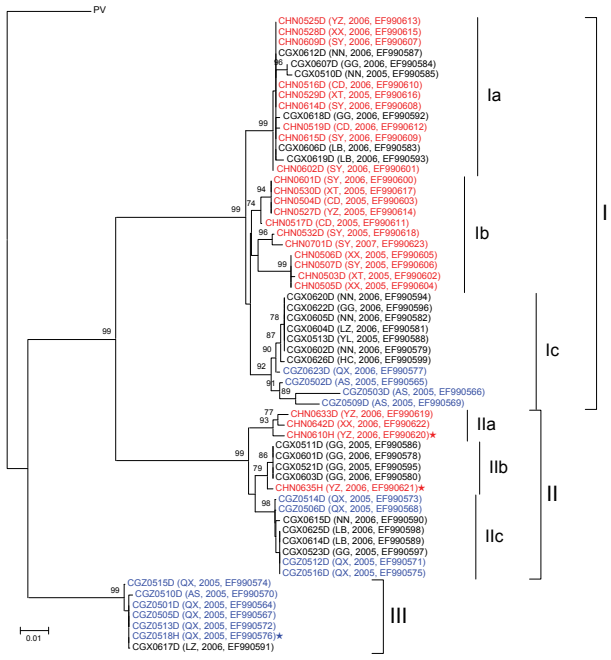

Figure 2. Neighbor-joining phylogenetic tree of 60 specimens of rabies virus from the People's Republic of China, 2005–2007, based on a 720-nt (nt 704–nt 1423) nucleoprotein (N) gene fragment of rabies virus rooted with the Pasteur strain of rabies virus (PV). Numbers at each node indicate degree of bootstrap support; only those with support >70% are indicated. Taxa are from Hunan Province are shown in red, taxa are from Guangxi Province in black, and taxa are from Guizhou Province in blue. City, year of sample collection, and GenBank accession no. are included for each taxon. Stars indicate human samples; others are dog samples. YZ, Yongzhou; XX, Xiangxi; SY, Shaoyang; NN, Nanning; GG, Guigang; CD, Changde; XT, Xiangtan; LB, Laibin; LZ, Liuzhou; YL, Yulin; HC, Hechi; QX, Qianxinan; AS, Anshun. Scale bar indicates nucleotide substitutions per site.

mens from Guangxi Province and 4 from Guizhou Province. Most (6/7) specimens in group III were from Guizhou Province; the other specimen was from Guangxi Province (Figure 1).

Human virus specimens showed close relationships to dog virus specimens from the same area (Figure 2). For example, specimen CHN0610H from a patient in Yongzhou in Hunan Province and specimen CHN0633D from a local dog were in subgroup IIa, and human specimen CGZ0518H from Qianxinan in Guizhou Province were in group III, which includes specimen CGZ0505D from a dog in the same city. However, specimen CHN0635H from a patient in Hunan Province is more closely related to specimens from dogs in Guangxi Province (e.g., CGX0601D).

Most lineages and sublineages in the 60 samples from southern China were 100% identical (Figure 2). Thus, we chose representative samples to conduct a comparative phylogenetic analysis with RABV sequences from other countries and reservoir hosts (Figure 3). All virus specimens from southern China clustered in genotype 1 (GT1) and constituted a major branch (Asian dog; Figure 3) that is clearly segregated from other lyssavirus genotypes (GTs 2–7).

Most of the lineages in the samples from China clustered with other dog-related RABV lineages from Asia, such as groups I and II, which are closely related to samples from Indonesia and Thailand, respectively. There was no close relationship between RABV from China and RABV from other countries in southern Asia, such as India and Sri Lanka.

Some RABVs from Guizhou and Guangxi provinces (group III; Figure 3) were related to cosmopolitan dog-related RABV variants, including nearly all vaccine strains and variants associated with skunks, wolves, and red foxes. In addition, group III showed a distant relationship with Arctic and Arctic-like RABVs. All virus specimens from China were phylogenetically distant from bat-associated variants and lineages from the United States, Mexico, and Brazil.

RABV vaccine strains isolated at different times in China showed close links with at least 2 of 4 groups. Vaccine strain CTN isolated in Shandong Province (in eastern China) in the 1950s may have evolved with group II, whereas strain 3aG, isolated in Beijing in the 1930s, and deer strain DRV, isolated in Jilin Province (in northeastern China), are closely related to group III (Figure 3).

Discussion

Guizhou, Guangxi, and Hunan provinces in China have reported the highest incidence of human rabies in recent years and were selected for national epidemic surveillance in 2005. According to national data on rabies surveillance, domestic dogs are the major infection source for humans and other domestic animals (*3*).

Phylogenetic analysis (Figure 2) showed that human RABV specimens generally belong to the same branch as dog RABVs from the same region, as exemplified by specimens HN0633D and HN0610H (subgroup IIa) and GZ0518H and GZ0505D (group III). This finding provides virologic evidence that domestic dogs are major reservoirs of human rabies epidemics (*2*). In addition, these results support the inference that RABVs from the same regions tend to group together and represent the same rabies focus or same geographically circumscribed outbreak (*20–22*).

We observed an infection rate of 2.3% in apparently healthy dogs from 15 cities in the 3 provinces. Previous surveys (*23*,*24*) in regions of high incidence of rabies showed different rates, ranging from 3.9% to 17.9% in dogs. Discrepancies in infection rates may be related to the sampling method (including nonclinically suspicious rabid dogs), different sample sizes, detection methods, and par-

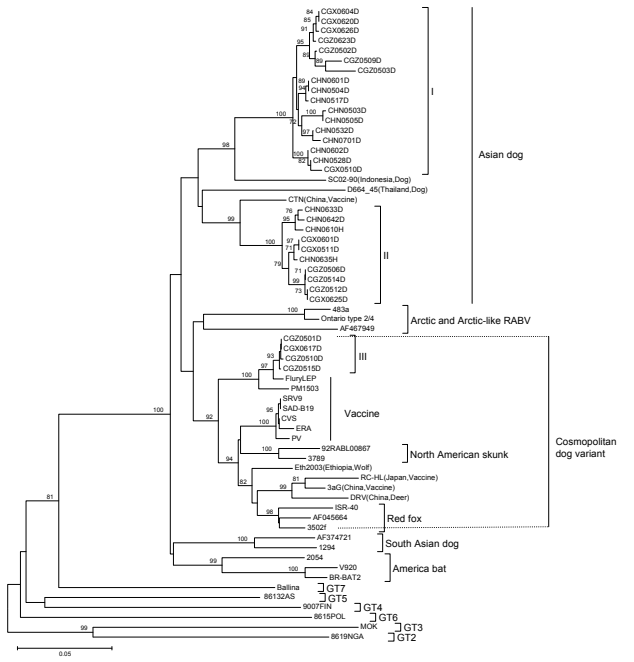

Figure 3. Neighbor-joining phylogenetic tree of 60 specimens of rabies virus (RABV) from the People's Republic of China, 2005–2007, and different genotypes (GTs) from other areas based on a 720-nt (nt 704–nt 1423) nucleoprotein (N) gene fragment of RABV. Numbers at each node indicate degree of bootstrap support; only those with support >70% are indicated. Scale bar indicates nucleotide substitutions per site.

ticular local variations. Regardless of the infection rate, the data indicate that rabies in dogs is prevalent in these regions and represents a cause of the high incidence of rabies in humans.

Phylogenetic analysis of specimens collected in the 3 provinces clearly shows that RABVs have a distinctive geographic correlation, in which group I was found predominantly in Hunan Province, group II in Guangxi Province, and group III in Guizhou Province (Figure 2). From an epidemiologic perspective, these groups may be interpreted as ongoing independent foci of dog rabies (or outbreaks).

Identity levels of deduced amino acid sequences of the 60 specimens ranged from 95.4% to 99.6%, which are higher than similarity values for corresponding nucleotide sequences. This finding indicates that most nucleotide variations in N gene coding sequence were synonymous mutations compatible with ongoing natural selection. Samples from different provinces had identical nucleotide sequences, which indicated that these viruses may have originated from the same outbreak or that a rabies focus had been spread between provinces (*13*,*14*).

Members of groups I and II were found in all 3 provinces selected for this study (Figure 2), which indicates that they have been spread throughout the region over time. The fact that subgroups Ic and IIa are integrated with virus samples from provinces distinct from their actual representative groups suggests that RABV translocation events are occurring between provinces (*25*). Phylogenetic inferences and epidemiologic and historical data suggest that ongoing rabies foci in Guangxi and Guizhou provinces, represented by subgroup Ic, had their origins in Hunan Province. Statistically supported independence of subgroup Ic within group I also suggests that such translocation events may have occurred in an isolated manner and may implicate dog RABV no longer circulating in Hunan Province. Similarly, subgroup IIa represents a rabies focus that had its origins in Guangxi Province and was then translocated to Hunan Province. Recent translocations from Hunan Province to Guangxi Province are represented by taxa CGX0612D, CGX0607D, CGX0510D, CGX0606D, and CGX0619D. Recent translocations from Guangxi Province to Hunan and Guizhou provinces represented by taxa CHNO635H, CGZ0514D, CGZ0506D, CGZ0512D, and CGZ0516D (Figure 2). Our results show trends in virus spread from current and historical occurrences in neighboring provinces.

Transprovincial spread of RABVs may be one of the forces responsible for exacerbating the dog rabies epizootic, as reflected by a human rabies epidemic. Specimen CGX0617D from Liuzhou in Guangxi Province (group III; Figure 2) is 100% identical to specimens from Guizhou Province, a finding that supports the suggestion that RABV was introduced into Liuzhou from Guizhou Province during the human rabies outbreak in 2004. Our findings are supported by previous studies in Guangxi Province (*26*,*27*), which suggested that different RABV lineages cocirculating in this province may correlate with an increased number of human rabies cases during 2003–2004 in Guangxi Province.

We sought to determine how transprovincial spread of RABVs occurred. The geographic nature of these rabies foci or groups suggests that dogs are not moving, per se, but that human-related activities may account for these phenomena. Persons in southern China are accustomed to eating dog meat because they believe it helps them resist dampness and cold in winter. In addition, many restaurants in cities and regions of southern China sell dog meat, which increases the demand for this type of meat. In rural areas of southern China, nearly every family owns several dogs, most of which are free-roaming, without special diets, and unvaccinated against rabies (to save costs). Sale of these dogs to restaurants can increase a farmer's income (average 12–15 US dollars/dog). Free-roaming and unvaccinated dog populations may increase the likelihood of transprovincial spread of RABVs. Other human-related activities, such as persons migrating with their dogs may also contribute to long-distance spread of rabies. Dogs purchased

by restaurants are soon killed for consumption. With the exception of butchers, there would be insufficient time to transmit RABV to other dogs and humans. Until now, persons in China who eat dog meat have not been not considered at risk for rabies because no related infections have been reported.

Specimens belonging to groups I and II, which are the predominant RABVs distributed across the 3 provinces, the Chinese vaccine strain CTN and strains from Southeast Asia form an isolated phylogenetic cluster. This finding demonstrates that strains from China and Southeast Asia may have the same origin (*11*,*14*,*28*–*30*). Southwestern China borders Vietnam, Laos, and Myanmar, countries into which persons in China have migrated over the past 2,000 years. Thus, dissemination of RABV has been historically linked across Southeast Asia.

Conversely, natural barriers such as the Himalayas and large rivers may have prevented RABV dissemination from the Indian subcontinent and central Asia into China (*20*). Our data indicate that RABV from these regions has not been recently transmitted into China. Enzootic dog RABV lineages from these regions (Indian dog and Arctic-like RABVs) are phylogenetically distinct from lineages in China, which supports this concept (*31*,*32*).

Groups I and II appear to have evolved from a common, long-term dog rabies enzootic in China. Group III appears to be more closely associated with widely spread dog-related RABVs represented by vaccine strains and other lineages established in wildlife in other regions (Figure 3). These results suggest 2 origins for rabies in dogs in the 3 provinces in southern China: an autochthonous origin and a more recent origin by introduction of a cosmopolitan dog RABV variant into China.

Approximately 57 years ago, Johnson (*33*) speculated that RABV strains from Europe were transmitted into China through Hong Kong and Shanghai. The attenuated 3aG strain, which was isolated in Beijing in 1931, and the DRV strain, which was isolated in Jilin Province in 2002, are closely related to group III. This finding implies that a group of viruses that originated in Europe is present in China and is still circulating. The hosts of this group include not only domestic dogs but also other mammals likely infected by rabid dogs (DRV strain). Alternatively, the similarity among some RABVs circulating in dogs in China and international vaccine strains (*34*) should motivate health authorities in China to revisit quality standards and adequacy for use of attenuated rabies vaccines to ensure that vaccine-related cases do not occur.

In conclusion, spread of RABVs from high-incidence regions, particularly by the long-distance migration or transprovincial movement of dogs caused by human-related activities, may be one of the causes of recent massive human rabies epidemics in southern China. We identified only 1 rabies case in a deer in China; it had evidence of spillover infection from a rabid dog. Although no evidence was found of independent rabies enzootics sustained by wildlife in China, multidisciplinary studies are required to improve rabies surveillance to identify potential alternative sources of human infection. Creation of a sensitive laboratory-based surveillance system for animal rabies detection is a prerequisite for enabling effective dog vaccination campaigns. Integration of enhanced surveillance, molecular analysis, local canine population management, and efficient rabies vaccination coverage in dogs are essential to prevent rabies from being transmitted by rabid dogs to humans.

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Serologic Evidence of Frequent Human Infection with WU and KI Polyomaviruses

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WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV) are novel human polyomaviruses. They were originally identified in human respiratory secretions, but the extent of human infection caused by these viruses has not been described to date. To determine the seroepidemiology of WUPyV and KIpyIV, we used an ELISA to screen serum samples from 419 patients at the St. Louis Children's Hospital and Barnes-Jewish Hospital during 2007–2008. The age-stratified deidentified samples were examined for antibodies to the major capsid proteins of WUPyV and KIPyV. Seropositivity for each virus was similar; antibody levels were high in the youngest age group (<6 months), decreased to a nadir in the next age group (6 to <12 months), and then steadily increased with subsequent age groups, eventually reaching a plateau of ≈80% for WUPyV and ≈70% for KIPyV. These results demonstrate that both KIPyV and WUPyV cause widespread infection in the human population.

WU polyomavirus (WUPyV) (*1*) and KI polyomavirus (KIPyV) (*2*) are newly described human polyomaviruses most closely related to JC virus (JCV) and BK virus (BKV). JCV and BKV are human pathogens that commonly infect the population. In the United States, seropositivity rates of 44%–75% for JCV and 63%–80% for BKV have been reported (*3*,*4*). Current models suggest that initial infection by BKV and JCV occurs asymptomatically during childhood; latency may establish in the kidneys and may reactivate during immune suppression. JCV causes a fatal demyelinating disease of progressive multifocal leukoencephalopathy in immunocompromised persons (*5*). BKV is associated with a number of renal and urinary tract infections including tubular nephritis, which can lead to allograft failure in renal transplant recipients (*6*), and hemorrhagic cystitis in hematopoietic stem cell transplant recipients (*7*). Another human polyomavirus, Merkel cell polyomavirus, was recently discovered and has tentatively been linked to Merkel cell carcinoma (*8*).

For KIPyV and WUPyV, neither disease association nor extent of infection in the human population has been established. Both viruses were originally identified in specimens from patients with respiratory illnesses of unknown etiology. Subsequent studies found WUPyV and KIPyV in the respiratory tract of patients with and without respiratory signs and symptoms (*9*–*11*), in fecal samples (*12*,*13*), and in lymphoid tissue from immunocompromised persons (*14*). Reported prevalence rates are 1%–9% for WUPyV and 0.5%–3% for KIPyV (*1*,*2*,*12*,*13*). The severity of diseases caused by BKV and JCV (*5*,*6*,*15*) raises the question of whether WUPyV and KIPyV can cause human disease. As a step toward determining the potential pathogenicity of these viruses, we developed serologic assays to assess the extent of infection by WUPyV and KIPyV in humans.

Materials and Methods

Plasmid Constructs, Protein Expression, and Purification

Genes encoding the major capsid proteins, KIPyV viral protein 1 (VP1) and WUPyV VP1, were cloned into the Gateway vector pENTR/SD/D-TOPO (Invitrogen, Carlsbad, CA, USA) by PCR from clinical samples. The primers were as follows: 5′-CACCATGAGCTGCACCCCGT-3′ (forward) and 5′-ATACATTCACTTTGAATTTTGTTGAG-3′ (reverse) for the KIPyV VP1 PCR and 5′-CACCA TGGCCTGCACAGCAAAGCCAGCC-3′ (forward) and 5′-TTATCCTTGTGTGTTTAGTATTGG-3′ (reverse) for the WUPyV VP1 PCR. Sequencing analysis showed that

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the KIPyV VP1 gene cloned was identical to that of the Brisbane 002 strain (GenBank accession no. ABR68682), except for 2 silent nucleotide mutations at positions 537 and 1005. For WUPyV, the gene encoding VP1 was identical to that of the B0 strain (GenBank accession no. ABQ09289). Positive clones containing the inserts were then transferred into the p-DEST15 plasmid (Invitrogen) by LR-homologous recombination to generate N-terminal–tagged glutathione S-transferase (GST)–WUPyV VP1 (plasmid NN003) and GST-KIPyV VP1 (NN006) constructs. N-terminal–tagged GST-VP1s from BKV, JCV, and simian virus 40 (SV40) were generously provided by Michael Pawlita (*16*), and GST-tagged microneme (Mic) protein encoded by *Toxoplasma gondii* was provided by David Sibley. VP1 was expressed in BL21(DE3)pLysS bacterial cells and affinity purified under native conditions by using the BugBuster GST-Bind Purification Kit (Novagen, Darmstadt, Germany) according to the manufacturer's suggested protocol.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Proteins were separated by electrophoresis in 4%–15% polyacrylamide gradient gels (no. 161-1122; BioRad, Hercules, CA, USA) by using Tris/glycine/sodium dodecyl sulfate (SDS) buffer (no. 161–0732; BioRad). The proteins were then either stained with Coomassie brilliant blue or transferred to a polyvinylidene difluoride membrane (no. LC2002; Invitrogen) for Western blot immunoassay. Membranes were blocked with 5% nonfat milk in phosphate-buffered saline with Tween 20 (PBS-T) for 1 h, then incubated with the primary antibody followed by peroxidase-conjugated Protein A/G (no. 32490; Pierce Biotechnology, Rockford, IL, USA). The proteins were visualized by using a SuperSignal West Pico kit (no. 34077; Thermo Scientific, Rockford, IL, USA). Membranes that were probed >1× were stripped with Restore Western Blot Stripping Buffer (no. 21059; Thermo Scientific) and reblocked with 5% nonfat milk in PBS-T between immunoassays.

Antibody Production

WUPyV VP1 peptide sequence (TAKPGRSPRSQPTRC) and KIPyV VP1 peptide sequence (CRPQKRLTRPRSQV) were each synthesized and injected into rabbits to produce polyclonal antibodies against WUPyV VP1 and KIPyV VP1 (service provided by GenScript, Piscataway, NJ, USA). Rabbit hyperimmune antiserum against the virus-like particles of BKV (BKVLP), JCV (JCVLP), or SV40 were kindly provided by Joakim Dillner (*17*). Peroxidase-conjugated goat anti-human immunoglobulin (Ig) G, (no. 31413) peroxidase-conjugated goat anti-rabbit IgG (no. 31463), and mouse anti-GST (no. 30001) antibodies were obtained from Thermo Scientific.

Serum Sample Analyses

We analyzed 419 deidentified serum samples from patients 1 day to 79 years of age at St. Louis Children's Hospital or Barnes-Jewish Hospital in St. Louis, Missouri, USA, from November 2007 through October 2008 for antibodies against VP1 of WU and KI polyomaviruses. Serum samples were kindly provided by Greg Storch (St. Louis Children's Hospital) and Mitchell Scott (Barnes-Jewish Hospital). The patients were age stratified (Table), and 30 samples were used for each age group, except for the group 6 to <12 months, for which 29 samples were available. Collection of samples and clinical data were approved by the Human Research Protection Office of Washington University in St. Louis, School of Medicine.

ELISA

To develop the WU ELISA and the KI ELISA, we used as positive controls 2 convalescent serum samples from a child known to be infected with WUPyV and rabbit hyperimmune serum for WUPyV VP1 or KIPyV VP1, and we used as negative controls rabbit preimmune serum and serum derived from pediatric patients (≤3 years of age). The optimal coating concentration of VP1, serum dilution, secondary conjugate dilution, and blocking reagent were determined by checkerboard titration experiments. Briefly, purified GST-VP1 (0.12 μg/well) was coated overnight at 4°C in PBS, pH 7.2, by using Maxisorp 96-well microtiter plates (Nunc, Naperville, IL, USA). Wells were washed 3× with PBS containing 0.05% PBS-T and blocked with 2% nonfat milk in PBS-T (PBS-TM) for 2 h at room temperature. Human serum samples (60 μL), diluted 1:100 in PBS-TM, were added to each well in triplicate and incubated for

Table. Age distribution of patients (419 samples) who were seropositive for WUPyV, KIPyV, or both, St. Louis, Missouri, USA, November 2007–October 2008*

Age group	No. (%) seropositive		
	KIPyV VP1	WUPyV VP1	Both
<6 mo	13 (43.3)	25 (83.3)	13 (43.3)
6 mo–<1 y	7 (24.1)	13 (44.8)	7 (24.1)
1 y	12 (40)	18 (60)	10 (33.3)
2 y	13 (43.3)	18 (60)	10 (33.3)
3 y	15 (50)	17 (56.7)	10 (33.3)
4 y	22 (73.3)	20 (66.7)	16 (53.3)
5 y	28 (93.3)	26 (86.7)	24 (80)
6–8 y	26 (86.7)	27 (90)	24 (80)
9–12 y	30 (100)	27(90)	27 (90)
13–19 y	28 (93.3)	28 (93.3)	27 (90)
20–34 y	21 (70)	30 (100)	21(70)
35–49 y	22 (73.3)	29 (96.7)	22 (73.3)
50–64 y	19 (63.3)	24 (80)	19 (63.3)
65–79 y	22 (73.3)	28 (93.3)	22 (73.3)
Total	278 (66.3)	330 (78.7)	252 (60.1)

*WUPyV, WU polyomavirus; KIPyV, KI polyomavirus; WUPyV VP1, WUPyV viral protein 1; KIPyV VP1, KIPyV viral protein 1.

4 h (or overnight) at 4°C. The plates were washed 6× with PBS-T, and a peroxidase-conjugated, secondary anti-IgG antibody, diluted 1:40,000 in PBS-TM, was added and incubated at 37°C for 2 h. After another washing step, plates were developed by adding peroxidase substrate tetramethyl benzidine (no. 34028; Thermo Scientific) for 15 min at room temperature. The reactions were stopped with H_2SO_4, and the absorbance was measured at 450 nm. For the blocking assays, serum samples were preincubated overnight at 4°C with, either singly or together in different combinations as indicated, recombinant GST-BKVP1, GST-JCVP1, GST-KIPyV VP1, GST-SV40VP1, GST-WUPyV VP1, or an unrelated GST-Mic encoded by *Toxoplasma gondii* (at 0.6 μg each, in solution), or in the blocking buffer alone. The ELISA was then used as described above.

Cutoff Value and Statistical Analysis

To calculate a cutoff value for the WU ELISA, we used 31 pediatric serum samples that gave signals below that of rabbit preimmune serum. Samples with absorbance intensity >3 SDs above the mean of these 31 samples (0.404 ± 0.103 SD) were considered positive. A parallel set of 31 negative samples (mean 0.286 ± 0.095 SD) were used to calculate a cutoff value for the KI ELISA. For each WU ELISA 96-well plate, the same negative control sample (serum from a 3-month-old child previously considered negative by initial ELISA experiments) and the same positive control sample (convalescent-phase serum from a patient previously found to be WU positive) were used to control for interplate variations. The cutoff value for percentage coefficient of variation of these 2 control samples was set <30%, as described by Jacobson (*18*). All blank wells had absorbance values <0.1.

Results

WUPyV VP1 and KIPyV VP1 Proteins as Target Antigens in ELISAs

WUPyV VP1 and KIPyV VP1 were expressed in bacteria as N-terminal, GST-tagged fusion proteins and subsequently purified by using glutathione-affinity chromatography. We used SDS polyacrylamide gel electrophoresis coupled with Coomassie blue staining to analyze the production and purification of the recombinant proteins (Figure 1, panel A). The purified GST-WUPyV VP1 or GST-KIPyV VP1 was then used as the capture antigen in ELISA to detect antibodies against WUPyV VP1 or KIPyV VP1, respectively.

The results of a WU ELISA using WU-hyperimmune rabbit serum and WU-positive human convalescent-phase serum are shown in Figure 1, panel B. Both the rabbit and human serum samples gave strong signals, which were effectively inhibited by preincubation with soluble GST-WUPyV VP1. By contrast, preincubation with GST alone had only marginal effects on the ELISA signal intensity. An ELISA performed on a GST-KIPyV VP1 coated plate using KI-hyperimmune rabbit serum also showed similar KI-specific binding activity (data not shown).

Detection of Antibodies against WUPyV VP1 and KIPyV VP1 in Human Serum Samples

Of the 419 serum samples analyzed, a representative WU ELISA result of 29 serum samples in the 3-year age group is shown in Figure 2, panel A. A range of absorbance

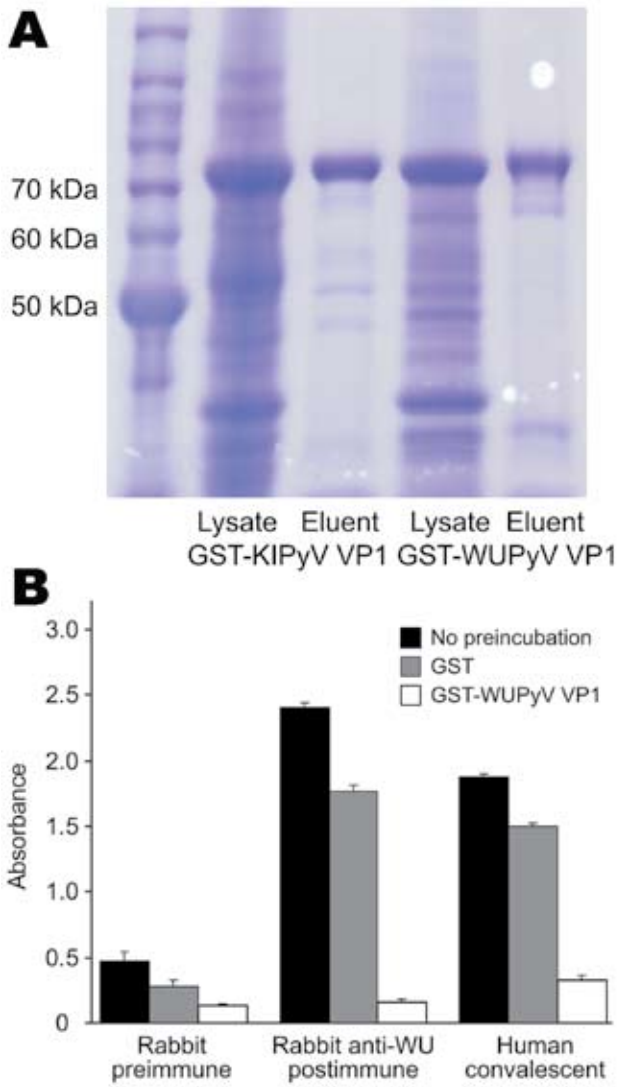

Figure 1. ELISA using WU polyomavirus (WUPyV) viral protein 1 (VP1) or KI polyomavirus (KIPyV) VP1 as the target antigen. A) Coomassie blue staining of a sodium dodecyl sulfate–polyacryliamide gel that contains bacterially expressed glutathione S-transferase (GST)–KIPyV VP1 and GST–WUPyV VP1 before and after glutathione-affinity purification. B) ELISA using rabbit hyperimmune serum and human WU polyomavirus convalescent-phase serum preincubated with buffer alone, GST protein, or GST–WUPyV VP1. Error bars indicate mean and SD.

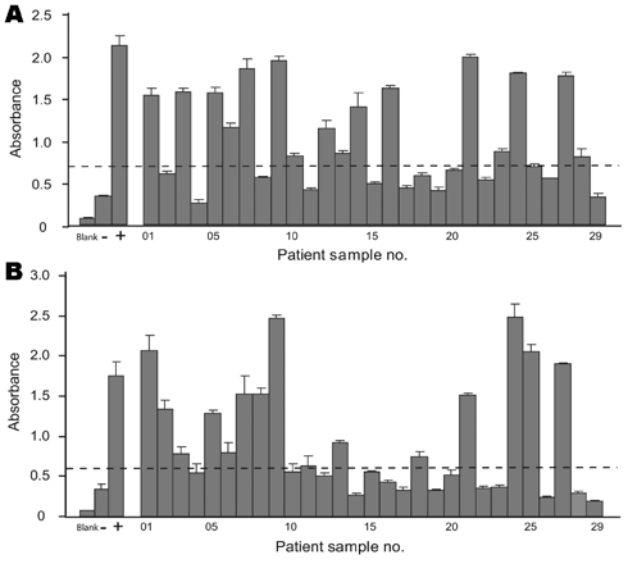

Figure 2. ELISA results from 3-year age group. A) 29 serum samples in the 3-year age group were assayed for antibodies against WU polyomavirus viral protein 1. B) KI polyomavirus ELISA results from the same 29 serum samples. The cutoff values for WU ELISA (0.713) and KI ELISA (0.572) are represented by dashed lines; –, negative control serum; +, positive control serum. Error bars indicate mean and SD.

intensities were observed; 17 samples were above the cutoff value. In a parallel KI ELISA conducted on this same set of serum samples, using rabbit serum immunized with a synthetic KIPyV VP1 peptide as positive control, 15 samples were considered ELISA positive (Figure 2, panel B).

Specificity of VP1-based WU ELISAs and KI ELISAs

WUPyV VP1 shares 65% amino acid identity with KIPyV VP1 and more limited similarity with JCVP1 (27%), BKVP1 (28%), and SV40VP1 (28%) (*1*). Previous serologic studies demonstrated that antibodies against VP1 from BKV, JCV, and SV40 polyomaviruses were cross-reactive with antigens from all 3 of these viruses (*4*,*19*). To assess the specificity of the WU ELISA and KI ELISA, we expressed and purified GST-tagged VP1 from BKV, JCV, and SV40 and performed blocking ELISAs as described above. Figure 3, panel A, shows a blocking WU ELISA of 3 serum samples that had been preincubated with, either singly or together in different combinations, GST-BKVP1, GST-JCVP1, GST-KIPyV VP1, GST-SV40VP1, and GST-WUPyV VP1; with an unrelated GST-Mic protein; or with the blocking buffer alone. Two WU-positive samples, C09 and C56, had absorbance values reduced to some extent after preincubation with different recombinant proteins. However, only when preincubated with the GST-WUPyV VP1 itself was the ELISA signal intensity strongly reduced; C56 was reduced by 83.5% and C09 by 79.6% compared with the buffer-alone samples. Of 34 randomly selected WU ELISA–positive samples tested, 32 showed inhibition levels >50% in the presence of soluble GST-WUPyV VP1. The 2 exceptions, A04 and A43, had limited inhibition of 42.5% and 36%, respectively. For these 2 samples, further dilution of the human serum enabled soluble GST-WUPyV VP1 to reduce the ELISA absorbance intensity by >50%, suggesting perhaps that high titers of antibodies against WUPyV VP1 were present in these 2 samples (data not shown). To define the specificity of the KI ELISA, we preincubated ELISA-positive samples with soluble GST-Mic, GST-WUPyV VP1, GST-KIPyV VP1, or the buffer alone. Figure 3, panel B, shows blocking results of 5 representative KI-positive samples. In each example, only preincubation with soluble GST-KIPyV VP1 substantially reduced the absorbance value, indicating the presence of specific antibodies against KIPyV VP1 in these samples.

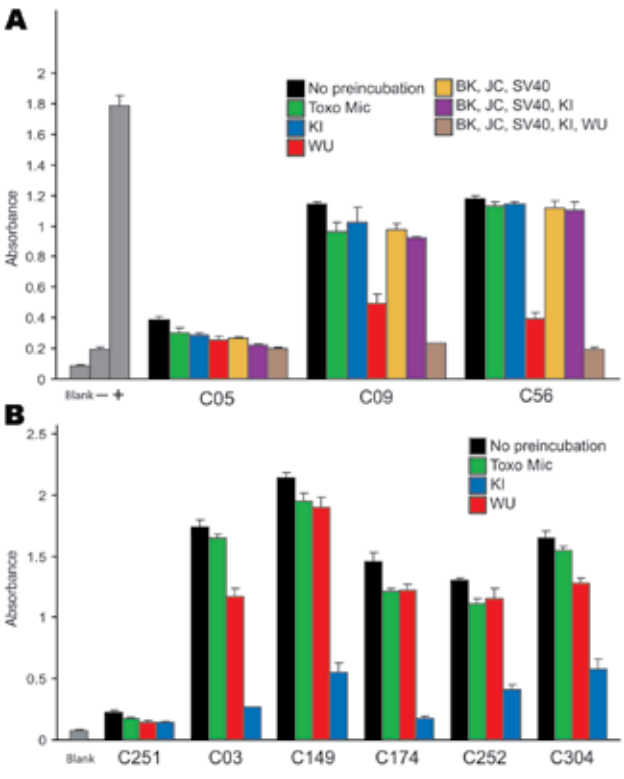

Figure 3. Effects of preincubation of polyomavirus viral protein 1 on WU ELISA and KI ELISA. Samples were preincubated with single or multiple proteins in different combinations or with the blocking buffer alone. Shown are results of blocking WU ELISA (A) and blocking KI ELISA (B) of representative serum samples. Proteins used in preincubation experiments are indicated by matching color scheme. Blocking data for WU ELISA–negative serum (C05) and KI ELISA–negative serum (C251) are shown for comparison. Toxo Mic, microneme protein from *Toxoplasma gondii*; SV40, simian virus 40. Error bars indicate mean and SD.

Detection of Antibodies against WUPyV VP1 and KIPyV VP1 by Western Blot

To confirm the results of our WU ELISAs and KI ELISAs and further assess the potential serum cross-reactivity with VP1 proteins of related polyomaviruses, we performed Western blot assays. The affinity purified GST-BKVP1 (69.3 kDa), GST-JCVP1 (68.8 kDa), GST-KIPyV VP1 (70.7 kDa), GST-SV40VP1 (69.3 kDa), and GST-WUPyV VP1 (69.0 kDa), which all migrated to their relatively expected position in an SDS-polyacrylamide gel (Figure 4, panel A), were used as target antigens.

Hyperimmune rabbit serum against BKVLP reacted strongly with BKVP1 and SV40VP1 and, to a lesser extent, with JCVP1. Anti-JCVLP rabbit serum reacted strongly to both JCVP1 and BKVP1 but rather weakly to SV40VP1. These results were consistent with those of previous studies describing cross-reactivity among the VP1 of BKV, JCV, and SV40 (*19*). Neither rabbit serum control, however, showed any cross-reactivity to WUPyV VP1 or to KIPyV VP1 (Figure 4, panel B).

A set of human serum samples was randomly selected for confirmatory Western blotting. Figure 4, panels C and D, shows the results of 3 representative samples. They included a sample that was positive by ELISA for antibodies against WUPyV VP1 and KIPyV VP1 (S20), a sample that was ELISA positive for WUPyV but negative for KIPyV (C258), and a sample that was positive by ELISA for KIPyV and negative for WUPyV (C244). In each case, Western blot analysis corroborated the ELISA results.

Seroprevalence Rates

Overall WUPyV seropositivity in this cohort was 78.7% (330/419), KIPyV seropositivity was 66.3% (278/419), and seropositivity for both viruses was 60.1% (252/419) (Table). In general, the rate of WUPyV seropositivity was slightly higher than KIPyV seropositivity throughout. Antibodies against WUPyV and KIPyV appeared to be maternally transmittable, on the basis of the high degree of seropositivity detected in the <6-month age group. The lowest seropositivity rates were observed for the age group 6 to <12 months (44.8% for WUPyV and 24.1% for KIPyV), consistent with the waning of maternal antibody levels. Infection rates for both viruses then increased rapidly, reaching 86.7% for WUPyV and 93.3% for KIPyV in the 5-year age group. For patients ≥6 years of age, average WUPyV seropositivity was 80% (193/240). In this dataset, KIPyV infection appeared to peak in the age group 5–19 years (average seropositivity rate 93%), but in patients ≥20 years of age, the average was 70% (Figure 5).

Discussion

We developed WUPyV-specific and KIPyV-specific ELISAs to assess the extent of human infection by

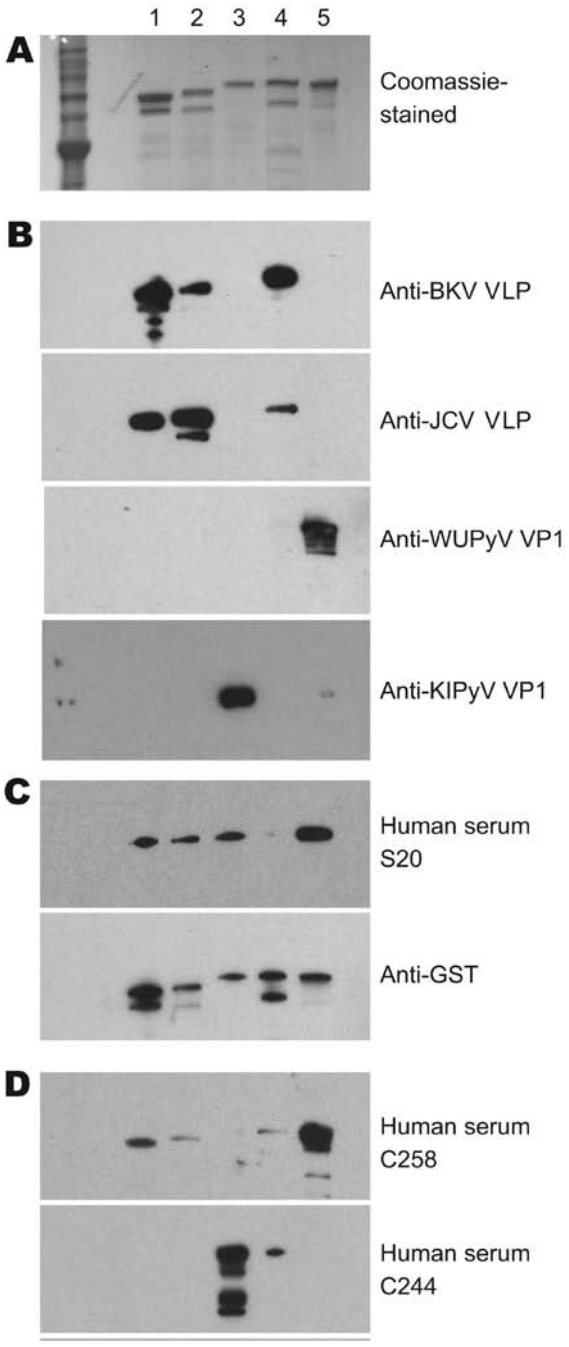

Figure 4. Results of patient serum sample Western blotting for polyomaviruses. A) Coomassie blue–stained image showing 5 types of purified glutathione S-transferase (GST)–tagged viral protein 1 (VP1) in a sodium dodecyl sulfate–polyacrylamide gel. Lane 1, GST-BKV VP1; lane 2, GST-JCV VP1; lane 3, GST–KI polyomavirus (KIPyV) VP1; lane 4, GST-SV40 VP1; lane 5, GST–WU polyomavirus (WUPyV) VP1. B) Western blot results using control rabbit antiserum against BK virus-like particles (BKVLP), JC virus-like particles (JCVLP), WUPyV VP1, or KIPyV VP1 as primary antibody. C) Western blot results for serum that was positive (S20) for WU polyomavirus and KI polyomavirus by ELISA. Antibody against GST was used as a loading control. D) Western blot result for serum that was ELISA positive for WU (C258) and KI (C244).

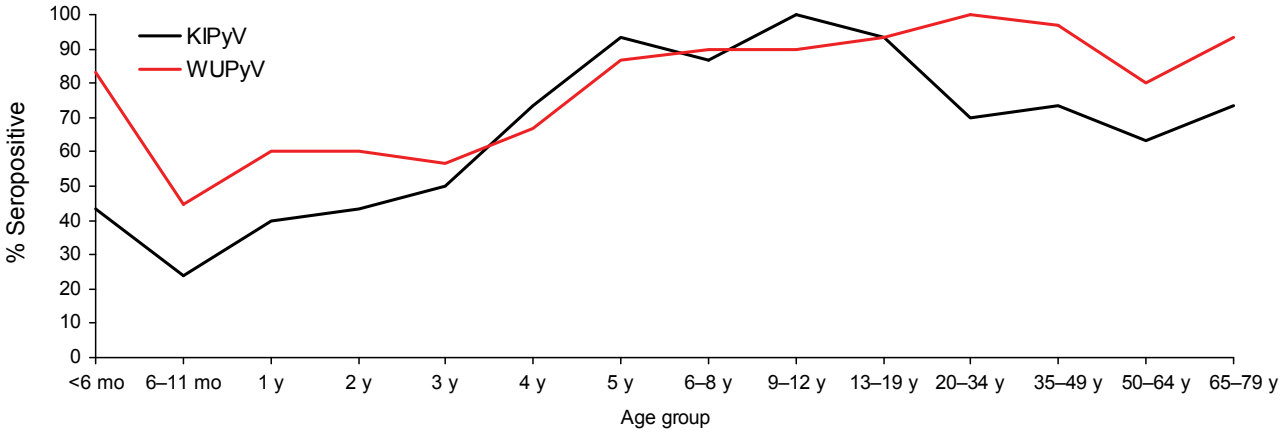

Figure 5. Percentage of serum samples positive for antibodies against WU polyomavirus (WUPyV) and KI polyomavirus (KIPyV), by patient age group.

WUPyV and KIPyV. Previous studies on BKV, JCV, and SV40 polyomaviruses indicated that the viral major capsid protein is immunodominant and that human serum can recognize the recombinant VP1 expressed in bacteria (*16*). We demonstrate that the full-length recombinant WUPyV VP1 and KIPyV VP1 expressed in bacteria are capable of detecting antibodies against WUPyV and KIPyV VP1, respectively, using either the ELISA or the Western blot assay format.

One issue that has confounded serologic analysis of human polyomaviruses in the past is antigenic cross-reactivity (*4*,*19*). We performed a number of experiments to define whether cross-reactivity exists between antibodies against WUPyV VP1 in human serum and the VP1 antigens of KIPyV, SV40, BKV, and JCV. Preincubation experiments demonstrated that the GST-VP1 from BKV, JCV, and SV40 reduced the absorbance signals only minimally, equivalent to the reduction observed when an irrelevant GST-fusion protein, GST-Mic, was used (Figure 3, panel A). These data indicate that BKV, JCV, and SV40 VP1s are not cross-reactive with antibodies against WUPyV VP1. This observation was further substantiated by Western blotting using rabbit serum against virus-like particles of BKV and JCV, which yielded no detectable cross-reaction with WUPyV VP1 or KIPyV VP1. Because WUPyV VP1 and KIPyV VP1 share 65% sequence identity, potential cross-reactivity between them was a concern. However, preincubation with GST-KIPyV VP1 reduced the WU ELISA absorbance signal by only 13.5% on average, compared with 10.4% on average when an irrelevant GST protein or VP1 of BKV, JCV, and SV40 were used. Similarly, KI ELISA showed an average of 16.9% and 9.4% signal reduction when samples were preincubated with GST-WUPyV VP1 or GST-Mic protein, respectively.

As a separate line of evidence, Western blotting with serum that was positive for antibodies against WUPyV VP1 and negative for antibodies against KIPyV VP1 (C258) and serum that was negative for WUPyV VP1 and positive for KIPyV VP1 (C244) yielded no evidence of cross-reactivity (Figure 4, panel D). Collectively, the ELISA preincubation experiments and the Western blotting demonstrated minimal, if any, detectable cross-reactivity between KIPyV and WUPyV within the sensitivity limits of these assays.

Our findings demonstrate high sustained rates of infection by WUPyV and KIPyV in this cohort. Although the serum samples were selected by using broad inclusion criteria (specimen volume >0.5 mL) from patients visiting St. Louis Children's Hospital and Barnes-Jewish Hospital, both tertiary referral hospitals, these results may be somewhat biased toward patients with some form of disease. Additional experiments with patient cohorts from different regions or with different inclusion criteria are necessary to generalize these results to the general population. The steep increase in seropositivity for WUPyV and KIPyV in patients 4–6 years of age raises the possibility that school attendance plays a role in facilitating transmission of these viruses. Serologic studies of other viruses such as bocavirus and BKV also showed elevated rates of infection in children of similar age (*20*–*22*). For KIPyV, the observed decrease in seropositivity rate for patients >19 years of age raises the possibility that antibodies against KIPyV wane over time. However, direct experimentation using longitudinally collected serum would be necessary to address this possibility.

In conclusion, we have established WUPyV-specific and KIPyV-specific ELISAs using VP1 as the capture antigen. We have demonstrated that cross-reactivity between antibodies against WUPyV VP1 (or KIPyV VP1) and VP1 from KIPyV (or WUPyV), BKV, JCV, or SV40 viruses is minimal. We also demonstrated that WUPyV and KIPyV cause widespread infection in humans. These observations roughly parallel the extent of human exposure to BKV and

JCV. Given that severe illnesses have been associated with infections by BKV and JCV, further studies are needed to determine whether WUPyV and KIPyV are similarly pathogenic.

Acknowledgments

We thank Steven Lawrence and Guang Wu for technical advice and support, and we thank Anne Gaynor for critical reading of the manuscript.

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Bordetella pertussis Strains with Increased Toxin Production Associated with Pertussis Resurgence

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Before childhood vaccination was introduced in the 1940s, pertussis was a major cause of infant death worldwide. Widespread vaccination of children succeeded in reducing illness and death. In the 1990s, a resurgence of pertussis was observed in a number of countries with highly vaccinated populations, and pertussis has become the most prevalent vaccine-preventable disease in industrialized countries. We present evidence that in the Netherlands the dramatic increase in pertussis is temporally associated with the emergence of *Bordetella pertussis* strains carrying a novel allele for the pertussis toxin promoter, which confers increased pertussis toxin (Ptx) production. Epidemiologic data suggest that these strains are more virulent in humans. We discuss changes in the ecology of *B. pertussis* that may have driven this adaptation. Our results underline the importance of Ptx in transmission, suggest that vaccination may select for increased virulence, and indicate ways to control pertussis more effectively.

Bordetella pertussis causes whooping cough or pertussis, a respiratory disease that is most severe in infants. Before childhood vaccination was introduced in the 1940s, pertussis was a major cause of infant deaths worldwide. Widespread vaccination of children reduced the incidence of illness and deaths caused by pertussis (*1*). However, globally pertussis remains 1 of the top 10 causes of death in children (*2*). Further, in the 1990s a resurgence of pertussis was observed in several countries with highly vaccinated populations (*3*,*4*), and pertussis has become the most prevalent vaccine-preventable disease in industrialized countries. In the Netherlands, the estimated incidence of infection was 6.6% per year for the 3–79-year age group from 1995 through 1996 (*5*). Similar percentages have been found in the United States (*6*). One of the hallmarks of the pertussis resurgence is a shift in disease prevalence toward older persons who have waning vaccine-induced immunity (*7*).

The reemergence of pertussis has been attributed to various factors, including increased awareness, improved diagnostics, decreased vaccination coverage, suboptimal vaccines, waning vaccine-induced immunity, and pathogen adaptation. The relative contribution of these factors may differ between countries and is the subject of ongoing debate. Pathogen adaptation is supported by several observations. We and others have shown that antigenic divergence has occurred between vaccine strains and clinical isolates with respect to surface proteins, which confer protective immunity: pertussis toxin (Ptx), pertactin (Prn), and fimbriae (*8*,*9*). Strain variation was shown to affect vaccine efficacy in a mouse model (*10*–*13*). Because adaptation may involve the structure of virulence factors (by antigenic variation) and their regulation, we extended our studies on the evolution of *B. pertussis* by investigating polymorphism in the promoter of Ptx (*ptxP*), a major virulence factor and component of all pertussis vaccines (*1*). We provide evi-

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dence that expansion of strains with increased Ptx production has contributed to the resurgence of pertussis in the Netherlands.

Methods

Pertussis Notifications

Pertussis became a notifiable disease in the Netherlands in 1976. Notifications are submitted online by local health authorities. Other notifiable diseases are also monitored through this system, which falls under the responsibility of the Dutch National Institute of Health and Environment (*3*).

Bacterial Strains

B. pertussis strains examined were obtained from 1935 through 2004. A total of 1,566 isolates, 879 from the Netherlands and 687 from other countries, were analyzed for polymorphism in *ptxP* (online Technical Appendix, available from www.cdc.gov/EID/content/15/8/1206-Techapp.xls). Eight strains isolated from patients in the Netherlands from 1999 through 2001 were selected to study Ptx and Prn production: B1834 (*ptxP1*), B1868 (*ptxP1*), B1878 (*ptxP1*), B1920 (*ptxP1*), B1836 (*ptxP3*), B1865 (*ptxP3*), B1917 (*ptxP3*), and B2030 (*ptxP3*) (Table 1).

Sequencing

The primers 5′-AATCGTCCTGCTCAACCGCC-3′ and 5′-GGTATACGGTGGCGGGAGGA-3′ were used for amplification and sequencing of *ptxP* and correspond, respectively, to bases 60–79 and 633–614 of the *ptx* sequence with GenBank accession no. M14378. The *ptx* gene cluster from the strains B1834 (*ptxP1*), B1920 (*ptxP1*), B1917 (*ptxP3*), and B1831 (*ptxP3*) was sequenced completely. The sequences of the *ptx* gene clusters from strains B1834, B1920, B1917, and B1831 can be found under the following accession numbers, respectively: FN252334, FN252335, FN252336, and FN252333. The *ptxP1-ptxP11* sequences have been assigned accession nos. FN252323, FN252322, FN252324, FN252325, FN252326, FN252327, FN252328, FN252329, FN252330, FN252331, and FN252332.

Pertussis Toxin and Pertactin Production

B. pertussis strains were grown on Bordet-Gengou agar plates supplemented with 15% sheep blood and incubated for 3 days at 35°C. Cells were harvested and suspended in 2 mL Verwey medium (*14*) per plate. Cells from 1 mL were collected by centrifugation and resuspended in Verwey medium to a concentration of 5×10^6 bacteria/mL. Subsequently, 100 μL of this suspension (5×10^5 CFU) was plated on Bordet-Gengou agar plates. After an incubation of 48 to 60 hours at 35°C, cells were harvested in 2.5 mL Verwey medium. The cell suspension was heat-inactivated for 30 min at 56°C and stored at 4°C. An ELISA was used to quantify Ptx and Prn. For Ptx, Maxisorp 96-well plates (Nunc International, Rochester, NY, USA) were coated with 100 μL of 0.04 mg/mL fetuin (Sigma-Aldrich, St. Louis, MO, USA) in 0.04 M carbonate buffer, pH 9.6, overnight at 4°C. For Prn, polystyrene 96-well plates (Immunolon II; Dynatech, Chantilly, VA, USA) were coated with 100 μL of a 2,000-fold dilution of polyclonal rabbit anti-Prn immunoglobulin (Ig)G (*15*) in 0.04 M carbonate buffer, pH 9.6, overnight at 20°C. Plates were blocked by incubation with 130 μL 1% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 1 hour at 37°C, after which plates were washed twice with PBS supplemented with 0.05% Tween. A 3-fold serial dilution of the heat-inactivated cell suspensions was made in 100 μL PBS supplemented with 0.1% Tween (PBST); 1 μg/mL of Prn and Ptx were used as reference. The suspensions were incubated for 1 hour at 37°C followed by 2 washings. The Prn monoclonal antibody (MAb) (PeM85) that was used binds to the linear epitope GGFGPGGFGP present in the repeat region 1 of all known Prn variants, except Prn13 (*15*). The Ptx MAb (3F10) binds to a conformational epitope in the *PtxA* subunit (*16*). All strains selected for the ELISA experiments produced Prn2 and PtxA1 (Table 1). The MAbs were diluted in PBST, added to the wells, and incubated for 1 hour at 37°C, followed by 2 washings. To detect bound MAbs, plates were incubated with horseradish peroxidase–conjugated polyclonal rabbit anti-mouse IgG (DakoCytmation, Glostrup, Denmark), diluted in PBST, for 1 hour at 37°C, and followed by 2 washings. The optical density at 450 nm was measured with a plate

Table 1. Characteristics of strains used for Ptx and Prn production experiments*

Strain	Year of isolation	Patient age, mo	*ptxP* allele	*prn* allele	*ptxA* allele
B1834	1999	28	PtxP1	Prn2	PtxA1
B1836	1999	3	PtxP3	Prn2	PtxA1
B1865	2000	2	PtxP3	Prn2	PtxA1
B1868	2000	35	PtxP1	Prn2	PtxA1
B1878	2000	45	PtxP1	Prn2	PtxA1
B1917	2000	44	PtxP3	Prn2	PtxA1
B1920	2000	9	PtxP1	Prn2	PtxA1
B2030	2001	3	PtxP3	Prn2	PtxA1

*Ptx, pertussin toxin; Prn, pertactin; *ptxP*, pertussin toxin promoter; *prn*, gene for pertactin; *ptxA,* gene for the A subunit of pertussin toxin.

reader (PowerWave HT 340; Biotek, Winooski, VT, USA) and the amount of produced Ptx and Prn were calculated using the KC4 program (Biotek).The ratio of Ptx and Prn production by *ptxP1* and *ptxP3* strains was calculated as follows: Ptx (or Prn) production *ptxP3* strains divided by Ptx (or Prn) production *ptxP1* strains.

Statistical Analyses

The significance of the increases in illness and death were calculated with the Fisher exact test. Ptx and Prn production was analyzed on the basis of the following considerations: 1) that there are random variations among experiments that influence Ptx and Prn production; 2) that there is a correlation between Ptx and Prn production; and 3) that the distribution Ptx and Prn measurements were skewed. To take into account these considerations regarding sources of random variation, a random intercept model was used and a logarithmic transformation was used before further analysis. Logarithmically transformed Ptx and Prn values were first analyzed with a random intercept model by using SAS PROC MIXED (SAS, Cary, NC, USA) and by using experiment as a random effect. We first tested whether there were differences between *ptxP1* and *ptxP3* strains in the production of Ptx and Prn by analyzing the logarithm of Ptx production and Prn production, respectively, as a dependent variable, and by using experiment as random effect and incubation time (in classes) and type (*ptxP1* or *ptxP3*) as fixed effects. To determine whether the ratio of production in *ptxP3* versus *ptxP1* strains differ significantly for Prn and Ptx, we further fitted a multivariate model with both factors (Ptx and Prn) as dependent variables, again using experiment as random effect, and allowing all variance parameters to be factor (Ptx or Prn) specific. In this model the interaction between *type* (*ptxP1* or *ptxP3*) with factor (Ptx or Prn) then gives the required P value.

Results

Polymorphism of the Pertussis Toxin Promoter

The synthesis and export of Ptx requires 14 genes, which are co-transcribed from *ptxP* (*17*). *ptxP* comprises a region of ≈170 bases upstream of the Ptx subunit gene *ptxA* and contains the RNA polymerase binding site and 6 binding sites for the BvgA dimer (*18*). BvgA is a global regulator of *B. pertussis* virulence genes, and cooperative binding of BvgA to *ptxP* is required for efficient transcription of ptx (*18*). We investigated polymorphism in *ptxP* by sequencing a DNA region of ≈380 bases upstream of *ptxA* by using a collection of 1,566 *B. pertussis* strains from 12 countries isolated during 1935–2004. Polymorphism was found to be restricted to the DNA region implicated in binding of RNA polymerase and BvgA. Eleven *ptxP* alleles were identified (Figure 1).

Geographic and Temporal Differences in *ptxP* Frequencies

Next we investigated geographic and temporal differences in *ptxP* frequencies. The following geographic regions were distinguished: the Netherlands, the continents of Africa, Asia, Europe (excluding the Netherlands), North America, and South America. Two periods, chosen on the basis of the appearance of *ptxP3* strains in the Netherlands, were compared: 1935 through 1990 and 1991 through 2004 (Table 2). Only strains from the later period were available from South America. Two *ptxP* alleles were found to predominate worldwide, *ptxP1* and *ptxP3,* and the remaining 9 alleles were pooled. Strains with *ptxP1* predominated in the first period and were found in lower frequencies in the second period (global frequencies were 88% and 47%, respectively). A reverse trend was observed for the *ptxP3* strains (global frequencies, 3% and 52%, respectively). In the first period, *ptxP3* strains were only detected in the Netherlands and the United States (frequencies were 3% and 13%, re-

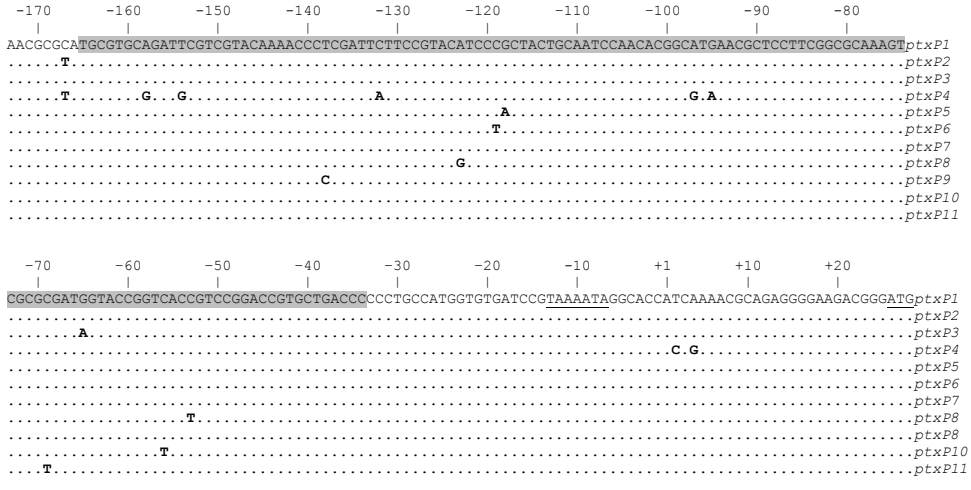

Figure 1. Alleles of pertussis toxin promoter (*ptxP*) observed worldwide. Bases are numbered –173 to +27 relative to the start of transcription (+1). The region to which 6 dimers of BvgA, the global regulator of *B. pertussis* virulence genes, bind is shaded. The –10 sequence motif and initiation codon are underlined. The DNA region –370 to –174, not shown here, was devoid of polymorphism. Locations of transcriptional signals and BvgA bindings sites are based on Bartoloni et al. (*16*).

Table 2. Worldwide frequencies of *ptxP1* and *ptxP3* during 1935–1990 and 1991–2004*

Region†	1935–1990				1991–2004			
	ptxP1	*ptxP3*	Other‡	N	*ptxP1*	*ptxP3*	Other‡	N
The Netherlands	89	3	8	265	47	53	0	614
Africa	100	0	0	11	100	0	0	7
Asia	100	0	0	12	83	13	3	30
Europe§	73	0	27	22	46	53	0	577
North America	50	13	38	8	20	80	0	10
South America	–	–	–	0	10	80	10	10
Total	88	3	9	318	47	52	1	1,248

**ptxP*, pertussis toxin promoter. Allele frequencies are given in percentages.
†The following countries represented the continents: Africa: Senegal; Asia: Japan and Australia; Europe: Denmark, Finland, France, Germany, Italy, Sweden; North America: USA; South America: Argentina.
‡Nine *ptxP* alleles were found in low frequencies: *ptxP2*, *ptxP4*, *ptxP5*, *ptxP6*, *ptxP7*, *ptxP8*, *ptxP9, ptxP10,* and *ptxP11*.
§Dutch strains were excluded.

spectively). The only region in which *ptxP3* strains were not detected was Africa, where only *ptxP1* strains were found. The minor *ptxP* alleles were observed in higher frequencies during 1935–1990 compared with 1991–2004 (global frequencies were 9% and 1%, respectively). The differences in *ptxP* allele frequencies may be due to sampling bias, geographic factors, or differences in vaccines, vaccination history, and vaccination coverage. Nevertheless, these data provide strong evidence that, in most parts of the world, *ptxP3* strains emerged recently and replaced the resident *ptxP1* strains. The *ptxP3* allele was first detected in a strain isolated in the United States in 1984.

To investigate if *ptxP1* and *ptxP3* alleles were linked to other polymorphisms in *ptx* genes, the gene clusters from 2 *ptxP1* and 2 *ptxP3* strains were sequenced. The *ptx* sequences were identical, except for a single point mutation in *ptxC*. The single nucleotide polymorphism (SNP) in *ptxC* has been described previously, does not result in a change in amino acid sequence, and is therefore most likely selectively neutral (*19*). To study the linkage, *ptxC* was sequenced in 249 *ptxP1* and 142 *ptxP3* strains. Linkage between *ptxP1-ptxC1* and *ptxP3-ptxC2* was 100% and 98%, respectively. Only 3 strains harbored the combination *ptxP3-ptxC1*.

Association of the *ptxP3* Allele with the Resurgence of Pertussis in the Netherlands

The availability of a large strain collection allowed us to analyze temporal trends in the Netherlands in more detail. From 1989 through 2004, a total of 99% of the strains harbored *ptxP1* or *ptxP3.* In this period, *ptxP1* was gradually replaced by *ptxP3,* which increased in frequency from 0% in 1989 to 100% in 2004. A close temporal relationship was shown between the increase in *ptxP3* frequency and mandatory pertussis notifications (Figure 2, panel A). Increased notifications were found in all age groups, however, the largest increase was among persons ≥5 years of age (Figure 2, panel B). The shift toward older age categories coincided with emergence of *ptxP3* strains. There was no change in age distribution from 1989 through 1992, which preceded the emergence of the *ptxP3* allele.

Ptx and Prn Production by *ptxP1* and *ptxP3* Strains

The effect of polymorphism in *ptxP* was assessed by determining the ratio of Ptx produced by *ptxP1* and *ptxP3* strains (Ptx produced by *ptxP3* strain / Ptx produced by *ptxP1* strain) after 48, 54, and 60 h growth on plates. In addition, we assessed the production of a second virulence factor, Prn, which is also regulated by *bvg*. No polymorphism was observed in the Prn promoter of the 8 strains analyzed. Data from 4 *ptxP1* and 4 *ptxP3* strains were pooled (Figure 3). The Prn ratios were slightly lower than 1, indicating that *ptxP3* strains produce slightly less Prn than *ptxP1* strains (average over all time points 0.94; p = 0.03).

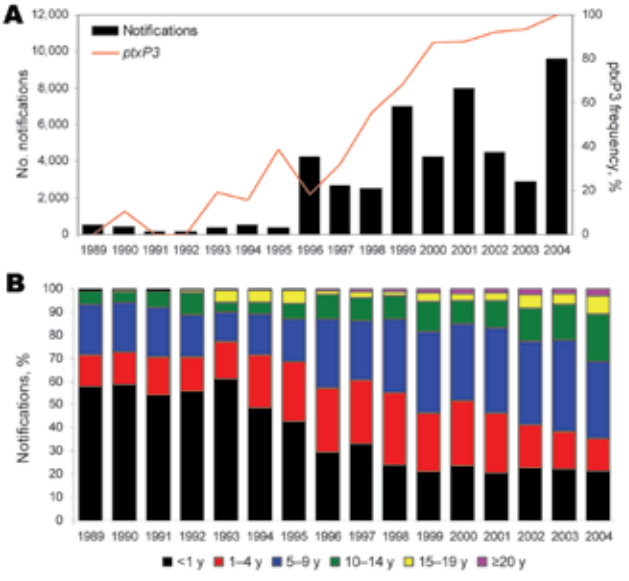

Figure 2. Relationship between the emergence of pertussis toxin promoter 3 (*ptxP3*) strains and the epidemiology of pertussis in the Netherlands, 1989–2004. A) Temporal trends in the frequencies of *ptxP3* strains and notifications. In this period 99% of the strains harbored either *ptxP1* or *ptxP3.* B) Shift in age-specific distribution of notifications.

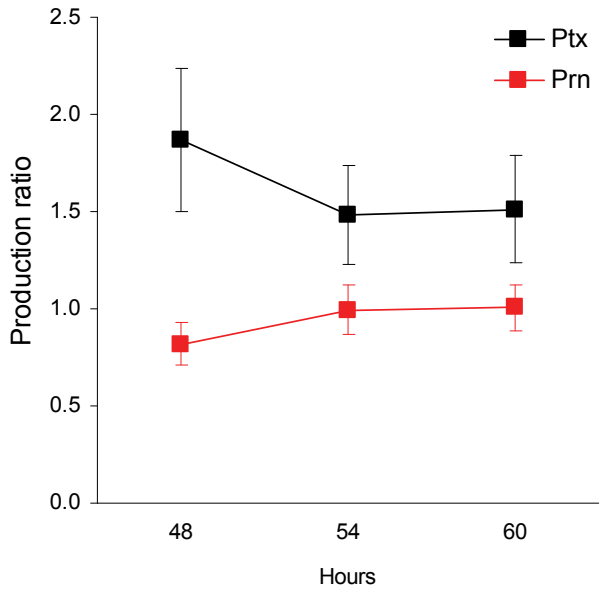

Figure 3. Production of pertussis toxin (Ptx) and pertactin (Prn) by pertussis toxin promoter 1 (*ptxP1*) and *ptxP3* strains. Strains were incubated for the 48, 54, and 60 h, after which the amount of Ptx and Prn was determined by ELISA. The production ratio was calculated as follows: *ptxP3* strain values/*ptxP1* strain values; 8 strains, 4 *ptxP1* strains and 4 *ptxP3* strains, were used. The experiment was performed 3 times. Error bars indicate 95% confidence intervals. The Ptx and Prn ratios were significantly different from 1 ($p<0.0001$ and 0.03, respectively).

In contrast, the Ptx ratio was significantly larger than 1 (average over all time points 1.62; $p<0.0001$), indicating that *ptxP3* strains produce more Ptx than *ptxP1* strains under the growth conditions tested. The Ptx and Prn ratios were significantly different ($p<0.0001$).

Evidence for Increased Virulence of *ptxP3* Strains in Humans

Ptx is assumed to contribute to the severity of infection. Therefore, we investigated whether *ptxP3* strains were more virulent in humans than *ptxP1* strains by comparing the incidence of hospitalizations, deaths, and lethality (ratio of deaths to hospitalizations) in the Netherlands during 2 periods, 1981 through 1992 and 1993 through 2004, with low (1.6%) and high (54.5%) *ptxP3* frequencies, respectively (Table 3). All 3 parameters showed a statistically significant increase (1.41, 10.21, and 7.23 times respectively; p values <0.0001, 0.0058, and 0.03, respectively), suggesting that *ptxP3* strains are more virulent in humans.

Discussion

The persistence of pertussis in the face of intense vaccination is unexpected because *B. pertussis* is extremely homogeneous (*19–21*), implying a limited ability to adapt. However, the Ptx promoter showed a relatively high degree of polymorphism, suggesting that fine tuning of Ptx production has adaptive value. Globally, 11 *ptxP* alleles were found in 1,566 strains, 8 of which occurred in the *B. pertussis* population in the Netherlands. Polymorphism was restricted to a region required for transcription of *ptx*. Silent *ptx* genes are found in the closely related species *B. parapertussis* and *B. bronchiseptica* (*17*). The silencing of *ptx* genes indicates that production of Ptx involves benefits and costs. Thus, production of Ptx is beneficial for the pathogen by suppressing host defenses but also involves metabolic costs and increases the number of immunologic targets. Ptx is a major antigen of *B. pertussis*, and Ptx antibodies are used in diagnosing pertussis cases.

Globally, *ptxP1* and *ptxP3* were the most prevalent *ptxP* alleles. In the Netherlands, during 1989–2004, *ptxP1* was completely replaced by *ptxP3*. The replacement of *ptxP1* strains by *ptxP3* strains in recent times is a global phenomenon because it has been observed in 11 countries representing 4 continents; Asia, Europe, and North and South America. Notably, *ptxP3* strains were not observed in Africa. A broad current distribution of *ptxP3* strains was also suggested by a recent study in which strains from 8 European countries were compared by pulsed-field gel electrophoresis (PFGE). One PFGE profile, BpSR11, predominated in 5 of the 8 European countries (*22*). We have found that in the Dutch population all BpSR11 strains carry the *ptxP3* allele (N = 18).

In the Netherlands, emergence of *ptxP3* strains was associated with increased notifications and a shift in disease prevalence toward older age categories. Changes in diagnostic procedures may have contributed to the latter 2 phenomena (*3*). However, hospitalizations, which are less sensitive to surveillance artifacts, also increased concurrently with the emergence of *ptxP3* strains (online Appendix Figure, available from http://www.cdc.gov/EID/content/15/8/1206-appF.htm). Furthermore, an extensive analysis of surveillance data confirmed a true increase in the pertussis incidence after 1995 in the Netherlands (*3*). The expansion of *ptxP3* strains was also associated with the resurgence of pertussis in Finland, where a large nationwide epidemic was observed in 2003 (*23*).

The SNP distinguishing the *ptxP1* and *ptxP3* alleles is located in a region involved in binding of BvgA, the global regulator of virulence gene expression in *B. pertussis*. We hypothesize that the *ptxP3* allele confers increased binding of BvgA compared to *ptxP1,* resulting in increased toxin production. When compared with *ptxP1* strains, *ptxP3* strains produced 1.62 times more Ptx. In contrast, the production of another *bvg*-regulated virulence factor, Prn, was slightly suppressed in *ptxP3* strains compared with *ptxP1* strains (factor 0.94), indicating that increased Ptx production cannot be explained by a global up-regulation of virulence genes.

Table 3. Increases in illness and death caused by pertussis in 2 periods with low and high frequencies of *ptxP3* strains in the Netherlands*

Parameter	*ptxP3* frequency, %	Hospitalizations/100,000	Deaths/100,000	Lethality†
1981–1992	1.6	1.38	0.00057	0.00041
1993–2004	54.5	1.95	0.00582	0.00299
Increase	33.1	1.41 (1.34–1.49)	10.21 (1.31–79.11)	7.23 (0.93–56.07)
p value		<0.0001	0.0058	0.03

**ptxP*, pertussis toxin promoter. Numbers in parentheses are 95% confidence intervals.
†Lethality = no. of deaths / no. of hospitalizations.

The expansion of *ptxP3* strains is remarkable and suggests that *ptxP3* increases strain fitness or is linked to other genetic loci that do so. Although we cannot exclude that other loci are involved in the expansion of *ptxP3* strains, several arguments underline the role of *ptxP3*. First, the high degree of polymorphism in the *ptxP* promoter indicates positive selection. Second, the increased Ptx production observed by *ptxP3* strains provides a rationale for its emergence. It has been well established that Ptx plays a central role in immune suppression. Ptx enhances colonization of naive and immune mice by targeting macrophages and neutrophils (*24*,*25*). Ptx also suppresses antibody responses (*26*). The *ptxP3* allele was found to be associated with 2 *ptxC* alleles, *ptxC1* and *ptxC2,* which are distinguished by a silent SNP. This finding suggests that the *ptxP3* allele is found in different genetic backgrounds, which may be explained by homoplasy or horizontal gene transfer. Both possibilities suggest that *ptxP3* confers increased fitness. In most strains (98%), *ptxP3* was linked to *ptxC2*. Furthermore, genomic profiling of Dutch *B. pertussis* strains indicates that *ptxP3* strains are closely related, and are characterized by a chromosomal deletion (*27*). Thus, it is likely that, in the Netherlands, *ptxP3* strains arose mainly by clonal expansion. We are analyzing a geographically more diverse strain collection to investigate this issue further.

Ptx has been suggested to increase severity of *B. pertussis* infections because the closely related *B. parapertussis,* which does not produce Ptx, generally causes less severe infections (*28*). Furthermore, Ptx causes leukocytosis in humans by inhibiting egression of leukocytes from the vasculature, and high levels of leukocytosis are associated with an increased mortality rate in infants due to pulmonary hypertension (*29*). Thus, the invasion of *ptxP3* strains may result in increased illness and death. Consistent with this assumption, we found that the emergence of *ptxP3* strains in the Netherlands was associated with increased incidence of hospitalizations and deaths and increased lethality. A recent Swedish study also suggested that *B. pertussis* strains differ in virulence. Infection with strains with PFGE profile BpSR11 was associated with a longer duration of hospital stay (*30*). As noted above, BpSR11 strains carry the *ptxP3* allele. An association between Fim2 and increased disease severity was found in a study in the UK (*31*). In contrast, the Swedish study found no association between Fim type and virulence (*30*). Nevertheless, it is conceivable that other polymorphic loci in *B. pertussis* may also affect virulence.

An important issue is whether vaccination has selected for the *ptxP3* strains. Several lines of evidence support this contention. First, *ptxP3* strains were not found in the prevaccination era. Furthermore, although *ptxP3* strains were found in high frequencies in vaccinated populations in the 1990s, they were not detected in Senegal, where vaccination was introduced in 1987 (*32*). Several studies have provided evidence that increased host immunity may select for higher virulence. Vaccination against 2 avian viruses, the Marek disease virus, and the infectious bursal disease virus, were associated with the emergence of more virulent strains (*33*). An important role of host immunity in selecting for virulence is also suggested by the co-evolution of the myxomatosis virus and rabbits (*34*). Furthermore, immune pressure was shown to select for more virulent *Plasmodium chabaudi* parasites in mice (*35*). Based on mathematical modeling, vaccines designed to reduce pathogen growth rate and/or toxicity may result in the evolution of pathogens with higher levels of virulence (*36*).

We propose that the crucial event, which shifted the competitive balance between *ptxP1* and *ptxP3* strains, was the removal by vaccination of immunologically naive infants as the major source for transmission, selecting for strains, which are more efficiently transmitted by primed hosts. Recent studies and historical data indicate an important role of naïve infants in transmission in unvaccinated populations. In a previously unvaccinated population, infant vaccination resulted in a reduction in pertussis in the vaccinated and unvaccinated parts of the population (*37*). Furthermore, in unvaccinated populations, 60%–80% of the pertussis cases were found in children 0–5 years of age, most of whom were probably immunologically naive (*32*,*38*). In most countries infants receive their first vaccination at the age of 2 or 3 months, essentially eliminating transmission by immunologically naive hosts. In primed hosts, increased Ptx production may delay an effective immune response (*24–26*) enhancing transmission, and hence, pathogen fitness. Increased Ptx production may also be beneficial for the pathogen because the host requires higher levels of antibodies against Ptx for toxin neutralization. The antigenic divergence observed between vaccine strains and circulating strains (*8*,*9*) may act synergistically

with the *ptxP3* polymorphism by enhancing transmission by hosts primed by vaccination. Pertussis among recently vaccinated children is rare, indicating that pathogen adaptation does not play a role unless immunity has waned. Thus, we propose that waning immunity and pathogen adaptation have contributed to the resurgence of pertussis, although other factors such as increased awareness and improved diagnostics have also played a role.

The effect of pathogen adaptation on disease impact may depend on factors such as vaccine coverage and the quality of the vaccine used, which may differ between countries. A relatively weak vaccine used in the Netherlands may have exacerbated the effect of the emergence of *ptxP3* strains on disease impact (*3*). Our results underline the important role of Ptx in the transmission of *B. pertussis* and suggest that an effective way to control pertussis is the improvement of current vaccines to induce Ptx-neutralizing antibodies which persist longer. An important question is whether other childhood vaccines also select for pathogens that are more efficiently transmitted by primed hosts, resulting in increased virulence.

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Dr Mooi is a senior scientist at the National Institute of Public Health and the Environment,the Netherlands. His current interests include the molecular epidemiology and evolution of *Bordetella* spp., particularly in the context of vaccination.

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Transgenic Mice Expressing Porcine Prion Protein Resistant to Classical Scrapie but Susceptible to Sheep Bovine Spongiform Encephalopathy and Atypical Scrapie

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How susceptible pigs are to infection with sheep prions is unknown. We show, through transmission experiments in transgenic mice expressing porcine prion protein (PrP), that the susceptibility of this mouse model to bovine spongiform encephalopathy (BSE) can be enhanced after its passage in ARQ sheep, indicating that the pathogenicity of the BSE agent is modified after passage in sheep. Transgenic mice expressing porcine PrP were, nevertheless, completely resistant to infection with a broad panel of classical scrapie isolates from different sheep PrP genotypes and with different biochemical characteristics. The atypical (Nor98 like) isolate (SC-PS152) was the only scrapie isolate capable of transmission in these mice, although with a marked transmission barrier. Unexpectedly, the atypical scrapie agent appeared to undergo a strain phenotype shift upon transmission to porcine-PrP transgenic mice and acquired new strain properties, suggesting that atypical scrapie agent may exhibit different phenotypes depending on the host cellular PrP or other genetic factors.

Transmissible spongiform encephalopathies (TSEs) are infectious diseases that affect humans and several livestock species, causing fatal neurodegeneration. TSEs are linked to the conversion of cellular prion protein (PrP^{C}) to the aberrant form associated with the disease (PrP^{SC}). Sheep scrapie, the most widely known TSE (*1*), has been documented in Europe for >2 centuries and is thought to have spread to other countries worldwide throughout the 1900s (*2*). Classical scrapie is caused by a variety of prion strains that can be distinguished by their biological and biochemical features (*3*), although several so-called atypical scrapie strains that have remarkably different biochemical and transmission characteristics have been recently described (*4*,*5*). Other TSEs include bovine spongiform encephalopathy (BSE), which reached epidemic proportions in Europe at the end of the past century due to the use of animal feed containing BSE-contaminated feedstuffs (*6*). A human variant of BSE, called variant Creutzfeldt-Jacob disease (vCJD) (*7*), was discovered in 1994 and reported in 1996 as linked to the BSE epidemic in the United Kingdom and elsewhere.

No reports exist of naturally occurring TSEs in pigs. However, the experimental inoculation of pigs and transgenic mice overexpressing porcine PrP has indicated that swine are susceptible to BSE infection by the parenteral route, although with a considerable transmission barrier (*8*,*9*). The oral transmission of BSE in pigs has not been demonstrated to date.

The potential spread of BSE to animals in the human food chain such as sheep, goats, and pigs needs assessing because a risk for human infection by animals other than BSE-infected cattle cannot be excluded. Moreover,

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the use of pigs as graft donors could cause concern, given a recent report of vCJD in the recipient of a porcine dura mater graft (*10*).

The transmission barrier limits TSE infection between different species. Sheep can be experimentally infected with BSE that is not easily distinguished from some scrapie strains showing a 19-kDa atypical proteinase K–resistant PrP (PrP^{res}) unglycosylated band (*11–13*). Susceptibility and resistance to TSE infection in sheep is determined by polymorphisms at PrP amino acid positions 136, 154, and 171; sheep have the VRQ and ARQ alleles that are most susceptible to scrapie infection (*14*). Although ARQ is considered to show the highest susceptibility to BSE infection (*15*), the ARR allele was until recently thought to confer full resistance to BSE and scrapie (*16,17*). However, the successful transmission of BSE prions to ARR/ARR sheep (*18*) and the detection of natural cases of classical scrapie in sheep with the ARR/ARR genotype (*19*) have shown that this resistance is penetrable. Moreover, the identification of previously unrecognized atypical scrapie strains in sheep with various genotypes, including ARR/ARR, further supports this statement (*20*,*21*).

Although only 1 case of BSE in a goat has been confirmed, several putative field cases of BSE infection affecting goats and sheep have been detected in Europe, and the infectious properties of the resulting TSEs are not well known (*22*,*23*). In addition, a rise in scrapie outbreaks among flocks in Europe has been described; it is possible that some cases of alleged sheep scrapie could be ovine BSE. In a previous report, we demonstrated that BSE experimentally passaged in homozygous ARQ sheep showed enhanced infectivity (compared with cattle BSE) as determined in transgenic mice expressing bovine PrP protein (*24*).

Previous experiments showed that transgenic mice expressing porcine PrP (PoPrP-Tg001) can be infected with cattle BSE, but that infection is limited by a strong barrier (*8*): only some BSE inocula were able to infect PoPrP-Tg001 mice in primary transmission experiments, and when transmission occurred only a reduced percentage of the inoculated mice were affected. In the present study, we used the PoPrP-Tg001 mouse model to compare the porcine PrP transmission barrier to BSE infection before and after passage in sheep. In parallel, we also analyzed the susceptibility of PoPrP-Tg001 mice to a broad panel of scrapie isolates from different ovine PrP genotypes and with different biochemical characteristics.

Materials and Methods

Transgenic Mice

The PoPrP-Tg001 mouse line was generated and characterized as previously described (*8*). These mice express porcine PrP protein under the control of the murine PrP promoter in a murine PrP0/0 background. The animals express ≈4× the level of porcine PrP in the brain compared with the levels expressed in pig brains.

TSE Isolates

Cattle BSE

Three isolates of different origins were used: cattle-BSE1, a pool of material from 49 BSE-infected cattle brains (TSE/08/59) supplied by the Veterinary Laboratory Agency (New Haw, Addlestone, Surrey, UK); cattle-BSE2, material obtained from the brainstem of 1 cow naturally infected with BSE supplied by the same agency (RQ 225:PG1199/00); and cattle-BSE0, an isolate obtained from the brainstem of 1 cow naturally infected with BSE (case 139) supplied by Institut National de la Recherche Agronomique (INRA) (Nouzilly, France).

Sheep BSE0

Sheep BSE0 came from a pool of brainstems from 7 ARQ/ARQ sheep experimentally infected by intracerebral inoculation with the same cattle-BSE0 described above. That work was part of the project "BSE in sheep" QLRT-2001-01309 (INRA, Nouzilly, France).

Sheep Scrapie Isolates

Eight scrapie isolates of different origins and biochemical characteristics obtained from sheep with different PrP genotypes were also used in this study. These isolates were SC-UCD-99, obtained from the brainstem of an Irish ARQ/ARQ sheep naturally infected with scrapie (provided by the Veterinary Research Laboratory, Abbotstown, Ireland); SC-Langlade, obtained from the brainstem of an ARQ/ARQ sheep from France naturally infected with scrapie (provided by INRA, Toulouse, France); SC-N662-97, obtained from the brainstem of an ARQ/ARQ sheep from Spain naturally infected with scrapie; SC-JR01, obtained from the brainstem of an infected VRQ/VRQ sheep provided by J. Requena (Santiago de Compostela University, Santiago de Compostela, Spain); SC-PS13, obtained from the brainstem of an ARQ/ARQ sheep from France naturally infected with scrapie (provided by INRA, Toulouse); SC-PS48, obtained from the brainstem of a VRQ/VRQ sheep from France naturally infected with scrapie (provided by INRA, Toulouse); SC-PS83, obtained from the brainstem of a ARR/ARR sheep from France naturally infected with scrapie (provided by INRA, Toulouse [*19*]); and SC-PS152, obtained from the brainstem of a AfRQ/AfRQ sheep from France naturally infected with atypical (Nor98-like) scrapie (provided by INRA, Toulouse).

SC-UCD/99 adapted to BoPrP-Tg110 was obtained after 2 subpassages of the SC-UCD-99 isolate in BoPrP-

Tg110 mice expressing bovine PrP (*24*). For subpassages, equivalent amounts of brain homogenates from all PoPrP-Tg001 mice collected from primary passage were pooled and used as inocula. Brainstem from healthy homozygous ARQ sheep was inoculated in PoPrP-Tg001 mice as a negative control.

All inocula were prepared in sterile 5% glucose as 10% homogenates. To minimize the risk for bacterial infection, we preheated inocula for 10 min at 70ºC before inoculation.

Transmission Studies

Groups of 12–20 mice (6–7 weeks of age) were housed according to the guidelines of the Code for Methods and Welfare Considerations in Behavioural Research on Animals (Directive 86/609EC). Mice were inoculated in the right parietal lobe by using a disposable 25-gauge hypodermic needle. Twenty microliters of 10% brain homogenate, containing similar amounts of PrP^{res} (as estimated by Western blot), was delivered to each animal.

The neurologic status of the inoculated mice was assessed twice a week. The presence of 3 of 10 signs of neurologic dysfunction established diagnostic criteria (*25*) was needed to score a mouse positive for prion disease. The animals were killed for ethical reasons when progression of the disease was evident or when considered necessary due to old age (650 days), and their brains were harvested for subsequent biochemical and histologic analysis.

PrP^{res} Assay

Frozen brain tissue samples from mice were homogenized in 5% glucose in distilled water by using grinding tubes (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 10% (wt/vol) with TeSeE Precess 48 Ribolyser OGER (Bio-Rad) according to the manufacturer's instructions. Brain samples were analyzed by using the TeSeE Western Blot 355 1169 kit (Bio-Rad) but with some adjustments for the different amount of sample used. To arrive at the volume proposed in the manufacturer's recommendations, 100 μL of the 10% brain homogenates to be tested was supplemented with 100 μL of 10% brain homogenate from PrP null mice (*26*). Processed samples were loaded on Criterion 12% acrylamide gels (165.6001; Bio-Rad) and electrotransferred to immobilon membranes (IPVH 000 10; Millipore, Billerica, MA, USA). For the immunoblotting experiments, Sha31 (*27*) and 12B2 (*28*) monoclonal antibodies (MAbs) were used at concentrations of 1 μg/mL. Immunocomplexes were detected by horseradish peroxidase–conjugated anti-mouse immunoglobulin G (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunoreactivity was visualized by chemiluminescence (Amersham Pharmacia Biotech).

Lesion Profiles and Paraffin-embedded Tissue Blots

All procedures involving mouse brains were performed as previously described (29). Briefly, samples were fixed in neutral-buffered 10% formalin (4% formaldehyde) before paraffin embedding. Once deparaffinated, 2 μm–thick tissue sections were stained with hematoxylin and eosin. Lesion profiles were established according to the standard method described by Fraser and Dickinson (*30*). For paraffin-embedded tissue (PET) blots, the protocol described by Andréoletti et al. (*31*) was used.

Results

Biochemical Properties of TSE Isolates

Samples of each TSE isolate were processed and analyzed by Western blotting. As shown in Figure 1, PrP^{res} in sheep BSE showed the characteristic unglycosylated band of 19 kDa (*32*); when compared with the original cattle BSE0 isolate, only slight differences could be observed in terms of the electrophoretic mobility of the PrP^{res}, probably due to PrP amino acid sequence differences. ARQ/ARQ isolates SC-UCD/99, SC-Langlade, and SC-662/97 showed a PrP^{res} unglycosylated band of 21 kDa, and isolate SC-PS13 from the same sheep PrP genotype showed a smaller band of 20 kDa. The SC-JR01 and SC-PS48 isolates from a VRQ/VRQ sheep showed unglycosylated bands of 21 kDa and 19 kDa, respectively. An unglycosylated band of 21 kDa was detected in the ARR/ARR SC-PS83 isolate. Finally, the SC-PS152 isolate, whose genotype is widely associated with Nor98 cases (*33*,*34*), showed the characteristic band pattern of the atypical (Nor98-like) scrapies (*4*,*35*).

All scrapie isolates showing a 20–21-kDa unglycosylated band and the atypical SC-PS152 isolate were recognized by the Sha31 antibody (Figure 1, panel A) and the 12B2 antibody (Figure 1, panel B), which probe

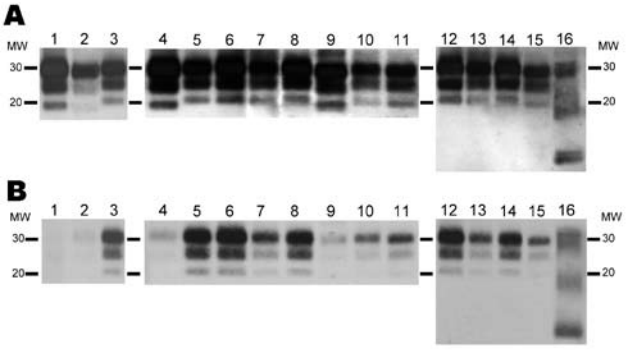

Figure 1. Electrophoretic profiles and antibody labeling of atypical proteinase K–resistant prion protein (PrP^{res}) detected with monoclonal antibodies Sha31 (A) and 12B2 (B) in different isolates used for inoculating porcine PrP transgenic mice. Panels A and B were loaded with the same quantities of extracted PrP^{res} from each sample. MW, molecular mass in kilodaltons.

the WGQGG epitope (amino acids 93–97 of sheep PrP). However, SC-PS48 and sheep-BSE (showing a 19-kDa unglycosylated band) were poorly recognized by 12B2 MAb (Figure 1, panel B), suggesting that the 12B2 epitope is not protected against digestion with proteinase K in these isolates, as in cattle BSE.

Susceptibility of PoPrP-Tg001 Mice to TSE Isolates

To evaluate the susceptibility of PoPrP-Tg001 mice to ARQ sheep BSE as opposed to the original cattle BSE, the BSE agent was inoculated in parallel before and after passage in ARQ/ARQ sheep in these mice. As shown in the Table, all PoPrP-Tg001 mice survived the cattle BSE0 infection and were culled without clinical signs at 650 days postinoculation (dpi), but when assessed for the presence of PrP^{res} in the brain, 3 (19%) were positive. In the second passage, all mice died at 197 ± 4 dpi. Similar results were obtained when 2 other BSE inocula (cattle BSE1 and cattle BSE2) were used (Table). In contrast, in PoPrP-Tg001 mice inoculated with BSE passaged in ARQ sheep (sheep BSE), an attack rate of 100% and survival time of 458 ± 11 dpi were observed, indicating that the PoPrP-Tg001 mice were fully susceptible to sheep BSE. Secondary subpassage in these mice led to a considerable reduction in the survival time (162 ± 4 dpi), which was maintained in subsequent subpassages. These results suggest the increased infectivity of BSE after passage in sheep in the PoPrP-Tg001 mouse model.

To evaluate the susceptibility of PoPrP-Tg001 mice to other sheep TSEs, we inoculated these mice with a panel of sheep scrapie isolates from different genotypes and with different strain properties. As shown in the Table, the atypical scrapie SC-PS152 isolate was the only one able to infect PoPrP-Tg001 mice at a low attack rate (16%) and survival time of 300 to 600 dpi. Secondary passage rendered a 100% attack rate and survival time of 162 ± 13 dpi. The other scrapie isolates included in the panel were not transmitted in the PoPrP-Tg001 mice either in primary or subsequent passages. We verified that the infectivity of the different scrapie isolate used in this work was sufficiently high for efficient transmission in transgenic mice expressing ovine PrP (data not shown).

PoPrP-Tg001 mice inoculated with healthy homozygous ARQ sheep brain material as controls were also euthanized after 600 dpi without showing clinical signs after first and second passages. None were positive for PrP^{res} in their brains.

Biochemical Characterization of PrP^{res} in Inoculated PoPrP-Tg001 Mice

PrP^{res} from BSE adapted to porcine PrP in PoPrP-Tg001 mice showed a different glycoprofile than the original inoculated BSE (Figure 2, panel C), although it preserved biochemical properties such as electrophoretic mobility (Figure 2, panel A) and lack of immunoreactivity to the 12B2 MAb (Figure 2, panel B). The 12B2 MAb is able to recognize porcine PrP^{C} as confirmed by Western blotting when samples not treated with proteinase K are used (data not shown).

In PoPrP-Tg001 mice, cattle BSE and sheep BSE agents produced identical PrP^{res} signatures and shared similar PrP^{res} biochemical properties (Figure 2, panel A). These features persisted after subsequent passages (data not shown).

In contrast, Western blot analysis of the porcine prion generated through the inoculation of atypical scrapie isolate (SC-PS152) showed a dramatic molecular shift after

Table. Transmission of cattle BSE, sheep BSE, and sheep scrapie isolates in transgenic mice expressing porcine prion protein*

			First passage		Second passage		Third passage	
TSE isolate	Genotype	PrP^{res}, kDa	Survival time, dpi	% (Attack rate)	Survival time, dpi	% (Attack rate)	Survival time, dpi	% (Attack rate)
Cattle-BSE1	–	19	>650	0 (0/12)†	269 ± 3	100 (10/10)	204 ± 12	100 (9/9)
Cattle-BSE2	–	19	498 ± 9	17 (2/12)†	198 ± 6	100 (15/15)	193 ± 17	100 (6/6)
Cattle-BSE0	–	19	>650	19 (3/16)†	197 ± 4	100 (12/12)	190 ± 10	100 (7/7)
Sheep-BSE0	ARQ/ARQ	19	458 ± 11	100 (15/15)	162 ± 4	100 (13/13)	166 ± 7	100 (7/7)
SC-662/97	ARQ/ARQ	21	>650	0 (0/10)	>650	0 (0/12)	ND	ND
SC-UCD/99	ARQ/ARQ	21	>650	0 (0/12)	>650	0 (0/9)	ND	ND
SC-Langlade	ARQ/ARQ	21	>650	0 (0/12)	>650	0 (0/12)	ND	ND
SC-PS13	ARQ/ARQ	20	>650	0 (0/12)	>650	0 (0/12)	ND	ND
SC-JR01	VRQ/VRQ	21	>650	0 (0/12)	>650	0 (0/12)	ND	ND
SC-PS83	ARR/ARR	21	>650	0 (0/12)	>650	0 (0/12)	ND	ND
SC-PS48	VRQ/VRQ	19	>650	0 (0/9)	>650	0 (0/10)	ND	ND
SC-PS152	AfRQ/AfRQ	≈7–14	300–600	16 (2/12)	162 ± 13	100 (9/9)	172 ± 16	100% (7/7)
SC-UCD/99 adapted to BoPrP-Tg110	—		>650	0 (0/13)	>650	0 (0/10)	ND	ND
Healthy sheep brain	ARQ/ARQ		>650	0 (0/14)	>650	0 (0/9)	ND	ND

*BSE, bovine spongiform encephalopathy; TSE, transmissible spongiform encephalopathy; PrP^{res}, atypical proteinase K–resistant prion protein; dpi, days postinoculation; ND, not determined.
†No. animals positive for the aberrant form of PrP associated with disease / no. inoculated animals.

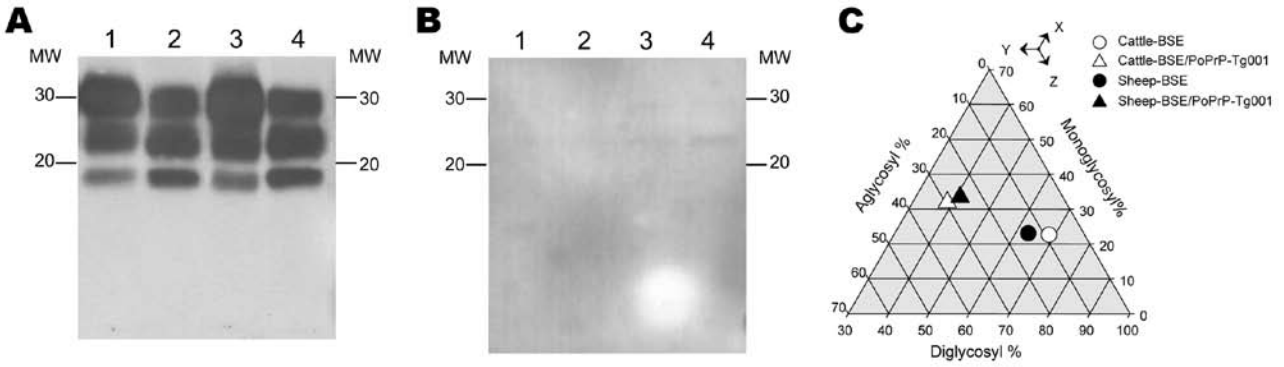

Figure 2. Brain atypical proteinase K–resistant prion protein (PrP^{res}) of porcine PrP transgenic mice infected with cattle bovine spongiform encephalopathy (BSE) (lane 2) or sheep BSE agents (lane 4). Electrophoretic profiles and antibody labeling of PrP^{res} detected with monoclonal antibodies Sha31 (A) or 12B2 (B). Profiles produced by cattle (lane 1) and sheep BSE (lane 3) before passage in the porcine mouse model are shown for comparison. MW, molecular mass in kilodaltons. C) Triangular plot of the glycosyl fractions of PrP^{res} after proteinase K digestion and Western blotting using the Sha31 antibody. Data shown are the means of 5 or more measurements obtained from density scans in 2 or more Western blots. To interpret the plot, read the values for the diglycosyl, monoglycosyl, and aglycosyl fractions along the bottom, right and left axes of the triangle, respectively. For each point, the sum of the 3 values is 100.

passage in the porcine PrP mouse model (Figure 3). A 3-band PrP^{res} pattern with an unglycosylated band of 19 kDa, which differed substantially from the molecular signature of atypical scrapie PrP^{res}, was observed Moreover, this porcine prion was indistinguishable from the porcine prion generated by inoculation of sheep BSE in terms of PrP^{res} electrophoretic mobility (Figure 3, panel A), 12B2 MAb immunoreactivity (Figure 3, panel B), or its glycoprofile (Figure 3, panel C), characteristics that were maintained in subsequent passages in the same mouse model.

Lesion Profile and PrPSc Deposition Pattern in Inoculated PoPrP-Tg001 Mice

Brain material from PoPrP-Tg001 mice inoculated with the BSE-infected brains of either cattle or sheep showed consistent similarities in lesion profiles and PrP^{Sc} deposition patterns in PET blots, although some differences could be observed, mainly in areas G7 and G9. These features persisted after a second passage in PoPrP-Tg001 mice (Figure 4). PrP^{Sc} was also detected in the spleens of PoPrP-Tg001 mice inoculated with either cattle BSE or sheep BSE in first and second passages (data not shown). In mice inoculated with the different classical scrapie isolates, no typical brain lesions associated with prion infection were detected. We observed no differences between animals inoculated with classical scrapie isolates and those inoculated with brain tissue from healthy homozygous ARQ sheep or noninoculated PoPrP-Tg001 mice (data not shown).

Although some similarities were observed between the brains of PoPrP-Tg001 inoculated with SC-PS152 and the

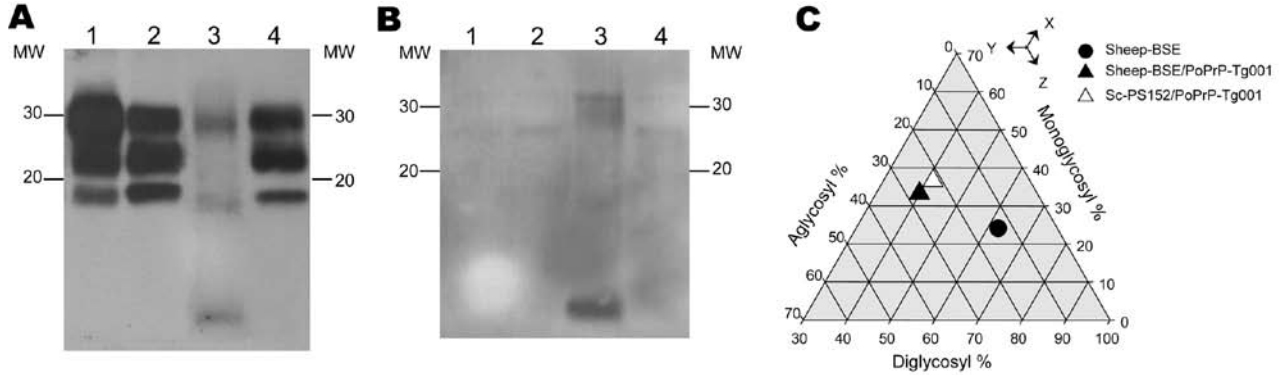

Figure 3. Brain atypical proteinase K–resistant prion protein (PrP^{res}) of porcine PrP transgenic mice infected with an atypical scrapie (SC-PS152) agent (lane 4) versus sheep bovine spongiform encephalopathy (Sheep-BSE) agent (lane 2). Electrophoretic profiles and antibody labeling of PrP^{res} detected with monoclonal antibodies Sha31 (A) or 12B2 (B). Profiles produced by atypical scrapie (SCPS152) (lane 3) and sheep-BSE (lane 1) before passage in the porcine mouse model are shown for comparison. MW, molecular mass in kilodaltons. C) Triangular plot of the glycosyl fractions of PrPres after proteinase K digestion and Western blotting using the Sha31 antibody. Data shown are the means of $\geq$5 measurements obtained from density scans in $\geq$2 Western blots. To interpret the plot, read the values for the diglycosyl, monoglycosyl, and aglycosyl fractions along the bottom, right and left axes of the triangle, respectively. For each point, the sum of the 3 values is 100.

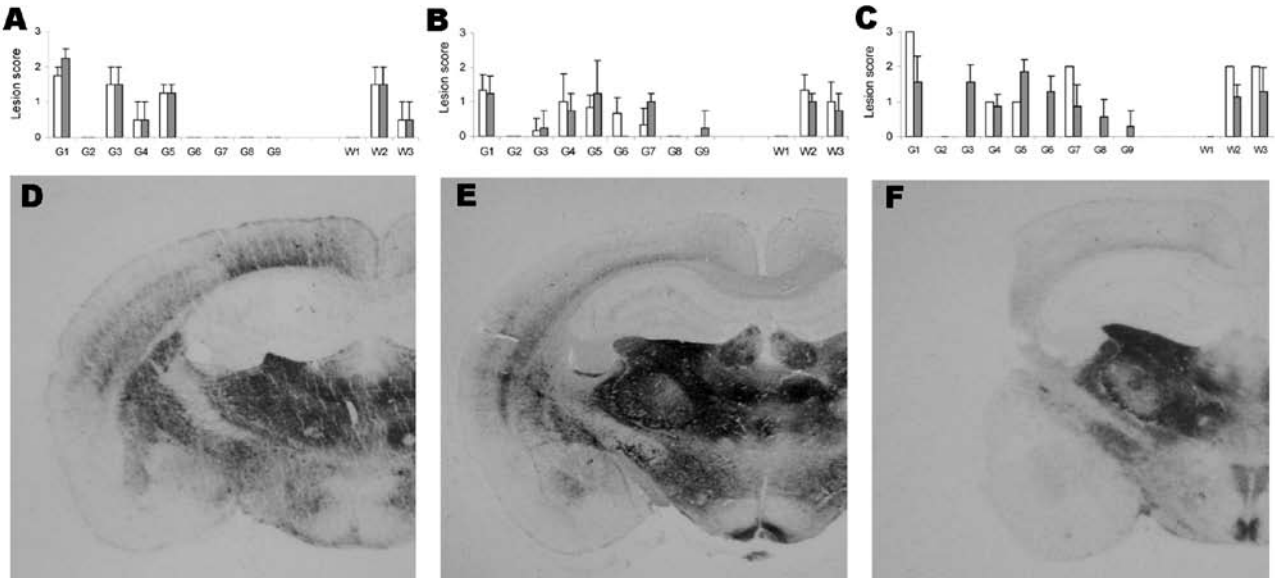

Figure 4. Lesion profiles and regional distributions of atypical proteinase K–resistant prion protein (PrP^{res}) in the brain of porcine PrP transgenic mice infected, either in 1st passage (white column) or in 2nd passage (black column) with cattle bovine spongiform encephalopathy (BSE) (panels A and D), sheep BSE (panels B and E), or atypical scrapie (panels C and F) agents. A–C) Lesion scoring of 9 areas of gray matter (G) and white matter (W) in mice brains: dorsal medulla (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), medial thalamus (G5), hippocampus (G6), septum (G7), medial cerebral cortex at the level of the thalamus (G8) and at the level of the septum (G9), cerebellum (W1), mesencephalic tegmentum (W2) and pyramidal tract (W3). Error bars indicate SE. D–F) Histoblots of representative coronal sections at the level of the hippocampus.

brains of those inoculated with cattle BSE and sheepBSE, some differences could be observed mainly in region G6 and G8 (Figure 4, panels A–C). Moreover, PrP^{SC} distributions in the PET blots showed some differences when compared with those observed in samples from both cattle and sheep BSE–inoculated mice (Figure 4, panels D–F). These differences mainly appeared in the cortex, the medial pretectal nucleus, the posterior commissure, the zona incerta and hypothalamic lateral area in which PrP^{SC} deposition was more intense in cattle and sheep BSE–inoculated mice than in the SC-PS152–inoculated animals.

Discussion

In this study, transgenic mice expressing porcine PrP (*8*) were used to assess the transmission capacity of a wide range of TSE agents from sheep. Our results indicated that none of the classical scrapie isolates tested was transmitted to our porcine PrP mouse model after intracerebral inoculation (Table), suggesting a highly (if not completely) resistance to the classical scrapie strains tested independently of their origin and biochemical signature. The absence of successful transmission of the SC-PS48 isolates with an unglycosylated bands of 19 kDa-like BSE suggests a BSE-unrelated origin for these BSE-like scrapie strains.

The atypical isolate SC-PS152 was the only scrapie isolate able to infect the Po-PrP mouse model after intracerebral inoculation (Table), albeit with a low efficiency of infection in the first passage (attack rate 16%). These results suggest the potential ability of atypical scrapie prions to infect pigs, although with a strong transmission barrier. Given the increasing number of atypical scrapie cases found in Europe and in North America, the potential ability of atypical scrapie to adapt to the pig becoming more easily transmitted could raise concerns about the potential danger of feeding ruminant meat and bone meal to swine.

In our transmission experiments, an obviously shorter survival period (458 ± 11 dpi) and an increased attack rate (100%) were observed in PoPrP-Tg001 mice inoculated with sheep BSE (Table) compared with those inoculated with the original cattle BSE (>650 dpi, 19%). These last figures correlate well with those reported for other cattle BSE isolates (Table). Differences in survival times were maintained after subsequent passages in this mouse model (Table), suggesting that the increased infectivity of sheep BSE cannot be linked to a higher infectious titer in the initial inoculum but must be the outcome of a modification in the pathogenicity of the agent. We can also rule out that the primary amino acid sequence of the ovine PrP^{SC} leads to more efficient conversion of porcine PrP^{C} because scrapie isolates from sheep with the same ARQ-PrP genotype were not able to infect these mice (Table). Taken together, the increased infectivity of sheep BSE in the por-

cine PrP mouse model must be considered as increased pathogenicity of the agent attributable to its passage in sheep. These features support previous results indicating that the BSE agent modifies its biological properties after passage in sheep, with the result that its pathogenicity increases in transgenic mice expressing bovine PrP (*24*). An increased pathogenicity of ovine BSE was also reported in conventional RIII mice when compared with retrospective cattle BSE experiments (*36*). In other prion strains, passage through an intermediate species has also been noted to alter host susceptibility (*37*).

The enhanced infectivity of the BSE agent after its passage in ARQ sheep raises concern about its potential danger for other species, including humans. This question, as well as others related to the infectivity of the new porcine prion generated in this study, is currently being addressed in transmission experiments using transgenic mice expressing human PrP.

Upon passages in porcine PrP transgenic mice, the BSE agent retained most of its biochemical properties, except for its PrP^{res} glycoprofile in which some differences were appreciable. Our comparative analysis of cattle BSE and sheep BSE upon transmission in porcine PrP transgenic mice showed that both agents exhibit similar molecular (Figure 2) and neuropathologic properties (Figure 4). These features were preserved after subsequent passages. These results suggest that, despite their modified pathogenicity, the 2 porcine prions generated share the same biochemical and neuropathologic properties, regardless of whether the BSE agent used to inoculate the mice was obtained from ARQ sheep or cows. In agreement with these results, the increased infectivity of sheep BSE previously observed upon transmission in bovine PrP transgenic mice was not reflected in its molecular or neuropathologic properties (*24*).

The atypical scrapie (SC-PS152) agent appeared to undergo a strain phenotype shift upon transmission to porcine PrP transgenic mice. Surprisingly, this novel strain phenotype was similar to that of sheep BSE propagated in the same mice in terms of several features: 1) survival times observed after stabilization in PoPrP-Tg001 mice (second passages) were similar (Table); 2) PrP^{res} molecular profiles of the 2 agents in porcine PrP mice were indistinguishable (Figure 3); and 3) vacuolation profiles observed in second passages largely overlapped (Figure 4).

These findings could reflect the evolutionary potential of prion agents upon transmission to a foreign host able to promote strain shift and emergence of new properties (*38,39*). The converging molecular, neuropathologic, and biological properties of atypical scrapie and sheep BSE upon propagation in porcine transgenic mice could be the consequence of a restriction imposed by the porcine PrP^{C}, which might only admit a few options as it changes its conformation to PrP^{SC}.

Our results could also suggest a common origin for sheep BSE and atypical scrapie agents, which may exhibit different phenotypes depending on the host PrP^{C} or other host factors. Although this last explanation seems to be less likely, so far we cannot draw any definitive conclusion on this issue. Whichever the case, the ability of an atypical scrapie to infect other species and its potential capacity to undergo a strain phenotype shift in the new host prompts new concerns about the possible spread of this uncommon TSE in other species as a masked prion undistinguishable from other strains.

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Slave Trade and Hepatitis B Virus Genotypes and Subgenotypes in Haiti and Africa

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In Haiti, >90% of the population descended from African slaves. Of 7,147 Haitian pregnant women sampled, 44% of hepatitis B virus (HBV) infections were caused by genotype A1, which today is found mainly in eastern Africa. Twenty percent belong to a rare subgenotype, A5, which has been found only in the former Bight of Benin, a former primary slave trading post. Haitian A subgenotypes appear to have separated early from the African subgenotypes; the most prevalent genotype and subgenotype in West Africa today (E and A3, respectively) are rare in Haiti. This difference indicates that the dominant subgenotypes in Africa emerged in the general population only after the slave trade and explains the low genetic diversity of genotype E. The high prevalence of HBV genotype E in much of Africa further suggests that HBV hyperendemicity is a recent phenomenon, probably resulting from extensive use of unsafe needles.

Because of a viral polymerase that lacks proofreading activity (*1*), hepatitis B virus (HBV) has evolved into at least 8 recognized genotypes, A–H (*2–4*), and a potential new genotype (tentatively designated genotype I) found mainly in Laos (*5*,*6*) but also in Vietnam (*7*). Except for genotypes E, G, and H, genotypes can be further divided into a variety of subgenotypes, sometimes with more or less geographic distribution. Genotype D strains are found almost worldwide (*8*), but subgenotype D1 occurs mostly in the Mediterranean and Middle East but also in Europe. D2 has been reported in India, Japan, Europe, and the United States; D3, mainly in South Africa and Brazil but also in Rwanda, Costa Rica, the United States, and Europe (*8–11*); and D4, in Australia, South Africa, Somalia, Rwanda, and Oceania (*8–10*).

In sub-Saharan Africa, genotypes E and A predominate. East of the E/A divide (*9*), subgenotype A1 is dominant in countries along the eastern coast from South Africa to the Horn of Africa (*12*). Although genotype A has been found on every continent, its genetic diversity is higher in Africa (4% over the complete genome) than in the rest of the world (3%). Five subtypes of HBV/A (A1–A5) have been proposed in Africa (*13*), whereas essentially only A2 and, to a lesser extent, A1 have been reported from other continents (*14*). Therefore, some researchers have suggested that genotype A has emerged in Africa (*15*) and, after a long evolution, has been introduced to other continents. However, despite the high genetic diversity of HBV/A in West Africa, this genotype is rare there. In contrast, genotype E has been found only in Africa, with some rare exceptions on other continents in persons with a link to Africans. Genotype E is found almost exclusively throughout the vast expanses of a crescent from Senegal in the west (*16*) to the Central African Republic in the east (*17*) and Namibia in the south (*13*). In comparison to HBV/A, the conspicuously low genetic diversity of HBV/E suggests its short natural history in Africa (*18*) and relatively recent introduction into the general population there (*18*). However, the recent presence of HBV/E in Africa contrasts sharply with its current high prevalence and extensive geographic distribution there. The wide spread of genotype E also seems difficult to reconcile with a long natural history of genotype A in Africa (*18*).

In Haiti, where >90% of the population descends directly from African slaves (*19*), we investigated the phylogeny of HBV to learn which genotypes may have been

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prevalent in Africa several centuries ago. The conspicuous absence of genotype E in Haiti suggests recent and rapid spread of genotype E in Africa during the past 200 years, probably as the result of public health interventions.

Materials and Methods

Serum samples were collected in 2006 after informed consent as part of a national survey to evaluate prevalences of human immunodeficiency virus infection, hepatitis B, and serologic syphilis among pregnant women at their first prenatal medical visit in 19 clinics throughout Haiti. Women were tested for hepatitis B surface antigen (HBsAg) by using the Murex HBsAg Kit (Abbott Laboratories, Ottiginies, Belgium). DNA was extracted from HBsAg–positive samples by using the QIAGEN DNA Blood Mini kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer's protocol. The complete HBV genome was amplified in 4 overlapping fragments (preS, S, X, and C) as described previously (*20*). Phylogenetic analysis and distance calculations were performed by using MEGA v.4 (*21*) with the neighbor-joining method of the Kimura 2-parameter model with 1,000 bootstrap replicates. Genotyping was performed by analyzing the complete genome or at least 1 of the 3 fragments of preS, S, or C genes. Subgenotyping was done on the full-length genome or on at least 2 complete fragments preS, S, or C. Sequences were submitted to EMBL/GenBank/DDBJ under accession nos. FJ692502–FJ692553 (Haiti S-fragment sequences), FJ692557–FJ692613 (Haiti complete genome sequences), and FJ692554–FJ692556 (Nigeria complete A5 sequences).

Results

Genotypes and Subgenotypes

In 7,147 blood samples of pregnant Haitian women, HBsAg prevalence was 5%, ranging from 1.0% to 8.5%, depending on the sampling clinic. Of 320 HBsAg–positive samples available, 247 (77.2%) were positive for at least 1 of the 4 overlapping PCR fragments (Table 1). Interpretable sequences from at least 1 of the 4 PCR fragments were obtained from 213 viruses. A total of 179 of these strains could be clearly assigned to a genotype by analyzing the complete genome or at least 1 of the 3 fragments of preS, S, or C genes. Of the 213 strains, 31 showed signs of mixed infection or recombination, and 3 strains were considered outliers because they could not be genotyped.

Phylogenetic analysis of the above 179 genotypeable strains (excluding mixed, recombinant, and untypeable strains) showed that 128 (71.5%) viruses belonged to genotype A; 40 (22.4%), to genotype D; and 11 (6.1%), to genotype E (Table 2). Genotype A strains were attributable to subgenotypes A1 (n = 77 [43.0%]) and A5 (n = 35 [19.6%]). Genotype D strains belonged to D4 (16.2%) and D3 (3.9%). Fifteen viruses of genotype A and 4 of genotype D could not be further subgenotyped (Table 2) because only partial gene sequences or single preS, S, or C fragments were obtained. In all of the above strains, genotypes of the different fragments agreed with each other. In addition, 31 viruses were suspected mixed genotype infections or recombinants; they were not included in the above analysis and are discussed later.

Table 1. Number of serum samples investigated, including suspected mixed and recombinant strains of HBV, Haiti*

Samples	No. samples (no. partial sequences)
No. serum samples (HBsAg positive) available	320
No. serum samples PCR positive	247
No. serum samples for which sequences were obtained†	182
Full-length genome	68
Full preS fragment	57 (37)
Full S fragment	67 (19)
Full X fragment	67 (20)
Full C fragment	20 (34)
No. serum samples of suspected mixed strains	25
Full-length genome	10
Full preS fragment	8 (3)
Full S fragment	12 (2)
Full X fragment	10 (2)
Full C fragment	3 (3)
No. serum samples of suspected recombinant strains	6
preS fragment	3
S fragment	–
X fragment	3
C fragment	–

*HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.
†No. serum samples includes 3 outliers and excludes mixed or recombinant strains.

Table 2. Prevalence of HBV genotypes and subgenotypes, excluding mixed or recombinant strains and untypeables, Haiti*

Genotype or subgenotype	No. (complete genomes; partial strains) of genotypeable or subgenotypeable strains	Genotypeable or subgenotypeable strains, %
A	128 (63; 65)	71.5
A1	77 (36; 41)	43.0
A2	1 (1; 0)	0.6
A5	35 (21; 14)	19.6
D	40 (5; 35)	22.4
D3	7 (2; 5)	3.9
D4	29 (3; 26)	16.2
E	11 (1; 10)	6.1
Total	179 (69; 110)	100

*HBV, hepatitis B virus.

Phylogenetic Analysis

Subgenotype A1

Phylogenetic analysis of A1 complete genome sequences showed that Haiti strains form several clusters (not necessarily supported by bootstrap values) within available full-length A1 strains (online Appendix Figure 1, available from www.cdc.gov/EID/content/15/8/1222-appF1.htm) from South Africa and other eastern African countries, as well as from the Philippines. Haiti's complete genome A1 strains showed a mean genetic diversity of 1.45% (maximum diversity of 3.86%) that rose to a mean genetic diversity of 2.49% (maximum 6.61% between FJ692589 and U87742) when all available A1 strains (mean 2.87%, maximum 7.64% between AY161140 and U87742) were included.

Subgenotype A5

A5 originally was proposed on the basis of the preS and preC/C gene fragments of 3 Nigerian strains (*20*). We present here the full-length sequences of the latter strains (accession nos. FJ692554–FJ692556) and compare them with all full-length A sequences from Haiti. Twenty-one sequences clustered with the only available A5 sequences from Nigeria. The overall mean intrasubgenotype diversity of A5 is 1.42% (maximum genetic diversity 2.89%). The mean intersubgenotype distance of the proposed A5 subgenotype was above the approximately 4% proposed for a new subgenotype (*3*,*22*) for subgenotypes A1 (4.1%), A2 (4.8%), and A3 (5.1%); it was 3.8% when compared with the previously proposed subgenotype A4 (*20*). Nevertheless, A5 strains from Haiti and Nigeria form 2 distinct phylogenetic subgroups within A5, supported by high bootstrap values (99%; Figure 1). These subgroups are separated by a mean, minimal, and maximum genetic distance of 2.28%, 1.71%, and 2.89%, respectively.

Genotype D

Because of low numbers of complete genome sequences available for genotype D in Haiti, we analyzed this genotype on the S fragment. In Haiti, most D strains belonged to D4 (29/179 [16.2%]). Besides a small cluster of D4 sequences from Australia and Papua New Guinea, a few

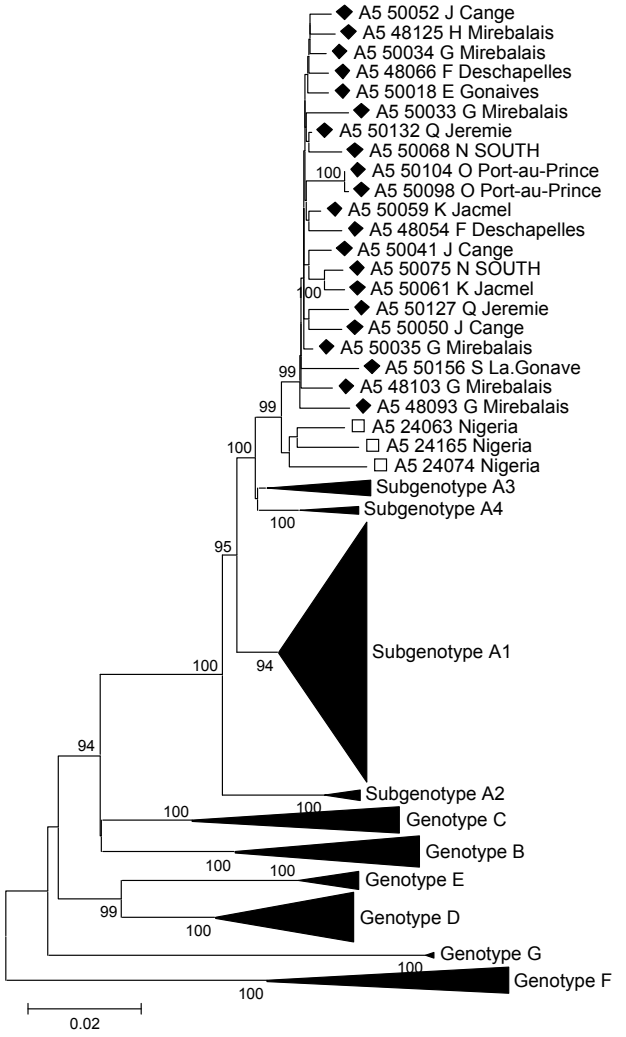

Figure 1. Phylogenetic analysis of selected sequences clustering with subgenotype A5, based on the complete genome. Diamonds indicate Haiti sequences; squares indicate Nigeria A5 strains. All complete A5 sequences available in GenBank are included. Scale bar indicates nucleotide substitutions per site.

D4 sequences from Rwanda (accession nos. FM200194, FM200212, and FM200213) (*9*) and single D4 sequences from Spain and France are available in GenBank. D4 sequences from Haiti were closely related to those from the latter 3 countries and somewhat separate from the Australian cluster (Figure 2, panel A). Despite the relatively high prevalence of D4 throughout most of Haiti, the mean genetic diversity of D4 S fragment sequences was only 0.39% (maximum diversity of 2.18%).

D3 (7/179, 3.9%) was less frequently found in Haiti than D4. Sequences seemed to form small geographic and genetic clusters, 1 of which most closely resembled strains from Rwanda (GenBank accession nos. FM200190, FM200191, FM200197, and FM200205) (*9*), but all D3 strains in Haiti were interspersed among strains from Brazil (Figure 2, panel B). Haiti D3 S fragment sequences showed a mean genetic diversity of 0.74% (maximum diversity of 1.44%).

Genotype E

The prevalence of genotype E sequences was surprisingly low, with only 11 (6.1%) of the 179 subtypeable strains being classified as this genotype. Available S-fragment sequences clustered with African HBV/E strains and were interspersed as individual strains among those HBV/E strains (online Appendix Figure 2, available from www.cdc.gov/EID/content/15/8/1222-appF2.htm). The mean and maximum genetic diversity of the Haiti S fragments were 0.76% and 1.93%, compared with 0.74% and 4.66% of all African genotype E strains.

Mixed Infections and Recombinations

We suspected mixed infections in 25 samples either because of at least 5 divergent nucleotides corresponding to discrepant (sub)genotypes in at least 1 overlapping region of the PCR fragments or because of divergent nucleotides within the fragments after additional PCR analyses**.** Mixed infections included all genotypes in Haiti, as well as 1 B4 (S-fragment) and 1 C (X-fragment) sequence. Six other strains showed possible recombinations within the preS fragment or the X fragment. PreS-fragment recombinants were based on HBV/E and HBV/A, whereas those in the X fragment emanated from HBV/G in recombination with genotypes D or A. One of these strains also showed signs of mixed infection. Although sequences were relatively short, recombination breakpoints seemed to be located around nucleotide 800 on the X gene; the location varied in the preS gene (nucleotide 330, 640, or 870).

Discussion

More than 90% of today's Haitian population is descended directly from African slaves (*19*) exported from the late 17th century through the early 19th century (*23*). Because vertical transmission and household transmission during early childhood are important routes of infection and are associated with excess risk for chronic disease, HBV is transmitted between generations (*24*,*25*). Thus, HBV strains in Haiti may to some extent reflect strains that were prevalent in Africa several centuries ago.

Subgenotype A1

Forty-three percent of African HBV sequences belong to subgenotype A1, the main African A subgenotype. This subgenotype was found in most eastern African countries (*13*) and dominated in this region for which larger sets of HBV strains have been characterized, including Somalia, Kenya (*13*), Rwanda (*9*), and South Africa (*13*). Subgeno-

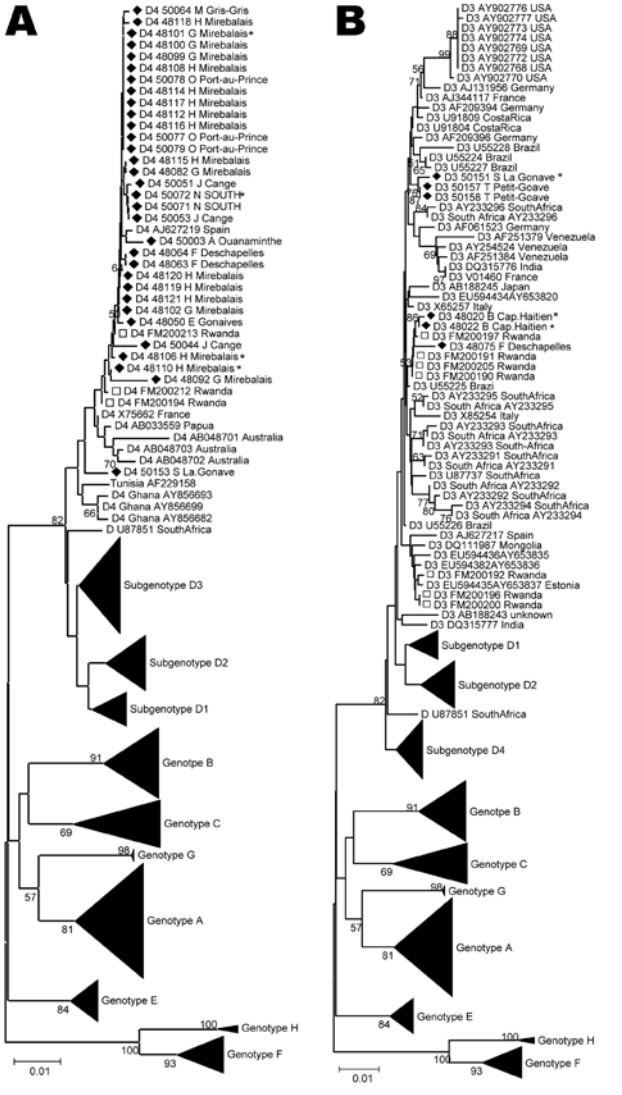

Figure 2. Phylogenetic analysis of selected sequences clustering with subgenotype D4 (A) or D3 (B), based on the S fragment, including potential mixed or recombinant strains (*). Diamonds indicate Haiti sequences; squares indicate Rwanda strains. Scale bar indicates nucleotide substitutions per site.

type A1 is essentially absent from West Africa (i.e., west of the African E/A1 genotype divide) (*9*) and from other continents. During the peak of Haiti's slave importation during the late 18th century, almost 60% of captives came from southeastern and central Africa (*26*,*27*) (Figure 3). Complete Haitian A1 strains (36/77 A1 strains) formed several small clusters within A1, at least the largest of which is supported by a bootstrap value of 95%. Thus, the phylogeny is highly suggestive of multiple early introductions into Haiti of distinct A1 strains from eastern Africa that continued to spread in Haiti's population.

A5, a New Subgenotype

One third of HBV/A strains clustered with a group of rare strains that have been found only in southwestern Nigeria (*20*). Because only preS and preC/C sequences had been available, these strains had only provisionally been assigned to a new subgenotype, A5, until full-length sequences would become available. Complete A5 genome sequences from Haiti (n = 21) and Nigeria (n = 3) presented in this study showed a mean intrasubgenotype diversity of 1.42%. The mean intersubgenotype distances of A5 are above the approximately 4% of the definition of a subgenotype (*3*) for subgenotypes A1 (4.1%), A2 (4.8%), and A3 (5.1%). Compared with the proposed subgenotype A4 (3.8%), the mean intersubgenotype diversity is only slightly <4%. Thus, together with high bootstrap support (99%), these strains fulfill the formal definition, proposed by Kramvis et al. (*13*), of a new subgenotype A5.

A5 has been found only in southwestern Nigeria, the former Bight of Benin, where the first wave of slaves brought to Haiti originated (*27*). Interestingly, all A5 sequences from Haiti clustered together, but somewhat separately (bootstrap support of 99%) from those from Nigeria, suggesting early evolutionary separation of the 2 clusters. This distinct clustering further corroborates early introduction of these strains to Haiti during the slave trade. A5 strains in Haiti were considerably more homogenous than A1 strains, possibly reflecting their geographic confinement to and homogeneity in Africa.

Compared with subgenotype A5, A1 showed only a slightly higher mean genetic distance between African and Haitian strains (2.62% for A1; 2.28% for A5). However, when each Haitian A1 group is considered as a separate and independent introduction, the mean genetic diversity of these groups is only 0.8%–1.6% (for the different groups), indicating more recent introduction of these strains into Haiti.

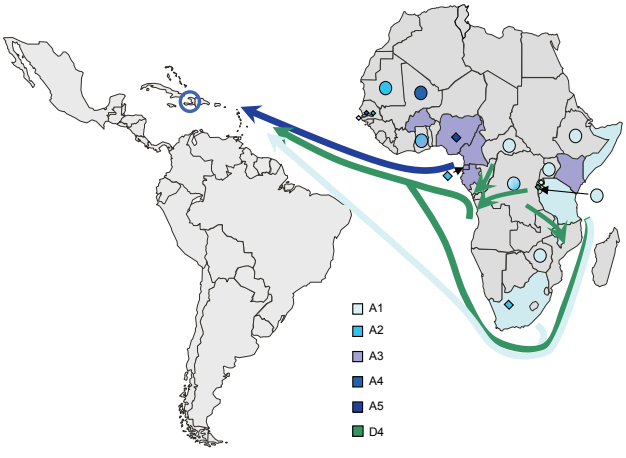

Figure 3. Distribution of hepatitis B virus A subgenotypes and D4 (only in Rwanda) in Africa and their potential routes of spread toward Haiti (color-coded arrows). Colored dots indicate African countries with ≤10 A strains available; full color indicates countries with >90% dominance of 1 subgenotype; or a 60%–90% predominance of 1 subgenotype, with minority subgenotypes shown as diamonds. Subgenotypes other than A1 and D4 are not shown for Rwanda. Sequences included were obtained from GenBank and unpublished data.

Genotype E

We have extensively investigated HBV genotypes in Nigeria (*18,20*; unpub. data). Of almost 300 sequences from both southern and northern Nigeria, 95% belong to genotype E and 4% to subgenotype A3. Thus, in Nigeria, the only location where HBV/A5 has been found, almost all HBV carriers are infected by genotype E, and the most prevalent none-E strains belong to subgenotype A3. In contrast, genotype A5 is rare and confined to Nigeria. Historical records (*27*) and the prevalence of A5 confirm that ancestors of the Haitian population came from the Bight of Benin, one of the most important slave trading posts. Nevertheless, only ≈6% of strains in Haiti belonged to genotype E. Single sequences were interspersed among current HBV/E sequences from Africa, with little genetic distance between them, suggesting that these HBV/E strains were introduced only recently into Haiti. The recent establishment HBV/E into Haiti strongly indicates that genotype E was essentially absent from West Africa when and where slaves were assembled for transport. Recent introduction of genotype E into the general West African population, as we have suggested previously (*18*,*20*), also would explain the low genetic diversity of this genotype.

Subgenotype A3

A3, the minority subgenotype in West Africa, was virtually absent from Haiti, suggesting that this subgenotype also arrived later in the Bight of Benin. Interestingly, Cameroon is the only country where genotypes E and A3 cocirculate at similarly high prevalences (*18,28*), suggesting that both E and A3 may cooriginate from this region.

Genotype D

More than 20% of sequences analyzed from Haiti belonged to genotype D (D3, D4). Subgenotype D4 strains

are rare in the world, but we found a surprising 17% prevalence in Haiti. Interestingly, some of the D3 and the D4 strains were closely related to recent strains from Rwanda (*9*). With prevalences of 15.6% of D3 and 6.7% of D4 (*9*), Rwanda is also the only country where sizeable percentages of these 2 subgenotypes were found, further corroborating an origin of these strains from that part of Africa. During the second half of the 18th century, slaves were, to a large extent, collected in west-central Africa and shipped either from the western coast (*23*) or the eastern coast (*23*,*27*) to the Caribbean. Thus, an African origin of the D strains seems likely (Figure 3).

Time of Evolution

Our results seem to agree with the time frame of the transatlantic slave trade. According to the simplest evolutionary model with a mutation rate of 4.2×10^{-5} (*29*), separation between Haitian and African A5 strains, with a mean genetic distance of 2.28%, would have occurred ≈270 years ago. When each Haitian A1 group is considered as a separate and independent introduction, the mean genetic diversity of 0.8%–1.6% corresponds to at least 100–190 years of evolution of each of the A1 groups in Haiti. These estimates for A1 and A5 seem to agree with the historical records that slaves (putatively infected with A5) from West Africa were introduced to Haiti ≈270 years ago, i.e., during the early phase (from the 1730s on) of the slave trade (*27*), and slaves (putatively infected with A1) from eastern Africa were exported to Haiti around the turn of the 18th century (*26*,*27*).

The apparent absence of old genotype E strains in Haiti indicates that it was rare in West Africa at the time of the slave trade and emerged in the general African population by the beginning of the 19th century, after the majority of the slave trade was suspended. Indeed, the mean genetic diversity (1.74%) would evolve over the complete genome in only ≈200 years, even from a single virus.

Given the recent introduction of genotype E, the excessively high prevalence of this genotype throughout the genotype E crescent is difficult to understand. If HBV antibody prevalence was as high at the time of the emergence of HBV/E as it is today, why did genotype E spread so much more efficiently than genotype A (subtype A3 or A5) in West Africa? No evidence exists to indicate that immunity to genotype A (e.g., through vaccines) does not protect against genotype E. The rare A/E recombinants do not suggest a long cocirculation of both genotypes at a high prevalence. A more likely scenario is that HBV was relatively rare in Africa until genotype E was massively spread by a new route of transmission. Much evidence points toward mass injection campaigns performed in the Belgian and French colonies at the turn of the 19th century. Treatment campaigns against yaws during the 1920s–1950s (*30*,*31*) and chemoprophylactic campaigns against sleeping sickness (*32*) were widespread and entailed sometimes reusing only a few syringes to treat, for example, 90,000 persons (*32*). Later, the extensive use of injectable antibiotics, vaccines, and other drugs (e.g., against syphilis) with unsafe needles further promoted HBV transmission (*30*,*31*). In Egypt, widespread transmission of hepatitis C virus has been linked to unsafe mass injection campaigns against schistosomiasis until the 1980s (*33*). Because HBV is estimated to be 10 times more transmissible than hepatitis C virus (*31*,*33*), injection with unsafe needles is a possible route of transmission of HBV (genotype E). In addition, early potentially contaminated vaccine preparations, as well as insect vectors, might be culpable in the spread of HBV infection.

The high prevalence of genotype A5 in Haiti strongly indicates that predecessors of the Haitian population came from the Bight of Benin. However, subgenotype A3 and genotype E, highly prevalent today in this part of Africa, are essentially absent in Haiti. This lack strongly indicates that HBV/E emerged only later in the general African population. The high prevalence of HBV/E in large parts of Africa further suggests that HBV hyperendemicity is a recent phenomenon and probably the result of the extensive use of unsafe needles.

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Recurrent Zoonotic Transmission of Nipah Virus into Humans, Bangladesh, 2001–2007

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Human Nipah outbreaks recur in a specific region and time of year in Bangladesh. Fruit bats are the reservoir host for Nipah virus. We identified 23 introductions of Nipah virus into human populations in central and northwestern Bangladesh from 2001 through 2007. Ten introductions affected multiple persons (median 10). Illness onset occurred from December through May but not every year. We identified 122 cases of human Nipah infection. The mean age of case-patients was 27 years; 87 (71%) died. In 62 (51%) Nipah virus–infected patients, illness developed 5–15 days after close contact with another Nipah case-patient. Nine (7%) Nipah case-patients transmitted virus to others. Nipah case-patients who had difficulty breathing were more likely than those without respiratory difficulty to transmit Nipah (12% vs. 0%, p = 0.03). Although a small minority of infected patients transmit Nipah virus, more than half of identified cases result from person-to-person transmission. Interventions to prevent virus transmission from bats to humans and from person to person are needed.

Human Nipah virus infection, characterized primarily by fever and encephalitis, was first recognized in a large outbreak of 276 reported cases in peninsular Malaysia and Singapore that occurred from September 1998 through June 1999 (*1,2*). Contact with sick pigs was the primary risk factor for infection (*3*). A newly identified porcine respiratory and neurologic syndrome developed in some pigs infected with the Nipah virus; this syndrome was characterized by fever, barking cough, behavioral changes, uncoordinated gait, spasms, and myoclonus (*4*). The outbreak of Nipah virus infection in humans ceased when the infected herds of pigs in the region were culled (*5*).

Substantial data implicate fruit bats (*Pteropus* spp.) as the natural reservoir host of Nipah virus. In initial studies after the Malaysia outbreak, 16/64 (25%) *P. vampyrus* and *P. hypomelanus* fruit bats had neutralizing antibodies to Nipah virus (*6*). Nipah virus was subsequently isolated from urine specimens collected under a *P. hypomelanus* roost from partially eaten fruit dropped during feeding activity in Malaysia (*7*) and from urine collected under a *P. lylei* roost in Cambodia (*8*). Nipah virus–specific RNA was identified in saliva and urine samples from *P. lylei* fruit bats in Thailand (*9*).

The nucleotide sequences of Nipah virus strains isolated from pigs and persons in Malaysia were remarkably similar (*5,10,11*) and suggest that the entire outbreak was caused by 1 or 2 closely related strains. Indeed, all human cases of Nipah infection in Malaysia and Singapore could have originated from a single or perhaps 2 introductions of Nipah virus from its bat reservoir into pigs (*10,12*).

In Bangladesh, by contrast, recurrent Nipah outbreaks have been recognized since 2001 (*13–17*), and the strains of Nipah isolates show substantial heterogeneity in their nucleotide sequences (*11*). This heterogeneity suggests repeated introductions of Nipah virus from its host reservoir into the human population in Bangladesh.

A single species of fruit bats of the genus *Pteropus*, *P. giganteus*, lives in Bangladesh and is widely distributed throughout the country (*18*). Blood samples from *P. giganteus* bats in Bangladesh and neighboring India commonly have antibodies to Nipah virus (*13,19*). The conditions that

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permit recurrent introduction of Nipah virus from fruit bats to persons in Bangladesh are unknown. Besides the tendency for Nipah virus outbreaks to reoccur in Bangladesh, a second notable difference in Nipah virus epidemiology in Bangladesh is that, in contrast to Malaysia, where person-to-person transmission of Nipah virus was not confirmed (*20*), person-to-person transmission has been repeatedly observed in Bangladesh (*15,16*).

The high prevalence of antibodies to Nipah virus among *Pteropus* spp. bats suggests that Nipah virus is well adapted to transmission between individual bats of this genus. We hypothesize that when a *Pteropus* spp. bat sheds Nipah virus in Bangladesh, this virus occasionally infects 1 or more persons. Once people are infected, the epidemic chain of transmission can be perpetuated by person-to-person transmission (*16*).

A more complete understanding of the character of Nipah virus infection in Bangladesh has been limited by the analysis of relatively small individual outbreaks. We combined data from the 7 recognized human outbreaks and the identified sporadic cases of Nipah virus in Bangladesh from 2001 through 2007. The objective was to describe the introduction of Nipah virus into the human population and the epidemiology of person-to-person transmission.

Methods

We reviewed available data from investigations of all of the human Nipah infections recognized in Bangladesh from 2001 through 2007. Information from the separate investigations was combined into a single database. Not all variables of interest were collected from the earliest outbreaks, but because many of the same investigators were involved across the outbreaks, data were collected in similar formats.

Persons were classified as being infected with Nipah virus if they had fever with new onset of altered mental status, seizures, or severe shortness of breath and either had specific antibodies against Nipah virus or were part of a cluster of similar case-patients in the same region, with at least 1 of the case-patients being Nipah-antibody positive. In addition, if a person had fever and immunoglobulin (Ig) M antibody to Nipah, that person was classified as being infected with Nipah virus.

We classified Nipah cases as part of a cluster if at least 1 other Nipah case was identified in the same community within 3 weeks of onset of illness. If no other cases appeared in the same community within 3 weeks, the Nipah case was classified as an isolated case.

We counted distinct introductions of Nipah virus into the human population. Each cluster of Nipah case-patients and each sporadic case was counted as a separate Nipah introduction.

We classified persons as primary case-patients if illness developed without known contact with any other Nipah case-patients, as secondary case-patients if Nipah disease developed 5–15 days after close contact with other Nipah case-patients, and as Nipah spreaders if at least 1 person with whom that person had close contact had Nipah illness develop 5–15 days after that contact. We collected geographic coordinates by using global positioning systems from the home of each case-patient.

Laboratory Methods

The field team, which involved a large number of different people over the course of the many outbreak investigations, centrifuged whole blood specimens and brought the separated serum on wet ice to the laboratory at the International Center for Diarrheal Diseases Research, Bangladesh (ICDDR,B), where it was stored at –70°C. Before 2007, serum samples were shipped on dry ice to the US Centers for Disease Control and Prevention (CDC) and tested with an IgM capture enzyme immunoassay (EIA), which detects Nipah IgM, and with an indirect EIA for Nipah IgG (*21*). Nipah (Malaysia prototype) virus antigen was used in both assays. In 2007, the Nipah antibody testing was conducted at the government of Bangladesh's Institute of Epidemiology Disease Control and Research using reagents provided by the Special Pathogens Branch of CDC in Atlanta. All positive samples were confirmed at CDC.

Statistics

We assessed whether differences in proportions were more extreme than would be expected by chance by using the χ^2 test or the Fisher exact test when the expected cell size was <5. The basic reproductive number (R_0) is the average number of persons infected by an infectious person during his or her entire infectious period when he/she enters a totally susceptible population (*22*). We estimated R_0 by dividing the number of persons infected by a primary case-patient by the total number of primary case-patients.

Ethics

Many of these data were collected as part of routine surveillance or emergency outbreak investigations, so study protocols did not undergo human subjects review. In 2005, a protocol for establishing a formal Nipah surveillance system and the strategy for outbreak investigations were reviewed and approved by the Ethical Review Committee of the ICDDR,B.

Results

A total of 122 Nipah case-patients were identified; 74 (61%) were male, and the mean of all case-patients age was 27 years (range 2–75). Eighty-seven (71%) of 122 died. Fifty-nine (48%) infections were serologically confirmed. Of the 63 case-patients that were not laboratory confirmed, 59 (94%) died before any blood was collected; 4 (6%) had

an initial serum specimen without detectable antibodies but died before a follow-up specimen could be collected.

We identified 23 introductions of Nipah virus into human populations in central and northwestern Bangladesh from 2001 through 2007 (Figure 1). Ten introductions involved clusters with a median of 10 affected persons (range 2–35). Thirteen Nipah introductions resulted in isolated human infections.

The onset of illness for the index cases for the 23 human Nipah introductions occurred from December 31 through April 20 (Figure 2). The probability that all 23 index cases would randomly occur in 5 contiguous months is $(5 / 12)^{23} \times 12 = 0.00000002$. The number of Nipah case-patients varied by year; most cases were recognized in 2004, and no cases were recognized in 2002 and 2006 (Figure 3).

In 62 (51%) Nipah case-patients, illness developed from apparent person-to-person transmission. None of these cases were professional healthcare workers exposed to Nipah case-patients on the job. The case-fatality ratio was similar for primary and secondary cases (75% vs. 66%, respectively; $p = 0.28$).

Nine case-patients (7%) were Nipah spreaders who transmitted Nipah infection to other persons. All Nipah spreaders died; 4 of these were classified as secondary cases. Nipah case-patients who had difficulty breathing during their illness were more likely to be Nipah spreaders (12% vs. 0%, $p = 0.03$). Similarly, Nipah case-patients who had cough were more likely to be Nipah spreaders (12% vs. 2%, $p = 0.080$). Patients classified as secondary cases were just as likely to have difficulty breathing as those classified as primary cases (65% vs. 66%, $p = 0.94$).

Nipah case-patients who died were more likely to be Nipah spreaders than were Nipah case-patients who survived (10% vs. 0%; Fisher exact test, $p = 0.057$). Persons who spread Nipah were no more likely to be primary Nipah case-patients than were Nipah patients who did not spread Nipah (56% vs. 49%, odds ratio 1.3, 95% confidence interval 0.34–5.1, $p = 0.74$). Nipah spreaders transmitted Nipah to a mean of 7 persons (range 1–22).

Among the 10 clusters, 5 involved person-to-person transmission ranging from 2 to 5 generations of transmission. The 60 primary Nipah case-patients infected 29 subsequent persons. The estimated basic reproductive number was 0.48.

Fourteen secondary Nipah case-patients had contact with a primary Nipah case-patient for ≤2 days. The median incubation period of these secondary cases was 9 days (range 6–11 days).

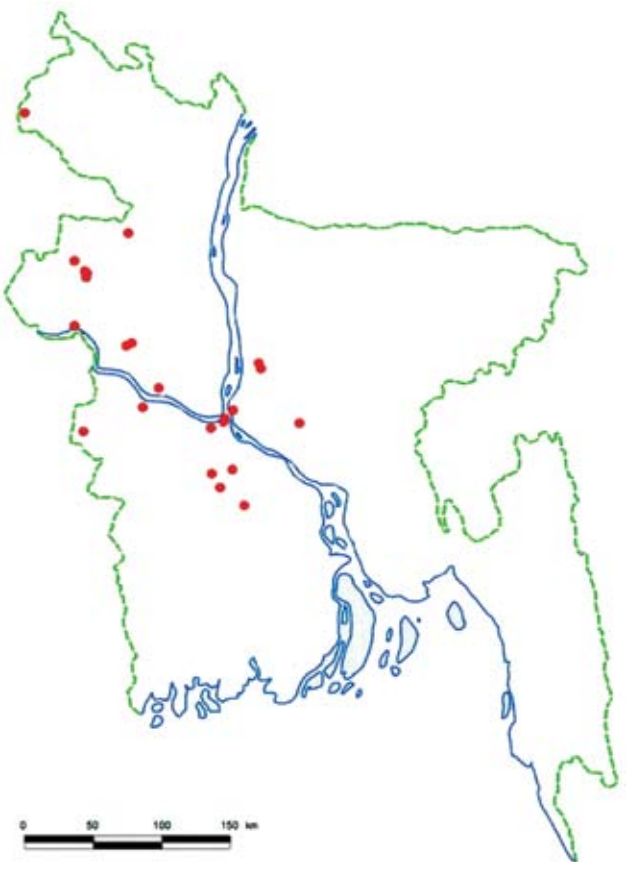

Figure 1. Locations of human Nipah virus introductions (red dots), Bangladesh, 2001–2007.

Discussion

These introductions of Nipah virus from its presumed reservoir in *Pteropus* bats to humans in Bangladesh were clustered, both in time of year as well as in specific years. Occurring during winter and spring in certain years, this clustering in Bangladesh suggests that the specific conditions necessary for Nipah virus transmission from bats to humans occurs only periodically. Perhaps shedding of Nipah virus by *Pteropus* bats is seasonal, and because of population dynamics and the accumulation of susceptible juvenile bats over time, transmission is quite low in some years compared with other years when widespread shedding and transmission occur.

The presumed wildlife reservoir of Nipah virus, bats of the genus *Pteropus*, is widely distributed across Bangladesh, the rest of the Indian subcontinent, and Southeast Asia (*18*). Wherever *Pteropus* bats have been collected and tested, they commonly have had antibodies against Nipah or a related virus (*8,9,13,23,24*). Human Nipah infections in Bangladesh, however, have been recognized in a confined geographic area. The Institute of Epidemiology Disease Control and Research in Bangladesh has national surveillance for disease outbreaks. Outbreaks involving the deaths of several previously healthy persons, which is characteristic of Nipah outbreaks, typically generate substantial local concern, media attention, and notification of local and

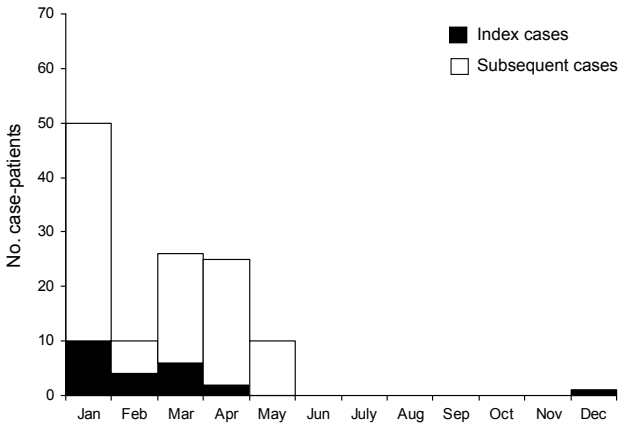

Figure 2. Human Nipah virus infections in Bangladesh, by month of illness onset, 2001–2007.

central health authorities. Despite investigations of outbreaks in all regions of the country, all confirmed Nipah outbreaks have occurred in the same central and northwestern regions (Figure 1). Notably, the only 2 outbreaks that have been reported from India have been in regions within 50 kilometers of the border with Bangladesh and immediately contiguous with the affected areas in Bangladesh (*25,26*). One hypothesis that would explain this geographic concentration of human cases is that the bats are attracted to specific foods available in these areas during the winter and spring; people living in these communities are occasionally exposed to foods contaminated with bat urine or saliva that contains Nipah virus. One outbreak of Nipah virus was associated with consumption of raw date palm sap, which is harvested from December through March in this region (*14*). In 21 of the 23 recognized index case-patients, Nipah virus illness developed during this harvest season.

Person-to-person transmission is a major pathway for human Nipah virus infection in Bangladesh, accounting for 51% of recognized cases in this review. By contrast, in Malaysia and Singapore, person-to-person transmission was not confirmed, although 1 nurse who cared for Nipah patients in an intensive care unit in Malaysia and reported no clinical illness had serologic evidence of Nipah virus infection and brain magnetic resonance imaging consistent with Nipah virus infection (*27*). Even if occasional unrecognized person-to-person transmission of Nipah virus occurred in the outbreak in Malaysia, person-to-person transmission is much more apparent and common in Bangladesh. Moreover, the outbreak in Siliguri, India, in 2001 was also characterized by widespread person-to-person transmission (*25*).

Three factors likely contributed to the higher frequency of person-to-person transmission of Nipah virus in Bangladesh than was observed in Malaysia. First, respiratory disease associated with Nipah infection was more common and more severe in Bangladesh compared with that in Malaysia and Singapore (*28–30*). Earlier studies demonstrated that Nipah virus was present in respiratory secretions of some Nipah virus–infected patients (*11,31*). We found that Nipah case-patients who had difficulty breathing were much more likely to be Nipah spreaders. Together, these findings suggest that when a Nipah virus–infected patient has a symptomatic respiratory tract infection associated with Nipah virus, the patient can shed an infectious inoculum of Nipah virus in his respiratory secretions. In the largest recognized Nipah outbreak in Bangladesh, touching a patient who had respiratory difficulties was a risk factor for developing Nipah infection (*16*). The personal care typically provided to ill and dying relatives in Bangladesh is likely a second important contributor to person-to-person transmission. This care is characterized by close physical interaction, frequent contact with a patient's saliva, and a lack of basic infection control practices because the paradigm of communicable disease is inconsistent with the prevailing cultural interpretation of illness (*32*). Third, all Nipah virus strains from human cases in Malaysia were genetically similar, in contrast to the marked diversity of the strains in Bangladesh (*11*). Some strains possibly possess genetic characteristics that facilitate person-to-person transmission.

Our 0.48 estimate of the basic reproductive number of Nipah virus in rural Bangladesh, a resource-poor setting with extremely limited infection control practices, suggests that Nipah virus is unlikely to cause a sustained human pandemic from person-to-person transmission. This conclusion is further supported by our observation of 23 separate introductions of the virus into the human population; none of these introductions resulted in sustained person-to-person transmission. Indeed, only 1 of the introductions exceeded 2 generations of transmission (*16*). However, we could study only 9 patients who transmitted the Nipah virus. Thus, our understanding of the variability in transmission of different strains of the virus in different contexts is limited.

This analysis has limitations. First, we almost certainly did not identify all human Nipah virus infections that occurred in Bangladesh from 2001 through 2007. Many persons in Bangladesh, especially the impoverished, do not access the formal healthcare system, even when seriously ill (*33,34*). The available data on human Nipah virus infection in Bangladesh are biased toward infections acquired in outbreaks recognized by public health authorities. Because meningoencephalitis is a common cause of hospitalization in Bangladesh, sporadic cases that are unrecognized as Nipah virus infection may be the more common presentation of Nipah virus infection in the country. Indeed, during 2004 and 2007, years when multiple outbreaks were iden-

tified, many patients who sought treatment for symptoms of encephalitis in hospitals located near identified outbreak areas were tested for Nipah virus. This testing identified several additional patients infected with the virus who lived quite a distance from the outbreak villages and who had no apparent connection to other cases. Thus, surveillance almost certainly underestimates the public health impact of Nipah virus infection in Bangladesh and may underestimate its geographic and seasonal range. Because outbreaks are more likely to lead to recognition of Nipah virus infection than sporadic cases and person-to-person transmission can occur only in clusters, the overall proportion of Nipah virus infections in Bangledesh that are transmitted person to person is probably <50%.

A second limitation is that the calculation of the basic reproductive rate assumed that all persons infected by a primary case-patient were identified. The investigation team could have failed to recognize all cases, especially in persons with milder or asymptomatic infection. In Malaysia, among 178 persons without symptoms who lived on farms where at least 1 person with confirmed Nipah encephalitis was identified, 20 (11%) had antibodies against Nipah virus (*3*). This possibility of asymptomatic infection suggests that our estimate of the basic reproductive rate is a minimal estimate. However, in outbreaks, when mild or asymptomatic persons were screened in Bangladesh, few additional Nipah cases were identified (J. Hossain, pers. comm.). Moreover, only patients who died transmitted the infection, so the possible infection of persons in whom mild illness developed is unlikely to contribute to the risk for pandemic transmission.

A third limitation is that we identified only 9 persons who transmitted Nipah virus and so have limited statistical power to assess their characteristics. Indeed, the association of cough with Nipah virus transmission and death with Nipah transmission are above the traditional guideline for statistical significance. However, the weight of the evidence, including an association with respiratory illness that meets the traditional criterion for statistical significance and the isolation of Nipah virus in respiratory secretions, suggests that person-to-person transmission occurs occasionally from virus-infected patients who are efficient respiratory transmitters of the virus.

A fourth limitation is that this analysis assumes that persons in whom Nipah illness developed 5–15 days after contact with a Nipah patient were considered infected by the contact. If the subsequent case-patient had a similar environmental exposure to Nipah virus as the initial case-patient, this approach may overestimate the proportion of cases that result from person-to-person transmission. However, Nipah virus is readily recovered from the saliva of infected persons (*11,31*), and epidemiologic studies of individual outbreaks implicate contact with Nipah-infected

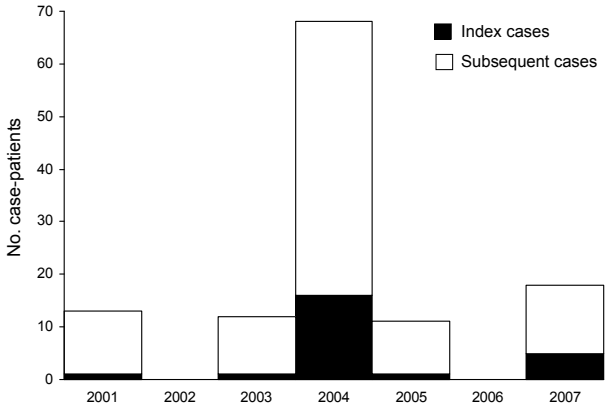

Figure 3. Human Nipah virus infections in Bangladesh, by year of illness onset, 2001–2007.

patients as a risk factor for transmission (*16*). Moreover, the observed 6–11-day window between exposure and disease is consistent with incubation periods for other human viral infections. Thus, person-to-person transmission is the most likely route of transmission in these scenarios.

The recurrent introduction of Nipah virus from its fruit bat reservoir to humans in Bangladesh causes outbreaks with high fatality rates and substantial neurologic sequelae among survivors (*35*). Fruit bats have a critical niche in the ecosystem, contributing to both plant pollination and distribution of seeds (*36,37*). Preventing human Nipah virus infections in Bangladesh is difficult because these infections are relatively rare compared with many other serious and more common health threats faced by the large population of this low-income country. Improved understanding of the mechanism of zoonotic transmission from bats to humans may help identify feasible approaches to prevent future introductions of the virus into the human population. Interventions to reduce the risk for pathogen exposure by minimizing saliva contact or improving hand hygiene among persons who care for seriously ill patients in severely resource-constrained settings could reduce person-to-person transmission of Nipah as well as transmission of other dangerous pathogens. In confirmed or highly suspected cases of Nipah virus infection, respiratory droplet precautions may also be warranted.

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National Outbreak of *Acanthamoeba* Keratitis Associated with Use of a Contact Lens Solution, United States

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the incidence and etiology of *Acanthamoeba* keratitis (AK) infection in the United States
- Identify the demographic and clinical characteristics of patients who acquired AK
- Identify the risk factors for AK among contact lens users.

Editor

Carol Snarey, Copyeditor, *Emerging Infectious Diseases. Disclosure: Carol Snarey has disclosed no relevant financial relationships.*

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An outbreak of *Acanthamoeba* keratitis, a rare, potentially blinding, corneal infection, was detected in the United States in 2007; cases had been increasing since 2004. A case–control study was conducted to investigate the outbreak. We interviewed 105 case-patients from 30 states and 184 controls matched geographically and by contact lens use. Available contact lenses, cases, solutions, and corneal specimens from case-patients were cultured and tested by molecular methods. In multivariate analyses, case-patients had significantly greater odds of having used Advanced Medical Optics Complete Moisture Plus (AMOCMP) solution (odds ratio 16.9, 95% confidence interval 4.8–59.5). AMOCMP manufacturing lot information was available for 22 case-patients, but none of the lots were identical. Three unopened bottles of AMOCMP tested negative for *Acanthamoeba* spp. Our findings suggest that the solution was not intrinsically contaminated and that its anti-*Acanthamoeba* efficacy was likely insufficient. Premarket standardized testing of contact lens solutions for activity against *Acanthamoeba* spp. is warranted.

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Acanthamoeba keratitis (AK), a painful corneal infection that may lead to vision loss or enucleation, is caused by the ubiquitous free-living *Acanthamoeba* spp. (*1–4*). AK occurs primarily among users of soft contact lenses (*5*), with an estimated US annual incidence of 1–2 cases per million contact lens users (*6*).

In May 2006, the Centers for Disease Control and Prevention (CDC) was contacted by the Illinois Department of Public Health regarding a possible increase in AK cases in the Chicago area during the preceding 2 years. Investigators at the University of Illinois at Chicago were conducting a case–control study to identify possible risk factors. In October 2006, CDC informally surveyed ophthalmologists across the country to ascertain whether cases of AK were increasing elsewhere; results were inconclusive. In January 2007, CDC initiated a retrospective survey of 22 ophthalmology centers nationwide. By early March 2007, results obtained from 10 centers in 9 states showed a rise in the number of culture-confirmed cases during 2004–2006 compared with 1999–2003.

On March 16, 2007, we initiated a national outbreak investigation. On May 23, a preliminary analysis compared data from the first 46 interviews of patients with culture-confirmed AK, with data obtained from 126 healthy adult contact lens users ascertained in a 2006 national outbreak investigation of *Fusarium* keratitis (*7*). The analysis indicated that the odds of having ever used Advanced Medical Optics Complete MoisturePlus (AMOCMP) multipurpose contact lens solution were 20× greater for AK case-patients than for controls. These results were communicated to the Food and Drug Administration (FDA) and were rapidly disseminated (*8*). On May 26, 2007, the company voluntarily recalled AMOCMP from domestic and international markets. Although public health action was taken on the basis of the preliminary analysis, we report here the results of a matched case–control study designed to verify the findings of the preliminary analysis, to identify additional risk factors for AK, and to guide recommendations to prevent future cases.

Methods

Case Definition and Case Finding

Case-patients had been given a diagnosis of AK by an ophthalmologist; had symptom onset on or after January 1, 2005; and had *Acanthamoeba* spp. identified from cultures of corneal specimens. Requests to report AK cases were disseminated through CDC's Epidemic Information Exchange system and through ophthalmology and optometry electronic mailing lists; websites; and associations at the national, state, and local levels. We also queried several referral microbiology laboratories and ophthalmology centers to find cases. Cases included in a concurrent study by University of Illinois at Chicago investigators were excluded (*9*).

Case-Patient Data Collection and Laboratory Investigation

We used standardized questionnaires to interview case-patients by telephone to obtain demographic characteristics, information regarding illness, contact lens–related product use, and hygiene practices and behavior during the month before symptom onset. An Internet-based visual aid was available to assist with specific product recognition. Ophthalmologists who were treating case-patients provided information by telephone- or self-administered questionnaires regarding diagnostic methods, treatment, and clinical outcomes.

Available clinical specimens (e.g., corneal scrapings or biopsy specimens, *Acanthamoeba* culture isolates) and environmental samples (e.g., opened and unopened contact lens solution bottles, lenses, lens cases) were sent to CDC laboratories. Specimens were processed for *Acanthamoeba* spp. by culture (*4*) and molecular analysis (*10*), including genotyping (*11*).

Case–Control Study

All interviewed case-patients were eligible. Control subjects had no history of AK and were ≥12 years of age. We attempted to match 3 controls to each case-patient by contact lens use (i.e., soft lenses, rigid lenses, or no contact lens use) and by geographic location using a reverse address directory to identify controls who resided near each case-patient. Because rigid lens use is uncommon, we did

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not attempt to obtain geographically matched controls for this group. Controls completed a standardized, telephone-administered questionnaire that asked about behavior and product use during the 1 month before their matched case-patient had symptom onset.

Data Analysis

Data were double-entered by using Visual FoxPro 8.0 (Microsoft Corp., Redmond, WA, USA) and analyzed by using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Conditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for univariate and multivariate analyses; significance was defined as $p<0.05$. All variables that were significantly associated with AK by univariate analysis were further investigated by using a multivariate model.

Results

Descriptive Epidemiology

Case-patients were enrolled during March 16–July 10, 2007. Of 221 AK patients identified from 37 states and Puerto Rico, 158 (71%) had infections that met the case definition. We interviewed 105 (66%) case-patients from 30 states (Figure 1) and the treating ophthalmologists of 92 case-patients (88%).

Times of symptom onset (Figure 2) did not show any obvious trends or a single period of peak exposure. The 105 case-patients were predominantly female (67 [64%]) with a median age of 29 years (range 12–76 years) (Table 1). Of these case-patients, 93 (89%) wore contact lenses (82 [88%] used soft contact lenses), and 87 (94%) reported using some type of cleaning or disinfecting solution (78 [90%]) used a multipurpose solution). Of the 6 contact lens users who did not report use of any cleaning or disinfecting solution, 1 used daily disposable lenses, 1 used extended-wear lenses that were replaced with no cleaning, 2 used only saline solution, 1 used saline solution and rewetting drops, and 1 did not recall which types or brands of solutions were used.

The most frequently reported symptoms among case-patients were pain, redness, sensitivity to light, and sensation of a foreign body (Table 1). The median time from onset of symptoms to initiation of anti-*Acanthamoeba* treatment was 49 days, range 4–197 days. At the time of their ophthalmologist interview, 24 (28%) of 85 had either undergone or were awaiting a corneal transplant, and 29 (41%) of 70 had a visual acuity of 20/200 or worse with best correction (i.e., legally blind) in the affected eye.

Case–Control Study

During June 14–July 10, >11,000 phone calls were made to obtain 184 controls matched to 91 case-patients; case-patients with no matched controls were excluded from subsequent analyses (Figure 3). Because of differences in possible exposures (primarily the use and type of contact lens solutions) between soft lens, rigid lens, and non–contact lens users, we further restricted the analysis to case-patients (n = 72) and controls (n = 140) who reported wearing soft contact lenses only. Separate analyses were performed among users of rigid contact lenses and non–contact lens users; however, sample sizes were small, and no associations were found. Users of soft contact lenses who were excluded for lack of a matched control were not significantly different from those included in the analysis with respect to age, sex, race, and ethnicity.

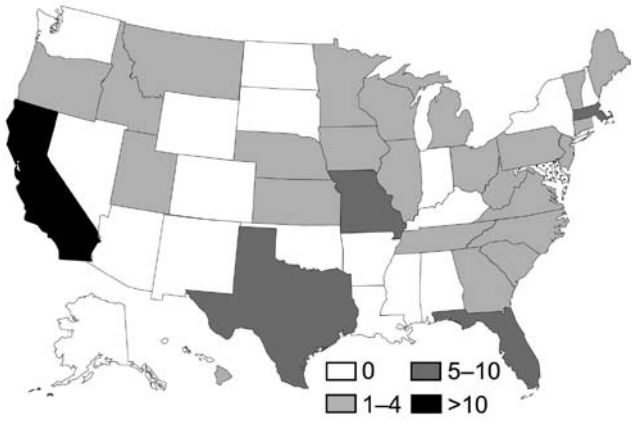

Figure 1. *Acanthamoeba* keratitis case-patients by state, USA (N = 105). *Number of interviewed case-patients per state. Because of incomplete case reporting and enrollment in case–control study, incidence rates were not calculated.

Matched univariate analysis of users of soft contact lenses (online Appendix Table, available from www.cdc.gov/EID/content/15/8/1236-appT.htm) indicated that any use of AMOCMP within the month before symptom onset was a substantial risk factor (OR 15.8, 95% CI 5.6–44.6). No other contact lens solutions were associated with disease. Variables in univariate analyses that were included in the multivariate modeling included the following: any use of AMOCMP, Hispanic ethnicity, age (12–17, 18–24, 25–34, 35–49, versus ≥50 years), male sex, history of ocular trauma, contact lens use ≤5 years, frequency of replacing old lenses with new ones, swimming in a lake or river while wearing lenses, washing the face while wearing lenses, lack of hand washing before inserting lenses, cleaning lenses at the bathroom sink, failure to always cap the solution bottle after use, and ever topping off solution (adding new solution to old solution in the lens case).

In multivariate analysis, only 3 exposures were statistically significant (Table 2). Case-patients had 16.9 times the odds of reporting any use of AMOCMP compared with controls (95% CI 4.8–59.5), 2.8 times the odds of reporting

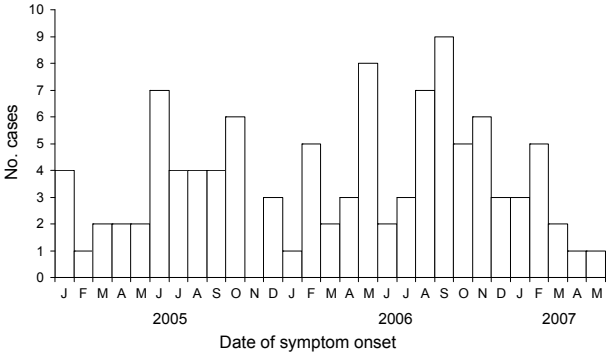

Figure 2. Symptom onset of cases of *Acanthamoeba* keratitis, by month and year, United States, 2005–2007 (N = 105).

ever topping off solution (95% CI 1.2–6.8), and 2.8 times the odds of having used contact lenses for ≤5 years (95% CI 1.0–7.6). Although age and sex were not significantly associated with AK in the multivariate analysis, they were included in the model to adjust for potential confounding.

Laboratory Investigation

We received 94 outbreak–related specimens from case-patients; 10 culture isolates, 4 corneal specimens, and 80 environmental specimens (48 lenses and/or cases, 32 bottles of solution, including 5 unopened bottles). Of the 4 corneal specimens, 1 (25%) was culture positive; that specimen and 2 others (overall 75%) were positive by real-time PCR. Among the 48 lenses and/or cases, 11 (23%) were positive by culture; real-time PCR detected an additional 3 positive samples (overall 29%). No bottles of solution were culture positive. Five opened AMOCMP bottles were positive by real-time PCR. Eight other AMOCMP bottles, including 3 that arrived in the laboratory unopened, were negative by PCR. The remaining bottles of solution (non-AMOCMP products) were all PCR negative. Of 22 different lot numbers for AMOCMP bottles used by case-patients before onset of symptoms, no lot number was repeated.

Acanthamoeba genotyping was performed on 22 outbreak–related isolates; 20 (91%) were T4 genotype, which is the most common genotype in the environment as well as in AK (*11*). Two environmental samples contained *Acanthamoeba* genotypes T3 and T14.

Discussion

This investigation of a national AK outbreak identified use of a single contact lens solution as the primary risk factor for infection. AMOCMP is a multipurpose solution used for disinfecting, rinsing, cleaning, and storing lenses. Our findings indicate that the strong association between AMOCMP and AK was unlikely to have resulted from intrinsic contamination. Case-patients had a wide geographic and temporal distribution, and there were no common lot numbers among AMOCMP bottles provided by case-patients. Although 3 opened bottles of AMOCMP tested positive by PCR for *Acanthamoeba* spp., all unopened

Table 1. Demographic and clinical characteristics of 105 patients with *Acanthamoeba* keratitis, United States, 2005–2007

Characteristic	No. (%) patients
Sex	
Female	67 (64)
Age, y*	
12–17	27 (26)
18–24	17 (16)
25–34	15 (14)
35–49	24 (23)
≥50	22 (21)
Contact lens wear	
Did not use contact lenses	12 (11)
Used contact lenses	93 (89)
Lens type	
Soft lenses	82 (88)
Rigid lenses	10 (11)
Hybrid lenses	1 (1)
Contact lens solution use among contact users†	
Did not use cleaning or disinfecting contact lens solution	6 (6)
Used any type of cleaning or disinfecting contact lens solution	87 (94)
Type of solution used‡	
Multipurpose solution	78 (90)
Hydrogen peroxide solution	6 (7)
Daily cleaner	11 (13)
Affected eye	
Right	53 (50)
Left	44 (42)
Both	8 (8)
Symptoms when treatment sought‡§	
Pain	78 (74)
Redness	78 (74)
Sensitivity to light	76 (72)
Sensation of foreign body	71 (68)
Increased tearing	59 (56)
Blurred vision	57 (54)
Discharge from eye	20 (19)
Clinical status¶	
Resolved with pharmacologic therapy	32 (38)
Currently receiving pharmacologic therapy	29 (34)
Corneal transplant performed	21 (25)
Corneal transplant planned	3 (4)
Most recent visual acuity with best correction in affected eye#	
20/20	17 (24)
20/25–20/100	24 (34)
20/≥200	29 (41)

*Median 29y, range 12–76y.
†During 1 month before illness onset; n = 93.
‡Not mutually exclusive.
§Median time from symptom onset to anti-Acanthamoeba therapy (n = 80) was 49 d (range 4–197 d).
¶At the time of treating ophthalmologist interview; n = 85.
#At the time of treating ophthalmologist interview; n = 70.

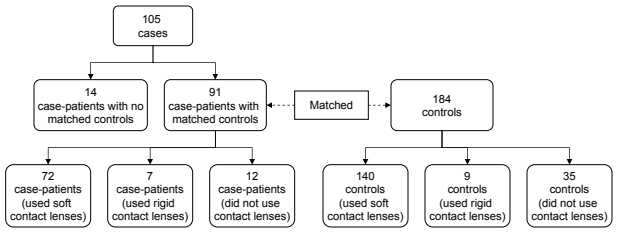

Figure 3. Matching of case-patients with *Acanthamoeba* keratitis and controls, United States, 2005–2007.

bottles were PCR negative, and all AMOCMP bottles were negative by culture. The positive PCR results most likely represent point-of-use contamination through contact with water or dirty hands. We suspect that AMOCMP was insufficiently active against *Acanthamoeba* spp., resulting in increased likelihood of infection among product users. The sources of *Acanthamoeba* spp. could have been varied and multiple because *Acanthamoeba* spp. are ubiquitous in the environment. A concurrent AK case–control study conducted in the Chicago area, which investigated 55 separate cases, also found AMOCMP to be the most important risk factor (*9*).

AMOCMP was introduced for sale in 2003, just before the nationwide increase in cases. This product differed from AMO Complete, the multipurpose solution that preceded it, by the addition of propylene glycol (a comfort enhancer) and taurine (a buffering agent) (*12*). After this outbreak, laboratory investigations found that propylene glycol may cause *Acanthamoeba* trophozoites to encyst, thereby making them resistant to disinfection (*12,13*). However, propylene glycol is not unique to AMOCMP and is an ingredient of at least 1 other multipurpose solution, as well as several brands of artificial tears. Published results on the performance of multipurpose solutions, including AMOCMP, against *Acanthamoeba* organisms have shown varying efficacy (*14–16*). Assessment of anti-*Acanthamoeba* solution efficacy is limited by a lack of standardized testing methods (*17*). FDA guidance and the International Organization for Standardization standards do not include anti-*Acanthamoeba* spp. testing (*18*). However, after this outbreak, the Ophthalmic Devices Panel of the FDA Medical Devices Advisory Committee has recommended adding *Acanthamoeba* spp. as a challenge organism for testing of contact lens solutions (*19*).

Several similarities exist between this AK outbreak and the 2006 *Fusarium* keratitis outbreak (*7*). Both outbreaks of serious corneal infections occurred primarily among users of soft contact lenses. The 3–4 year duration of the AK outbreak spanned the timeframe of the *Fusarium* outbreak (June 2005–June 2006). In each outbreak, the primary risk factor was use of a certain multipurpose solution; Bausch and Lomb Renu with MoistureLoc (RML) was recalled in April 2006 after its use was identified as a major risk factor for *Fusarium* keratitis. Neither investigation found evidence of intrinsic microbial contamination of the solution; instead, insufficient antimicrobial efficacy was hypothesized to be the primary driving force behind each epidemic. In both outbreaks, the practice of topping off solution in the lens case also emerged as an important risk factor. After the *Fusarium* keratitis outbreak, tests simulating the reported practices of the case-patients found that topping off reduced the antimicrobial efficacy of RML (*20*). Together, these outbreaks raise concern about the safety of multipurpose contact lens solutions and related consumer behavior. FDA recently discussed these concerns and recommended changes to premarket testing and labeling for contact lens solutions, including more explicit warnings against topping off the solution (*19*).

This investigation yielded several notable negative findings. No use of contact lens solutions other than AMOCMP was identified as a risk factor, including the 1 other multipurpose solution that contained propylene glycol. Contact lens characteristics that have been hypothesized to increase the risk for AK, such as lens material and FDA lens group (*21,22*), were not associated with AK in this study; nor was there statistical interaction between use of AMOCMP and characteristics of soft contact lenses. Although rubbing and rinsing of lenses during the disinfection process have been shown in laboratory studies to decrease *Acanthamoeba* contamination of lenses (*23*), we did not find these practices to be protective. Although poor contact lens hygiene is widely recognized as a risk factor for AK (*24–26*), in this study only the topping off of old solution emerged as an important behavioral risk factor. We speculate that the association between AK and contact lens use for ≤5 years may reflect a wide range of poor hygiene practices among new contact lens users. Individual hygiene lapses may not be prevalent enough to emerge as important risk factors, but in aggregate, poor hygiene practices might be more common among users of new lenses.

We also found that a wide range of behaviors that can result in exposure of contact lenses to water (e.g., showering with lenses in, rinsing lenses or cases with tap water) was not associated with AK in this study. Although such practices are generally considered to be important risk factors for AK (*2,24,26*), a separate case–control study among contact lens users found that water exposure was not a risk factor (*27*).

Some researchers have suggested that municipal water treatment type may play a role in the development of AK (*28*). *Acanthamoeba* organisms are present in the water of many households (*29–32*). A temporal association was noted between an increase in AK cases in the Chicago area (*28*) and the implementation of the Environmental Protec-

Table 2. Multivariate analysis of demographic characteristics and exposures among 72 *Acanthamoeba* keratitis case-patients and 140 controls who used soft contact lenses, United States, 2005–2007*

Demographic characteristic or exposure†	No. (%) case-patients, n = 72	No. (%) controls, n = 140	Adjusted matched OR (95% CI)‡
AMOCMP	40 (55.6)	15 (10.7)	16.9 (4.8–59.5)
Ever top off or reuse old solution in case	40 (57.1)	30 (22.7)	2.8 (1.2–6.8)
≤5 y as contact lens wearer	37 (51.4)	32 (22.9)	2.8 (1.0–7.6)

*OR, odds ratio; CI, confidence interval; AMOCMP, Advanced Medical Optics Complete MoisturePlus.
†During 1mo before symptom onset. Missing values excluded from analysis.
‡Adjusted for age and sex. p<0.05.

tion Agency Disinfectants and Disinfection Byproducts Rule, which was aimed at decreasing potentially harmful disinfection byproducts in water (*33*). To comply with this rule, many municipal water supply systems have switched from using chlorine to using chloramine as a residual drinking water disinfectant. However, no change in water disinfection type was made by the Chicago Department of Water Management during the period of interest. Chicago-area water has been continuously disinfected with chlorine for >30 years (A. Stark, City of Chicago Department of Water Management, pers. comm.). A preliminary analysis (J. Verani, unpub. data) conducted during the early phase of this investigation found that only 12 (29%) of 41 case-patients for whom water treatment data were available received household water from chloraminated systems during the month before symptom onset, compared with an estimated 32% of the general US population (*34,35*). These findings suggested that water disinfection type was not an important risk factor in this outbreak.

This study had several limitations. First, because AK culture is a highly specific, but insensitive, diagnostic tool (*36*), and because preferred diagnostic methods vary by medical center, inclusion of only patients with culture-confirmed cases may have introduced regional testing bias and underestimated the scope of the outbreak. Second, the response rate among persons approached for control interviews was low; therefore, demographic differences between cases and controls may have been due to selection bias among controls. Third, recall bias may have been introduced as we asked participants to report on contact lens product use and behavior during the previous 2 years. Fourth, misclassification bias may have been introduced because at least 2 case-patients appeared to not differentiate between use of saline and cleaning or disinfecting solutions. Fifth, because >40% of case-patients and all controls were interviewed after AMOCMP was recalled in May 2007, reporting bias may have been introduced.

Despite these limitations, among users of soft contact lenses, case-patients had almost 17 times the odds of reporting any AMOCMP use compared with matched controls, validating the results of the preliminary analysis comparing AK cases to *Fusarium* keratitis investigation controls (*8*). The use of this existing *Fusarium* comparison data enabled rapid public health action months before the case–control study was completed. Recent associations of 2 distinct multipurpose solutions with outbreaks of rare corneal infections highlight the need for improved surveillance to promptly detect contact lens–related outbreaks and raise concerns about the effectiveness of multipurpose solutions. Continued monitoring of AK case trends to assess the impact of the AMOCMP recall and research on the anti-*Acanthamoeba* efficacy of AMOCMP and other solutions are under way. Our findings highlight the importance of promoting healthy habits among contact lens users, particularly discouraging the practice of topping off solutions and reinforcing safe hygienic practices among new users of contact lenses, as well as the need for standardized anti-*Acanthamoeba* testing of contact lens solutions.

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Entomologic and Virologic Investigation of Chikungunya, Singapore

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Local transmission of chikungunya, a debilitating mosquito-borne viral disease, was first reported in Singapore in January 2008. After 3 months of absence, locally acquired Chikungunya cases resurfaced in May 2008, causing an outbreak that resulted in a total of 231 cases by September 2008. The circulating viruses were related to East, Central, and South African genotypes that emerged in the Indian Ocean region in 2005. The first local outbreak was due to a wild-type virus (alanine at codon 226 of the envelope 1 gene) and occurred in an area where *Aedes aegypti* mosquitoes were the primary vector. Strains isolated during subsequent outbreaks showed alanine to valine substitution (A226V) and largely spread in areas predominated by *Ae. albopictus* mosquitoes. These findings led to a revision of the current vector control strategy in Singapore. This report highlights the use of entomologic and virologic data to assist in the control of chikungunya in disease-endemic areas.

Chikungunya is a mosquito-borne infectious disease caused by chikungunya virus (CHIKV), which belongs to the family *Togaviridae* and genus *Alphavirus*. CHIKV causes a nonfatal, self-limiting disease characterized by abrupt onset of high fever, severe arthralgia, or arthritis, often associated with skin rash.

CHIKV was first isolated during an outbreak in Tanganyika (now Tanzania) in 1952–1953 (*1*). The virus is believed to have originated in Africa and subsequently was introduced into many regions of Asia (*2*). The first CHIKV isolation in Asia was in Thailand in 1958 (*3*), followed by India in 1963 (*4*). A 2002–2003 serosurvey on 531 healthy young adults in Singapore showed a low prevalence (0.3%) of chikungunya antibodies (*5*). Although CHIKV has caused several large-scale epidemics in Asia and the Pacific region, it largely was neglected until its reemergence in the Indian Ocean Islands in early 2005 (*6*). Since then, CHIKV has caused outbreaks in India (*7*), Sri Lanka (*8*), Singapore (*9*), Malaysia (*10*), and Italy (*11*), focusing global attention on this newly emerging disease.

CHIKV is an enveloped, positive strand RNA virus with a genome of ≈11.8 kb (*12*). Phylogenetic analysis of the CHIKV genome has identified 3 lineages; West African, Asian and East, and Central and South African (ECSA) (*13*). The Asian lineage circulated in Asia until it was replaced by the ECSA type, which emerged during the 2005–2006 outbreaks in the Indian Ocean Islands and India (*6*).

Unlike in Africa, where the virus is maintained in a sylvatic cycle, chikungunya in Asia has been an urban disease, typically found in dengue-endemic areas and transmitted largely by *Aedes aegypti* mosquitoes. However, the predominant *Aedes* sp. in locations such as Réunion Island, where chikungunya emerged in 2005, was *Ae. albopictus* (*14*). The spread of chikungunya into rural areas during the later stages of outbreaks in India further confirmed the potential of *Ae. albopictus* mosquitoes in transmitting CHIKV (*15*). These changes were concurrent with the emergence of a strain having an alanine to valine substitution at codon 226 (A226V) of the envelope 1 (*E1*) gene in Réunion Island (*16*) and India (*17*). This mutation is known to increase the transmissibility of the virus by *Ae. albopictus* mosquitoes (*18*).

Because there is no licensed vaccine or specific drug therapy available to cure the illness, intervention relies upon vector control and minimizing mosquito-human contact. The first chikungunya outbreak in Singapore during

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January 2008 was successfully contained by combining aggressive vector control operations with active case detection and isolation of patients (*9*). On February 21, 2008, 24 days (2 incubation periods) after the last reported case, the outbreak was declared closed (*9*). After 3 months of no cases, local chikungunya cases resurfaced in May 2008, causing an outbreak that is yet to be resolved. This outbreak coincided with a rise in chikungunya incidence in Malaysia (*10*). In this report, we focus on the virologic and entomologic investigations carried out in Singapore, which assisted in the effort against the emergence of chikungunya in 2008.

Methods

Case Surveillance

Singapore initiated a chikungunya surveillance system in late 2006. The medical community was apprised by the Ministry of Health to look out for chikungunya cases among febrile patients, especially when associated with symptoms and signs (e.g., arthralgia, rash) suggestive of chikungunya (*9*). At the Environmental Health Institute (EHI), a national public health laboratory, an active laboratory-based surveillance was set up among a network of general practitioners. Confirmed cases were categorized as imported or local based on detailed travel history. Virologic analysis described in this study was performed on samples received by the EHI as part of the national public health surveillance program designed for chikungunya in Singapore.

Laboratory Diagnosis

Diagnosis of chikungunya was confirmed by detection of a fragment of the nonstructural protein 1 gene of CHIKV by a real-time reverse transcription–PCR (RT-PCR) protocol described previously (*19*). CHIKV RNA was extracted from serum by using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), and the amplification was performed in a LightCycler 2.0 system by using Light Cycler RNA Master SYBR Green I kit (Roche Diagnostics GMbH, Mannheim, Germany) according to manufacturers' instructions. All tests included 2 negative controls: a PCR control and a negative extraction control of DNAse/RNAse-free water. The positive control was RNA extracted from a CHIKV culture with a known PFU titer determined by plaque assay. The presence of CHIKV was determined based on the melting peaks (83.07°C–84.17°C) of the positive control amplifications.

Design of Specific Primers for Sequencing

All primers were essentially constructed towards strains of the Indian Ocean and Central African origin using Gene Runner 3.05 (Hastings Software, Inc., Hastings, NY, USA) and Primer Select 5.03 (DNASTAR Inc., Madison, WI, USA) software. The primer sequences used are listed in Table 1.

Sequencing of the E1 Gene

Complimentary DNA was synthesized as described in SuperScript III First-Strand synthesis system for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA). All templates were purified with the QIAquick PCR purification kit (QIAGEN) before sequencing. Sequencing was performed using BigDye Terminator Cycle Sequencing kit, according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

The nucleotide sequences were assembled using the SeqMan II version 5.03 (DNASTAR) and aligned using Clustal W multiple alignment tool in the BioEdit Sequence Alignment Editor version 7.0.9.0 (*20*). The phylogenetic tree was inferred based on the 1,002-nt sequence of the *E1* gene from aa residues 91 to 424, using the maximum-likelihood (ML) method as implemented in PAUP* version 4.0b10 (*21*). Bootstrapping to access the robustness of the ML tree topology was performed using the neighbor-joining method under the ML criterion based on 1,000 replicates.

Entomologic Surveillance

Seven local transmission clusters representing major local outbreaks were selected for entomologic investigation: Little India (1°18′24″N, 103°50′57″E), Queen Street (1°17′ 52″N, 103°51′05″E), Teachers' Estate (1°23′0″N, 103° 49′43″E), Kranji (1°25′30″N, 103°45′43″E), Sungei Kadut (1°25′1″N, 103°45′2″E), Mandai Estate (1°24′31″N, 103°45′34″E), and Bah Soon Pah Road (1° 24′45″N, 103°49′E) (Figure 1). These areas were classified naturally into urban (Little India and Queen Street), suburban

Table 1. Primers for DNA template synthesis and sequencing of chikungunya virus, Singapore*

Name/genomic position†	Sequence (5′ → 3′)
ChikE1/9870F	ACAAGCCCTTATTCCGCTG
ChikE1/9994F	TACGAACACGTAACAGTGATC
ChikE1/10246F	TACCCATTTATGTGGGGC
ChikE1/10378F	GCATCAGCTAAGCTCCGC
ChikE1/10397R	ACGCGGAGCTTAGCTGAT
ChikE1/10521R	ACCTTTGTACACCACAATT
ChikE1/10643F	CACAACTGGTACTGCAGAGACC
ChikE1/10710R	GCCAGATGGTGCCTGAGA
ChikE1/10965F	GAAAGGCAAGTGTGCGGT
ChikE1/10993R	TCATCGAATGCACCGCAC
ChikE1/11232F	CACGGGAGGTGTGGGAC
ChikE1/11238R	TCCCGTGATCTTCTGCACC
ChikE1/11359R	GTGTGTCTCTTAGGGGACACATA

*Chik, chikungunya; F, forward primer; R, reverse primer.
†Genomic position of chikungunya virus (GenBank accession no. DQ443544.2) to which the first base (5′ end) of the primer corresponds.

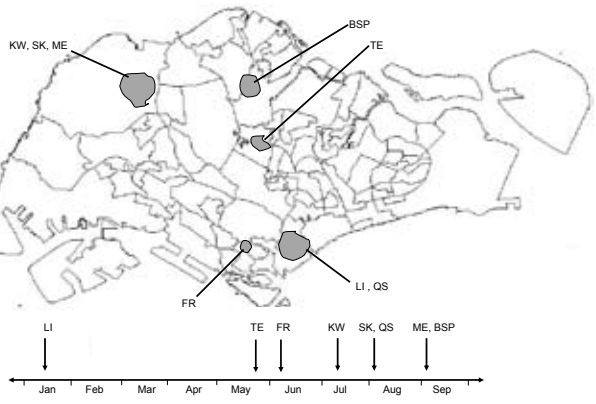

Figure 1. Geographic and temporal distribution of 123 indigenous chikungunya cases in Singapore. Shading indicates the 7 cluster areas where entomologic investigation was carried out. Data include cases reported through September 2008. The arrows in the timeline shown below the map indicate the months of occurrence of the local outbreaks from the beginning of January to the end of September 2008. BSP, Bah Soon Pah Road; FR, Farrer Road; KW, Kranji Way; LI, Little India; ME, Mandai Estate; QS, Queen Street; SK, Sungei Kadut; TE, Teachers' Estate.

(Teachers' Estate) and rural (Kranji, Sungei Kadut, Mandai Estate and Bah Soon Pah Road). The georeferenced *Aedes* larvae collection data from the chikungunya clusters were extracted from the Geographic Information System (ArcGIS) database of the National Environmental Agency, Singapore. The database, which is a part of the national vector control program, was assembled based on routine vector surveillance data obtained daily through area-wide inspection for mosquito breeding by ≈500 vector control officers.

The ultimate objective of this routine exercise was to identify as many active breeding places as possible in all residential and nonresidential premises within each cluster area. The collected larvae were separated into species based on morphologic identification before their numbers were counted. For this study, larval surveillance data were expressed as the larval abundance index, the ratio between the numbers of *Ae. aegypti* and *Ae. albopictus* larvae collected. For a single case, the number of larvae found within a 200-m radius of the case was used to calculate the larval abundance index, whereas the number of larvae found within the boundary of the cluster area was used in widespread clusters. Larval data collected 3 months before and after the first case reported from each cluster were used to calculate the index.

In each cluster area selected, adult mosquito surveillance was also conducted to determine the *Aedes* spp. composition and to confirm the presence of CHIKV in identified mosquitoes. Adult mosquitoes were collected using the sweep-net method, the Biogents (BG) Sentinel Trap (Biogents AG, Regensburg, Germany) or both. In each area, adult mosquito surveillance was conducted within 1-week from the beginning of the outbreaks, usually at the location from where the highest number of cases was reported. The survey was conducted once in all areas, except for Kranji Way, where it was carried out twice with a gap of 1 week between each collection. The number of locations surveyed ranged from 1 to 25 premises in each area, with higher number of premises in urban areas and lower numbers in rural areas in general. However, if a single case was reported from a cluster area, the adult mosquito survey was conducted in a few randomly selected premises within the neighboring area of the index case, even if it was an urban area. The sweep net method was performed in Little India and Teachers' Estate areas. The BG Sentinel traps were deployed in Queen Street, Sungei Kadut, Mandai Estate, and Bah Soon Pah Road areas. The number of traps deployed in each area ranged from 4 to 15 traps, with a trapping duration of 12 to 24 hours on each occasion. The sweep net method and BG Sentinel traps were used in Kranji Way. Adult *Aedes* mosquitoes were crushed individually in minimum essential medium before RT-PCR was performed as for serum samples. The isolated viruses were sequenced and analyzed as described above.

Results

Chikungunya Cases

From December 2006 through December 2007, a total of 1,375 samples were tested at the EHI for chikungunya; 10 of these cases were positive by PCR or immunoglobulin M testing. Epidemiologic investigation showed that all these cases were imported from India, Maldives, Sri Lanka, and Indonesia, which generally reflected the regional distribution of chikungunya during that time.

More than 7,000 samples from general practitioners, hospitals, and active case detection were tested from January through September 2008. In January 2008, the first locally acquired case of chikungunya was detected in the Little India area by a general practitioner involved in the chikungunya surveillance network (Figure 1). A total of 13 locally acquired chikungunya cases were confirmed by PCR before the outbreak was finally brought under control.

Between the first episode of transmission and May 2008, 6 cases imported from Sri Lanka (n = 2), Indonesia (n = 3), and Malaysia (n = 1) were diagnosed. By June, the number of imported cases increased, and the local scene remained relatively quiet with only 2 episodes of local transmission in Teachers' Estate area in late May (2 cases) and Farrer Road area in early June (1 case) (Figure 1). Both of these episodes were in suburban residential areas. Active case detection did not show any additional cases associ-

ated with those 2 episodes. Locally acquired cases occurred again in July 2008 coinciding with a rise in imported cases from Malaysia. By the end of September 2008, there was a cumulative total of 231 cases comprising 108 imported and 123 locally acquired infections. Of the imported cases, 92% (n = 99) had travel history to Malaysia, largely to the state of Johor, whereas the 123 local cases were distributed across 25 different locations.

After July 2008, transmission was more active in rural industrial and farming areas of Singapore, with the biggest clusters being in Kranji, Sungei Kadut, and Bah Soon Pah Road (Figure 1). Notably, during the active case surveillance using PCR, 2 viremic cases were found 1 day before the onset of clinical manifestations, with viral loads of 750 pfu/mL and 40 pfu/mL of blood, determined by using an external standard curve generated by plotting 10-fold serially diluted virus from a concentration of 10^8 pfu/mL, against respective crossing-point values of real-time PCRs.

Virologic Investigation

The *E1* gene of CHIKV from 85 imported and locally acquired infections was analyzed. Because there were several groups of similar sequences, the phylogenetic tree was constructed by using only 17 sequences that represented in all imported as well as locally acquired strains at different time points. The tree also included 5 CHIKV from Sri Lanka sequenced at the EHI and 17 global sequences retrieved from the GenBank database (Figure 2). Phylogenetic analysis showed that all viruses reported in Singapore after January 2008, except 1, were related to the ECSA genotype. CHIKV isolated from the remaining infection was of Asian lineage and was imported from Indonesia (Figure 2). All ECSA-type viruses formed a distinct clade, together with isolates from India, Sri Lanka, Italy, and the Indian Ocean Islands (Figure 2). In the phylogenetic tree, the viruses isolated during the first outbreak in the Little India area clustered closely with those reported in India in 2006. One isolate from an imported case from Maldives also clustered within this group. In contrast, viruses isolated during the second local episode in the Teachers' Estate in May 2008 and all other areas from July 2008 grouped with those imported from Malaysia. Similarly, CHIKV isolated during the third local episode in the Farrer Road area in June 2008 clustered separately with isolates from Sri Lanka (Figure 2).

CHIKV isolated during the first local outbreak was wild-type (alanine) at aa residue 226 (A226) of the *E1* gene, whereas, those detected during the second, third, and subsequent local episodes contained valine (A226V). Besides A226V, CHIKV isolated during the second local outbreak showed 2 synonymous mutations at nucleotide positions 300 (C300T) and 363 (A363G) of the *E1* gene, which were not present in viruses involved in the first and third

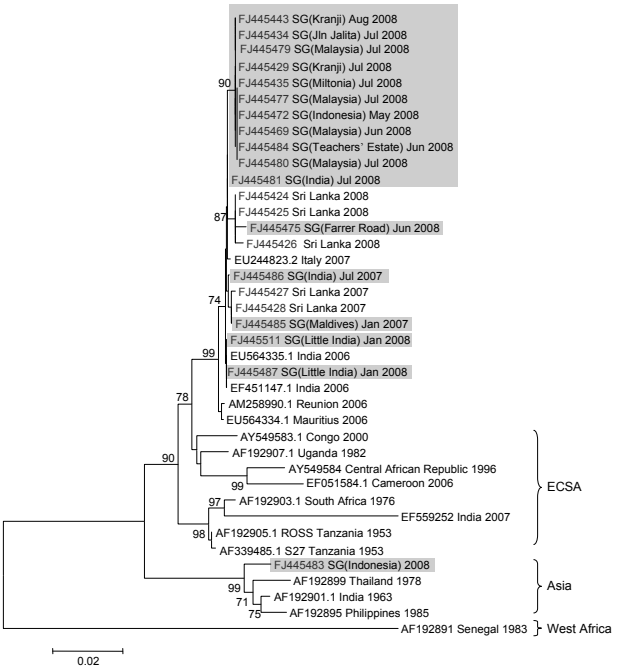

Figure 2. Phylogenetic analysis of the chikungunya virus (CHIKV) envelope 1 (*E1*) gene. The maximum-likelihood method was used to construct the phylogenetic tree by using 1,002 nucleotides of the sequence of the *E1* gene from codons 91 to 424. The tree included 17 isolates detected in Singapore (shaded), 5 Sri Lankan isolates sequenced at the Environmental Health Institute, and 17 global sequences selected to represent all known phylogenetic lineages. In the tree, all sequences are labeled with GenBank accession numbers and country of origin, and are isolated by year/month. In addition, all locally acquired and imported Singapore isolates are labeled with the reported area and country of origin, respectively, within parentheses. Only the bootstrap values >70 are shown on branches. Scale bar indicates nucleotide substitutions per site. ECSA, East, Central and South African genotype; SG, Singapore.

outbreaks. Of these isolates, C300T was unique to CHIKV strains imported from Malaysia. C300T and A363G were also found in all viruses detected in imported cases from Malaysia after June 2008. Similarly, CHIKV isolated in the third local episode was unique because it showed 2 synonymous mutations at nucleotide positions 105 (A105G), 1308 (C1308T) and a nonsynonymous mutation at nucleotide position 633 (A633C [K211N]) of the *E1* gene, the combination of which was unique to CHIKV isolates from Sri Lanka. Therefore, we defined the combinations of C300T + A363G and A105G + A633C + C1308T as genetic signatures of isolates from Malaysia and Sri Lanka, respectively. These observations demonstrated that the first 3 episodes of chikungunya transmission in Singapore were most likely due to independent importations of distinct viruses from different geographic locations.

Entomologic Investigation

Aedes larval collection data showed that *Ae. albopictus* was the predominant species in all cluster areas, except Little India, an urban area where the first outbreak occurred (Table 2). In the Little India cluster, larval abundance index in the Clive Street area (2.14:1) was even higher than the generalized ratio for the whole cluster (1.77:1). The Clive Street area is a highly urbanized area and reported the highest number of chikungunya cases (n = 10) within the Little India cluster. This observation was further strengthened by adult mosquito surveillance, which yielded only *Ae. aegypti* in the Little India cluster. In contrast, *Ae. albopictus* (n = 164) was the only *Aedes* sp. caught in other cluster areas (Table 2). Adult *Ae. albopictus* mosquitoes from the Kranji Way and Bah Soon Pah Road areas were positive for CHIKV by RT-PCR. In Kranji Way, 7 (9.1%) of 77 female *Ae. albopictus* mosquitoes were positive for CHIKV, whereas 6 (13.5%) of 45 mosquitoes were positive in the Bah Soon Pah Road area. The *E1* gene sequences of those 13 *Ae. albopitcus*-borne CHIKV were identical to sequences of strains imported from Malaysia. All mosquito-borne viruses possessed the A226V substitution.

Discussion

Chikungunya is an emerging infectious disease of public health importance in Singapore. Owing to Singapore's small size, tropical climate, presence of the vectors, and high population density, timely and effective disease control is required to minimize the risk for chikungunya outbreaks. Since its emergence on the local scene in January 2008, entomologic and virologic investigations have been used to elucidate the origin of the current outbreak of chikungunya in Singapore.

Phylogenetic data showed that the first, second, and third episodes of local transmission from January 14, 2008 to June 9, 2008, were due to 3 genetically distinct viruses of different geographic origins. The first outbreak in the Little India area in January 2008 was due to a CHIKV strain of Indian origin, whereas the second episode (2 cases) in Teachers' Estate area in May was due to a strain closely related to viruses detected in cases imported from Malaysia. On the other hand, the CHIKV strain of the third episode (1 case) in Farrer Road area in June was closely related to isolates from Sri Lanka. According to epidemiologic data, no locally acquired chikungunya cases occurred between the first and the second episodes. Similarly, no cases were reported between the second and third episodes. Therefore, the possibility that CHIKV involved in the first outbreak evolved into genetically distinct strains detected in the second and third episodes was highly unlikely.

The unique genetic signatures among these viruses and the lack of local transmission between episodes indicated that the first 3 local episodes were most likely due to independent importations of CHIKV, most likely from India, Malaysia, and Sri Lanka. This finding was further supported by the fact that 6 imported cases reported during the first and second episodes included cases imported from Malaysia and 2 from Sri Lanka. However, all cases reported after July 2008 were due to a single strain, which was closely related to CHIKV detected in cases imported from Malaysia. This strain was genetically close to the virus that caused the second episode. Recently, it was reported that the 2007 Chikungunya outbreak in Malaysia was due to a virus of the ECSA lineage (*10*). This evidence points to the interconnectedness of simultaneous chikungunya outbreaks in Singapore and Malaysia, which is not unexpected given the close proximity and porous borders between these 2 countries.

Entomologic surveillance showed a difference between the vector species involved in the first and subsequent outbreaks in Singapore. All adult mosquitoes caught in the vicinity of the first outbreak area (Little India) were *Ae. aegypti*. The larval surveillance data also showed the predominance of *Ae. aegypti* mosquitoes in this area (Table 2). Little India is generally a highly urbanized area with sparse vegetation, which could explain the presence of more *Ae. aegypti* vectors than *Ae. albopictus*. On the other hand, subsequent chikungunya episodes were seen in less-urbanized areas (Table 2) where *Ae. albopictus* was the predominant

Table 2. Summary of the characteristics and entomologic data of chikungunya cluster areas, Singapore

Location	Type	No. cases*	Adult female mosquito collection†		*Aedes* larval abundance index‡
			Aedes aegypti	*Ae. albopictus*	
Little India	Urban	13	10	0	1.77:1 (826:466)
Queen Street	Urban	1	0	2	0:1 (0:127)
Teachers' Estate	Suburban	1	0	10	0.03:1 (40:1,261)
Kranji Way	Rural	41	0	77	0.04:1 (1,129:26,546)
Sungei Kadut	Rural	33	0	7	0.001:1 (70:77,086)
Mandai Estate	Rural	11	0	23	0.02:1 (30:1,260)
Bah Soon Pah Road	Rural	21	0	45	0:1 (0:3,465)

*Numbers are preliminary data from press releases.
†Species of adult mosquitoes collected in each location where entomologic surveillance was conducted. The numbers do not necessarily represent adult mosquito density in each area as the numbers of traps and man-hours committed were not consistent.
‡*Aedes* larval abundance index is expressed as the ratio between the number of *Ae. Aegypti* and *Ae. albopictus* larvae collected through routine surveillance, 3 months before and up to 3 months after the detection of the first case at respective locations. Number of larvae (*Ae. aegypti; Ae. albopictus*) collected in each cluster is shown in parentheses.

vector species. Detection of CHIKV in *Ae. albopictus* mosquitoes further confirmed its role in CHIKV transmission in less urbanized areas. In general, large clusters of chikungunya were seen in less urbanized areas, with a high *Ae. albopictus* mosquito density near human habitations.

Of note, CHIKV strains isolated from Little India, an *Ae. aegypti* mosquito–abundant area, showed alanine at codon 226 (A226) of the *E1* gene. In contrast, all CHIKV strains isolated during subsequent episodes showed A226V substitution and were distributed in areas that were mainly inhabited by *Ae. albopictus* mosquitoes. Recently, Tsetsarkin et al. showed that CHIKV strains with A226V substitution replicate better in *Ae. albopictus* mosquitoes than does the wild-type strain (*18*). Their findings indicated that although the transmission potential of the wild-type virus is optimum for *Ae. aegypti* mosquitoes, A226V substitution confers greater vector competence in *Ae. albopictus* mosquitoes, making the latter species a better vector of the mutated strain than *Ae. aegypti* (*18*). This finding may result in selection for the mutated strain in areas where *Ae. albopictus* mosquitoes are abundant. Although the competence of *Ae. aegypti* mosquitoes in transmitting the virus with A226V in Singapore remains uncertain, the known evidence may therefore explain why the mutant virus with A226V caused outbreaks in less urbanized areas in Singapore where *Ae. albopictus* mosquitoes dominate but had little effect on urbanized areas where *Ae. aegypti* mosquitoes dominate. Similarly, this finding could also explain the distribution of the wild-type (A226) strain in urban areas, where *Ae. aegypti* mosquitoes are predominantly found. A similar observation has also been made in India, where the emergence of CHIKV with A226V was first reported in rural areas of Kerala region that are predominantly inhabited by *Ae. albopictus* mosquitoes (*15*). The low transmission rate of the mutant virus in urban and suburban Singapore could also be due to the aggressive dengue control program, which targets mainly urban and suburban parts of the country.

Based on these observations, the National Environment Agency's *Aedes* spp. control strategy was revised and expanded, especially in areas where *Ae. albopictus* mosquitoes are present. Because *Ae. albopictus* are generally outdoor mosquitoes, in contrast to *Ae. aegypti*, measures such as outdoor fogging and residual spray of external walls were conducted in chikungunya outbreak areas. The phylogenetic data was mainly used to trace the possible origins of viral strains causing the local chikungunya episodes. The longitudinal monitoring of *E1* gene sequences of CHIKV is in progress to monitor local transmission of chikungunya in Singapore. Our results showed that Singapore, being a travel hub and a cosmopolitan city, is vulnerable to multiple importations of CHIKV. The aggressive A226V variant of the ECSA genotype that has established itself in the region is posing a challenge to Singapore. Because *Ae. albopictus* is a common vector species in the region, the establishment of the A266V CHIKV variant in the region may continue to pose challenges in the years to come.

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Reproducibility of Serologic Assays for Influenza Virus A (H5N1)

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Hemagglutination-inhibition (HI) and neutralization are used to evaluate vaccines against influenza virus A (H5N1); however, poor standardization leads to interlaboratory variation of results. A candidate antibody standard (07/150) was prepared from pooled plasma of persons given clade 1 A/Vietnam/1194/2004 vaccine. To test human and sheep antiserum, 15 laboratories used HI and neutralization and reassortant A/Vietnam/1194/2004, A/turkey/Turkey/1/2005 (clade 2.2), and A/Anhui/1/2005 (clade 2.3.4) viruses. Interlaboratory variation was observed for both assays, but when titers were expressed relative to 07/150, overall percentage geometric coefficient of variation for A/Vietnam/1194/2004 was reduced from 125% to 61% for HI and from 183% to 81% for neutralization. Lack of reduced variability to clade 2 antigens suggested the need for clade-specific standards. Sheep antiserum as a standard did not reliably reduce variability. The World Health Organization has established 07/150 as an international standard for antibody to clade 1 subtype H5 and has an assigned potency of 1,000 IU/ampoule.

Influenza viruses agglutinate erythrocytes by binding to cell surface sialic acid. Agglutination may be blocked by strain-specific antibody detectable in hemagglutination-inhibition (HI) tests (*1*). Because serum HI titers correlate with protection (*2*), they are used to evaluate immunogenicity of influenza vaccines (*3–5*). However, conventional HI is generally insensitive for the detection of antibody to avian strains (*6,7*). Alternative serologic assays, including neutralization and HI with horse erythrocytes (hHI), are used to evaluate vaccine for pandemics (*7–9*). HI sensitivity for avian influenza increases when erythrocytes that express sialic acid containing α2,6-galactose linkages are used; these erythrocytes are preferentially recognized by avian hemagglutinin (*8,9*). Virus neutralization can be developed for any influenza subtype, although use of live virus may require heightened biocontainment.

Variability of influenza serologic assay results is partly attributed to differences in protocols and expression of endpoints (*10,11*). Assay variability limits comparison of candidate influenza virus subtype H5N1 vaccines in different clinical trials, posing challenges for licensure, particularly if specific seroprotective titers are required as endpoints (*3–5*). The use of bioassay standards to improve interlaboratory agreement is well recognized (*12*,*13*). However, the antigenic diversity of subtype H5N1 viruses (*14*) may pose challenges in maintaining relevant strain-specific antibody standards. We assessed the reproducibility of neutralization and hHI tests for influenza virus A (H5N1) and evaluated the suitability of a standard (freeze-dried plasma pool, obtained from persons vaccinated with clade 1 subtype H5N1, called 07/150) for detection of antibody.

Methods

Serum Samples

We used 14 serum samples (coded A–N) from persons who had received nonadjuvanted or adjuvanted split-product vaccine derived from reassortant clade 1 virus (A/

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Vietnam/1194/2004 or A/Vietnam/1203/2004). Samples included 3 prevaccination and postvaccination paired samples and 4 postvaccination samples. Postvaccination samples were generally obtained within 42 days of vaccination. Samples A and L were identical duplicates from a person primed with clade 0 vaccine and boosted with adjuvanted clade 1 vaccine. Sample K was from a person known to show nonspecific assay reactivity for antibodies to H5. Sample M was pooled negative human serum. Sheep antiserum to influenza subtype H5N1 (samples O and P) was supplied by the US National Institute of Allergy and Infectious Diseases and the UK National Institute for Biologic Standards and Controls, respectively. Serum P was produced by intramuscular administration of 20 μg hemagglutinin (HA) from A/Vietnam/1194/NIBRG-14 with Freund complete adjuvant, followed by 3 more injections with 10 μg HA with Freund incomplete adjuvant. Serum was collected 5 weeks after the first vaccination. Serum O was produced in a similar manner, with bromelin-cleaved purified HA. A single animal was vaccinated with A/turkey/Wisconsin/68 H5 bromelin-cleaved HA and received booster vaccinations with purified HA from A/Vietnam/1203/2004.

Candidate Standard 07/150

Standard 07/150 contained freeze-dried plasma from 9 persons who had received inactivated whole-virus A/Vietnam/1194/NIBRG-14 vaccine. Four donations (total volume 3 L) were obtained from Omninvest, Hungary (vaccine contained aluminum phosphate), and 5 donations (total volume 2 L) were obtained from Sinovac, People's Republic of China (vaccine contained aluminum hydroxide). Persons gave informed consent after studies had received approval by appropriate ethics committees. Donations were negative for antibodies to HIV-1, HIV-2, hepatitis B surface antigen, and hepatitis C RNA. Plasma was pooled and freeze dried at the National Institute for Biologic Standards and Controls, according to standard procedures (*15*) to produce 1-mg ampoules and stored at –20°C. Stability studies found no significant change in titers after 8 months at –20°C, +4°C, or +20°C when compared with samples stored at –70°C.

Virus Reagents

Reassortant subtype H5N1 influenza viruses were prepared by reverse genetics from wild-type viruses, amplified in 10-day-old embryonated hens' eggs, and stored at –80°C. Each virus passed internationally approved safety testing (*16*), which permitted use at Biosafety Level 2–enhanced facilities. The National Institute for Biologic Standards and Controls supplied NIBRG-14 (A/Vietnam/1194/2004, clade 1) and NIBRG-23 (A/turkey/Turkey/1/2005, clade 2.2) viruses, and the Centers for Disease Control and Prevention supplied IBCDC-RG5 (A/Anhui/1/2005, clade 2.3.4).

Study Design

Fifteen laboratories from 9 countries agreed to participate and were assigned a code from 1 to 15. One additional laboratory returned titers from 1 neutralization and 1 pseudotype assay and was excluded from analysis.

The participating laboratories were sent reagents on solid CO_2, asked to store serum at –20°C and viruses at –70°C, and instructed to reconstitute 07/150 with 1 mL distilled water and to test it and the serum for antibodies to each antigen, using hHI and neutralization, on at least 3 separate occasions. Suggested protocols were supplied, but participating laboratories could use in-house assays.

Statistical Analyses

Neutralization and hHI data consisted of replicate absolute titers, expressed as the reciprocal of serum dilution, and represented the last dilution giving a positive response from a doubling-dilution series. If the initial dilution did not give a positive response, the titer was recorded as less than the reciprocal initial dilution, e.g., <10 if the starting dilution was 1:10. Serum was interpreted as negative if no titer was detected and positive if any titer was detected. For calculation, negative titers were assigned the value of half the minimum detectable titer, and titers greater than the final dilution were assigned a value 2× the largest titer. These values represent the hypothetical adjacent dilution steps in the doubling-dilution series. This convention enables comparison of overall mean titers among groups on a consistent basis.

We calculated the geometric mean titer (GMT) for each serum, virus, and assay combination. Overall titers were calculated as the GMT of the individual laboratory means. Interlaboratory variation was expressed as percentage geometric coefficient of variation (%GCV) between the individual laboratory GMTs. The distribution of hHI or neutralization titers does not represent a continuous variable, and the results from using different viruses within laboratories are not independent. Thus, use of parametric modeling techniques, such as analysis of variance, to characterize intra- and interlaboratory variability was precluded.

To assess intralaboratory variation, we calculated the percentage of endpoints of replicate tests for identical serum samples A and L that differed >2-fold or >4-fold for each antigen and assay in each laboratory. We also compared the percentage of replicate tests returned for all serum samples and postvaccination samples that differed by >2-fold or >4-fold for each antigen and assay.

To assess interlaboratory variation, we compared differences between hHI and neutralization GMTs for 07/150 by different laboratories by using a paired nonparametric Wilcoxon signed-rank test for each antigen separately. For each laboratory, the difference in GMT between hHI and neutralization for NIBRG-14 was calculated, and these dif-

ferences were compared with zero by using the Wilcoxon signed-rank test. Similarily, the results for hHI with each antigen were compared, taking the laboratory differences between the hHI GMT for NIBRG-14 and NIBRG-23 and the differences between NIBRG-14 and IBCDC-RG5 and comparing these differences with zero. The same was done for neutralization assays. We also compared differences among overall (for all laboratories) mean GMT for all serum samples by using a paired nonparametric Wilcoxon signed-rank test for each antigen separately; e.g., for NIBRG-14, the difference between the overall mean GMT for hHI and neutralization was calculated for each sample, and these differences were compared with zero. The nonparametric tests use the ranks of observed titers to calculate the significance of differences among groups and are unaffected by the value chosen to represent titers below the initial dilution or greater than the highest dilution used in the individual assays.

To assess a standard's ability to improve interlaboratory agreement, we expressed titers relative to 07/150 by taking the ratio of the GMT for a sample to the GMT for 07/150 and multiplying it by an assigned value for 07/150. The assigned value was the overall GMT by hHI and neutralization. The effect on interlaboratory agreement and %GCV is independent of the value chosen.

To evaluate improvement in interlaboratory agreement from expressing titers relative to 07/150 (or sheep antiserum), we calculated %GCV between laboratory GMTs, both absolute and relative, for each sample. We then calculated the difference between the %GCV of the laboratory GMT of absolute titers and the %GCV of the laboratory GMT of relative titers. Using the Wilcoxon signed-rank test for each antigen separately, we compared these differences with zero.

Results

Assays

All participating laboratories returned at least 3 replicates by both assays, except laboratory 11, which did not perform hHI. Negative serum M was excluded because all titers were negative, except in laboratory 3, which reported 1 positive (titer 45) and 2 negative neutralization titers.

Intralaboratory Reproducibility: Identical Samples A and L

The numbers of intralaboratory comparisons of samples A and L differing by >2-fold were 1 (2.4%), 2 (4.8%), and 1 (2.4%) of 42 for NIBRG-14, NIBRG-23, and IBCDC-RG5, respectively, by hHI compared with 3 (7.1%), 3 (7.1%), and 1 (2.4%) of 42 by neutralization. Overall, 4 of 126 (3.1%) comparisons of identical samples by hHI differed by >2-fold compared with 7 of 127 (5.5%) by neutralization. No samples differed by >4-fold by either assay.

Intralaboratory Reproducibility: Replicate Assays

The proportion of serum samples for which replicate assays differed by >2-fold and >4-fold was assessed in each laboratory for all serum and postvaccination serum samples (Figure 1). By hHI, 13 of 14 (93%) laboratories reported >90% replicate titers within a 2-fold range. By neutralization, intralaboratory reproducibility was more variable; 9 of 15 (60%) laboratories reported >90% replicate titers within a 2-fold range. For postvaccination serum, greater variability was found with neutralization; laboratories 7, 14, and 15 reported >25% replicates differing by >2-fold. Three laboratories reported 3.7%–7.4% of replicate samples differing by >4-fold by either assay.

Interlaboratory Reproducibility of Absolute Titers: 07/150

All laboratories reported positive titers to 07/150, but variation was marked (Table 1). By hHI, the GMTs, ranges, and %GCVs in all laboratories to NIBRG-14, NIBRG-23, and IBCDC-RG5 were 140 (25–406; 16-fold difference, 112%), 102 (25–320; 13-fold difference, 109%), and 91 (25–256; 10-fold difference, 101%), respectively. By neutralization, the GMTs, ranges, and %GCVs of titers to NIBRG-14, NIBRG-23, and IBCDC-RG5 were 518 (127–2032; 16-fold difference, 120%), 291 (52–1810; 35-fold difference, 140%), and 299 (80–806; 10–fold difference, 109%), respectively. Titers were higher with neutralization than with hHI ($p = 0.003$, NIBRG-14; $p = 0.005$, NIBRG-23; $p = 0.005$, IBCDC-RG5; Wilcoxon signed-rank test for all comparisons). The GMT for NIBRG-14 was higher than that for clade 2 viruses (hHI: $p = 0.039$, NIBRG-23; $p = 0.028$, IBCDC-RG5; neutralization: $p = 0.004$, NIBRG-23; $p = 0.008$, IBCDC-RG5; Wilcoxon signed-rank test).

Interlaboratory Reproducibility of Absolute Titers

GMT and %GCV for each serum sample, virus, and assay were calculated (Table 2). For postvaccination serum, both assays showed higher titers to NIBRG-14 than to clade 2 viruses. For all serum, neutralization gave higher titers than hHI ($p = 0.001$, NIBRG-14; $p = 0.008$, NIBRG-23; $p = 0.001$, IBCDC-RG5; Wilcoxon signed-rank test) and fewer negative values but displayed more range variation, particularly in prevaccination serum. Absolute titers for sheep serum samples O and P were highly variable; %GCV was 147%–582% for hHI and 117%–283% for neutralization.

When summarized over all serum samples, the best interlaboratory agreement was for IBCDC-RG5 by hHI and neutralization; %GCVs were 108% and 112%, respectively (Table 3). The worst interlaboratory agreement was for

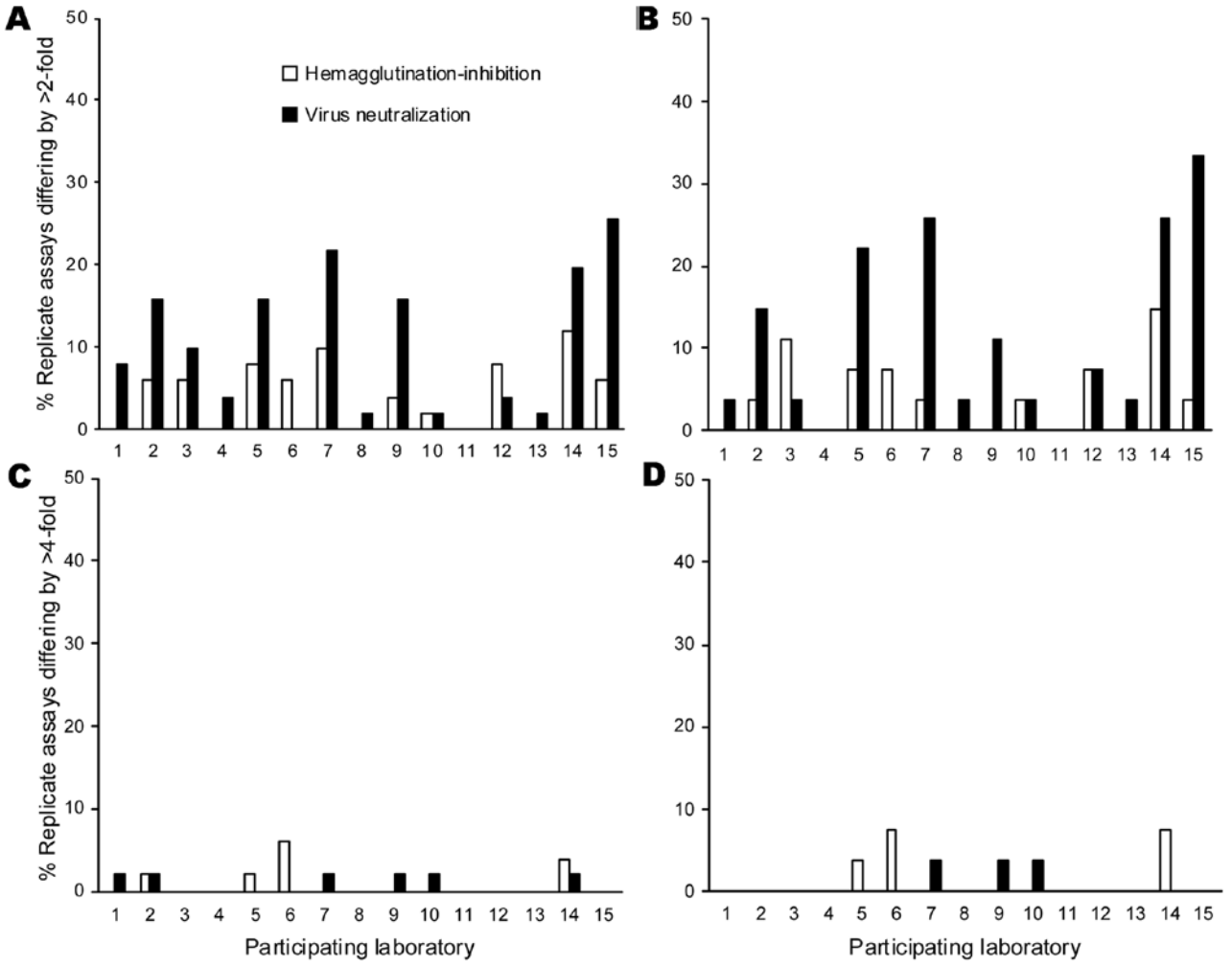

Figure 1. Intralaboratory reproducibility showing proportion (%) of replicate assays differing by >2-fold (A, B) and >4-fold (C, D) by horse hemagglutination-inhibition and neutralization assays for each participating laboratory for all serum samples (A, C) and postvaccination serum samples (B, D). Laboratory 11 did not return hemagglutinin-inhibition result.

neutralization with NIBRG-23 and NIBRG-14; %GCVs were 185% and 183%, respectively.

Reproducibility of Relative Titers: 07/150 or Sheep Serum as Standard

To evaluate the ability of 07/150 to improve interlaboratory agreement, GMTs were expressed relative to 07/150 for each sample (Table 2) and then summarized for all samples (Table 3). For all serum, interlaboratory reproducibility improved significantly for NIBRG-14; the median %GCV for hHI decreased from 125% to 61% ($p = 0.001$) and for neutralization from 183% to 81% ($p = 0.002$, Wilcoxon signed-rank test). However, for clade 2 viruses, interlaboratory variation did not change significantly. Figures 2 and 3 display the range of absolute and relative 07/150 titers for each antigen in serum F (shown as an example of postvaccination serum with midpoint GMT and wide range of values).

For sheep antiserum, the interlaboratory variability was increased because some laboratories reported negative hHI titers, resulting in high %GCV when test serum samples were expressed relative to them (Table 3). However, when these laboratories were excluded from analysis, the interlaboratory variation for NIBRG-14 by hHI became comparable to that found for 07/150. Laboratory 5 reported negative hHI titers for serum P; when that laboratory was excluded from analysis, the range of %GCV by hHI improved from 689%–796% to 51%–71%. Laboratories 5, 6, and 12 reported negative hHI titers for serum O; when they were excluded, the range of %GCV improved from 306%–442% to 39%–113%. When neutralization titers were expressed relative to serum O, interlaboratory variation to NIBRG-14 was reduced, in contrast with serum P, for which interlaboratory variation by hHI or neutralization did not improve for any antigen, even when laboratory 5, which failed to detect antibody in this sample, was excluded.

Table 1. Geometric mean titers (reciprocal shown) and geometric coefficient of variation for the candidate antibody standard 07/150, measured by each participating laboratory for each influenza virus*

	Assay tested and virus used (phylogenetic clade)					
	hHI†			Neutralization†		
Laboratory no.	NIBRG-14‡§ clade 1	NIBRG-23‡ clade 2.2	IBCDC-RG5§ clade 2.3	NIBRG-14‡§ clade 1	NIBRG-23‡ clade 2.2	IBCDC-RG5§ clade 2.3
1	101	160	80	480	605	419
2	25	25	101	1016	640	806
3	403	320	160	1810	1810	226
4	160	80	80	336	233	469
5	406	256	256	450	270	632
6	80	160	202	320	320	202
7	101	63	63	127	101	80
8	160	80	80	320	160	160
9	160	183	115	160	52	80
10	160	80	105	960	210	733
11¶	ND	ND	ND	640	254	320
12	403	160	202	320	160	160
13	80	32	25	640	320	320
14	160	127	25	2032	905	508
15	101	63	63	731	313	471
Overall GMT	140	102	91	518	291	299
GCV, %	112	109	101	120	140	109

*GMT. geometric mean titer; GCV, geometric coefficient of variation; hHI, hemagglutination-inhibition assay using horse erythrocytes; neutralization, virus neutralization assay; ND, not done.
†GMT neutralization vs. hHI (NIBRG-14, p = 0.003; NIBRG-23, p = 0.005; IBCDC-RG5, p = 0.005 by Wilcoxon signed-rank test).
‡GMT NIBRG-14 vs. NIBRG-23 (hHI, p = 0.039; neutralization, p = 0.004 by Wilcoxon signed-rank test).
§GMT NIBRG-14 vs. IBCDC-RG5 (hHI, p = 0.028; neutralization, p = 0.008 by Wilcoxon signed-rank test).
¶Laboratory 11 did not return hHI data.

Relationship between hHI and Neutralization

Because a serum HI titer ≈40 is considered seroprotective (*2*), the establishment of a consistent equivalence factor between an hHI titer of 40 and neutralization would be useful. However, the relationship of hHI and neutralization is dependent on the virus-serum-laboratory combination and cannot be generalized. Equivalence factors display large differences of 0.1–40.3 based on absolute titers and 0.3–6.3 based on titers relative to 07/150 for NIBRG-14 (online Appendix Table 1, available from www.cdc.gov/EID/content/15/8/1250-appT1.htm).

Serum K: False-positive Serum

Serum K was prevaccination serum from a person with detectable antibodies against influenza subtype H5 (by neutralization and hHI in the trial laboratory) but no known exposure to influenza subtype H5N1. Using NIBRG-14, NIBRG-23, and IBCDC-RG5 as test antigens, 13/14 (93%), 12/14 (86%), and 3/14 (21%) laboratories reported positive titers by both hHI and neutralization, respectively.

Assay Operating Protocols

Thirteen laboratories supplied hHI protocols. Although similar (online Appendix Table 2, available from www.cdc.gov/EID/content/15/8/1250-appT2.htm), they differed in some respects: pretest serum hemabsorption, erythrocyte suspension concentration (<1% vol/vol or ≥1% vol/vol), and time and temperature of erythrocyte-settling period (60 or ≥120 min, 4°C or room temperature). Although no relationship between protocol and intralaboratory reproducibility was found, laboratories that used lower erythrocyte concentrations or read plates at 4°C tended to report higher titers. Laboratories that performed pretest hemabsorption tended to report lower titers.

Thirteen laboratories supplied neutralization protocols (online Appendix Table 3, available from www.cdc.gov/EID/content/15/8/1250-appT3.htm) that were grouped into 3 broad methods: use of cell suspension for virus infection with short incubation time to endpoint (<26 hours), use of cell suspension with long incubation (>3 days), and use of cell monolayer for infection with long incubation (>3 days). Although no parameters were clearly associated with reproducibility, laboratories that used monolayers tended to report lower titers than those that used cell suspensions, and those that used longer incubation times had more interlaboratory variation by more frequently reporting titers at either end of the range (i.e., highest or lowest) than laboratories that used shorter times. Expression of initial serum dilution varied among laboratories as dilution of test serum was calculated either before or after the addition of virus.

Discussion

Having effective vaccines against influenza virus A (H5N1) is a public health priority. However, interlaboratory assay variation limits comparison of vaccine strategies without direct comparative studies. We compared the re-

Table 2. Geometric mean titers and percentage coefficient of variations of absolute titers and titers relative to candidate antibody standard 07/150 for each serum sample for each influenza virus by hHI and neutralization*†

	Virus assay, antigen, and clade					
	hHI (GMT, %GCV, %GCV relative to 07/150)			Neutralization (GMT, %GCV, %GCV relative to 07/150)		
Serum sample	NIBRG-14 clade 1	NIBRG-23 clade 2.2	IBCDC-RG5 clade 2.3	NIBRG-14 clade 1	NIBRG-23 clade 2.2	IBCDC-RG5 clade 2.3
Prevaccination						
B	7, 110, 72	7, 111, 86	6, 112, 85	12, 176, 202	12, 141, 173	10, 68, 223
N	6, 31, 46	5, 29, 18	5, 22, 0	14, 175, 228	13, 160, 215	10, 186, 250
J	10, 111, 123	8, 81, 155	6, 43, 96	19, 175, 228	18, 218, 309	11, 102, 287
Postvaccination						
Low						
C	34, 84, 36	17, 29, 18	10, 126, 164	63, 183, 80	27, 201, 236	23, 122, 206
D	15, 141, 105	8, 110, 127	6, 68, 45	19, 232, 248	15, 215, 288	12, 98, 226
High						
E	104, 133, 58	60, 96, 36	8, 128, 141	148, 191, 81	87, 159, 42	20, 118, 223
F	78, 97, 55	16, 141, 116	20, 104, 105	83, 144, 62	18, 152, 234	35, 147, 196
G	281, 152, 61	147, 102, 131	44, 163, 144	504, 132, 37	274, 217, 83	140, 103, 45
H	95, 118, 66	42, 144, 116	18, 106, 111	130, 166, 74	91, 157, 33	34, 127, 154
I	351, 125, 60	93, 91, 47	107, 75, 44	379, 199, 71	106, 207, 191	161, 136, 41
A	391, 138, 55	335, 114, 34	448, 119, 51	1,389, 86, 45	1,313,125, 76	2,893, 78, 63
L	391, 147, 61	398, 145, 42	480, 108, 44	1,453, 104, 63	1,520, 101, 85	3,097, 73, 52
False positive (K)	37, 120, 34	24, 136, 135	8, 81, 117	52, 185, 143	44, 185, 53	13, 112, 298
Sheep						
O	48, 262, 285	30, 245, 338	17, 147, 319	216, 139, 53	148, 170, 33	49, 117, 156
P	1,857, 582, 535	1,171, 496, 487	1,338, 487, 545	7,317, 148, 196	732, 283, 144	3,806, 145, 59

*GMT geometric mean titer; %GCV, percentage geometric coefficient of variation; hHI, hemagglutination-inhibition assay using horse erythrocytes; neutralization, virus neutralization assay. (Paired samples were B and C, N and F, and J and H.)
†Overall serum samples, GMT by neutralization vs. hHI (p = 0.001, NIBRG-14; p = 0.008, NIBRG-23; p = 0.001, IBCDC-RG5; Wilcoxon signed-rank test).

producibility of hHI and neutralization against a candidate standard. Overall, both assays were consistent, although neutralization displayed more intralaboratory variability than did hHI; 3 of 15 laboratories reported >2-fold differences in >25% of identical replicates.

Titers determined by neutralization were higher and had a greater range than those determined by hHI, which suggests that neutralization may be more sensitive, particularly with low-titered serum. However, for some prevaccination serum, e.g., sample N, 6 (40%) laboratories reported neutralization titers of 20–160 but negative hHI titers, which suggests nonspecific reactivity or that neutralization detects functionally different antibodies than HI. This finding is consistent with findings of seroprevalence surveys in which titers to influenza virus subtype H5N1 may be detected by neutralization but not HI or Western blot among some persons with no exposure to subtype H5N1 (*7*). Sample K was from a person who had no known exposure but had detectable antibodies against H5. Most (93%) laboratories detected anti-H5 reactivity to NIBRG-14 by neutralization in this sample, but fewer (21%) detected antibodies to IBCDC-RG5. Studies suggest that antibodies against subtypes H1N1 and H3N2 detected by neutralization may be more strain specific than those detected by HI (*10*,*17*); however, we did not observe this difference.

Consistent with previous serologic comparisons (*10,11*), interlaboratory variation was noted when absolute titers for the same serum samples were compared. Neutralization displayed more variability and had differences of 35-fold (%GCV 68%–232%) compared with differences of 16-fold (%GCV 22%–163%) for hHI. Although difficulty of measuring hHI values due to fragility of erythrocytes has been noted, the intralaboratory reproducibility of hHI appears better than that of seasonal HI (*10*,*11*). Both assays for subtype H5N1 compared favorably with those for subtype H3N2 evaluated previously, which found 128-fold (%GCV 138%–261%) and 724-fold differences (%GCV 256%–359%) with HI and neutralization, respectively (*10*), and up to 32-fold differences (%GCV 90%–128%) with HI to human influenza subtypes H1N1, H3N2, and B viruses (*11*).

Although HI is straightforward, most laboratories preferred their own assays. Variable parameters that may influence hHI include pretest serum hemabsorption (lowers titers) and erythrocyte suspension (higher concentration lowers titers). Because no common neutralization protocols exist, laboratories have developed their own protocols, which creates potential for variability. Because operator inexperience may have influenced reproducibility of assays for subtype H3N2 (*10*), laboratories were selected for expertise in serologic testing for H5. Although most used microneutralization based on an assay described by the World Health Organization (*18*), protocols differed by starting dilution of serum; preparation of cells; and virus inocula-

Table 3. Interlaboratory geometric coefficients of variation of absolute titers and titers and relative to candidate antibody standard 07/150 and sheep serum summarized over all serum for each influenza virus tested by hHI and neutralization*

%GCV, virus strain	hHI, all serum samples, median (min–max)	Neutralization, all serum samples, median (min–max)
%GCV of absolute titer		
A/Vietnam/1194/NIBRG-14 clade 1	**125 (31–582)†**	**175 (86–232)†**
A/Turkey/23/NIBRG-23 clade 2.2	114 (29–496)	170 (101–283)
A/Anhui/IBCDC-RG5 clade 2.3	108 (22–487)	112 (68–147)
%GCV of titers relative to 07/150		
A/Vietnam/1194/NIBRG-14 clade 1	**61 (34–535)**	**77 (37–285)**
A/Turkey/23/NIBRG-23 clade 2.2	106 (18–487)	144 (33–309)
A/Anhui/IBCDC-RG5 clade 2.3	105 (0–545)	196 (41–298)
%GCV of titers relative to serum P		
A/Vietnam/1194/NIBRG-14 clade 1	**796 (39–1,020)**	**249 (162–381)**
A/Turkey/23/NIBRG-23 clade 2.2	689 (7–953)	237 (90–844)
A/Anhui/IBCDC-RG5 clade 2.3	752 (0–1,005)	195 (66–263)
A/Vietnam/1194/NIBRG-14 clade 1 (excluding laboratory 5)	**68 (32–174)**	**255 (162–396)**
A/Turkey/23/NIBRG-23 clade 2.2 (excluding laboratory 5)	70 (7–222)	237 (90–844)
A/Anhui/IBCDC-RG5 clade 2.3 (excluding laboratory 5)	51 (0–148)	195 (66–262)
%GCV of titers relative to serum 0		
A/Vietnam/1194/NIBRG-14 clade 1	**442 (21–725)**	**78 (41–213)**
A/Turkey/23/NIBRG-23 clade 2.2	373 (20–804)	111 (29–204)
A/Anhui/IBCDC-RG5 clade 2.3	306 (0–812)	199 (44–323)
A/Vietnam/1194/NIBRG-14 clade 1 (excluding laboratories 5, 6, 12)	**39 (24–91)**	**82 (34–225)**
A/Turkey/23/NIBRG-23 clade 2.2 (excluding laboratories 5, 6, 12)	113 (22–225)	97 (25–186)
A/Anhui/IBCDC-RG5 clade 2.3	100 (0–198)	194 (47–299)

*%GCV, geometric coefficient of variation; hHI, hemagglutination-inhibition assay using horse erythrocytes; neutralization, virus neutralization assay; min–max, minimum–maximum. **Boldface** indicates homologous clade 1 strain.
†%GCV absolute titer vs. relative 07/150 for NIBRG-14 (hHI $p = 0.001$, neutralization $p = 0.002$; Wilcoxon signed-rank test).

tion, incubation, and endpoint estimation. Laboratories that performed assays with virus infection of cell monolayers generally reported lower titers than those that used suspensions. Assays with long incubation times and non-ELISA endpoints (e.g., cytopathic activity) were associated with greater interlaboratory variation than ELISAs with shorter incubation times. A biostandard should reduce variation associated with assay differences because standardization of protocols may be limited by local availability of reagents.

Expression of the initial serum dilution, which clearly influences absolute titers, should be standardized. Although HI titers are typically expressed as the serum starting dilution before any addition of virus, calculation of starting dilutions for neutralization varies among laboratories. We propose that the calculated starting dilution for seasonal and avian influenza neutralization be expressed as serum dilution before the addition of virus (e.g., 5 μL serum in 45μL diluent plus 50 μL virus solution is considered as 1:10) as it is with HI.

Because the correlation between serum antibodies detected by hHI and protective efficacy against influenza subtype H5N1 is unclear, by default, immunogenicity criteria established for seasonal vaccines (*3*–*5*) are used for subtype H5N1 vaccines despite the lack of established immune correlates for neutralizing antibodies. Although hHI and neutralization titers correlate closely (*9*,*19*), this and other studies (*10*) find that the relationship depends on individual laboratory-antigen-serum combinations and cannot be generalized.

A potential limitation to this study is that 07/150 was derived from recipients of adjuvanted whole-virus vaccine but test serum samples were obtained from persons who received plain or adjuvanted split-product vaccines. Interlaboratory agreement improved when NIBRG-14, but not heterologous antigens, was used, which suggests that 07/150 is clade specific. Although no association between vaccine formulation and %GCV was noted in test serum, the quality and cross-reactivity of antibodies induced by whole-virus vaccine may differ from quality and cross-reactivity induced by alternative formulations including adjuvanted, subunit, or recombinant vaccines. To reduce potential variation in antibody isotypes, we obtained day-42 post-vaccination samples when possible; however, the avidity of antibody to hemagglutinin or presence of antibody against denatured viral proteins after whole-virus vaccination (*20*) could influence the effectiveness of 07/150 against test serum. Differences among vaccine formulations should be examined, if possible, during evaluation of clade 2 standards; however, because production requires substantial donations of plasma, providing separate standards for specific vaccine formulations is impractical.

The overall reproducibility of sheep antiserum raised against clade 1 H5 hemagglutinin was poor; reported titers ranged widely. Because some laboratories failed to detect

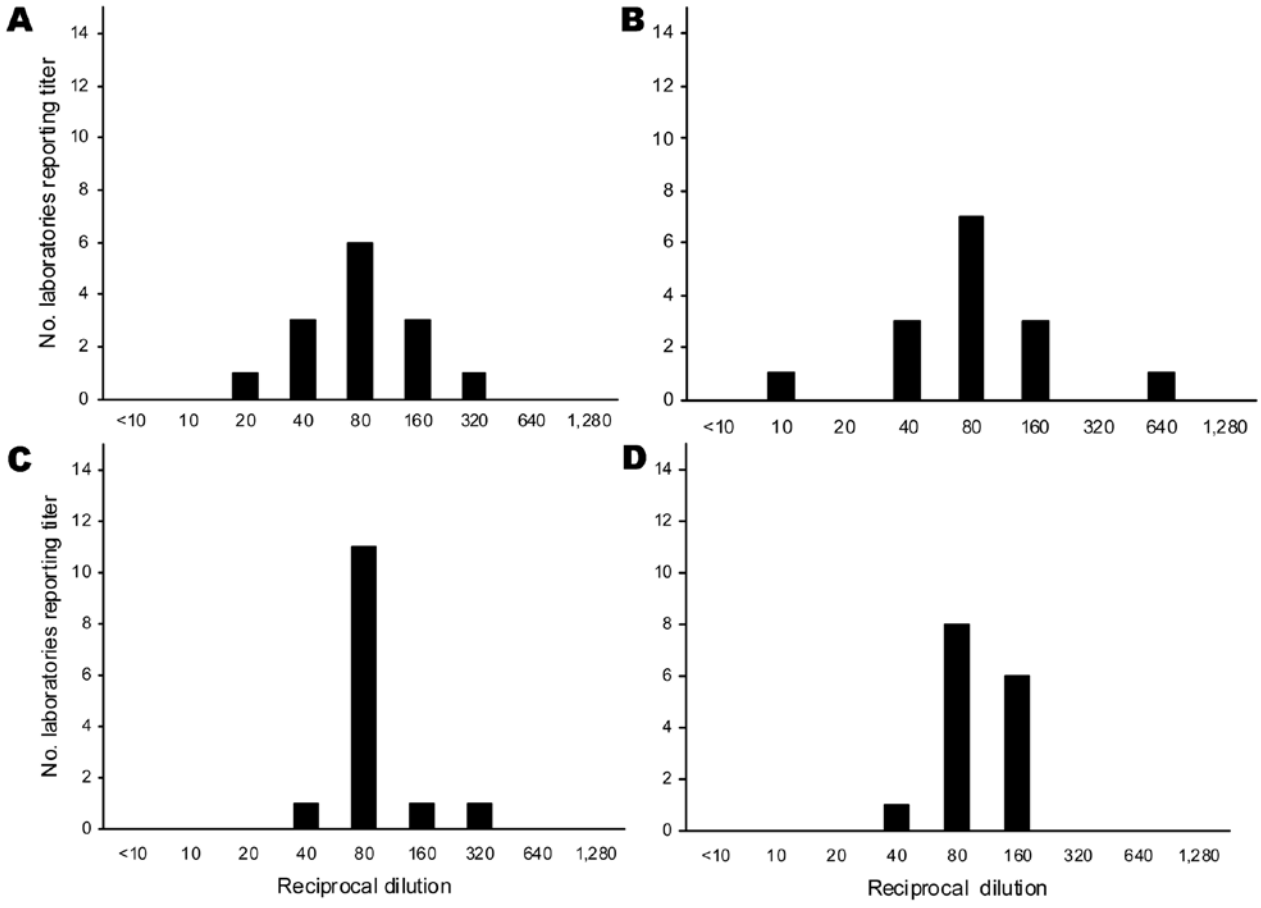

Figure 2. Range of hemagglutination-inhibition (HI) and neutralization titers to clade 1 homologous NIBRG-14 virus in postvaccination serum sample F: the number of laboratories reporting specific titer dilution of absolute titers and titers relative to 07/150. A) Absolute horse HI titers, B) absolute neutralization titers, C) titers relative to 07/150 horse HI titers, D) titers relative to 07/150 neutralization titers.

antibodies in sheep antiserum, the expression of relative titers did not reduce %GCV. When these laboratories were excluded from analysis, sheep serum improved interlaboratory agreement to NIBRG-14 by hHI but not by neutralization or for clade 2 antigens. This finding suggests that if assays can detect antibodies, sheep antiserum is a useful internal control; however, its role as an international standard is limited if some hHI assays appear unable to detect antibody titers. The reason for this discrepancy is unexplained because no clear association with assay method has been found. The antibody repertoire induced by cleaved hemagglutinin in Freund adjuvant in sheep antiserum will differ from that induced in humans by purified antigens. An alternative animal source and/or production method may be more reliable.

The World Health Organization Expert Committee on Biologic Standards has accepted 07/150 as an antibody standard for clade 1 H5 hemagglutinin and has assigned an arbitrary value of 1,000 IU. The assigned value of 1,000 IU is equivalent to an hHI titer of 140 (i.e., GMT to NIBRG-14 found across study laboratories), giving a seroprotective titer for 07/150 of ≈285 IU. For neutralization, a standard value of 1,000 IU for 07/150 would be equivalent to a neutralization GMT of 518. Because the relationship between hHI and neutralization is inconsistent and immune correlates are lacking, assigning a seroprotective level to neutralization is not possible. Useful information may be obtained by retesting serum from completed trials of clade 1 subtype H5N1 vaccine candidates against 07/150. Regulators will be required to discuss the interpretation of a standard before vaccine licensure for clinical use.

For standardizing serologic assays that use different influenza (H5N1) clades, a reliable animal serum source would be most convenient, but failure of some laboratories to detect antibody in sheep antiserum limits their use. The production of clade-specific standards for subtype H5 viruses will require human plasma donations, which can only be produced after initial clinical trials have been conducted. This requirement must be considered in future vaccine studies.

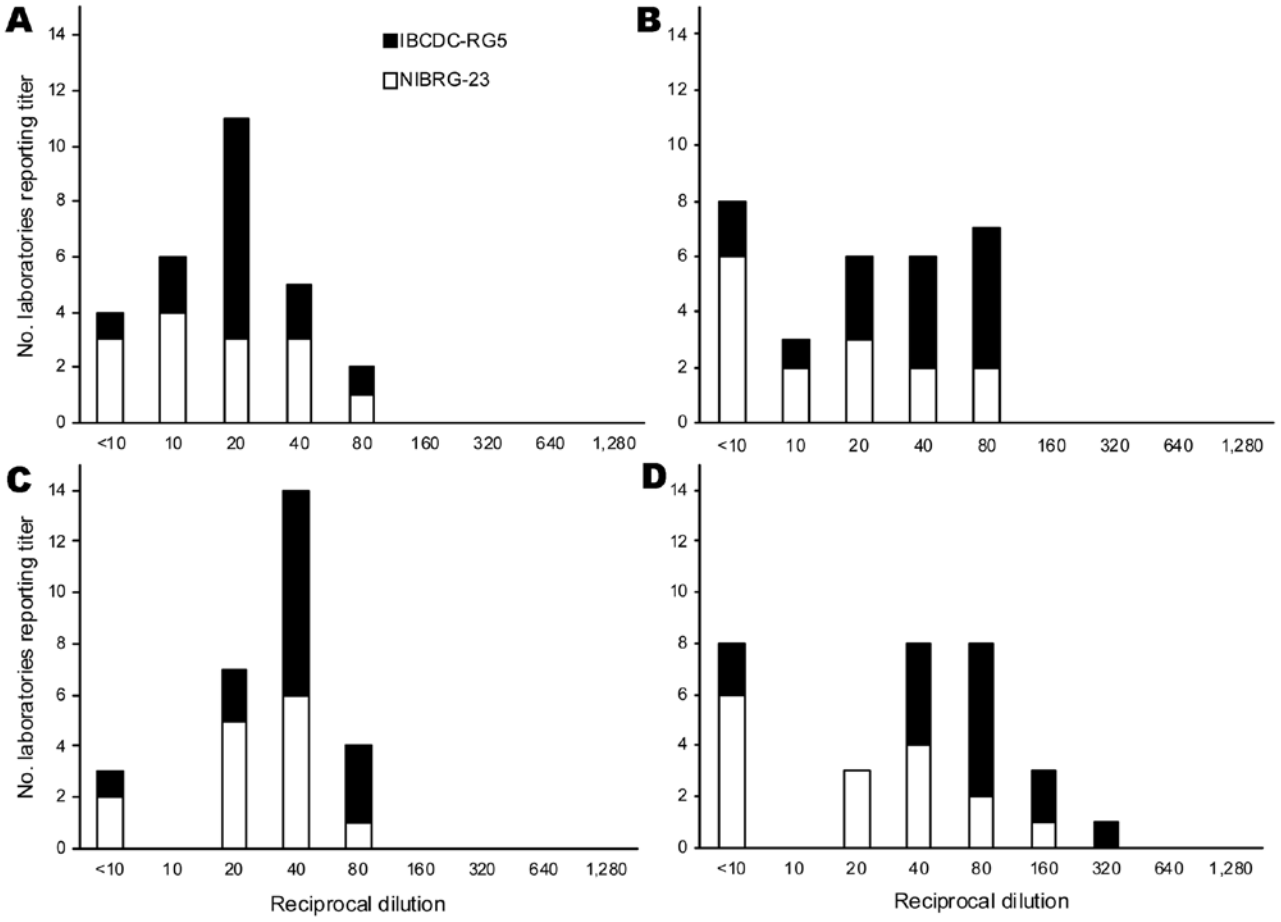

Figure 3. Range of hemagglutination-inhibition (HI) and neutralization titers to clade 2.2 and 2.3 heterologous NIBRG-23 and IBCDC-RG5 viruses in postvaccination serum sample F: the number of laboratories reporting specific titer dilution of absolute titers and titers relative to 07/150. A) Absolute horse HI titers, B) absolute neutralization titers, C) titers relative to 07/150 horse HI titers, D) titers relative to 07/150 neutralization titers.

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Increase in Pneumococcus Macrolide Resistance, United States

Stephen G. Jenkins and David J. Farrell

During year 6 (2005–2006) of the Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin surveillance study, 6,747 *Streptococcus pneumoniae* isolates were collected at 119 centers. The susceptibility of these isolates to macrolides was compared with data from previous years. Macrolide resistance increased significantly in year 6 (35.3%) from the stable rate of ≈30% for the previous 3 years (p<0.0001). Macrolide resistance increased in all regions of the United States and for all patient age groups. Rates were highest in the south and for children 0–2 years of age. Lower-level efflux [*mef*(A)]–mediated macrolide resistance decreased in prevalence to ≈50%, and highly resistant [*erm*(B) + *mef*(A)] strains increased to 25%. Telithromycin and levofloxacin susceptibility rates were >99% and >98%, respectively, irrespective of genotype. Pneumococcal macrolide resistance in the United States showed its first significant increase since 2000. High-level macrolide resistance is also increasing.

Antimicrobial drug treatment of community-acquired respiratory tract infections (RTIs) is usually initiated when the causative pathogen has not been documented. Treatment is therefore chosen empirically on the basis of potential pathogens and their antimicrobial susceptibility. *Streptococcus pneumoniae* is the major pathogen responsible for community-acquired RTIs (*1*), and treatment guidelines advise the use of agents that provide adequate coverage of this pathogen (*2*).

Although macrolides such as azithromycin and clarithromycin are active against *S. pneumoniae* and are in widespread clinical use, increasing in vitro bacterial resistance may have compromised their use. Resistance to macrolides in *S. pneumoniae* increased steadily during the 1990s; however, recent surveillance studies indicate that resistance may have plateaued at ≈30% in the United States (*3–5*). Although the link between in vitro resistance and clinical outcome is not fully understood, recent studies provide evidence that infection with macrolide-resistant pneumococci is a notable risk factor for failure of macrolide therapy in community-acquired RTIs (*6–9*).

Resistance to macrolides in *S. pneumoniae* is mediated by 2 major mechanisms: target modification caused by a ribosomal methylase encoded by the *erm*(B) gene or drug efflux encoded by the *mef*(A) gene. High-level macrolide resistance (MIC required to inhibit growth in 90% of organisms [MIC_{90}] ≥32 μg/mL) is usually associated with *erm*(B), whereas *mef*(A)-mediated resistance, the most prevalent mechanism in the United States (*10*), usually results in lower-level resistance (MIC_{90} 1–4 μg/mL) (*11,12*). Results from the Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin (PROTEKT US) surveillance study, covering isolates collected during 2000–2004, indicate that the prevalence of *mef*(A) is decreasing, and isolates harboring *erm*(B) and *mef*(A) genes are becoming increasingly common (*13*). In addition, isolates carrying only the *mef*(A) gene showed a higher-level resistance (MIC_{90} = 16 μg/mL) than observed previously (*10*). This analysis reports results from year 6 of PROTEKT US (2005–2006), focusing on macrolide-resistance rates and mechanisms in *S. pneumoniae* isolates collected from patients with community-acquired RTIs.

Methods

To reduce bias when interpreting trends, we restricted the analysis to *S. pneumoniae* isolates collected from the 119 centers that had previously provided isolates for year 5

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of the study. Isolates were collected from patients in whom clinical acute/chronic bacterial sinusitis, acute/chronic otitis media, acute bacterial exacerbations of chronic bronchitis, chronic obstructive pulmonary disease, or community-acquired pneumonia had been diagnosed. Specimen sources included ear, blood, bronchoalveolar lavage, sinus aspirate, and sputum. Isolates were included from adults and children.

MICs for the antimicrobial agents were determined at the Central Microbiology Institute (CMI; Portland, OR, USA) by using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (*14*) and were interpreted by using CLSI breakpoints (*15*). The breakpoints used for amoxicillin-clavulanate were ≤2 μg/mL (susceptible), 4 μg/mL (intermediate), and ≥8 μg/mL (resistant). Breakpoints used for cefpodoxime were ≤0.5 μg/mL (susceptible), 1 μg/mL (intermediate), and ≥2 μg/mL (resistant). Erythromycin-resistant (MIC ≥1 μg/mL) isolates were analyzed for the presence of *erm*(B), *mef*(A), and *erm*(TR) macrolide resistance genes by using a multiplex TaqMan PCR assay (*16*).

Results

Of 6,747 *S. pneumoniae* isolates collected at 119 centers in year 6 of PROTEKT US, 2,381 (35.3%) showed in vitro resistance to erythromycin; this result compares with 1,907/6,257 (30.5%) in year 5. Resistance rates for azithromycin and clarithromycin in year 6 were 35.3% and 35.2%, respectively. Erythromycin resistance was stable at ≈30% in years 3, 4, and 5. Analysis of isolates from centers common to years 3–6 showed a significantly higher rate for year 6 than for years 3–5 ($p<0.0001$ by χ^2 test).

Erythromycin resistance varied considerably by geography; the highest rates were in the North Central, Southeast, and South Central regions (Table 1). However, the rate of resistance increased from year 5 and year 6 in all 6 regions (Table 1).

Erythromycin resistance increased from year 5 to year 6 in all patient age groups; the highest rates of resistance occurred in isolates collected from children 0–2 years of age (year 5: 423/882 [48.0%]; year 6: 533/1,058 [50.4%]) (Figure 1).

Erythromycin resistance was less frequent in isolates collected from blood than in those collected from other sources (487/1,801 [27.0%] vs. 1,894/4,946 [38.3%]). The proportion of erythromycin-resistant *S. pneumoniae* (ERSP) isolates exhibiting high-level resistance to erythromycin (MIC ≥32 μg/mL) was 18.0% in year 6 compared with 13.4% in year 5.

Coresistance to penicillin (oral penicillin V for nonmeningitis isolates) was exhibited by 14.8% of ERSP isolates collected in year 6 compared with 13.2% of ERSP isolates collected at the same centers in year 5. Among all *S. pneumoniae* isolates, resistance to amoxicillin-clavulanate increased from 5.2% in year 5 to 8.1% in year 6; resistance to the third-generation oral cephalosporin, cefpodoxime, increased less substantially (19.4% in year 5 vs. 20.5% in year 6).

Genotyping

The distribution of genotypes among ERSP isolates changed from year 5 to year 6. Lower-level efflux *mef*(A)-mediated macrolide resistance decreased, while high-level *erm*(B) with or without *mef*(A) increased (Table 2). In year 6, just over half of ERSP isolates showed *mef*(A) resistance; nearly one quarter were positive for *erm*(B) and *mef*(A). Analysis of isolates from centers common to years 3–6 of the study indicated a significant decreasing prevalence of *mef*(A) and significantly increasing prevalence of *erm*(B) ± *mef*(A) ($p<0.0001$ and $p = 0.0033$, respectively, by χ^2 test).

ERSP isolates from patients 0–2 years of age showed the highest frequency of the *erm*(B) + *mef*(A) genotype (38.6% in year 6 compared with 35.5% in year 5). With the exception of years 3–4, the proportion of isolates harboring *erm*(B) and *mef*(A) throughout the 6 years of the PROTEKT US study has trended upward (Figure 2).

Most (398/575 [69.2 %]) of the *erm*(B) + *mef*(A) isolates from year 6 were serotype 19A; most of the remainder (154/575 [26.8%]) were serotype 19F. Overall, 72.8% of year 6 ERSP isolates were susceptible to amoxicillin-cla-

Table 1. Erythromycin resistance among *Streptococcus pneumoniae* isolates, year 5 (2004–2005) and year 6 (2005–2006) of the PROTEKT US surveillance study*

US region†	Isolates, no. resistant/no. submitted (%)	
	Year 5	Year 6
Northeast	518/1,931 (26.8)	662/2,102 (31.5)
North Central	467/1,314 (35.5)	568/1,395 (40.7)
Northwest	94/417 (22.5)	108/422 (25.6)
Southeast	340/998 (34.1)	419/1,064 (39.4)
South Central	402/1,149 (35.0)	529/1,368 (38.7)
Southwest	86/448 (19.2)	95/396 (24.0)
Total	1,907/6,257 (30.5)	2,381/6,747 (35.3)

*PROTEKT US, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin, United States.
†States submitting isolates for testing included Northeast: CT, DE, IN, MA, MD, MI, NJ, NY, OH, PA, RI, VT, and DC; North Central: IA, IL, KS, MN, MD, ND, NE, SD, and WI; Northwest: AK, ID, MT, OR, WA, and WY; Southeast: FL, GA, KY, NC, SC, VA, and WV; South Central: AL, AR, LA, OK, TN, and TX; Southwest: AZ, CA, CO, NM, NV, and UT.

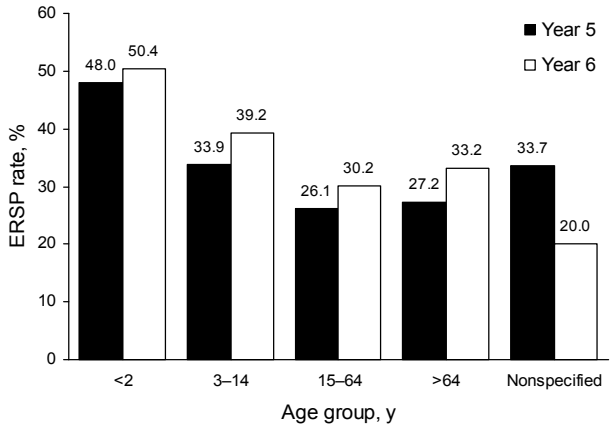

Figure 1. Increased prevalence of erythromycin-resistant *Streptococcus pneumoniae* (ERSP), by age group, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin, United States surveillance study, years 1–6 (2000–2006).

vulanate. However, amoxicillin-clavulanate susceptibility varied considerably between genotypes; <10% of isolates carrying *erm*(B) and *mef*(A) genes were susceptible to this agent, compared with >90% of isolates harboring either gene alone (Table 3). MIC_{50} and MIC_{90} for amoxicillin-clavulanate among *erm*(B) + *mef*(A) ERSP isolates were both ≥8 μg/mL; these were also the values for 19A and 19F strains. By contrast, >98% of ERSP isolates were susceptible to levofloxacin and >99% were susceptible to telithromycin. The genotypic mechanism of erythromycin resistance had little impact on susceptibility to either of these agents (Table 3).

Discussion

These findings from PROTEKT US indicate that pneumococcal macrolide resistance has demonstrated its first significant increase since the study began in 2000 (*4,13,17*). Whether the increase from ≈30% to 35% represents the start of a new upward trend will become evident only when results of surveillance studies in future years become available. However, it is worth noting that another smaller US surveillance study recently reported an azithromycin resistance rate of 34% in *S. pneumoniae* isolates collected during the same 2005–2006 respiratory infection season as this analysis (*18*). A further sustained rise in macrolide resistance would be a major cause for concern because macrolides, such as azithromycin and clarithromycin, remain in widespread use for the treatment of community-acquired RTIs in the United States.

The increase in ERSP isolates from year 5 to year 6 in all 6 regions of the country indicates a lack of specific local factors that might explain this sudden increase. Even so, resistance continued to be higher in some regions (Southeast, North Central, and South Central) than in others. Higher rates of macrolide resistance in the southern states aligns with a recent retrospective cohort study involving 1,574 patients with pneumococcal bacteremia, which identified residence in the southern United States as a risk factor for infection with macrolide-nonsusceptible pneumococci (*9*).

Other potential explanations for the increase in macrolide resistance include increased use and/or inappropriate prescription of macrolides. Pneumococcal macrolide resistance in *S. pneumoniae* has been linked in several studies with increased consumption of macrolides in general and of azithromycin in particular (*19*). Other factors associated with pneumococcal macrolide resistance are recent use of antimicrobial drugs, age extremes, and daycare attendance (*6,8*). However, which (if any) of these factors might explain the trends reported here are not clear.

Another concern arising from this report is the continuing change in the distribution of macrolide-resistance genotypes. Although *mef*(A), usually associated with lower-level macrolide resistance, remains the most prevalent genotype, it now accounts for only about half of all ERSP isolates. Isolates carrying *mef*(A) continue to be replaced by strains that harbor *mef*(A) and *erm*(B) genes. Data from the first 4 years of PROTEKT US showed that the proportion of *S. pneumoniae* isolates positive for *erm*(B) and *mef*(A) genes increased from 9.3% to 19.1% from Fall of 2000 through spring of 2001 and the same for subsequent years through spring of 2004 while isolates positive for the *mef*(A) gene decreased over this time from 69.0% to 60.7% (p = 0.03) (*10*). A Canadian study found a significant increase of 8% (from 4% to 12%) in the prevalence of dual *erm*(B) and *mef*(A)-positive *S. pneumoniae* isolates (p<0.05) between Fall and spring seasons of each year

Table 2. Macrolide resistance genotypes, year 5 (2004-2005) and year 6 (2005–2006) of the PROTEKT US surveillance study*

Genotype	No. isolates (% of ERSP)†	
	Year 5 (n = 1,907)	Year 6 (n = 2,381)
erm(B)	310 (16.3)	448 (18.8)
mef(A)	1,172 (61.5)	1,282 (53.8)
erm(B) + *mef*(A)	377 (19.8)	575 (24.1)
erm(TR)	2 (0.1)	0
Ribosomal mutations	26 (1.4)	41 (1.7)

*PROTEKT US, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin, United States; ERSP, erythromycin-resistant *Streptococcus pneumoniae.*
†A total of 20 isolates in year 5 and 35 isolates in year 6 were not viable for genotyping.

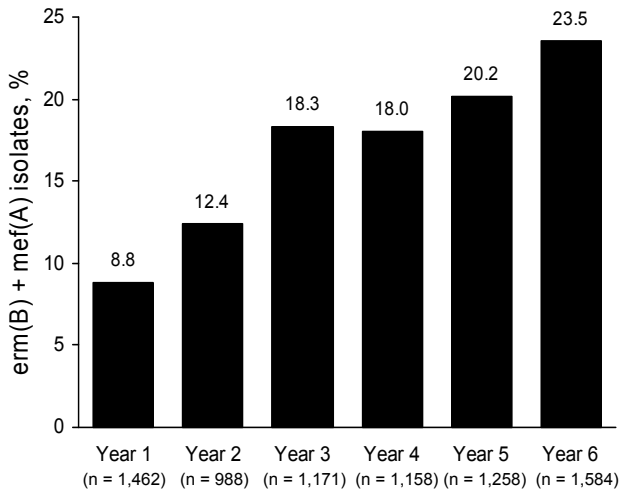

Figure 2. Increased prevalence in the *erm*(B) + *mef*(A) macrolide resistance genotype from year 1 (2000–2001) to year 6 (2005–2006), Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin, United States surveillance study.

(1998–2004); this increase coincided with a 17% decrease in high-level *erm*(B)-mediated resistance and a 5% increase in the proportion of isolates carrying only the *mef*(A) gene (*20*). The latest PROTEKT US data show that these trends are continuing; nearly one quarter of year 6 ERSP isolates have both resistance genes, and the frequency of this genotype is approaching 40% in isolates from children.

The increased prevalence of isolates harboring *erm*(B) and *mef*(A) genes is most likely due to the recent expansion in the US and elsewhere of a multidrug-resistant serotype 19A pneumococcal clone that carries both resistance genes (*21,22*). The expansion of this clonal variant resistance provides at least a partial explanation for the greater frequency of high-level erythromycin resistance observed in year 6 compared with that of the previous year. Most *erm*(B) + *mef*(A) strains show high-level resistance to macrolides (MIC ≥32 μg/mL). In addition, although *mef*(A) is traditionally associated with lower-level macrolide resistance (MIC 1–4 μg/mL), recent data suggest that the macrolide MICs for a growing proportion of *mef*(A) isolates exceed 16 μg/mL (*10*).

Because most *erm*(B) + *mef*(A) strains show multidrug resistance (*21*), their increased prevalence may compromise the effectiveness of other commonly used antimicrobial therapies. For example, although >90% of all *S. pneumoniae* isolates collected in year 6 of PROTEKT US exhibited in vitro susceptibility to amoxicillin-clavulanate, <10% of *erm*(B) + *mef*(A) isolates tested in this analysis were susceptible to this agent. Moreover, the MIC distribution for amoxicillin-clavulanate within the *erm*(B) + *mef*(A) isolates (MIC_{50} and MIC_{90} both ≥8 μg/mL) suggests that no oral β-lactam antimicrobial drug may be available that can provide adequate concentrations to eradicate these increasingly prevalent strains. On the other hand, the fluoroquinolone levofloxacin and the ketolide telithromycin continue to show good activity against ERSP isolates, with little impact of resistance genotype on their respective activities.

Our study is subject to several potential limitations. A major potential limitation inherent in surveillance studies that measure in vitro antimicrobial drug resistance is their clinical relevance. Although an association between in vitro resistance and adverse clinical outcome remains generally unproven for most respiratory infections, an increasing number of studies indicate that infection with macrolide-resistant pneumococci is associated with clinical failure (*6–9,22*). Furthermore, clinical failures have been associated with *mef*(A)- and *erm*(B)-mediated resistance (*6,23,24*). A second potential limitation of this study is the derivation of resistance rates from collection centers where a predetermined number of isolates were to be collected and may not entirely reflect those found more widely.

These data from PROTEKT US year 6 indicate that in vitro pneumococcal macrolide resistance may not have plateaued as previously thought. Continued surveillance of erythromycin resistance in general, and of highly resistant *erm*(B) + *mef*(A) strains in particular, is warranted.

Acknowledgments

We thank colleagues throughout the United States for the supply of bacterial isolates as part of the PROTEKT US study

Table 3. Antimicrobial susceptibility of erythromycin-resistant isolates, by genotype, year 6 (2005–2006) of the PROTEKT US surveillance study*

	Amoxicillin–clavulanate					Levofloxacin					Telithromycin				
	Susceptibility, %			MIC, μg/mL		Susceptibility, %			MIC, μg/mL		Susceptibility, %			MIC, μg/mL	
Genotype	S	I	R	MIC_{50}	MIC_{90}	S	I	R	MIC_{50}	MIC_{90}	S	I	R	MIC_{50}	MIC_{90}
erm(B)†	96.2	0.9	2.9	0.12	2	98.2	0.2	1.6	1	1	99.1	0.7	0.2	0.03	0.12
mef(A)‡	92.1	3.7	4.2	0.25	2	98.9	0.1	1.0	1	1	99.6	0.4	0	0.25	0.5
erm(B) + *mef*(A)§	9.7	9.9	80.3	≥8	≥8	98.8	0.2	1.0	1	1	99.1	0.5	0.3	1	1

*PROTEKT US, Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin, United States; S, susceptible; I, intermediate; R, resistant.
†n = 448.
‡n = 1,282.
§n = 575.

and the Quotient Bioresearch Ltd and CMI PROTEKT teams who performed the MIC determinations and genotyping.

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Dengue 1 Virus and Dengue Hemorrhagic Fever, French Polynesia, 2001

Bruno Hubert and Scott B. Halstead

An epidemic of dengue 1 virus (DENV-1) occurred in French Polynesia in 2001, 4 years after a DENV-2 epidemic that ended in 1997. Surveillance data from hospitalized case-patients showed that case-patients with dengue hemorrhagic fever (DHF) exhibited a bimodal age distribution with 1 peak among infants 6–10 months of age and a second peak at 4–11 years of age. The relative risk of DHF developing in children born before rather than after the DENV-2 epidemic was 186 (95% confidence interval 26–1,324). Among children born toward the end of the DENV-2 epidemic, a strong temporal association was found between the month of birth and the risk of being hospitalized for DHF. This study documents epidemic pathogenicity associated with the sequence of DENV-2 infection followed by DENV-1 infection.

Four dengue virus serotypes (DENV-1 to DENV-4) are transmitted to humans. In some infected persons a milder form, dengue fever (DF), may develop, whereas in a smaller proportion, the severe disease, dengue hemorrhagic fever (DHF), develops, which is characterized by an excessive capillary permeability that may lead to shock (dengue shock syndrome [DSS]) and death.

Island populations provide unique opportunities to study the epidemiology and pathogenesis of introduced pathogens. Of note have been the dengue epidemics that have succeeded the introduction of DENV-1 into Cuba and its transmission during 1977–1978 to nearly 45% of the population. An Asian genotype of DENV-2 was introduced into Cuba in 1981 and caused a major epidemic of DF and DHF across the population of a wide range of ages, beginning with those 3 years of age (*1*). In 1997, an Asian DENV-2 virus was again introduced into the city of Santiago de Cuba and DF and DHF were again observed, this time in persons ≥20 years of age (*2*). In 2001, DENV-3 appeared in Havana and its environs, and again DF and DHF cases were observed only in adults (*3*). The epidemiologic situation in French Polynesia resembles that of Cuba because, over the past 50 years, at least 10 different DENVs have resulted in epidemics (*4*). Each epidemic was associated with the recovery of only 1 serotype and generally was succeeded during interepidemic periods by low transmission of that same DENV.

A recent DENV-1 epidemic has provided an opportunity to relate the occurrence of DHF to a history of exposure of the population to another dengue serotype. Four years after a large DENV-2 epidemic that began in 1996 and affected all of French Polynesia, ending in 1997, in January 2001 DENV-1 virus was identified on Bora Bora Island. Over the next 10 months, this epidemic spread to all of French Polynesia. The number of dengue-like syndromes diagnosed by general practitioners in the Society Islands was estimated to be 33,000 (16% of the overall population) (*5*). We report an analysis of the distribution of hospitalized case-patients by date of birth and our conclusion that severe disease in 2001 resulted from infections in the sequence of DENV-2 followed by DENV-1.

Methods

Geographic Background

French Polynesia (235,000 inhabitants in 2001), located 4,400 km southeast of Hawaii, includes 4 archipelagos. The most highly populated, Society Archipelago (202,000 inhabitants), includes 7 inhabited islands including Tahiti and Bora Bora. The 3 other archipelagoes, considered as

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outer islands, are the Marquesas, Tuamotu, and Australes Islands (Figure 1).

History of Dengue Activity in French Polynesia

Ten outbreaks with DENVs isolated have been documented since 1944 (Table). Except for a DENV-2 epidemic in 1971 and a DENV-3 epidemic in 1990, the severity of disease in other epidemics was mild (*4*). In August 1996, cases of DF were reported to the Directorate of Health for French Polynesia. These were shown to be caused by DENV-2. The scope and duration of the epidemic are illustrated in Figure 2.

Surveillance

In 2001, surveillance of DF cases in hospitalized patients was established in all 7 hospitals in French Polynesia, 4 of them on Tahiti Island, and the 3 others in Raiatea (close to Bora Bora), Moorea, and Hiva Oa. Hospital-based physicians were asked to complete a questionnaire that included demographic, clinical, and biologic information on each patient admitted with a diagnosis of dengue regardless of severity. Completed questionnaires were sent to the epidemiologic unit of the Directorate of Health at the time the patient was discharged from the hospital.

Case Definition

Hospitalized case-patients were classified as having DF, DHF, or DSS, according to World Health Organization guidelines (*6*). Because the tourniquet test was rarely performed, case-patients with a history of fever, thrombocytopenia, and evidence of plasma leakage but without spontaneous bleeding phenomena were classified as having DHF grade I.

Laboratory Studies

Confirmation of DENV infection was obtained from case-patients by reverse transcription–PCR or virus isolation at an early stage of the disease or by immunoglobulin (Ig) M and IgG detection during hospitalization. Second serum specimens were rarely obtained after hospitalization. Laboratory analyses to detect antibodies against DENV by using IgM capture and IgG ELISAs were performed as previously described (*7*). Isolation and identification of the virus were performed at the Laboratoire de Recherche en Virologie Médicale, Institut Louis Malardé, Papeete, Tahiti. Results are reported elsewhere (*8*).

Analysis

DHF incidence rates were computed by reported age and, for some analyses, by year and month of birth. A more in-depth analysis by month of birth was performed on hospitalized children with DENV-1 infections born in 1996 or 1997. The hypothesis being tested was that, as the 1996–7 DENV-2 epidemic waned, infection rates with this serotype will decline and, during the 2001 DENV-1 epidemic, the disappearance of those immune to DENV-2 will correlate with a reduction in severe cases. As a proxy of the monthly evolution of the risk of being DENV-2 infected from August 1996 through June 1997, we used the monthly cumulative percentage of patients still not infected among the 2,035 DENV-2 confirmed cases during this period (Figure 2).

Statistical Analysis

All reported p values are 2-sided. Statistical analyses were performed with Epi-Info V6.4d (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

During the 2001 epidemic, 1,379 persons were hospitalized with a diagnosis of possible DENV infection

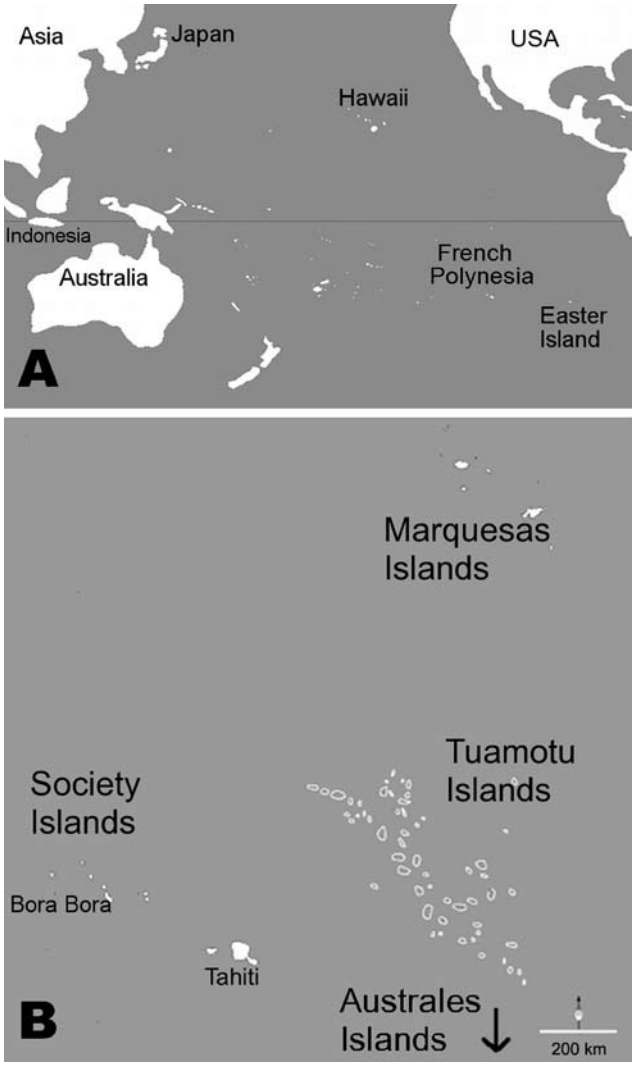

Figure 1. A) French Polynesia in the South Pacific. B) Archipelagoes and main islands of French Polynesia.

Table. History of outbreaks with dengue viruses with documented serotype in French Polynesia*

Year	Dengue virus serotype
1944	1
1964–1965	3
1969	3
1971	2
1975–1976	1
1979	4
1988–1989	1
1989–1990	3
1996–1997	2
2001	1

*Source: (*4*).

(9/1,000 population). Among these, 256 were confirmed by virus isolation or reverse transcription–PCR and 420 additional cases by serologic analysis. All viruses and RNA recovered were DENV-1. Among hospitalized case-patients, 746 (54%) were classified as DF, 157 (11%) as DHF grade I, 198 (14%) as DHF grade II, and 278 (20%) as DSS (DHF grade III or IV). The overall incidence rate of DHF was 2.7 per 1,000 population. Eight fatal cases were reported; 7 were DSS cases in patients 5–12 years of age.

DHF Case-Patients by Age

Ninety-seven percent of DHF cases occurred among persons <20 years of age. The age distribution of hospitalized and of DHF case-patients exhibited a bimodal distribution with 1 peak in infants, followed by a 3-year gap in severe cases, an increase in severe cases among 4 year-olds and a steep increase in 5–11 year age group (Figure 3, panel A). Among children 5–11 years of age, a linear declining trend was observed with the DSS rate falling from 11.6/1,000 to 3.1/1,000 ($p<0.001$, by χ^2 test for trend). Among the 63 hospitalized children <2 years of age, 27 had DHF. Seventy percent of hospitalized case-patients and, among hospitalized patients, 88% of DHF case-patients were 4–11 months of age; the incidence peaked at 8 months of age (Figure 3, panel B). Among those 12–24 months of age, only 2 DHF case-patients were observed.

DHF Case-Patients by Date of Birth

DHF attack rates fell sharply among children born earlier than 1989 (the year of a previous DENV-1 epidemic) (Figure 4). In 2001, only 1 child born during 1997–1999 (after the DENV-2 epidemic) had DHF. The relative risk for DHF to develop in a child that was born in 1990–1996 (499/34,000) versus being born in1997–1999 (1/12,900) was 186 (95% confidence interval 26–1,324). Among children born during the second half of 1996, the DHF incidence rates decreased by month of birth and fell to 0 among those born in January 1997. This decrease was parallel to the risk of being subsequently infected by DENV-2 during 1996, with a 2-month delay between birth and the risk for infection (Figure 5).

Discussion

Although the evidence linking DHF/DSS with a second dengue infection is substantial, the proposed pathogenetic mechanisms of DHF/DSS that explain why dengue infections occur in the presence of circulating antibodies are still controversial (*9*). Two theories, not mutually exclusive, are frequently cited. The most commonly accepted is the antibody-dependent enhancement hypothesis in which dengue disease severity is modified by enhanced infections in monocytes and macrophages that result from infection by immune complexes formed by virus and antibodies raised from prior infection with a heterologous DENV or passively acquired at birth (*10*). The second theory is that DENVs differ in virulence or fitness. DHF risk is found most notably during secondary DENV-2 and DENV-3 infections, whereas some genotypes, notably the American

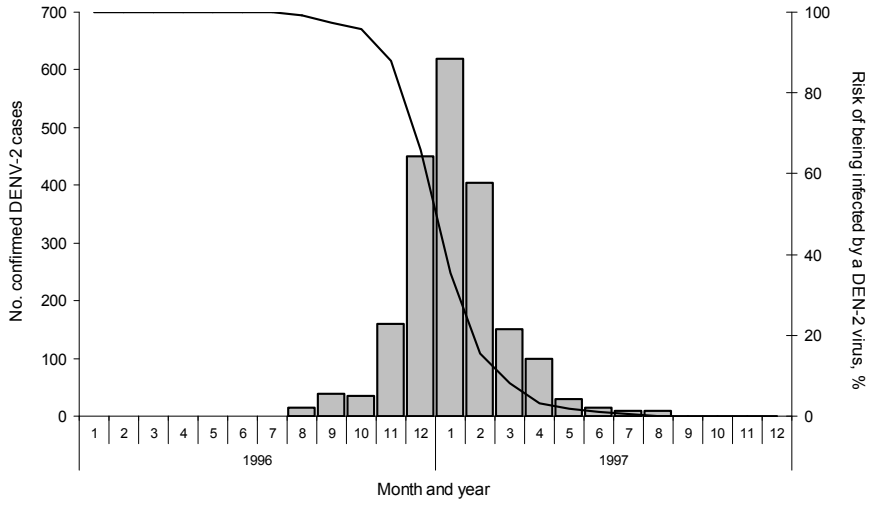

Figure 2. Epidemic curve of confirmed dengue cases, dengue virus 2 (DENV-2) epidemic of 1996–1997 (gray bars), and theoretical risk of being infected with DENV-2, August 1996–June 1997 (line), French Polynesia.

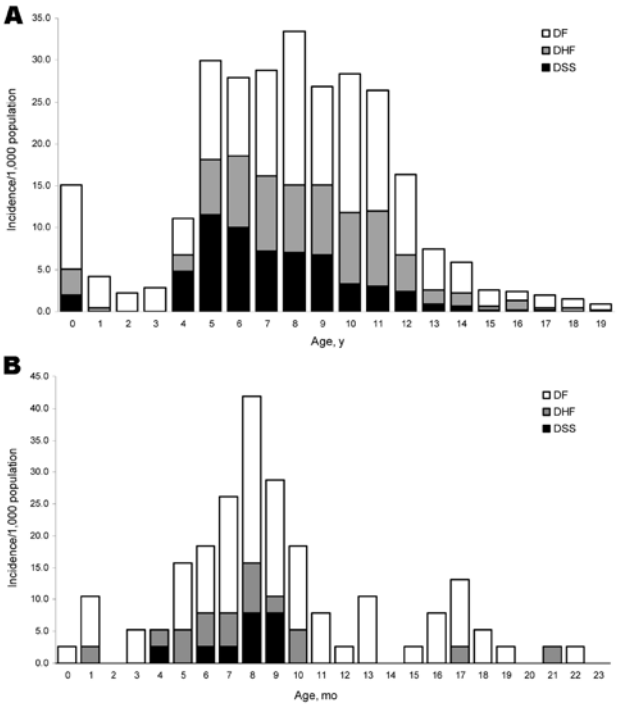

Figure 3. Age-specific hospitalization for dengue fever (DF) and dengue hemorrhagic fever (DHF), incidence rates/1,000 children, French Polynesia, 2001. A) Children (<20 years of age); B) toddlers (<24 months). DSS, dengue shock syndrome.

genotype DENV-2, have reduced severity potential compared with Asian genotype viruses (*11–13*). Both hypotheses are consistent with an increased circulation of DENVs in the world with a possible selection of the fittest strains and an increased probability of secondary infections.

We describe the bimodal nature of age distribution in DHF in French Polynesia. This phenomenon was described in Thailand in 1962–1964 (*14*) and in Cuba in 1981 (*1*). This feature, unique among human infectious diseases, supports the hypothesis that DHF in infants is explained by enhanced dengue infections resulting from passively acquired maternal dengue antibodies (*15*). Among hospitalized infants with DF/DHF observed in French Polynesia in 2001, the age distribution of DHF cases was similar to age distributions of case-patients hospitalized in 4 Southeast Asian countries where all 4 dengue serotypes are highly endemic. In French Polynesia, as in Thailand, the incidence of DHF was 15/1,000 in 8-month-old infants (*16*). The age distribution of infant case-patients has been attributed to the catabolism of maternal antibodies following parturition (*17*). This degradation of antibodies creates 3 successive phases of risk for dengue infection or disease in the infant: 1) a protection phase between birth and the age of 5 months, 2) a period that follows in which the antibodies no longer neutralize but rather facilitate severe infections, and finally, 3) the complete disappearance of these antibodies when the child is ≈11 months of age, leaving the child open to a so-called normal infection. These data and interpretations were confirmed in recent studies on infant DHF in Vietnam (*18,19*).

In this study, the incidence of DHF in children >1 year of age, when arranged by date of birth, demonstrated the major risk factor was to have been born before the DEN-2 epidemic. In addition, children born before 1989, and exposed to an earlier DENV-1 epidemic, had a reduced risk of being hospitalized during the 2001 epidemic and were apparently protected by having acquired DENV-1 immunity. An even more striking result is the decreasing incidence rate of DHF by month of birth among children born at the interface between high and low transmission of DENV-2 epidemic. This association was even stronger if a 2-month delay between birth and risk for infection is considered. Explanations include the possibility that very young infants may be shielded from mosquito bites or that maternal antibodies are highly protective during this interval (*20*).

Another notable finding in this study is that the incidence of DSS steadily declined among children 5–11 years of age. This finding of a decline resembles an observation made concerning the 1981 DHF epidemic in Cuba: that DHF/DSS age-specific incidence rates were inversely related to age (*21*). This finding is also consistent with results of studies about normal human endothelial function. Gamble et al. found a marked decline in filtration capacity

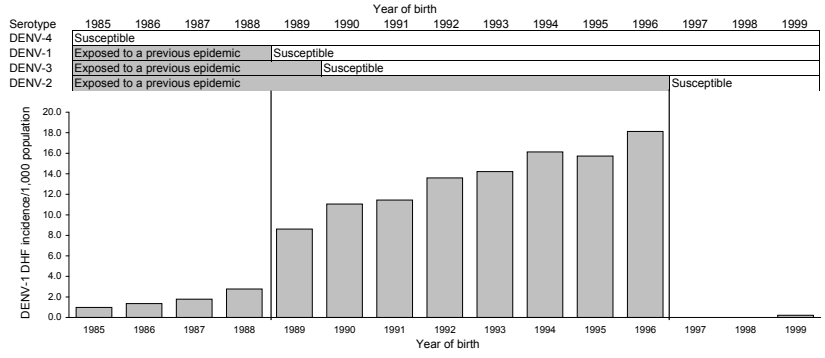

Figure 4. Dengue virus 1 (DENV-1) dengue hemorrhagic fever (DHF) incidence rates (per 1,000 population), according to year of birth and indication of the immune status against each serotype based on exposure to a previous epidemic, French Polynesia, 2001.

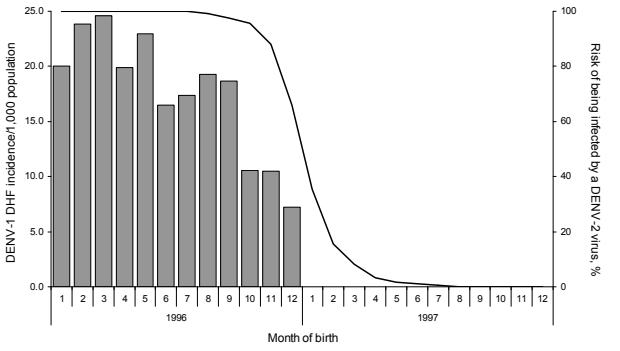

Figure 5. Dengue virus 1 (DENV-1) dengue hemorrhagic fever (DHF) incidence rates among children born in 1996–1997, according to their month of birth (gray bars). The theoretical risk of being infected with DENV-2 from August 1996 through June 1997 is also shown (line).

(a factor describing vascular permeability) in the first 2 decades of life and hypothesized that the incidence of DSS might be age dependent (*22*). Bethell et al. demonstrated that children with DHF are more likely than adults to progress to the DSS state because their baseline microvascular permeability is higher (*23*).

Based upon envelope glycoprotein sequence analyses, the 2001 Tahitian DENV-1 strain was found to be similar to strains isolated in Indonesia in 1988 and was likely of Southeast Asian origin, in contrast to the 1988–1989 Tahitian DENV-1 strain, which was found to be of Asian-American origin (*8*,*24*). A complete nucleotide sequence analysis confirmed that the same DENV-1 virus spread from French Polynesia to Hawaii and Easter Island (*25*). It is noteworthy that in those sites where no other DENVs have circulated for many years, DHF cases were not observed during these DENV-1 outbreaks (*26*,*27*). This finding is in agreement with our observation that severe disease did not develop in any children, 1–3 years of age, who were born after the DENV-2 epidemic in French Polynesia, although the youngest children are at highest risk for dengue vascular permeability syndrome (*21*). This comprises supportive evidence that the DENV-1 circulating in Tahiti in 2001 was not inherently virulent.

Earlier epidemic occurrences of DHF have been primarily associated with DENV-2 or DENV-3. The large Cuban epidemic of 1981 was DENV-2, which followed DENV-1 (*1*). Notably, in the 1980 dengue outbreak in Rayong, Thailand, no severe disease was observed in any infection sequence that ended with DENV-1, including DENV-2, followed by DENV-1 (*28*). Although, in other years and places, secondary DENV-1 infections have resulted in DHF, we found epidemic DHF that accompanied sequential infection in the specific sequence of DENV-2 followed by DENV-1 (*29*,*30*). This outbreak resulted in a DHF/DSS incidence rate of 2.7/1,000 population, similar to the 3.2/1,000 rate observed during a large epidemic in Thailand in 1987 in which secondary DENV-2 and DENV-3 virus infections predominated (*6*). The reasons for changes in pathogenicity of secondary DENV-1 infections require further careful study.

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Strategy to Enhance Influenza Surveillance Worldwide[1]

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The emergence of a novel strain of influenza virus A (H1N1) in April 2009 focused attention on influenza surveillance capabilities worldwide. In consultations before the 2009 outbreak of influenza subtype H1N1, the World Health Organization had concluded that the world was unprepared to respond to an influenza pandemic, due in part to inadequate global surveillance and response capacity. We describe a sentinel surveillance system that could enhance the quality of influenza epidemiologic and laboratory data and strengthen a country's capacity for seasonal, novel, and pandemic influenza detection and prevention. Such a system would 1) provide data for a better understanding of the epidemiology and extent of seasonal influenza, 2) provide a platform for the study of other acute febrile respiratory illnesses, 3) provide virus isolates for the development of vaccines, 4) inform local pandemic planning and vaccine policy, 5) monitor influenza epidemics and pandemics, and 6) provide infrastructure for an early warning system for outbreaks of new virus subtypes.

The emergence of a novel strain of influenza virus A (H1N1) in April 2009 and its subsequent rapid global spread have focused attention on influenza surveillance capabilities worldwide (*1*). A consultation convened by the World Health Organization (WHO) in 2005 had previously concluded that the world was unprepared to respond to an influenza pandemic, due in part to inadequate global surveillance and response capacity (*2*). The International Health Regulations 2005 call for strengthened surveillance for all events that may constitute a "public health emergency of international concern"; such events include individual human cases of influenza caused by a new subtype of influenza virus A (*3*). As part of the International Health Regulations 2005 core surveillance and response capacity requirements, each Member State must develop and maintain capabilities to detect, assess, and report disease events nationally and internationally to WHO within 48 hours of confirmation. However, reviews of national pandemic planning indicate that surveillance systems are often inadequate to support current preparedness strategies (*4–8*). WHO has existing surveillance guidelines to help Member States implement universal surveillance for novel and pandemic influenza (*9*), but the guidelines lack the specificity that would enable many countries to establish operational surveillance plans.

Quality influenza surveillance systems are needed to enable countries to better understand influenza epidemiology, including disease incidence and severity, and help them implement appropriate prevention strategies. The challenges experienced by the United States and Mexico to rapidly determine the extent and severity of illness of the 2009 novel influenza A (H1N1) outbreak highlighted the need for systems that can reliably produce these estimates. Furthermore, global strategies to address other vaccine-preventable diseases have acknowledged the importance of establishing local disease burden (effects, severity, amount of illness, and costs) as a first step toward decisions about the introduction of vaccines into new countries. We describe a generic guideline for collecting data on severe acute re-

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spiratory infection (SARI), influenza-like illness (ILI), and laboratory-confirmed influenza that can be implemented in limited-resource settings.

Current Situation

Global Influenza Surveillance

For 60 years, the WHO Global Influenza Surveillance Network (GISN) has provided virologic information used in the biannual process of selecting strains for the Northern and Southern Hemisphere influenza vaccine formulations. However, its capacity to provide epidemiologic data or an alert of an emerging pandemic is limited. GISN currently comprises 122 National Influenza Centers in 87 countries and 4 WHO Collaborating Centers for Reference and Research on Influenza (*10*). Although this system has proven to be valuable, tropical and resource-limited countries (particularly in Africa) are underrepresented (*11*).

Influenza in Developing Countries

Virus transmission or clinical presentation may be altered by differences in cultural practices, the environment, geography, human genetics, and social structures. Enhanced influenza surveillance can permit assessment of a number of factors that may affect disease activity: population density, differences in prevalence and spectrum of chronic illness, proximity of the young and elderly, low proportion of elderly in the population, low school attendance, and school schedules that may not correspond with peak transmissibility season. The effectiveness of control measures such as social distancing and vaccination may differ between developed and developing settings because of these factors.

Available epidemiologic evidence suggests that influenza is common in tropical regions and contributes substantially to disability and use of healthcare resources (*12–16*). Data describing the seasonality and epidemiology of influenza in tropical areas are limited; however, some tropical countries report year-round human influenza activity (*12*), unlike in temperate regions where transmission occurs with marked seasonality. Because of these limited data, most of the understanding of seasonal influenza is derived from epidemiologic data collected in western Europe and North America. Nevertheless, estimates of a pandemic impact indicate that most deaths will be in developing countries and that more than half will occur in southern Asia and sub-Saharan Africa (*17*). A better understanding of the epidemiology of influenza in these areas would facilitate country-appropriate pandemic planning and vaccine policy development.

Objectives

The most efficient process for producing high-quality epidemiologic data for influenza-associated illness is sentinel surveillance. The primary limitation of most existing influenza sentinel-site networks that track ILIs has been that they often provide little epidemiologic data, do not produce data on disease incidence, and are focused on mild disease, which supports the notion that influenza is a benign disease. We propose that influenza surveillance should capture severe influenza outcomes as a primary measure. Hospital-based sentinel surveillance is the most efficient way to collect clinical data and laboratory specimens from persons with a prevalent and severe infectious disease.

Carefully placed sentinel sites can provide adequate information on the epidemiology of influenza without the need for comprehensive national case ascertainment or reporting. Placing surveillance sites where population data are known would permit calculation of population-based estimates of disease rates according to age and other demographic variables. In addition, collection of clinical specimens from persons from whom epidemiologic data are also collected would ensure virus strain surveillance and provide isolates that can be used for vaccine development.

A sentinel surveillance system can be used to monitor >1 disease, can be sustainable, and can integrate with and build upon existing systems. The system objectives are 1) describe the disease impact and epidemiology of severe, acute, febrile respiratory illness and define the proportion that is associated with influenza; 2) provide influenza virus isolates for monitoring changes in viral antigens and development of new vaccines; 3) contribute data for local pandemic planning and making decisions regarding vaccine policy; 4) provide infrastructure for an early warning system for outbreaks of new subtypes of influenza A viruses and new strains of existing subtypes; and 5) serve as a monitoring tool for pandemic influenza.

Components and Processes

Case Definitions

These surveillance guidelines use the existing WHO case definition for ILI and incorporate WHO guidance to define SARI in adults and children (Table 1). The case definitions fit within the existing framework for pandemic early warning, use existing definitions for ease of adoption, and rely on physical examination findings that do not require laboratory or radiographic criteria. In addition, SARI definitions may capture a broad spectrum of severe influenza-associated illness, including exacerbations of asthma, chronic obstructive pulmonary disease, and decompensated congestive heart failure, which may account for ≈75% of hospitalized influenza patients (*16,20,21*).

Sentinel Site Selection

Ideally, sites should represent a wide cross-section of ethnic and socioeconomic groups and should be in differ-

Table 1. Influenza sentinel surveillance case definitions*

Case	Definition criteria
Influenza-like illness	ALL OF THE FOLLOWING • Sudden onset of fever >38°C, AND • Cough or sore throat, AND • Absence of other diagnoses
Severe acute respiratory infection in persons >5 years of age	ALL OF THE FOLLOWING • Sudden onset of fever >38°C, AND • Cough or sore throat, AND • Shortness of breath or difficulty breathing, AND • Requires hospitalization
Severe acute respiratory infection in persons ≤5 years of age	EITHER IMCI criteria for pneumonia Any child 2 mo to 5 y of age with cough or difficult breathing and: • breathing faster than 60 breaths/min (infants <2 mo) • breathing faster than 50 breaths/min (2–12 mo) • breathing faster than 40 breaths/min (1–5 y) OR IMCI criteria for severe pneumonia Any child 2 mo to 5 y of age with cough or difficult breathing and any of the following general danger signs: • unable to drink or breastfeed • vomits everything • convulsions • lethargic or unconscious • chest indrawing or stridor in a calm child AND Requires hospital admission

*Surveillance guidelines use the existing World Health Organization (WHO) case definition for Influenza-like Illness (*19*), and incorporate WHO guidance to define severe acute respiratory infection in adults and children (*9,18,19*). IMCI, Integrated Management of Childhood Illness.

ent climatic regions. Placement of sites in areas where the population denominator can be ascertained or estimated will facilitate incidence estimates. Ultimately, the choice of sentinel hospitals will often be based on practical issues such as human resources, communication infrastructure, and availability of specimen transport and testing. There is no ideal number of surveillance sites; the number chosen by a particular country will depend in part on sustainability and resources available.

Data Collection

Minimum data elements are outlined in Table 2. Data collected should be adequate for routine public health surveillance and description of key epidemiologic features of disease. Data can be broadened to include clinical signs and symptoms, potential exposures, laboratory data, and therapies.

Specimen Collection

Respiratory specimens should be collected early from all SARI patients, following established protocols (*24*). If resources do not allow collection from all patients, an unbiased systematic sampling scheme should be established. To develop quality estimates of incidence and severity, data and specimens from all or most SARI patients from a few facilities would be preferred over a small sample of SARI patients from multiple facilities.

Because seasonality, attack rates, and public health priorities differ from country to country, there is no generic number of specimens to be collected by each site. The number must be determined by the primary surveillance objective (e.g., understanding of seasonality, risk factor analysis, or determination of clinical outcomes) and must represent climatic and geographic regions. For example, a country with coastal, mountainous, and tropical regions may have different influenza activity in each region and may thus require more surveillance sites and increased specimen collection than neighbors or similarly sized countries. Therefore, the number of specimens collected must be approached on a case-by-case basis and depends on objectives of a country, country-specific geographic and climatic issues, and public health priorities.

Integration into National Reporting Systems

In countries with established national disease reporting systems, such as the Integrated Disease Surveillance Reporting system used in Africa (*25*), sentinel surveillance for SARI can be incorporated into the existing system. Because Integrated Disease Surveillance Reporting is generally a passive surveillance program, a few select sites should serve as embedded sentinel sites; intensive training and close follow-up should be conducted to ensure the quality of the reported data.

Outpatient Surveillance

The highest priority should be to collect data on SARI cases because they contain the most influenza-associated disability and premature death. However, if resources permit, data collection at sentinel sites should be expanded to include ambulatory patients with ILI. Because the number of cases at ambulatory care sites is likely to be large, case counts would be aggregated, and clinical specimens and epidemiologic data would be collected from only a small sample of patients. Weekly case counts should be categorized by age group according to well-studied age-range categories (6–23 months, 2–4 years, 5–17 years, 18–49 years, 50–64 years, and ≥65 years) (*26*). Patients chosen to give detailed epidemiologic data and clinical specimens should be selected in as unbiased a manner as possible. The selection protocol must take into account local health-seeking behavior, such as differential use of evening and weekend clinics. Ideally, the weekly total number of patients seen by clinics would also be collected by age group to allow for proportion of ILI to be calculated. Rapid system expansion can compromise the quality of collected data; therefore, ILI surveillance should emphasize quality data collection from a few well-run sites.

Table 2. Sample data collection from cases of severe acute respiratory infection and influenza-like illness*

Recommended essential minimum data for SARI surveillance
General information
• Unique identification number
• Medical record number
• Name (of patient and parent's name, if a minor)
• Date of birth
• Sex
• Address
• Date of onset of symptoms
• Date of collection of epidemiologic data
• Suspected novel influenza case
• Inpatient or outpatient
Clinical signs and symptoms
• Fever >38°C
• Cough
• Sore throat
• Shortness of breath/difficulty breathing
• Other clinical danger signs (*19,22,23*)
Type of specimen collected and date of collection
• Throat swab specimen, date of collection
• Nasal swab specimen, date of collection
• Other specimen (if collected), date of collection
Preexisting medical conditions
• Liver disease
• Kidney disease
• AIDS, cancer, or other immunocompromised state
• Neuromuscular dysfunction
• Diabetes
• Heart disease
• Lung disease
• Smoking history
Optional data collection for SARI surveillance
General information
• Diarrhea
• Encephalopathy
Exposure
• Occupation of patient
• Part of an outbreak investigation
• Contact with sick or dead poultry or wild birds
• Contact with friend or family who has SARI
• Travel in an area known to have endemic circulation of avian influenza (H5N1)
• Other high-risk exposure (e.g., eating raw or undercooked poultry products in an area of influenza virus [H5N1] circulation)
Vaccine/treatment history
• Vaccination against influenza within the past year
• Currently taking antiviral medicine

*SARI, severe acute respiratory infection; ILI, influenza-like illness.

Laboratory Testing

Clinical specimens should be collected from a high proportion of SARI patients and a systematic sample of ILI patients. These specimens can be processed in sentinel site laboratories, but further analyses may require their transport to additional laboratories. Ideally, specimens would be tested for evidence of influenza viruses by reverse transcription–PCR (RT-PCR). A subset of specimens should undergo viral culture and antigenic characterization. Surveillance data should be submitted to WHO FluNet, and, if possible, national laboratories should work with a WHO Collaborating Center laboratory to submit sample virus isolates for vaccine strain selection.

In countries where influenza spreads in seasonal epidemics, it may be adequate to collect less epidemiologic data and fewer specimens for laboratory testing by sampling a smaller proportion of SARI patients during the non-influenza season. Knowledge of SARI rates outside influenza season will permit comparisons between peak season and baseline rates. Non–influenza season rates of SARI can also be monitored by public health authorities, because anomalies in SARI rates could represent outbreaks in need of investigation. However, high-quality, year-round data will be required for >1 season before assumptions can be made about seasonality in a region.

Nasal and nasopharyngeal specimens have a higher yield for influenza virus detection in ILI cases than do oropharyngeal specimens (*27*). However, the relative sensitivity of nasal versus oropharyngeal swabs to detect influenza virus infection in SARI cases is unknown. If both are collected, specimens can be placed in the same tube of viral transport media for processing. If SARI patients are intubated, endotracheal aspirates can also be used. Specimens can be frozen at –70°C for storage and possible future assessment of other respiratory pathogens.

The sensitivity and specificity of any test for influenza will depend on the laboratory performing the test, the

quality of the clinical specimen, the manner in which the specimen is processed, and the type of specimen collected. Generally, RT-PCR testing of respiratory specimens is the most sensitive laboratory test for influenza virus, but it is relatively expensive and is not useful for antigenic characterization (*28*). If the proper primers and probes are used, RT-PCR can determine influenza virus A subtype and can detect novel influenza virus A subtypes. Fluorescent antibody tests, although less expensive, are less sensitive and specific than RT-PCR (*27*). Rapid point-of-care tests are less sensitive and specific than RT-PCR or fluorescent antibody tests and are not generally recommended for use by sentinel surveillance. Virus culture has been the diagnostic standard for identifying influenza virus. Culture sensitivity depends on proper specimen handling and the experience of the laboratory. Virus culture should be performed on at least a sample of specimens to provide material for antigenic determination and potential isolates for vaccine production.

Data Analysis and Reporting

Timely analysis and reporting of surveillance data will facilitate treatment decisions by clinicians and control measures by public health officials. It will also encourage continued reporting of cases by clinicians in the surveillance system. Weekly reports of clinical and laboratory confirmed case counts should be disseminated throughout the surveillance system to participating healthcare providers and all stakeholders during peak seasons. The frequency of reports and the extent to which they are disseminated will depend on data timeliness and public health priorities. Sentinel surveillance reporting mechanisms should use existing public health communications systems and augment other reporting mechanisms such as FluNet through WHO GISN (*29*).

Basic analyses of surveillance data should include weekly frequencies of SARI and laboratory-confirmed influenza cases as well as the proportion of tested patients, by age group, who are influenza virus positive. If possible, proportions of SARI and influenza cases per total of weekly sentinel hospital admissions should be reported. Reports with case frequencies and proportions during prior weeks and years will demonstrate trends over time. At least once annually, analyses of surveillance data to determine risk factors for disease should be reported. These reports should use collected data on concurrent conditions and population-based rates, if these can be determined.

Understanding the epidemiology of severe influenza-associated disease is essential for decisions related to vaccine recommendations. These data are prioritized in the guidelines because many developing countries have limited funds and competing healthcare priorities. However, data collected during SARI surveillance alone will be inadequate to describe aspects of influenza epidemiology such as transmission dynamics, costs, and occurrence of mild disease.

Evaluation and Quality Assurance

The usefulness of surveillance data will depend directly on the quality of the data; every system should have a quality assurance program. Quality indicators will reflect such attributes as system acceptability, timeliness, completeness, and representativeness of collected data. These attributes should be assessed routinely. In addition, the system should undergo regular data audits and systematic field evaluation. In 2001, the Centers for Disease Control and Prevention published comprehensive guidelines for the evaluation of public health surveillance systems (*30*). These guidelines serve as a template for sentinel surveillance evaluation and quality recommendations. Several key quality indicators are recommended in the following section and in Table 3.

Data Validity

Regular field evaluations and audits at a facility level must be a standard component of the system. This process can determine that cases are being counted appropriately, that reported cases meet the case definition, and that sampling procedures are being used uniformly without evidence of bias. Data values recorded in the surveillance system can be compared with standard chart-review values by a retrospective review of a sample of medical records. If a sampling procedure is used for specimen collection, audits can ensure that procedures are uniform and unbiased. Additionally, audits can determine whether clinical specimens are being taken, stored, processed, tested (if appropriate), and shipped properly and in a timely manner from all those who meet sampling criteria.

Observance of expected trends in reporting and disease activity can provide an additional means of assessing data quality. Although it is not possible to define expected values for some parameters, such as the percentage of specimens testing positive for influenza virus or the number of SARI cases occurring in a given facility, aberrations in the data over time or substantial differences between facilities can signal problems at a given site. Trends assessed may include number of cases reported by month, number of specimens submitted by month, percentage of influenza-positive specimens, and number and percentage of SARI and ILI cases tested.

Timeliness

To be useful, collection and reporting of surveillance data must be timely. Timeliness of the following activities is appropriate for routine measurement as quality indicators for surveillance sites: data reporting, specimen shipment to

the laboratory for testing, receipt of specimens by the laboratory, laboratory processing and testing of specimens, and reporting of laboratory results.

One way to quantify timeliness is to calculate the percentage of times that a site achieves targets for specific intervals, for example, the percentage of times that a site sends reports or specimens to the appropriate place within a specified time frame. A hypothetical system may choose as a goal that 80% of data reports be sent within 48 hours of the reporting deadline or that 80% of specimens be shipped within 48 hours of specimen collection. Likewise, for the laboratory, the percentage of samples that are tested and have final results within a target time frame can be calculated. Targets will depend on site-specific circumstances and public health priorities.

A similar quality metric that can be used is the calculation of the average time to accomplish surveillance activities. For example, a hypothetical site that is chronically late in sending data every month might average several days between the deadline for receipt (the day of the week or month on which reports are due) and actual receipt of data. For laboratory specimen processing, the average number of days between receipt of specimens and the reporting of the results can be measured and followed similarly. Site time averages can be compared to identify sites that are underperforming and to target improvements. Either percentages of sites achieving timeliness targets or time lag averages can also be used as a quality metric to be followed over time.

Completeness

Indicators of completeness can be determined by analyzing reported data. They may include percentage of reports received from each site with complete data, percentage of total expected data reports received, and percentage of total expected cases that have specimens submitted to the laboratory (depends on sampling scheme devised for sites).

Pandemic Early Warning Systems and Monitoring

Emergence of new subtypes of influenza virus A in human populations is unusual and unlikely to be detected by a sentinel surveillance system, except by chance or if transmission is sustained. Control of a pandemic caused by the introduction of a new subtype of influenza virus A will require early detection and recognition of the event. Although sentinel surveillance as a stand-alone system may not accomplish this, it has value in establishing the infrastructure necessary to respond to a pandemic. In addition to providing a basic understanding of the epidemiology of influenza transmission and risk, a routine reporting system would produce an infrastructure for reporting, specimen processing and testing, and data collection and analysis. It would make data interpretation more routine (and thus more manageable in the face of a pandemic emergency) and drive interest in influenza-associated disease and vaccination.

After a novel strain of influenza emerges, monitoring its course is necessary to determine whether cases are increasing or decreasing, to detect changes in patient age distribution or other epidemiologic characteristics, to detect changes in mortality rates, and to monitor changes in susceptibility to antiviral agents. In the midst of an outbreak, national monitoring may not be necessary or feasible, and most, if not all, critical information can be gained from a few sentinel sites. Emergence of a new strain of influenza increases the data needs of health policy makers. Historical

Table 3. Influenza surveillance evaluation and recommended quality indicators*

- 1. Timeliness
  - a. Several time intervals are appropriate for routine measurement as quality indicators. These include the duration of time from
    - i. Target date for data reporting from the sentinel site to the next administrative level until the actual reporting date
    - ii. Target date for data reporting from the next administrative level to the national level until the actual reporting date
    - iii. Date of specimen collection at facility until shipment to laboratory
    - iv. Date of result availability in laboratory until date of report to referring institution and physician
    - v. Date of receipt of specimen in the laboratory until result availability
  - b. Metrics. Two metrics can be used to reflect timeliness indicators:
    - i. Percentage of time that a site achieves target for timeliness
    - ii. Average number of days for each interval over time for each site
- 2. Completeness
  - a. Percentage of reports received from each site with complete data
  - b. Percentage of data reports that are received
  - c. Percentage of reported cases that have specimens collected
- 3. Audit. Regular field evaluations and audits at facility level of a subset of medical records to ensure
  - a. Cases are being counted appropriately and not being underreported
  - b. Reported cases fit the case definition
  - c. Epidemiologic data are correctly and accurately abstracted
  - d. Respiratory samples are being taken, stored, processed, tested, and shipped properly and in a timely fashion from all those who meet sampling criteria
  - e. Sampling procedures are being done uniformly without evidence of bias
- 4. Data to be followed and observed for aberrations over time
  - a. Number of cases reported by month for each site
  - b. Number of specimens submitted by month for each site
  - c. Percentage of specimens that are positive for influenza
  - d. Number and percent of ILI and SARI cases tested

*ILI, influenza-like illness; SARI, severe acute respiratory illness.

surveillance data for comparison can facilitate the understanding of answers to critical questions such as severity of the outbreak related to a new strain and its potential to adversely affect healthcare delivery. An existing surveillance infrastructure also provides the platform needed to describe the clinical course of emerging pathogens, risk factors for severe outcomes, and effectiveness of control measures.

Conclusions

Surveillance for SARIs can provide critical understanding of the contribution of influenza infection to the global burden of disease, provide a platform for the study of other common respiratory pathogens, and strengthen public health infrastructure. Such a system should be a part of a routine surveillance program to provide data needed for allocation of scarce healthcare resources.

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Invasive Group B Streptococcal Disease in the Elderly, Minnesota, USA, 2003–2007

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In Minnesota, incidence of invasive group B streptococcal disease was 3 times greater in older adults in long-term care facilities than in older adults in community settings (67.7/100,000 vs. 21.4/100,000) during 2003–2007. The overall case-fatality rate was 6.8%, and concurrent conditions were common among both groups.

Invasive group B streptococcal (GBS) disease is a major cause of illness and death in older adults (*1*). A 2- to 4-fold increase in invasive GBS disease among US adults has been reported since the 1980s (*2*), and incidence increased 32% in adults from 1999 through 2005 (*1*). The objective of this study was to characterize the incidence and epidemiology of GBS disease in Minnesota among the elderly in long-term care facilities (LTCFs) and in the community.

The Study

The Minnesota Department of Health conducts statewide, population-based surveillance for GBS disease as part of the Centers for Disease Control and Prevention Active Bacterial Core Surveillance Network/Emerging Infections Program. Invasive disease is defined as isolation of GBS bacteria from a normally sterile site, such as blood, pleural fluid, cerebrospinal fluid, joint fluid, or bone (*3*). To ensure completeness of reporting, the Minnesota Department of Health audits laboratories to identify all GBS bacteria–positive cultures from normally sterile sites. For each case, a standardized case report form is completed by hospital infection control practitioners. GBS isolates are sent to the Minnesota Department of Health Public Health Laboratory for susceptibility testing using broth microdilution. Erythromycin-resistant, clindamycin-susceptible isolates are tested for inducible clindamycin resistance by double-disk diffusion (D test). Interpretation is based on Clinical and Laboratory and Standard Institute protocols (*4*). Serotyping is performed at the Centers for Disease Control and Prevention by latex agglutination tests with rabbit antiserum to GBS capsular polysaccharide types Ia, Ib, and II–VIII (*5*). When latex tests are indeterminate, the Lancefield method is used (*6*).

The study comprised all Minnesota residents aged ≥65 years with invasive GBS disease during 2003–2007. LTCF residence was defined as living in an LTCF before the date of first positive culture. Resident addresses were checked by a reverse-address directory to determine whether they corresponded with the address of an LTCF. All other residents were defined as community dwelling. Incidence was calculated using 2000 census data. Analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA); the χ^2 test was used to evaluate differences in proportions for discrete variables.

A total of 723 cases of invasive GBS disease among persons ≥65 years of age were reported; 596 (82.4%) cases occurred among community residents, and 127 (17.6%) occurred among LTCF residents (Table 1). The overall incidence rate was 24.3 cases per 100,000 persons. Incidence did not vary significantly by year but did increase with age (19.3/100,000 at 65–74 years, 26.3/100,000 at 75–84 years, and 36.9/100,000 at ≥85 years; χ^2 for trend = 44.4, $p<0.001$) and was higher among LTCF residents than among community residents (67.7/100,000 vs. 21.4/100,000; $p<0.001$). The overall case-fatality rate was 6.8 (8.7% LTCF vs. 6.4% community). Case-fatality rates increased as age increased (6.0% at 65–74 years, 6.8% at 75–84 years, and 8.2% at ≥85 years).

The most common clinical presentation reported was bacteremia without focus (50.2%), followed by pneumonia (10.9%). LTCF residents (18.9%) were more likely than community residents (9.2%) to have pneumonia ($p = 0.002$) (Table 1). Blood (84.0%) was the most common site for isolation of GBS bacteria, followed by joint fluid (10.2%) and bone (3.3%). Other sites included peritoneal fluid (1.4%), pleural fluid (0.7%), and cerebrospinal fluid (0.4%).

Data on concurrent conditions were collected for 96 (75.6%) of 127 LTCF case-patients and 448 (75.2%) of 596 community case-patients. Of these, 176 (32.3%) had only 1 concurrent condition, 166 (30.5%) had 2 concurrent conditions, and 145 (26.6%) had ≥3 concurrent conditions. LTCF residents (94.8%) were more likely than community residents (88.4%) to have a documented concurrent condition ($p = 0.06$) (Table 1). Among case-patients with known concurrent condition status, 41% had diabetes mellitus and 30% had coronary artery disease; similar proportions were

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Table 1. Comparison of LTCF residents and community-dwelling elderly persons with invasive GBS disease, Minnesota, 2003–2007*

Characteristic	LTCF elderly, no. (%)	Community elderly, no. (%)
Total no. case-patients	127	596
Male gender	63 (49.6)	338 (56.7)
Type of infection†	63 (49.6)	297 (49.8)
Bacteremia without focus	24 (18.9)	55 (9.2)
Pneumonia	17 (13.4)	120 (20.1)
Cellulitis	5 (3.9)	47 (7.9)
Septic arthritis	5 (3.9)	24 (4.0)
Osteomyelitis	6 (4.7)	20 (3.4)
Abscess	1 (0.8)	3 (0.5)
Meningitis	13 (11.0)	58 (10.2)
Other or >2 types	7 (5.5)	27 (4.5)
Concurrent condition data collected	96 (75.6)	448 (75.2)
No concurrent conditions	5 (5.2)	52 (11.6)
1 concurrent condition	34 (35.4)	142 (31.7)
2 concurrent conditions	34 (35.4)	132 (29.5)
≥3 concurrent conditions	23 (24.0)	122 (27.2)
Concurrent condition types‡	39 (40.6)	186 (41.5)
Diabetes	26 (27.1)	135 (30.1)
ASCVD	25 (26.0)	67 (15.0)
Congestive heart failure	13 (13.5)	23 (5.1)
Stroke	15 (15.6)	37 (8.3)
COPD	13 (13.5)	126 (28.1)
Cancer	44 (45.8)	195 (43.5)
Died	11 (8.7)	38 (6.4)

*LTCF residents were a median of 84 years of age; community residents, a median of 76 years of age ($p<0.05$). LTCF, long-term care facility; GBS, group B streptococcal; ASCVD, atherosclerotic cardiovascular disease; COPD, chronic obstructive pulmonary disease.
†A single patient may have had >1 type of infection.
‡Percentages are of those with concurrent condition data collected.

noted among LTCF and community case-patients. Congestive heart failure (26.0% vs. 15.0%, $p = 0.009$), stroke (13.5% vs. 5.1%, $p = 0.003$), and chronic obstructive pulmonary disease (15.6% vs. 8.3%, $p = 0.026$) were more common among LTCF residents. Cancer was more common among community residents (28.1% vs. 13.5%, $p = 0.003$) (Table 1). Cellulitis as a manifestation of invasive GBS disease was more likely in residents with diabetes than in those without diabetes (24.4% vs. 16.3%, $p = 0.019$).

GBS serotypes were obtained for 654 (90.5%) of 723 case-patients. Five serotypes, Ia (21.1%), Ib (11.0%) II (11.8%), III (11.3%), and V (35.0%), accounted for 94.6% of LTCF case-patients and 89.7% of community case-patients. Antimicrobial drug susceptibility data were obtained for 655 (90.6%) of 723 case-patients. All isolates were susceptible to penicillin. Susceptibility to erythromycin and clindamycin decreased during 2003–2007 (Table 2). Sixty percent of erythromycin-resistant, clindamycin-susceptible isolates had inducible clindamycin resistance as evidenced by a positive D test. During 2004–2005, 78% of erythromycin-resistant, clindamycin-susceptible isolates had inducible clindamycin resistance compared with 46% from 2006–2007 ($p = 0.003$). Serotype V was associated with higher rates of resistance than other serotypes to both erythromycin (46.7% vs. 27.9%, $p<0.001$) and clindamycin (28.4% vs. 12.9%, $p<0.001$). Serotype V was also associated with higher rates of inducible clindamycin resistance (88.6% vs. 43.4%, $p<0.001$).

Conclusions

We found that rates of invasive GBS disease were substantial among the elderly and 3× higher among LTCF residents than elderly persons living in the community. These results are supported by an earlier report from Maryland that also found increased incidence of invasive GBS infections in LTCF residents (*7*). The reason for increased incidence in LTCF residents is not fully known. However, concurrent conditions, such as advanced age, diabetes, cirrhosis, and stroke, are known risk factors for GBS infection (*8*). In our study, concurrent conditions were common among both groups, however, concurrent condition types differed by group. Although higher rates of GBS disease among LTCF residents may be caused in part by differences in underlying concurrent conditions, other factors not collected as part of this study may also play a role. These factors include use of invasive devices (urinary catheters, intravenous catheters) and the possible role of person-to-person transmission of GBS bacteria in LTCF settings.

Case-fatality rates in this study were lower than those reported in other studies. National surveillance data (*1*) have shown a case-fatality rate of 13.1% for persons ≥65

Table 2. Susceptibility of invasive GBS disease to erythromycin and clindamycin, Minnesota, 2003–2007*

Susceptibility	No. (%) case-patients					p value†
	2003 (N = 133)	2004 (N = 127)	2005 (N = 131)	2006 (N = 143)	2007 (N = 121)	
Erythromycin susceptible	95 (71.4)	86 (67.7)	87 (66.4)	90 (62.9)	71 (58.7)	0.023
Clindamycin susceptible	114 (85.7)	105 (82.7)	108 (82.4)	116 (81.1)	92 (76.0)	0.057

*GBS, group B streptococcal.
†χ^2 test for trend.

years of age, and a similar study among LTCF residents showed a case-fatality rate of 16.7% (*7*).

Macrolide resistance is common, and increases in clindamycin resistance continue to occur among GBS strains. In the era of methicillin-resistant *Staphylococcus aureus* infections, nonpenicillin agents, such as clindamycin, are increasingly being used for empiric treatment of skin and soft tissue infections, but they may not provide adequate coverage if these infections are caused by GBS bacteria. Although β-lactams remain the preferred therapy for GBS infections, strains with elevated penicillin MICs have recently been reported (*9–11*).

The prevalence of serogroup V in this study is consistent with findings from other studies of adult populations (*12*,*13*) that show the recent emergence of this serotype. Serotype V has been associated with higher rates of antimicrobial drug resistance (*14*); thus, following trends in serotype prevalence may be useful. High rates of antimicrobial drug use in the elderly may result in further selection of serotype V, and resistance may increase in other serotypes. Molecular studies may be useful to further evaluate strains because serologically nontypeable strains contained specific capsular polysaccharide genes, including those for V (*15*). LTCF residents and persons with concurrent conditions should have high priority for vaccine administration after a vaccine becomes available. Vaccines should be multivalent; based on predominant serotypes, currently Ia, Ib, II, III, and V; and effective and immunogenic for older adults.

Dr Kothari recently completed an Infectious Diseases Fellowship at the University of Minnesota. His research interests include the epidemiology of infections among residents of long-term care facilities, with a focus on antimicrobial drug resistance and appropriate use of antimicrobial drugs in this population.

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Epidemiologic Study of *Vibrio vulnificus* Infections by Using Variable Number Tandem Repeats

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A 3-year environmental and clinical *Vibrio vulnificus* survey using simple-sequence repeats typing shows that *V. vulnificus* biotype 3 constitutes ≈21% of the bacterium population in tested aquaculture ponds as opposed to ≈86% of clinical cases. Simple-sequence repeats proved to be a useful epidemiologic tool, providing information on the environmental source of the pathogen.

Vibrio vulnificus is a highly invasive human pathogen and presents a food safety issue worldwide. Human infections caused by *V. vulnificus* are primarily caused by contaminated seafood consumption or contaminated skin wounds, which can lead to septicemia, wound infections, and high hospitalization and fatality rates (*1*,*2*). *V. vulnificus* strains are biochemically divided into 3 biotypes (BTs). BT3, found in Israel, is associated with infections caused by contaminated fish (*3*). Until now, only 3 BT3 isolates had been isolated from the environment in direct contrast with their large clinical numbers (*3*,*4*).

BT3 is a clonal group, which various molecular methods have failed to differentiate among its strains, (*4*,*5*) with the exceptions of rep-PCR (*6*), simple-sequence repeats (SSR) (*7*), and recently pulsed-field gel electrophoresis (PFGE) (*8*). SSR analysis of *V. vulnificus* was highly discriminative among BT3 strains (*7*). These SSR markers have been used for typing and for epidemiologic studies in many bacterial species (*9*,*10*). We present results from a 3-year monitoring program of clinical and environmental *V. vulnificus* using SSR as an epidemiologic genotyping tool.

The Study

A total of 414 *V. vulnificus* isolates were studied, including a reference panel of 32 strains previously studied (*7*). A total of 360 environmental *V. vulnificus* isolates were successfully retrieved from September 2004 through October 2006 from artificial fish ponds and stores in the western Galilee region of Israel (from 21 samplings), and 22 clinical isolates were retrieved from nearby hospitals during matching years (Tables 1, 2). Fish samples were collected and gills and fins/scales were pooled from ≈10 *Tilapia* spp. (300–400 g). Each sample was incubated in 0.5 L modified alkaline peptone water with 4% NaCl, pH 6.9, at 37°C for 16 to 18 h. Samples were diluted in saline and streaked on thiosulfate-citrate-bile-salts-sucrose (TCBS) agar. Suspected colonies were further grown on chromogenic agar (CHROMagar Microbiology, Paris, France), and validated by amplification of *V. vulnificus*–specific gene vvh (*7*). All *V. vulnificus* colonies were green on TCBS agar. Notably, not all bacterial isolates showed the expected turquoise-colonies on CHROMagar but rather pale white colonies. The latter colonies were further identified as BT3. All other isolates, which were BT1, showed the expected turquoise-colony phenotype. No BT2 isolates were found. However, 6 previously studied isolates (*7*) showed the expected turquoise-colony.

SSRs were used to genetically characterize 254 clinical and environmental *V. vulnificus* isolates (Table 1), including 32 previously studied isolates (*7*). DNA extraction, PCR and primers, SSR sizing, and statistical analysis were conducted as previously described (*7*). Capillary electrophoresis was performed by using a 3130 Genetic Analyzer and analyzed with GeneMapper-v4.0 (Applied-Biosystems Inc., Foster City, CA, USA). Two to 34 alleles were detected at the 12 SSR loci among the isolates. Environmental isolates were selected from 21 samples with an average 8.5 isolates per enrichment. We removed 71 isolates that had identical SSR genotypes and originated from the same enrichment from the analysis because they were probably clones. Thus, 183 isolates were discriminated to 170 SSR types. SSR variation data was used to calculate genetic relationships among isolates. A genetic distance matrix was generated followed by cluster analysis (*7*). The resulting dendrogram (Figure 1) showed clear separation between BT3 isolates and the others (average genetic distance of 0.825 ± 0.101). Genetic distances among BT3 isolates were rather low (average 0.369 ± 0.174) relative to high genetic distances (average 0.804 ± 0.149) found among isolates of the other biotypes, in accordance with our previous analysis of 32 isolates (*7*). The new studied isolates showed a variety of SSR genotypes and were spread throughout the dendrogram (Figure 1). Further analysis using eBURST (*12*) showed similar grouping results (data not shown) (*7*).

Differentiation of SSR alleles results at locus VV0401 into environmental types (E-types, ≥12 repeats), and clinical types (C-types, <10 repeats) was tested (*13*). Of the clinical isolates, 44 isolates (98%) exhibited the expected

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Table 1. *Vibrio vulnificus* isolates from Israel that were genetically analyzed, 2003–2006*

Isolate identification	Biotype	Date	Origin/hospital name	IMH no.†
Environmental				
VVyb1	3	2004	Store: yb1-yb53	
VVyb2–VVyb58, (VVyb38, VVyb45 missing)	1		Pond: yb54-yb58	
VVyb63, VVyb66, VVyb67 VVyb71–VVyb73, VVyb83, VVyb86–Vvyb93, VVyb95–Vvyb109, VVyb111–VVyb126	3	2005	Store: yb87-yb126, yb158-yb193	
VVyb59–VVyb62, VVyb64,VVyb65, VVyb68–VVyb70, VVyb74–VVyb82, VVyb84, VVyb85, VVyb159, VVyb162–VVyb164, VVyb167–VVyb170, VVyb172–VVyb182, VVyb187, VVyb189–VVyb193	1		Pond: yb62-yb86	
VVyb94, VVyb110, VVyb158, VVyb160, VVyb161, VVyb165, VVyb166, VVyb171, VVyb183–VVyb186, VVyb188	ND			
VVyb127–Vyb133, VVyb137–VVyb157	3	2006	Store: yb127-yb134, yb194-yb206	
VVyb134–VVyb136, VVyb195–VVyb208, VVyb210–VVyb216, VVyb218–VVyb221, (VVyb201, VVyb212 missing)	1		Pond: yb135-yb157, yb207-yb221	
VVyb209, VVyb217	ND			
v232‡	3	2003 Dec	Fish	8/03e
Clinical§				
v233	3	2006 May	Rivka Ziv	1/06
v234	3	2006 Sep	HaEmek	2/06
v235	ND	2006 Oct	Western Galilee	3/06
v236	3	2005 Feb	HaEmek	1/05
v237	3	2005 Jun	Western Galilee	2/05
v238	3	2005 Jun	Western Galilee	5/05
v239	3	2005 Aug	Rambam	6/05
v240	3	2005 Oct	Western Galilee	7/05
v241	3	2005 Nov	Rambam	8/05
v242	3	2005 Nov	Rambam	9/05
v243	3	2005 Dec	Carmel	10/05
v244	3	2005 Nov	Western Galilee	11/05
v245	3	2005 Jun	Carmel	3/05
v246	ND†	2005 Jun	Rambam	4/05
v247	3	2004 Jun	HaEmek	2/04
v248	3	2004 Jun	HaEmek	3/04
v249	3	2004 Jun	HaEmek	4/04
v250	3	2004 Jul	Rambam	5/04
v251	3	2004 Aug	Western Galilee	6/04
v252	1	2004 Aug	Western Galilee	7/04
v253	3	2004 Oct	Carmel	9/04
v254	3	2003 Dec	Barzilai	8/03

*IMH, Israeli Ministry of Health; ND, not determined.
†All clinical isolates are part of the IMH collection.
‡Previously studied (*7*).
§All clinical isolates are associated with fish.

C-type allele/repeat but only 69 isolates (33%) of the environmental isolates showed the E-type allele/repeat, rejecting the null hypothesis ($p<0.0001$), using Pearson χ^2 test. Notably, 97% of 110 BT3 isolates (clinical/environmental) showed the C-type allele/repeat, in contrast to BT1 isolates (72 isolates, 2%). If, C-type allele/repeat at VV0401 is an indication of potential pathogenicity of *V. vulnificus* strains, then our results further support the high virulence of BT3. However, additional studies are needed to confirm the relationship of this locus to pathogenicity.

Three clinical BT3 isolates exhibited identical SSR genotypes and 2 clinical BT3 isolates had a genotype related to 5 environmental isolates sampled on related dates from nearby regional areas (Figure 1, panel B). One clinical

Table 2. Environmental *Vibrio vulnificus* isolates obtained in Israel from artificial fish ponds and fish stores, 2004–2006

Dates	No. samples	No. isolates	No. *V. vulnificus**	Biotype 3, %	Biotype 1, %†
2004 Sep–Oct	7	58	58	2	98
2005 May–Oct	5	280	166	28	72
2006 Mar–Oct	9	251	136	21	79
Total	21	589	360		

*Tested by specific amplification of *vvh* gene.
†Including isolates not determined.

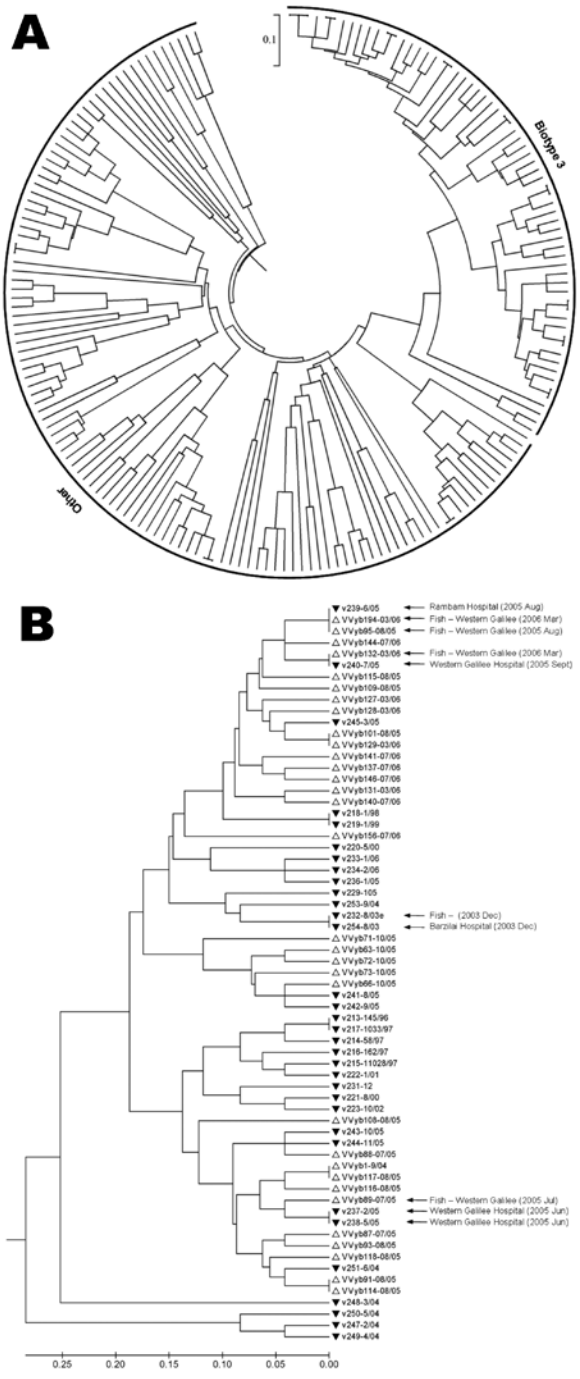

Figure 1. A) Genetic relationships based on simple-sequence repeat (SSR) variation data among 183 *Vibrio vulnificus* isolates including 135 new environmental, 22 new clinical, and 26 previously studied isolates. B) A subtree enlargement of panel A displaying a set of 65 *V. vulnificus* biotype 3 isolates. Similar clinical and environmental isolates, showing an epidemiologic connection, are indicated by arrows. The genetic-distance matrix was generated based on 212 polymorphic points (the sum of alleles across 12 SSR loci). Genetic relationships are based on unweighted pair group method with arithmetic mean cluster analysis of SSR variation using MEGA4 software (*11*). Scale bar represents genetic distance.

BT3 isolate v239 (August 2005) showed SSR genotypes identical to the environmental isolate VVyb95 obtained in the same month (August 2005) and to VVyb194 (obtained in March 2006). A second BT3 clinical isolate v240 (October 2005) and the fish-pond isolate VVyb132 (March 2006) had identical SSR genotypes. Moreover, these 2 results suggest survival of *V. vulnificus* strains through the winter season, either in a viable nonculturable state (*14*) or as viable cells in sediment that can serve as a shelter for some subpopulations (*15*). The latter scenario is more probable in artificial fish ponds because water circulation is high throughout the growth period and pond sediment remains untouched. A third clinical isolate was analyzed earlier and showed an epidemiologic connection: v254 isolate was obtained from an injured woman (injured by a fish) in December 2003. Analysis of microbial flora on the fish (found in the woman's freezer) identified a *V. vulnificus* BT3 isolate, v232. These 2 isolates showed identical SSR genotypes, confirming the fish as the origin of infection (Figure 1, panel B). Additionally, clinical BT1 isolate v252 (August 2004), showed similar SSR genotypes, 1 repeat difference in 1 locus, to environmental isolate VVyb50 (October 2004).

To strengthen our typing results, we compared the epidemiologic SSR results to those of PFGE in 12 representative BT3 isolates. PFGE was performed and analyzed as described previously (*8*). Results for PFGE were generally similar to results for SSR (Figure 2). PFGE patterns were similar (≥85%) between isolates. Identical PFGE patterns and SSR genotypes were seen in isolates v239 (clinical) and VVyb95, VVyb194 (environmental), as were isolates v232 (fish) and v254 (clinical). Identical PFGE patterns and single-locus variants in SSR genotypes were seen in clinical isolate v237 and the environmental isolates VVyb89 and VVyb1. Notably, VVyb1, which was isolated a year earlier, differentiated from VVyb89 by another single repeat in an additional single locus, confirming higher resolution of SSR method. Finally, identical SSR genotypes and PFGE patterns that differed by 1 band were seen in v240 and VVyb132.

Conclusions

The developed isolation and enrichment procedures obtained large numbers of BT3 and BT1 from the environment. Results showed that although BT3 makes up only ≈21% of the *V. vulnificus* isolates from fish, BT3 accounts for ≈86% of the clinical cases and thus could imply high pathogenicity for this group (*4*). Genetic analysis of this large survey confirms the distinctness (clonality) (*5*) of BT3 and the high resolution power of the SSR (*7*).

SSR genotyping of *V. vulnificus* was used to determine the genetic relatedness between clinical and environmental isolates and identify the source of contamination. SSR

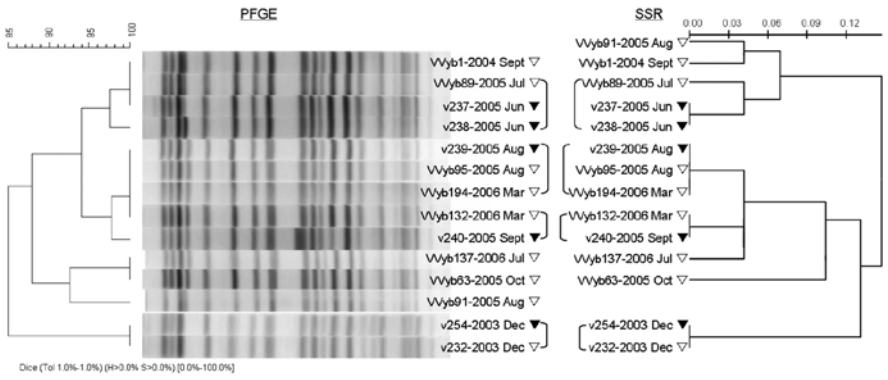

Figure 2. Genetic relationships showing the epidemiologic connection among 12 clinical and environmental *Vibrio vulnificus* biotype 3 isolates based on pulsed-field gel electrophoresis (PFGE) analysis compared to analysis at 12 single-sequence repeat (SSR) loci. PFGE profiles were compared by using the Dice coefficient followed by unweighted pair group method with arithmetic mean clustering (tolerance, 1.0%). Scale bars represent pattern similarity (% for PFGE and genetic distance for SSR).

can serve as an epidemiologic tool to indicate the infection source of pathogens such as *V. vulnificus*, and can potentially provide knowledge for preventive steps in terms of public health.

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Dr Broza was a PhD student in the laboratory of Applied Genomics at the Faculty of Biotechnology, and Food Engineering, Technion-Israel Institute of Technology, at the time of this study. This work was part of his dissertation on the human pathogen *V. vulnificus*. His current research is studying nanomaterial-based sensors for detection of disease biomarkers through breath samples.

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Challenges of Investigating Community Outbreaks of Cyclosporiasis, British Columbia, Canada

Lena Shah, Laura MacDougall, Andrea Ellis, Corinne Ong, Sion Shyng, Linda LeBlanc, and the British Columbia Cyclospora Investigation Team[1]

Investigations of community outbreaks of cyclosporiasis are challenged by case-patients' poor recall of exposure resulting from lags in detection and the stealthy nature of food vehicles. We combined multiple techniques, including early consultation with food regulators, traceback of suspected items, and grocery store loyalty card records, to identify a single vehicle for a cyclosporosis outbreak in British Columbia, Canada, during 2007.

Cyclospora cayetanensis is an emerging coccidian parasite that causes outbreaks of protracted and relapsing gastroenteritis (*1*,*2*). Delays in clinical diagnosis caused by the waxing and waning symptoms of *Cyclospora* infection, coupled with a long incubation period (median 7 days) and concealed food vehicles (e.g., herbs), result in poor recall of food exposures. Therefore, outbreaks in which no common meal is eaten are even more difficult to solve. In 2007, the British Columbia Centre for Disease Control used early collaboration with the Canadian Food Inspection Agency (CFIA), grocery card shopping records, and product traceback for several suspected items simultaneously to successfully implicate a vehicle in a community *Cyclospora* outbreak.

The Study

From May 1 through July 30, 2007, a total of 29 cases of locally acquired *Cyclospora* infection were reported in British Columbia (Figure 1; Table 1). An initial investigation was conducted around the 6 laboratory-confirmed case-patients reported in the last 2 weeks of May and the first week of June (phase 1). No common exposure was reported, and case reports subsided. During the last week of June, case reports resumed, and phase 2 of the investigation was initiated. A total of 19 confirmed and 4 probable cases were identified with symptom onsets during June 28–July 20, 2008. Fifty-three percent of these cases occurred in male patients. No case-patients were hospitalized. Average time from symptom onset to positive laboratory result was 17 days (range 6–31 days).

During phase 2, a total of 17 confirmed case-patients were interviewed with hypothesis-generating questionnaires about items eaten in the 2 weeks before symptom onset. The instrument included questions about restaurant history with meal details; grocery stores frequented; and yes/no questions about >70 fruits and vegetables, 8 herbs, and 16 mixed foods (e.g., salsa, pesto) previously implicated in outbreaks of foodborne disease. No common restaurants or events were identified.

Frequently reported foods were compared with population controls from Canadian (Waterloo, Ontario) and American (Oregon; US Foodborne Diseases Active Surveillance Network [FoodNet]) published food consumption surveys (*3*–*5*). Although such measurements may be limited by the timing of questionnaire administration and the recall period considered, they can be useful comparators during the hypothesis-generating stages of an investigation. By the end of phase 2, strawberries, cilantro, and sweet basil were reported more often than expected by case-patients (Table 2). Garlic and red peppers also were commonly eaten by case-patients; however, population comparisons were unavailable. Eighty-eight percent of case-patients reported having eaten romaine lettuce; 85% of controls in the Waterloo survey (*4*,*5*) had eaten lettuce of any type, and romaine lettuce consumption was much less commonly reported in the FoodNet survey (*3*) (Table 2). Other foods assessed were not eaten more often than expected. A formal case–control study was considered premature in the early stages of phase 2 because no strong hypothesis emerged from early interviews and comparisons to population controls. We further explored the plausibility of various hypotheses through a combination of methods described below that allowed room for additional hypotheses to emerge or existing hypotheses to strengthen as cases accrued.

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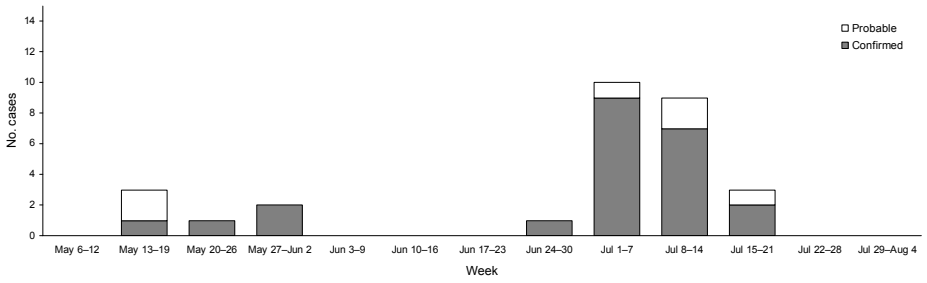

Figure 1. Confirmed and probable cases of cyclosporiasis (N = 29), by date of onset, British Columbia, Canada, May–August 2007.

Detailed questionnaires asked whether foods were eaten in a restaurant or were store-bought and about type of packaging and method of preparation (because *C. cayetanensis* is heat-sensitive) (*6*). We reinterviewed early case-patients using the second questionnaire and interviewed later case-patients using both questionnaires. In phase 1, garlic eaten at restaurants by all 4 persons with confirmed infections were traced back to different suppliers; only 1 case-patient ate raw garlic in a restaurant. Three case-patients also reported eating cooked garlic at home; cooking would have inactivated the pathogen.

Early and proactive collaboration with CFIA involved a general assessment of the country of origin and distribution patterns for frequently eaten foods. According to CFIA records, romaine lettuce and red peppers sold during the exposure period were not imported from a known *Cyclospora*-endemic country and were widely distributed in Canada and the United States. This was not consistent with case distribution (R. Cardinal, CFIA, pers. comm.).

Because interviews, population control comparisons, and product distribution limited suspected foods to strawberries, cilantro, and basil, we began preliminary traceback of all 3 suspected items. Environmental health officers and regional CFIA staff interviewed grocery store owners, restaurant managers, and distributors to trace produce to its supplier. Local strawberries eaten by case-patients from 3 small markets were traced back to 2 local farms in geographically separate regions of British Columbia. Cilantro eaten by case-patients was traced to 2 suppliers; both supplied home-grown rather than imported produce. Of 14 case-patients with confirmed basil exposures, 8 (57%) ate only organic basil supplied by distributor A. Additionally, 4 (29%) reported multiple basil exposures, including exposure to organic basil from distributor A (Figure 2). In British Columbia, organic basil enjoys a smaller market share than the conventional product.

In phase 2, 12 (71%) of 17 case-patients reported shopping at grocery C. Records of any grocery store purchases for the households of 8 consenting case-patients were obtained through grocery C's savings card program; other case-patients were not cardholders. All purchase histories were requested for 1 month before symptom onset to account for the typical incubation period plus product shelf life. Records from 3 (38%) case-patients showed purchases of the same organic basil supplied by distributor A. Two case-patients had bought organic basil on the same day at the same location. Of the remaining 5 case-patients who recalled purchasing organic basil but whose consumer card records did not confirm it, 2 had not used their cards for large portions of the incubation period.

We collected supplier information for organic basil during a visit to the distribution warehouse and local farm site of distributor A. The remaining 2 (14%) case-patients with basil exposure previously unlinked to distributor A were confirmed through trace-forward from distributor A. The first had eaten organic basil at a smaller market supplied by distributor A under another trade name. The second had eaten conventional basil from a grocery store supplied by distributor A. Distributor A confirmed using organic basil to supplement conventional basil shipments when supply was low. Late summer outbreaks of cyclosporiasis in British Columbia are unusual; distributor A confirmed that imported product was used throughout the summer in 2007 because of a poor local growing season.

All case-patients in phase 2 who recalled basil exposure (82%) could have been exposed to organic basil from distributor A. Once this common vehicle was identified,

Table 1. Cyclosporiasis case definitions, British Columbia, Canada, May 1–July 31, 2007

Case type	Definition
Confirmed	British Columbia residents who had not traveled outside Canada and the United States within 2 weeks before symptom onset and in whom *Cyclospora* species oocysts* were detected by microscopy on or after May 1, 2007
Probable	Clinical illness compatible with *Cyclospora* infection (i.e., watery diarrhea and/or bloating, cramps, loss of appetite, loss of weight), no travel outside Canada and the United States within 2 weeks before symptom onset, and onset of symptoms within a week of a person with whom they shared food exposures and who had a laboratory-confirmed case

*Laboratory-confirmed cases were reported to public health by medical diagnostic laboratories and specimens forwarded for confirmation to the public health reference laboratory. The morphologic identification of *Cyclospora* spp. oocysts included shape (spherical), size (8–10 μm in diameter), oocyst wall (well-defined), internal contents with refractile globules, autofluorescence, and modified acid-fast or safranin staining (*1*).

Table 2. Food items eaten more often by case-patients* than by population controls, British Columbia, Canada, May 1–July 31, 2007*

Food item	No. (%) case-patients	Controls, %	
		Waterloo survey	US FoodNet
Strawberries	16–17 (94–100) (1 unsure)	32	28
Romaine lettuce	15 (88)	Any lettuce, 84	37
Garlic	12 (71)	NA	NA
Red peppers	14 (82)	NA	NA
Cilantro	12–15 (70–88) (3 unsure)	8	NA
Basil	14–16 (82–94) (2 unsure)	12	NA

*Case-patients were 17 persons with laboratory-confirmed cases interviewed during phase 2 (June 24–July 21, 2007). US FoodNet, Foodborne Diseases Active Surveillance Network; NA, not applicable.

CFIA conducted a full traceback of organic basil by using formal documentation including invoices, shipment numbers, and airway bills. The suspected imported basil was no longer available for testing. Using distributor A invoices, we identified a specific shipment of organic basil imported from 1 of 2 Mexican supplier farms, and CFIA notified Mexican authorities. The Mexican farm was located in a region previously linked to cyclosporiasis outbreaks (R. Cardinal, CFIA, pers. comm.).

Conclusions

Detailed interviews, modified traceback of several suspected items, and information about product distribution and market share led to organic basil as a primary hypothesis. Food regulators could pinpoint a specific shipment and trace it to its origin because consumer cards provided the exact purchase dates for basil that case-patients could not recall. Overall, the approach used in this investigation increased the work load typically requested of team members during foodborne outbreaks. However, this combination of investigative methods successfully identified a single vehicle during a community cyclosporiasis outbreak where a common menu was not available.

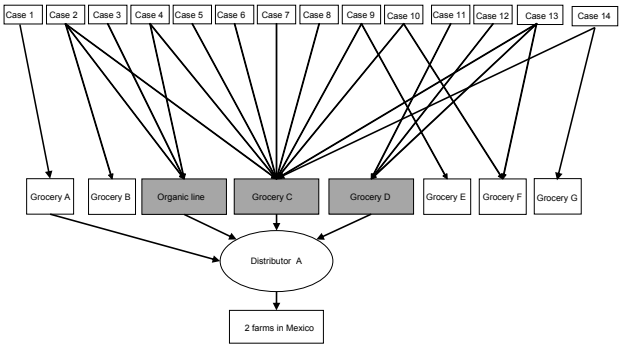

Figure 2. Traceback of basil eaten by persons with confirmed cyclosporiasis (N = 14), British Columbia, Canada, May–August 2007.

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Ms Shah is a field epidemiology trainee in the Canadian Field Epidemiology Program. Her research interests include methods for investigating infectious disease outbreaks and for studying community transmission of pathogens.

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Shiga Toxin–producing *Escherichia coli*, New Mexico, USA, 2004–2007

Sarah Lathrop, Karen Edge, and Joseph Bareta

Sporadic infection with Shiga toxin–producing *Escherichia coli* (STEC) in New Mexico increased from 0.9 cases per 100,000 population (95% confidence interval [CI] 0.5–1.36) in 2004 to 1.7 (95% CI 1.14–2.26) in 2007. Non-O157 STEC was more common in nonwhite residents, children <5 years of age, and urban residents.

The epidemiology of infections and hemolytic uremic syndrome (HUS) caused by Shiga toxin–producing *Escherichia coli* (STEC) O157:H7 are well described (*1*–*4*). Non-O157 STEC infection also is associated with severe illness and HUS but often is underdiagnosed and less well understood (*3*–*7*). Studies in Europe indicate that non-O157 STEC infections occur more frequently than do STEC O157 infections (*3*). STEC O157 infection has been a notifiable disease in the United States since 1994, but non-O157 STEC infection became reportable only in 2000 (*8*). To understand trends in STEC O157 and non-O157 infections and their epidemiology in New Mexico, we analyzed population-based data from the active surveillance of clinical laboratories. This surveillance was performed as part of the Centers for Disease Control and Prevention's (CDC's) Foodborne Diseases Active Surveillance Network (FoodNet), to which New Mexico began contributing data in 2004.

The Study

All STEC isolates or broths were sent for confirmation to the Scientific Laboratory Division of the New Mexico Department of Health, where Shiga toxin expression was confirmed by enzyme immunoassay (EIA); the broth was then cultured and preliminarily serotyped. Pulsed-field gel electrophoresis was performed on all isolates. The laboratory submitted non-O157 STEC isolates to CDC for additional serotyping and PCR testing for toxin genes.

This analysis comprised only sporadic cases of STEC. Negative binomial regression models were used to calculate incidence rates and assess differences in risks for STEC O157 and non-O157 infections. Variables were reporting year, patient age, race/ethnicity, sex, and rural versus urban residence (*8*); $p \leq 0.05$ was considered statistically significant.

During 2004–2007, New Mexico FoodNet identified 111 cases of laboratory-confirmed sporadic STEC infection; 40 (36%) were STEC O157, and 71 (64%) were non-O157 STEC (Table 1). Six additional cases were outbreak associated. Incidence increased from 0.93 cases per 100,000 population (95% confidence interval [CI] 0.5–1.36) in 2004 to 1.07 per 100,000 (95% CI 0.61–1.52) in 2005 and 1.84 per 100,000 (95% CI 1.25–2.44) in 2006. The rate fell slightly in 2007, to 1.70 per 100,000 (95% CI 1.14–2.26) population, resulting in a test of trend that approached statistical significance ($p = 0.09$). From 2004 through 2007, sporadic STEC infections increased 94%. A total of 18 STEC serotypes were identified during this time. The primary se-

Table 1. Demographic characteristics of case-patients who had laboratory-confirmed STEC infections, New Mexico, USA, 2004–2007*

Characteristic	No. case-patients		
	O157 STEC	Non-O157 STEC	Total
Age group, y†			
<1	3	3	6
1–4	3	26	29
5–10	10	7	17
11–18	4	12	16
19–29	5	7	12
30–39	4	2	6
40 –49	1	2	3
50–59	1	5	6
≥60	9	7	16
Sex			
Male	18	34	52
Female	22	37	59
Race/ethnicity			
White non-Hispanic	17	17	34
White Hispanic	11	12	23
White, unknown ethnicity	5	4	9
Native American	3	10	13
African American	1	0	1
Other	0	5	5
Unknown	3	23	26
Type of county			
Urban	29	64	93
Rural	11	7	18
Year of diagnosis			
2004	7	11	18
2005	10	11	21
2006	14	23	37
2007	9	26	35
Total no. case-patients	40	71	111

*STEC, Shiga toxin–producing *Escherichia coli*; O157, serotype O157:H7.
†Median age (range) of patients: STEC O157–infected, 18 y (4 mo–78 y); non-O157 STEC–infected, 10 y (5 mo–70 y); total, 13.3 y (4 mo–78 y).

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rotypes responsible for the increase were STEC O157, O26, O111, and O103, constituting 75% of all STEC cases reported. Incidences of STEC serotypes O26, O111, and O103 combined increased 300% from 2004 through 2007. The proportion of non-O157 STEC ranged from 52% in 2005 to 74% in 2007.

Although STEC O157 was the 1 serotype most often identified, infections caused by non-O157 STEC (all serotypes) were diagnosed more frequently. STEC O26 (18%) and O111 (13%) were the most commonly identified non-O157 STEC serotypes. Other STEC O serotypes (O103, O121, O46, O177, and O91) were responsible for 33% of all STEC infections. Most isolates were positive for toxin gene stx_1 (86%), intimin (66%), and enterohemolysin A (81%).

STEC O157 infection was significantly more likely to be diagnosed in adults (half of all cases, one third of non-O157 STEC cases) than in children (<18 years of age) ($p = 0.01$). Non-O157 STEC serotypes were most commonly identified in children 1–4 years of age. STEC O157 infections occurred most commonly in children 5–10 years of age, followed by adults >60 years. Sex distributions were similar for patients with STEC O157 and non-O157 infections (55% female vs. 45% male and 52% vs. 48%, respectively). White non-Hispanics, which constitute 43% of New Mexico's population, made up 31% of all confirmed STEC infections in New Mexico; white Hispanics (42%) made up 21% of confirmed STEC cases; and Native Americans (10%) made up 12% of cases (Table 1).

More laboratory-confirmed STEC infections were diagnosed during the summer months than during the rest of the year. STEC O157 cases were diagnosed more frequently in September (10 cases); non-O157 STEC cases were most frequently diagnosed in June and July (9 cases each).

Although STEC O157 infections was diagnosed in more persons than were non-O157 STEC infections (28% vs. 16%), the difference was not significant. Patients with STEC O157 infections stayed in the hospital a mean of 6 days (median 4), compared with a mean of 4.5 days (median 3) for patients with non-O157 STEC infections, also not significant. All 5 reported cases of HUS were caused by STEC O157.

International travel was related to STEC infection for 12 (11%) patients. Two (5%) STEC O157 cases were travel related, as were 4 (10%) cases each of STEC O111 and O26; and 1 (1%) case each of STEC O128 and O103. Travel to Mexico was documented for 10 of the 12 travel-associated cases.

Most (99 [89%]) persons with STEC were from New Mexico's urban counties. We calculated incidence rate ratios between STEC O157 and non-O157 infections during 2004–2007 (Table 2), while adjusting for variables that were significant in negative binomial models, including race (white non-Hispanic vs. nonwhite), age (<5 years vs. ≥5 years), and county (urban vs. rural). More patients with non-O157 STEC infection during this time were nonwhite, <5 years of age, and residents of urban counties. Patients infected with STEC O157 were more likely to be white non-Hispanic, ≥5 years of age, and residents of rural counties.

Conclusions

The data collected by New Mexico's FoodNet surveillance network indicate that sporadic STEC cases increased substantially from 2004 through 2007. Reports of STEC O157 infection doubled from 7 to 14 from 2004 to 2006 but dropped to 9 in 2007. However, the number of non-O157 STEC cases continued to climb and accounted for most of the increase in overall STEC rates in New Mexico during this time, similar to rates in Connecticut and other FoodNet sites (*4*,*9*).

Non-O157 STEC infections ranged from 52% to 74% of all Shiga toxin–positive cases diagnosed each year and 64% of all identified STEC cases. New Mexico was second only to Colorado (2.12 cases per 100,000 population) among FoodNet sites for non-O157 STEC incidence in 2007 (*10*). Similar to serotypes reported from other locations, STEC serotypes O26, O111, and O103 made up most non-O157 infections in New Mexico (*1*), especially O26 and O111 (18% and 13% of all cases, respectively).

Table 2. Comparisons of risk for infection with STEC O157 versus non-O157 STEC types, by demographic characteristic, averaged population, New Mexico, USA, 2004–2007*

Characteristic	Unadjusted incidence rate ratio† (95% CI)	p value	Adjusted incidence rate ratio† (95% CI)	p value
Race				
Other	1		1	
White	3.21 (1.42–7.27)	0.01	3.03 (1.34–6.90)	0.008
Age, y				
<5	1		1	
≥5	2.61 (1.09–6.22)	0.03	2.74 (1.15–6.54)	0.031
County				
Urban	1		1	
Rural	1.96 (0.98–3.92)	0.05	1.84 (0.91–3.69)	0.089

*STEC, Shiga toxin–producing *Escherichia coli*; O157, serotype O157:H7; CI, confidence interval.
†Corrected for overdispersion by using negative binomial distribution.

As previously reported (*1*), non-O157 STEC infections occurred commonly in young children in this study. They also occurred at a higher rate for non-white New Mexico residents. Another recent study similarly found higher shigellosis rates in counties with higher proportions of Hispanics (*11*). This study concurs with others that have reported finding the highest rates of STEC O157 rates in rural communities (with increased opportunities for animal contact) and in the West (*12*,*13*).

Year-to-year increases in numbers of non-O157 STEC infections, both nationally and in New Mexico, must be interpreted with caution because of changes in laboratory testing practices. Although the state's largest clinical laboratory performed EIAs before active surveillance was implemented in 2004, other laboratories throughout the state might have changed their testing practices.

Risk factors for non-O157 STEC infections may be similar to those for STEC O157 infections (*9*), but additional studies are needed to elucidate differences and similarities between them, as well as among non-O157 STEC serotypes (*14*). Clinical laboratories should simultaneously screen for Shiga toxin and culture all positive isolates to determine the true incidence of non-O157 STEC infections.

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Chronic Invasive Aspergillosis caused by *Aspergillus viridinutans*

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Aspergillus viridinutans, a mold phenotypically resembling *A. fumigatus*, was identified by gene sequence analyses from 2 patients. Disease was distinct from typical aspergillosis, being chronic and spreading in a contiguous manner across anatomical planes. We emphasize the recognition of *fumigati*-mimetic molds as agents of chronic or refractory aspergillosis.

Aspergillus fumigatus is the most common cause of invasive aspergillosis afflicting various immunocompromised patients. We report 2 cases of documented invasive disease in children in the United States caused by *A. viridinutans*, a *fumigati*-mimetic mold, which produced clinical manifestations distinct from *A. fumigatus*.

The Patients

Patient 1 was a 14-year-old boy with p47^{phox}-deficient chronic granulomatous disease. He had *Staphylococcus aureus* liver abscesses at ages 5 and 10 years, *Burkholderia cepacia* complex pneumonia at age 6 years, and *Serratia marcescens* pneumonia at age 12 years. In February 2004, while the patient was receiving itraconazole prophylaxis, right-sided chest pain and fever developed. A computed tomography (CT) scan showed a 2-cm right middle lobe nodule adjacent to the cardiac border and mediastinal lymphadenopathy abutting the superior vena cava and anterior pericardium (Figure 1). Lymph node biopsy yielded a mold morphologically identified as *A. fumigatus*. Treatment with voriconazole was initiated. Repeat imaging 1 week later showed slight enlargement of the mediastinal mass. One month later, there was continued enlargement of the lung nodule and mediastinal adenopathy with necrosis. Caspofungin was added. Two weeks later, a new right middle lobe infiltrate was noted. Antifungal therapy was changed to posaconazole. Over the next 2 months, there was expansion of the right lung infiltrates and lymphadenopathy. Treatment was modified to posaconazole and caspofungin. Serial imaging over the next 3 weeks showed regression of the lung consolidations and mediastinal mass. Four months later, with ongoing resolution of the thoracic disease, the patient began receiving a maintenance dosage of posaconazole. As of 5 years later, he had experienced no recurrence.

Patient 2 was an 8-year-old boy with hyperimmunoglobulin-E (Job) syndrome due to mutation in signal transducer and activator of transcription-3 in whom a right-sided pulmonary abscess developed and failed to improve after 1 month of antibacterial therapy. New left lung nodules were biopsied and specimens yielded a mold morphologically identified as *A. fumigatus*. During 3 months of treatment with voriconazole, the bilateral pulmonary lesions cavitated. Two months later, left lower lobe wedge resection yielded the same mold. Diaphragmatic injury required primary closure with sutures. One month later, enlargement of the residual left lung lesions prompted change in therapy to amphotericin B and caspofungin. Following the patient's transfer to the National Institutes of Health, imaging showed left apical and lower lobe cavitary masses, left pleural mass and effusion, and a right upper lung cavity with nodule. Samples taken from the left lower cavity and pleura demonstrated septated branching hyphae; specimens grew a mold resembling *A. fumigatus*. Amphotericin B–related nephrotoxicity prompted change of treatment drug to posaconazole. Tissues obtained through subsequent surgical debridements of lung, pleural, diaphragmatic, and subpulmonic abscesses, left lower lobe segmentectomy, and decortication all grew a mold morphologically consistent with *A. fumigatus*. The patient's medical regimen was changed to posaconazole, caspofungin, and flucytosine. Persistent pleural tube drainage yielded the same fungus, despite treatment with amphotericin B and granulocyte intrapleural infusions. Systemic granulocyte infusions, adjunctive granulocyte-colony stimulating factor, and interferon-γ led to transient stabilization of disease. However, progressive splenic abscess formation contiguous with the diaphragm was noted (Figure 2). Seven months later, Guillain-Barré syndrome without a clear cause developed in the patient. The patient died of progressive respiratory failure with aspiration of abscess cavity content. Autopsy demonstrated extensive fungal abscesses in the lungs and left pleural space that extended into the airways, diaphragm, and spleen.

Isolates were initially identified by morphologic criteria using standard clinical mycology laboratory media and incubation conditions. Subsequently, multilocus sequence analyses were performed and targeted the internal tran-

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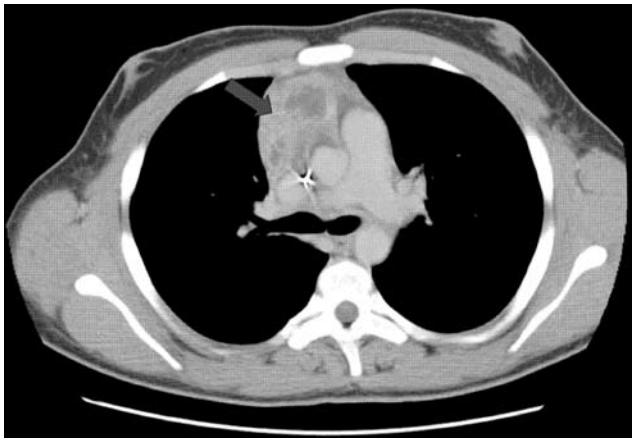

Figure 1. Computed tomography scan of thorax showing extension of infection with *Aspergillus viridinutans* into mediastinal structures (arrow).

scribed spacer (ITS) 1 and 2 regions flanking 5.8S rDNA, (partial) β-tubulin gene (*benA*), and (partial) calmodulin gene as previously described (*1–4*). Sequences were assembled by using Lasergene version 7.0 (DNASTAR; www.dnastar.com), compared with sequences in GenBank by using BLAST (www.ncbi.nlm.nih.gov/BLAST), and realigned relative to best-matched sequences by using MegAlign (DNASTAR). Sequencing of ITS assigned the isolates to *Aspergillus* section *Fumigati* but could not provide further intrataxon discrimination. The β-tubulin sequence from patient 1 had 96.3% and 99.5% similarity with *A. viridinutans* type strain IMI062875T (AF134779) and strain IMI182127 (AF134777), respectively. Three isolates from patient 2 were tested, demonstrating 96.3% and 98.4% similarity with *A. viridinutans* type strain CBS127.56T (AF134779) and NRRL6106 (AF134778), respectively. The calmodulin sequences from patient 1 and patient 2 demonstrated 99.8% and 99.0% similarity, respectively, to *A. viridinutans* IMI182127 (DQ094888). The 2 patients' isolates were 98.6% similar to each other. The strains were designated NIHAV1 (from patient 1) and NIHAV2 (from patient 2). The deduced amino acid sequence from NIHAV1 had 100% similarity with strain IMI182127 (AF134777); NIHAV2 differed by 1 aa with strain NRRL6106 (AF134778). NIHAV1 and NIHAV2 differed by 1 aa (98.8% similarity). Sequences obtained for NIHAV1 *benA*, NIHAV1 calmodulin, NIHAV2 *benA*, and NIHAV2 calmodulin have been deposited in GenBank under accession nos. GQ144441, GQ144442, GQ144440, and GQ144443, respectively.

Antifungal drug susceptibility testing was performed by broth microdilution at the Fungus Testing Laboratory, University of Texas Health Sciences Center, using Clinical Laboratory Standards Institute's contemporary method M38A (Table). Isolates underwent extended incubation to ensure sufficient conidia for testing. Although no clinical interpretive breakpoints are established, most clinical isolates identified as *A. fumigatus* have MICs to amphotericin B and voriconazole ≤0.5–1 mg/L (*5–7*). The *A. viridinutans* isolates, however, demonstrated significantly higher MICs to amphotericin B and voriconazole, agents recommended as first-line therapy for treatment of aspergillosis (*8*). This in vitro resistance may explain in part the refractory disease seen clinically. Furthermore, synergistic studies of isolates from patient 2 with the combination of either posaconazole and terbinafine or amphotericin B and terbinafine produced an indifferent effect.

Conclusions

Differentiation of species within *Aspergillus* section *Fumigati* using phenotypic features alone is difficult. Our isolates resembled *A. fumigatus* but demonstrated slower growth at 37°C and markedly reduced sporulation. Based on multilocus sequence analyses, however, these isolates are *A. viridinutans*, a mold phenotypically resembling but phylogenetically distinct from *A. fumigatus*. Although the ITS region failed to distinguish these isolates from *A. fumigatus*, weak intrataxon discriminatory capacity has been previously reported for this locus (*9*). β-tubulin and calmodulin sequences confirmed its identification as *A. viridinutans*. Sequence divergence within our isolates is consistent with findings from previous studies demonstrating intraspecific genetic variability within the *A. viridinutans* species (*10–12*).

A. viridinutans was originally isolated from rabbit dung in Australia and has since been identified in soil samples globally (*12,13*). Although *A. viridinutans* has been reported from retrospective analysis of culture collections (*9–11*), its clinical relevance has not been defined. These cases demon-

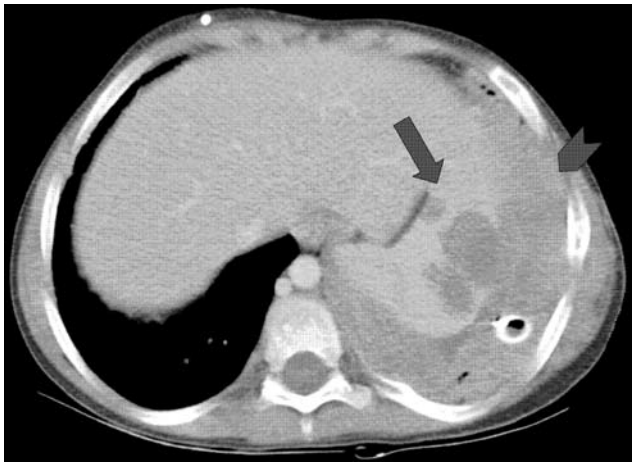

Figure 2. Computed tomography scan showing infection with *Aspergillus viridinutans*, originating in the lungs, extending into the diaphragm (arrowhead), and producing hypodense splenic lesions (arrow).

Table. Antifungal drug susceptibility results of *Aspergillus viridinutans* isolates relative to *A. fumigatus* reported at 48 hours*

Isolate	Amphotericin B MIC, mg/L	Itraconazole MIC, mg/L	Voriconazole MIC, mg/L	Posaconazole MIC, mg/L	Caspofungin MEC, mg/L	Terbinafine MIC, mg/L
Patient 1	4	1	1	0.06	0.25	ND
Patient 2†	2–8	8	2–4	≤0.016–0.5	0.06–0.25	0.125–1
A. fumigatus B-5233‡	0.5	0.5	0.5	0.125	0.25	2

*MEC, minimal effective concentration; ND, not determined.
†Composite results of 3 isolates from the patient.
‡Clinical isolate from a patient with a fatal case of aspergillosis (courtesy of K. J. Kwon-Chung, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA).

strate that *A. viridinutans* can cause a distinct form of aspergillosis, characterized by chronicity, propensity to spread in a contiguous manner across anatomical planes, and relative refractoriness to antimycotic drugs. In contrast, invasive aspergillosis affecting neutropenic chemotherapy or transplant patients is typically an acute disease with predilection for angioinvasion and hematogenous dissemination; successful response to antifungal therapy most commonly occurs within the first 6 weeks of treatment (*14*). However, *A. viridinutans* infections in 2 patients with distinct primary immunodeficiencies suggest that these clinical phenomena reflect inherent pathogenic features of the mold, rather than manifestations due to a specific immune defect. A similar constellation has been described for another cladistically related *fumigati*-mimetic, *Neosartorya udagawae*, in patients with chronic granulomatous disease or myelodysplasia (*15*), which may suggest pathogenetic differences between subgroups within section *Fumigati*. Thus, genotypic-based identification of *fumigati*-mimetic fungi may have implications for clinical course and management.

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Legionella pneumophila in Rainwater on Roads

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During rain, transient puddles form on roads, and this water is splashed into the air by moving vehicles. To determine whether this water contains *Legionella pneumophila,* we collected samples from roads. We found that *L. pneumophila* are abundant in these puddles, especially during warm weather.

Legionella pneumophila bacteria are a major cause of severe community-acquired pneumonia; in recent years the numbers of reported cases of legionellosis have increased substantially where testing is available (e.g., United States, Europe, Japan). However, the source and mode of transmission for sporadic cases are often obscure. Several studies have indicated an association between rain and legionellosis (*1–5*). We recently reported a case of legionellosis in a commercial truck driver in Japan who became infected during the rainy season (*6,7*). No particular environmental risk factors were noted, although he had certain host risk factors such as middle age (mid 50s) and heavy smoking. All specimens collected from his home environment had negative *L. pneumophila* culture growth.

In this study, we investigated the prevalence of *L. pneumophila* in puddles on asphalt roads. Our hypothesis was that after rain *L. pneumophila* grows prolifically in puddles on asphalt roads and can be spread by moving cars, which may increase the amount of organism in the environment.

The Study

From July through October 2007, we collected 45 samples of rainwater from 4 points on 1 asphalt road in Tokyo, Japan. After rainfall, 100-mL water samples were suctioned from road surfaces into sterile syringes and then stored in sterile bottles. Also, sterile flat containers were placed beside the roads for 1 day so that raindrops were collected directly. Swabs from roads were also collected when it was sunny. Meteorologic data for the sample collection dates were obtained from the Japan Meteorological Agency. Water samples were concentrated and mixed with equal volumes of 0.2 M KCl–HCl buffer (pH 2.2). The mixture was allowed to stand at room temperature for 15 min before being spread onto WYOα plates (Eiken Chemical Co., Tokyo, Japan). The plates were then incubated for at least 7 days. Smooth colonies that were grayish white or gray-blue-purple were subcultured on *Legionella* agar (Becton-Dickinson, Mountain View, CA, USA) supplemented with L-cysteine and ferric pyrophosphate and on nutrient agar (Eiken Chemical co.). Colonies that grew on only *Legionella* agar were subsequently identified by PCR. The following primers were used to detect the 430-bp sequence of the 16S r RNA-encoded gene: forward primer 5′-GAGGGT TGATAGGTTAAGAGC-3′ and reverse primer 5′-CGGTCA ACT TATCGCGTT TGCT-3′ (*8*). Serotyping was also performed by a slide agglutination test with commercial specific antiserum (Denka Seiken Co., Ltd, Tokyo, Japan). The detection limit of the procedure was 20 CFU/100 mL. A loop-mediated isothermal amplification (LAMP) kit (Eiken Chemical Co.) was also used to detect *Legionella* spp. (*9*). To confirm the presence of free-living amebae (FLA) DNA, we used PCR with forward primer P-FLA-F: 5′-CGCGGTAATTCCAGCTCCAATAGC-3′ and reverse primer P-FLA-R: 5′-CAGGTTAAGGTCTCGTT CGTTAAC-3′ targeted at conserved stretches of *Acanthamoeba* 18S rDNA (*10*). For molecular typing, we used repetitive-element PCR with 18-mer degenerate primers REP1R-Dt (3′-CGGNCTACNGCNGCNIII-5′) and REP2-Dt (3′-CATCCGGNCTATTCNGCN-5′) (N = A, C, G, and T; I = inosine) (*11*).

We took samples from puddles at 18 points as a primary survey. Simultaneously, as fixed-point observations, we set 4 points on a road and collected samples when puddles were formed. In the primary survey, *L. pneumophila* were detected by culture in 7 of 18 samples taken from puddles. *Acanthamoeba* spp. were detected by PCR in 4 of 18 puddles. *L. pneumophila* were not detected by culture of swab samples obtained from asphalt surfaces on sunny days, although they were detected in 2 of 12 samples tested by the LAMP method. *Acanthamoeba* spp. were also detected by PCR from 2 of 12 swab samples (Table). In the fixed-point observations, 16 (35.6%) of 45 puddle samples were positive by culture. According to serogroup typing of 150 strains of *L. pneumophila* isolated, the most prevalent serogroup was serogroup 1 (n = 56 [37.3%]) (Table). *L. pneumophila* were detected by culture in 3 (15.8%) of 19 samples from puddles when mean temperature was <20°C, in 6 (42.9%) of 14 samples when mean temperature was 20°C–25°C, and in 7 (58.3%) of 12 samples when mean

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Table. *Legionella* spp. and *Acanthamoeba* spp. isolated from asphalt road sources, Tokyo, Japan, July–October 2007

Source	Positive samples, no. (%)
Primary survey	
Puddles on roads (n = 18)	
*L. pneumophila**	7 (38.9)
Acanthamoeba spp.†	4 (22.2)
Rainwater (n = 10)	
*L. pneumophila**	0
Legionella spp.‡	1 (10.0)
Acanthamoeba spp.†	3 (30.0)
Swabs from roads on sunny days (n = 12)	
*L .pneumophila**	0
Legionella spp.‡	2 (16.7)
Acanthamoeba spp.†	2 (16.7)
Fixed-point observation of puddles on roads	
L. pneumophila concentration (n = 45)	
<20 CFU/100 mL	29 (64.4)
20–99 CFU/100 mL	7 (15.6)
100–999 CFU/100 mL	7 (15.6)
≥1,000 CFU/100 mL	2 (4.4)
L. pneumophila serogroups (n = 150 strains isolated)	
1	56 (37.3)
3	50 (33.3)
2	15 (10.0)
6	12 (8.0)
5	11 (7.3)
Others	6 (4.0)

*Tested by using culture method.
†Tested by using PCR method.
‡Tested by using a loop-mediated isothermal amplification method.

temperature was >25°C (p = 0.043) (Figure 1). With regard to molecular typing by repetitive-element PCR, organisms of lanes 1–5 were all detected on the same day in the same puddle and identified as *L. pneumophila* serogroup 1. Although lanes 1–3 appeared to have different patterns, lanes 1 and 4 and lanes 3 and 5 appeared indistinguishable (Figure 2).

Isolation of *L. pneumophila* in puddles of rainwater on asphalt roads indicates that the organisms may be spread from wet roads into the air by moving vehicles. Thacker et al. reported an outbreak of legionellosis after a heavy rainstorm (*1*). In our study, *L. pneumophila* were not isolated by culture of direct rainwater samples or of swabs collected from road surfaces on sunny days, but they were detected in these 2 sample types by the LAMP method. Factors known to support colonization of *L. pneumophila* are humidity, stagnation, scales (residues, deposits), sediments, and temperature (*12*). When *L. pneumophila* are exposed to unsuitable conditions, it changes its metabolism to reduce growth activity and remains viable but not culturable. *Acanthamoeba castellanii* have been shown to resuscitate *L. pneumophila* in this viable but nonculturable state (*13*). We identified *Acanthamoeba* spp. by PCR from raindrops and from swabs of asphalt pavement surface on sunny days. In a previous study, we demonstrated that at temperatures >25ºC, *L. pneumophila* could replicate in amebae and that at temperature <20ºC, amebae digested *L. pneumophila* and eliminated them through the process of encystation (*14*). It is possible that *L. pneumophila* are in a viable but nonculturable state in raindrops in the air or that they survive on asphalt on sunny days and return to a proliferative state in rainwater on asphalt roads, especially during warm weather.

L. pneumophila may be splashed from puddles into surrounding air. In this regard, Fisman et al. identified an association between legionellosis and precipitation (odds ratio 2.48, 95% confidence interval [CI] 1.30–3.12) and increased humidity (odds ratio per 1% increase in relative humidity 1.08, 95% CI 1.05–1.11) 6–10 days before identification of infected patients (*3*). Hicks et al. reported that a 1-cm increase in rainfall was associated with a 2.6% (risk ratio 1.026, 95% CI 1.012–1.040) rise in incidence of legionellosis (*4*). The increase in reported legionellosis patients may result partly from growth of *L. pneumophila* in puddles on roads, although the possibility of an increase in *L. pneumophila* in tap water after rain cannot be excluded. Because airborne *L. pneumophila* can survive longer at high relative humidity (*15*), after rain the organism may be sprayed into the air and increase both on the ground and in the air.

Conclusions

The frequent presence of *L. pneumophila* in puddles of rainwater on asphalt roads, especially during warm weather, indicates the possibility of frequent contact with *L. pneumophila*–containing aerosols. To decrease illness and death from legionellosis, community physicians should consider the possibility of legionellosis for pneumonia patients, even those who had not traveled or visited spa fa-

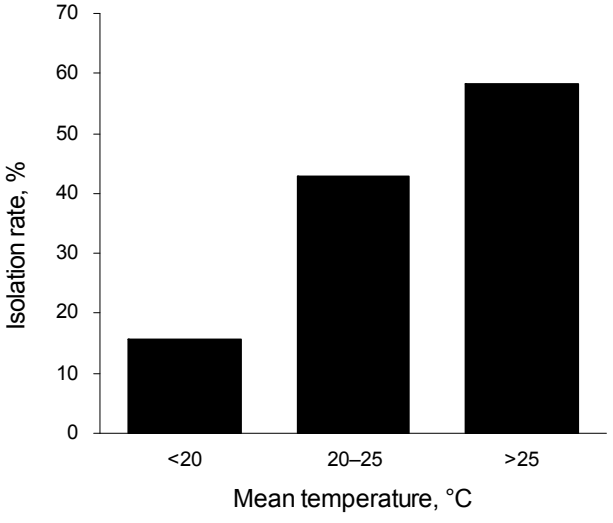

Figure 1. Isolation rate (%) of *Legionella pneumophila* according to mean environmental temperature on sampling date, Tokyo, Japan.

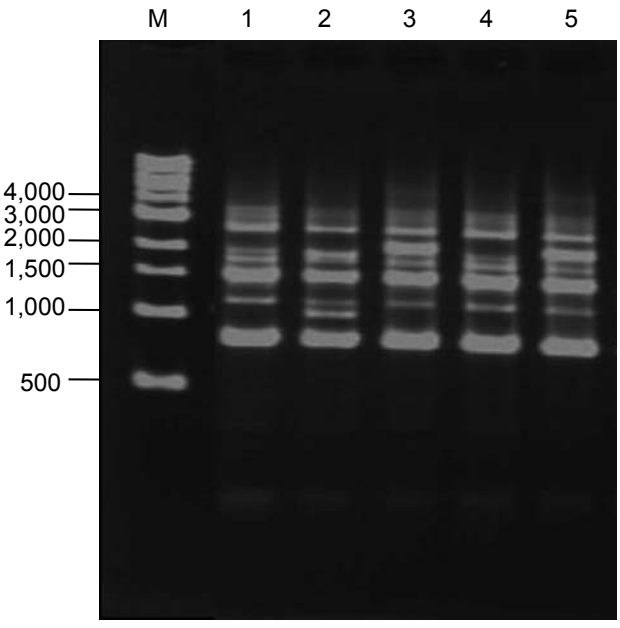

Figure 2. Repetitive-element PCR DNA fingerprints of *Legionella pneumophila* serogroup 1 isolates (lanes 1–5) from the same puddle of rainwater on an asphalt road in Tokyo, Japan. Lane M shows DNA reference marker sizes (New England BioLabs, Inc., Ipswich, MA, USA) in basepairs.

cilities, especially during warm, rainy weather. To prevent legionellosis, persons must consider their risk factors; *L. pneumophila* may be more ubiquitous than previously thought.

Dr Sakamoto is a medical doctor and a researcher at the Research Institute for Humanity and Nature. His field of specialization is public health, especially legionellosis.

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West Nile Virus from Blood Donors, Vertebrates, and Mosquitoes, Puerto Rico, 2007

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West Nile virus (WNV) was isolated from a human blood donor, a dead falcon, and mosquitoes in Puerto Rico in 2007. Phylogenetic analysis of the 4 isolates suggests a recent introduction of lineage I WNV that is closely related to WNV currently circulating in North America.

The first human cases of West Nile virus (WNV) in the Caribbean were documented in the Cayman Islands and the Florida Keys in 2001 (*1*). Antibody-positive animals were described in 2002 in Guadeloupe, the Dominican Republic, Jamaica, and eastern Mexico (*2–4*). Most of the sequencing information and phylogenetic analysis are primarily on isolates from the United States. Viral isolates from outside the United States were obtained from Mexico in 2003 and Argentina in 2006 (*2,5*). Although many studies have described seropositive animals in the Americas and the Caribbean, isolates were not commonly obtained for virus characterization.

The presence of WNV antibodies in nonmigratory birds was first reported in Puerto Rico in 2004 (*6*). In response to this discovery, serum samples from 345 healthy, unvaccinated horses and 14 dead horses were tested by plaque reduction neutralization test (PRNT) during 2004–2005. Three horses from Fajardo municipality were confirmed seropositive for WNV by PRNT. Additionally, samples from 408 free-ranging chickens were tested by hemagglutination inhibition assay and two chickens from Arecibo municipality were seropositive. However, the sample volumes were insufficient for confirmation by PRNT (Centers for Disease Control and Prevention [CDC], unpub. data). During 2004–2006, no additional WNV-positive samples were detected. We hypothesized that the virus could have been circulating on the island without causing detectable illness or death in humans, horses, or birds.

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The Study

To test this hypothesis, we selected a study site in Ceiba and Naguabo municipalities, where the first birds positive for WNV were detected (*6,7*). Sixty sentinel chickens were placed in groups of 5 in 12 locations in accordance with Institutional Animal Care and Use Committee policies as previously described (*7*). The first seroconversion occurred in June 2007, one year after the initiation of the surveillance. Up to 43% of the chickens seroconverted in June and July 2007, as detected by immunoglobulin M ELISA (*8*); the percentage declined during September through December (Figure 1). During the period of transmission, miniature light/CO_2 traps and gravid traps from CDC were placed near the chicken cages to capture mosquitoes. Samples from pools of *Culex nigripalpus*, *Cx. bahamensis*, and *Cx. quinquefasciatus* mosquitoes were positive for WNV RNA, as detected by real-time reverse transcription–PCR (RT-PCR) (*7*). Sixteen WNV isolates were obtained by inoculating either C6/36 (*Aedes albopictus*) or Vero (African green monkey kidney) cell cultures with mosquito pool extract.

In addition to the study site, WNV activity was detected throughout the island from June through mid-September 2007. In June 2007, WNV in 3 human blood donations from San Juan, Gurabo, and Vega Baja municipalities were detected by the American Red Cross (ARC) screening. Two blood donation samples were positive for WNV by RT-PCR, and 1 sample yielded an isolate of WNV confirmed by immunofluorescence (*9*). In September 2007, an encephalitic horse died in Cabo Rojo municipality and a dead falcon (*Falco sparverius*) was reported from the neighboring Mayaguez municipality. Brain tissue from the horse and falcon were WNV positive by RT-PCR, and an isolate was obtained from the falcon. Neurologic symptoms were reported in 3 other horses in these municipalities, but no specimens were available for testing.

WNV isolates from the falcon, human blood donation, sentinel chicken (*7*), and a pool of *Cx. nigripalpus* mosquitoes were sequenced to assess their phylogenetic relationships (Figure 2). Viral RNA was extracted from first passage Vero cell supernatant using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA). RT-PCR was performed by using the OneStep RT-PCR kit (QIAGEN) with WNV-specific primers (*9*), and DNA bands were excised and purified using the QIAquick gel-extraction kit (QIAGEN). The 2,004-nt premembrane-envelope (prM-E) genes of both DNA strands were directly sequenced by using the BigDye Terminator v3.1 Cycle Sequencing kit on a 3130X Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

These newly obtained WNV sequences were aligned with other WNV sequences from GenBank database by ClustalW in MEGA (www.megasoftware.net) (*10,11*).

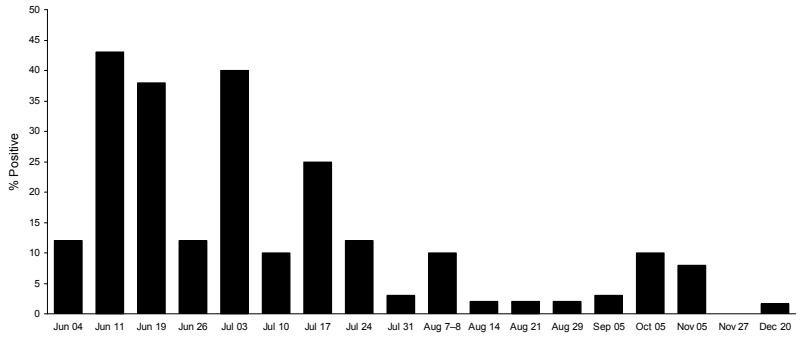

Figure 1. Summary of surveillance data of West Nile virus (WNV) in chickens in Puerto Rico in 2007. Percentage of anti-WNV immunoglobulin (Ig) M–positive chickens per week from June 4 through December 20. Chickens were bled weekly during the beginning of transmission and monthly starting in September 2007. Sixty chickens were placed in the sentinel surveillance sites as previously described by Barrera et al. (*7*).

Neighbor-joining (NJ) and minimum-evolution analyses were also performed in MEGA. Maximum-likelihood (ML) analysis was performed by using PAUP* (*12*) under the best-fit TrN model selected by Modeltest 3.7 (*13*). Bootstrapping was performed (1,000 replications) by the NJ method using the substitution model specified for the ML analysis. The ML phylogeny analysis is depicted in Figure 2. On the basis of previous work, most North American WNV isolates fall into 3 clades: Eastern United States (including NY99); North American, 2002–2004 (the dominant clade); and an intermediate clade. All phylogenetic analyses yielded similar tree topologies with high (95%–96%) bootstrap support for placement of the Puerto Rican WNV isolates into a monophyletic group within the intermediate clade identified by Davis et al. (*14*).

Within the West Nile virus Puerto Rico (WNV_PR) group, the isolates from all 4 sources were identical at the amino acid level, and only 2-nt differences were observed, prM-A114G in PR_2007_Falcon and E-C276T in PR_2007_Human. PR_2007_Sentinel and PR_2007_*Culex* isolates were identical at the nucleotide level. The WNV-PR viruses differed from the consensus of the other sequences analyzed at 3 nucleotides: E-T201C, E-G537A, and E-A561G. None of these substitutions resulted in an aa difference. Consistent with a previous report (*7*), all WNV-PR isolates contained the valine to alanine substitution at the E-159 characteristic of WNV isolated in North America after 2001. Overall sequence homology of WNV-PR viruses to NY99 was 99.7% nt and 99.8% aa.

Conclusions

The chicken surveillance in Puerto Rico demonstrated active WNV transmission in the Caribbean and a pattern of transmission with peak activity occurring from early June through late July in 2007 and decreasing from August through December 2007. The peak WNV activity in chickens coincided with the human, falcon, and horse infections. Although blood donations to the ARC from Puerto Rico had been consistently screened for WNV since June 2003 (≈80,000 donations/year), WNV was first detected in 3 donations in June 2007 <10 miles from one another and >40 miles east of the sentinel chicken site (*15*). Moreover, the WNV-infected falcon and horse died on the western region of the island in September. Together, these findings support seasonality in WNV transmission in Puerto Rico. Alternatively, WNV might be reintroduced to Puerto Rico sporadically with long periods of low or no transmission, which could explain the apparent absence of zoonotic or human transmission from the end of 2004 through May 2007.

To assess the relationship of WNV_PR with other WNV strains, we sequenced isolates obtained from the sentinel chicken, human, falcon, and *Culex* spp. mosquito pool. The 2,004-nt prM-E region was sufficient based on previous evidence to produce an accurate phylogeny (*14*). The high sequence homology between PR_2007 viruses was expected, given that previous studies found little genetic difference in strains isolated in the same period and geographic space (*14*). Our analysis suggests the introduction of a single clade of WNV in Puerto Rico before June 2007. Unfortunately, determining the exact origin of the PR_2007 isolates was not possible in this analysis.

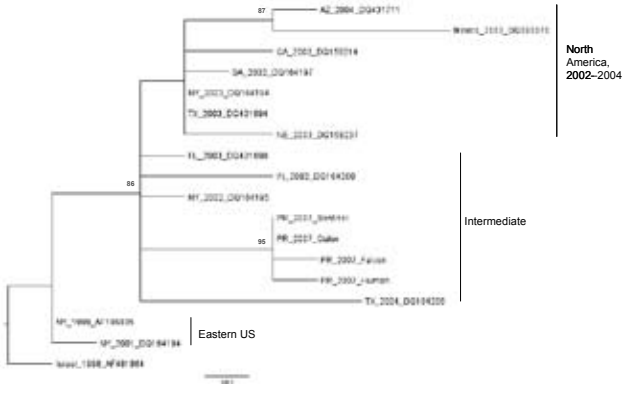

Figure 2. Identification of West Nile virus (WNV) in Puerto Rico. WNV premembrane-envelope maximum likelihood phylogenetic tree of WNV isolates collected from 1998 through 2007, demonstrating the relationship of viruses from Puerto Rico to other lineage I isolates. Viruses are labeled by place and year of isolation and GenBank accession no. FJ799714–FJ799717 (Fiji). Clade names are consistent with those used by Davis et al. (*14*). Numbers indicate the neighbor-joining bootstrap values for groups in the tree and are shown when >70. Scale bar indicates nucleotide substitutions per site.

Active transmission of WNV in Puerto Rico indicated that this virus has established a life cycle of natural, amplifying hosts and arthropod vectors with horses and humans as dead-end hosts. Sequence analysis of 4 isolates indicates that PR_WNV was derived from a recent introduction of lineage I WNV and is closely related to WNV currently circulating in North America based on the location of the isolates in the phylogenetic tree. Additional WNV isolates from Puerto Rico are necessary to assess the impact of WNV on the ecology and human disease in the tropics.

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Dr Hunsperger is a virologist and the chief of the Serology Diagnostics and Viral Pathogenesis Research Laboratory at the Centers for Disease Control and Prevention in San Juan, Puerto Rico. Her primary research interest is the pathogenesis of dengue viruses and West Nile virus.

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Lobomycosis in Man and Lobomycosis-like Disease in Bottlenose Dolphin, Venezuela

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We report 1 case of lobomycosis caused by *Lacazia loboi* in a fisherman and 1 case of lobomycosis-like disease in a bottlenose dolphin (*Tursiops truncatus*) along the coast of Venezuela. These findings suggest that the marine environment is a likely habitat for *L. loboi* and a reservoir for infection.

Lobomycosis (lacaziosis) is a chronic, granulomatous, fungal infection of the skin and subcutaneous tissues that affects humans and members of the family Delphinidae (*1–6*). It is caused by the noncultivable yeast-like organism (*Lacazia loboi*) of the order Onygenales (*7*).

Rare in humans, lobomycosis was first reported in Recife, Brazil, in 1930 (*1*) and subsequently in other countries in South and Central America, where it seems to be endemic (*4*). It was also recently reported in Europe, Canada, the United States, and South Africa, mostly in persons who had traveled to Central or South America or had contact with an infected dolphin (*8–10*). Geographic and climatic conditions associated with endemic human lobomycosis are those of tropical continental areas that are generally located 200–250 m above sea level and characterized by dense vegetation, annual rainfall ≤2,000 mm, mean temperature of 24°C, and mean relative humidity >75% (*4*).

Cases of lobomycosis have been found in bottlenose dolphins (*Tursiops truncatus*) and Guiana dolphins (*Sotalia guianensis*) in North and South America since the 1970s and are being increasingly reported (*2*,*3*,*5*,*6*). The disease in these mammals is characterized by white to pink, verrucous lesions, often in pronounced relief that may ulcerate and form large plaques. Lobomycosis-like disease, a syndrome pathoanatomically consistent with lobomycosis but for which a histologic diagnosis is lacking, has been found in coastal bottlenose dolphins from Colombia, Ecuador, Peru, and Brazil, Guiana dolphins in Brazil (*5*,*11*), and Indo-Pacific bottlenose dolphins (*T. aduncus*) in the tropical lagoon of Mayotte in the Indian Ocean (*12*).

L. loboi cells from lesions in bottlenose dolphins were smaller than those found in infected tissues in humans, which suggests that the organism may not be identical in the 2 hosts (*13*). Serologic data have indicated that dolphins and humans are infected with similar *L. loboi* strains (*14*). The possibility of humans acquiring lobomycosis from dolphins appears low; only 1 documented case of disease transmission from a rescued dolphin to its attendant occurred in the early 1970s (*9*). The ecology of lobomycosis in humans and odontocetes seems to be unconnected. Infections occur mostly in persons inhabiting the Amazon Basin and in inshore and estuarine dolphins in North and South America (*3–6*). We report cases of lobomycosis in a fisherman and lobomycosis-like disease in a bottlenose dolphin along the coast of Venezuela.

The Cases

Human

A 62-year-old fisherman from the central coast of Venezuela (Puerto Cruz and Chichiriviche de la Costa, 10°32′N, 67°14′W) was examined during a fieldwork expedition in March 2008. He had extensive lesions on the left ear and recalled that his illness began when he was ≈52 years of age and had accidentally injured the posterior portion of the helix of that ear with a fishhook. A small, solitary, hard nodule subsequently developed and was later accompanied by similar satellite lesions that tended to become confluent and form harder nodules, sometimes hyperchromic with flat and shiny surfaces (Figure 1, panel A). These nodules slowly invaded the entire free border, posterior aspect, and lobule of the ear and caused occasional pruritus and a tingling sensation. Because of the diffuse infiltration of the ear, the condition was initially diagnosed as diffuse cutaneous leishmaniasis or lepromatous leprosy.

Microscopic findings included a granulomatous reaction indicated by lymphohistiocytic elements with large numbers of multinucleated Langhan-type giant cells and numerous isolated yeast-like organisms with a birefringent membrane isolated or in chains alternating with some piriform elements showing simple gemation (Figure 1, panels B, C). The patient refused otoplasty and was treated with itraconazole; some nodules partially regressed.

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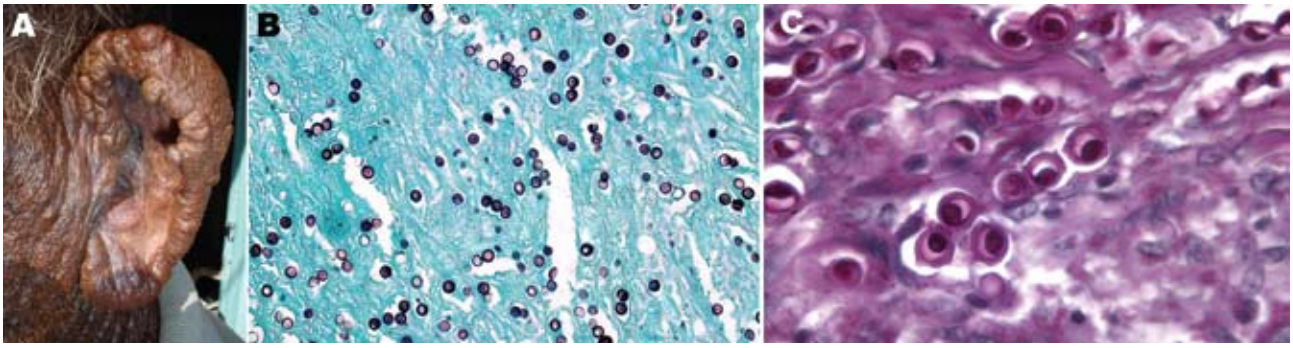

Figure 1. A) Multiple, confluent, keloid-like, hyperchromic nodules with flat shiny surfaces involving the entire free border, posterior aspect, and lobule of the left ear of a fisherman, Venezuela. B) Numerous *Lacazia loboi* tissue-phase organisms within the stroma. Note the typical chain pattern showing simple gemation budding (Gomori-Grocott stain, magnification ×100). C) Yeast cells showing typical double refraction of the membrane and protoplasmic bodies within cells (periodic acid–Schiff stain, magnification ×600).

Dolphin

On June 28, 2004, an adult male, likely inshore, bottlenose dolphin, which had recently died, was found on a beach of La Restinga National Park (11°01′N, 64°10′W) on Margarita Island, Venezuela. The dolphin was 3.8 m long and was emaciated. Several teeth were missing, especially at the distal end of the beak, and an 8-cm *Conchoderma auritum* stalked barnacle was attached to the right 10th mandibular tooth. The dolphin had severe lobomycosis-like disease with a large number of white, gray, and pink proliferating, congregating lesions, some bleeding, with keloidal and verrucous characteristics that formed rosettes on the beak, back, flanks, dorsal fin, tailstock, and tail (Figure 2). The dorsal fin was severely affected and the asymmetric distribution of the lesions caused the fin to bend. Granulomas extended into the oral cavity between the maxillar teeth and the palate. Unfortunately, because of a variety of factors, including a lack of field sampling capabilities, presence of crowd, and limited beach access for transport, no necropsy was conducted and no samples were available. However, the severe emaciation suggested that the dolphin had a chronic debilitating disease. Whether its poor health status favored the wide dissemination of lobomycosis-like disease or whether lobomycosis-like disease was the primary undermining factor remains unknown.

Conclusions

We report lobomycosis in a fisherman and lobomycosis-like disease in a bottlenose dolphin along the coast of Venezuela. The fisherman likely contracted the disease

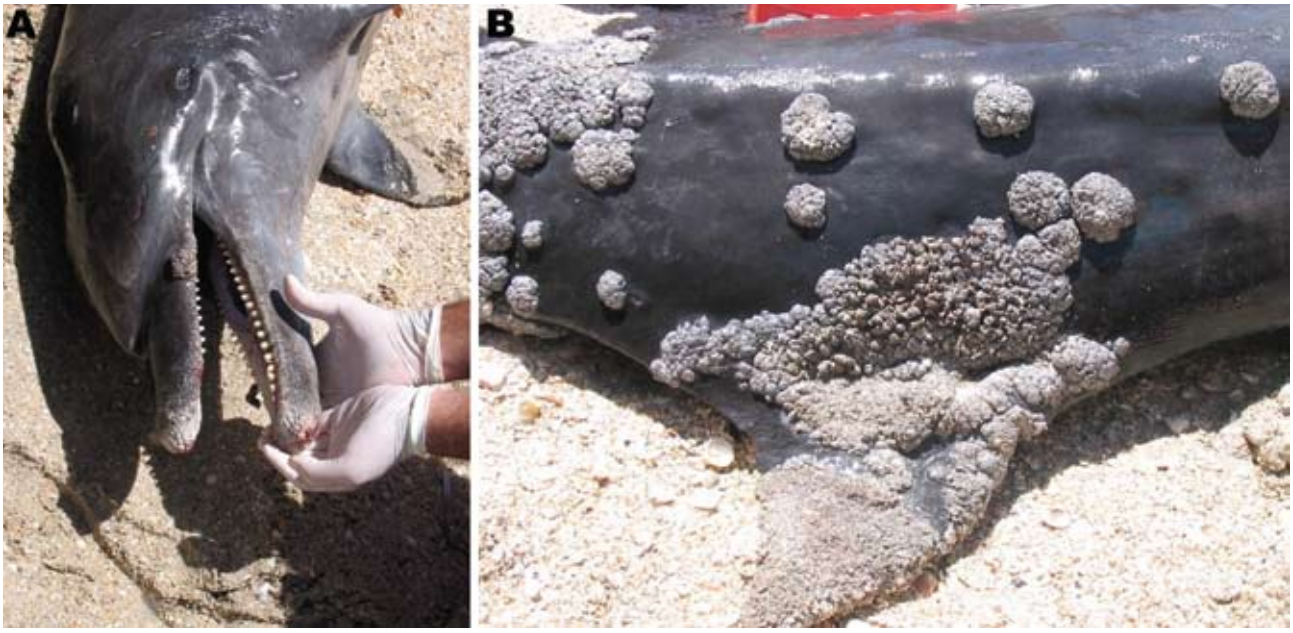

Figure 2. Extensive lobomycosis-like disease on the beak (A) and dorsal fin (B) of a bottlenose dolphin (*Tursiops truncatus*) stranded on Margarita Island, Venezuela.

from the marine environment after pathogen inoculation with a fishing hook. He had never visited the Amazon Basin. Although the human and dolphin cases were probably not related, they suggest the role of the marine environment as a likely natural habitat for *L. loboi* and as a reservoir for infection. Along the central coasts of Venezuela and Margarita Island, temperatures range from 22°C to 28°C, annual rainfall ranges from 0 mm to 500 mm (Margarita Island) or >500 mm (central coast), and the mean relative humidity is ≈50%.

Many aspects of transmission, pathogenesis, and ecology of lobomycosis are still poorly understood. Transmission of lobomycosis among Delphinidae may occur by contact, as suggested by the endemic status of the disease in bottlenose dolphins in the Indian River Lagoon in Florida, USA, and possible transmission from mother to calf in an Indo-Pacific bottlenose dolphin from the Mayotte Lagoon (*5*,*12*). Humans may also acquire the infection through rare contact with infected free-ranging Delphinidae. The disease signs and pathologic changes are similar in humans and dolphins. In humans, lobomycosis is associated with an apparent partial deficit of cell-mediated immunity and no alterations of humoral immunity (*15*). In dolphins, the disease is related to a substantial decrease in CD4+ helper T-lymphocytes and CD19+ and CD21+ B cells (*6*). Lesions are also similar in humans and cetaceans, although they tend to be larger in cetaceans. These lesions cover a wide and pleiomorphic clinical spectrum, ranging from the typical smooth and shiny nodular lesions with keloidal aspect to the extensive and confluent verrucous lesions. They occur predominately on the most exposed and cooler areas (*4*,*6*): i.e., head, back, dorsal fin, flanks, caudal peduncle, and tail in dolphins; and lower limbs, outer ears, upper limbs, and face in humans.

The apparent emergence of lobomycosis, lobomycosis-like disease, and other skin diseases in coastal cetaceans from South America and the Indian Ocean (*5*,*11*,*12*) is cause for concern. This emergence may be indicative of increased biological contamination and environmental changes, including climatic changes worldwide, which may represent a potential threat to human health.

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Predictors of Death after Severe *Streptococcus pyogenes* Infection

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An evaluation of the relative importance of host and pathogen factors on the survival rates of patients with invasive *Streptococcus pyogenes* infection found a number of clinical and demographic factors to be associated with risk for death. Some evidence suggested a seasonal pattern to patient survival rate.

Infectious diseases caused by *Streptococcus pyogenes* are among the most acutely life threatening. Although invasive *S. pyogenes* infections are uncommon (3 per 100,000 population annually in the United States, United Kingdom, and Australia) (*1*–*3*), the case-fatality rate is high relative to many other infections (*1,3,4*). Identification of specific host and pathogen characteristics with poor survival rates in patients who have these infections could help identify potential pathogenic mechanisms for further research at a cellular level, potentially resulting in identification of novel therapeutic targets.

The Study

As part of a European study of severe *S. pyogenes* infections, the United Kingdom undertook enhanced surveillance during 2003–2004 (*2,5,6*). Case-patients were defined as persons with *S. pyogenes* isolated from a sterile site or from a nonsterile site if the patient had pneumonia, necrotizing fasciitis, puerperal sepsis, meningitis, septic arthritis, or if streptococcal toxic shock syndrome (STSS) developed (*7*). Methods are reported elsewhere (*2,8*).

To identify patient outcome, data from England and Wales were linked to death registrations obtained from the Office for National Statistics by using probabilistic methods (*9*). Deaths were identified that occurred up to 30 days after diagnosis of infection; time between diagnosis and death was measured from the date the culture-positive specimen was taken.

Data were analyzed by using STATA statistical software version 8.2 (Stata Corporation, College Station, TX, USA). A nonproportional test for equality of survivor function (Peto-Peto-Prentice) was used to assess differences between subgroups, with Cox proportional hazards regression used for multivariable analysis.

Of the 3,566 case-patients with severe *S. pyogenes* infection, 3,422 (96%) had sufficient identifiers to be linked to death registrations and were used for all further analyses. Overall, 698 (20%) case-patients died within 30 days after collection of culture-positive specimens. Risk for death was highest within 1 day after specimen collection (11% [375]), extending to 16% (559) for the first 7 days, beyond which risk for death dropped substantially.

Analysis of the certified cause of death identified an infectious underlying cause in 280 (50%) deaths occurring from any International Classification of Diseases, 10th Revision, condition classification within 7 days. *S. pyogenes* infection was specified as the cause of death in 5 of these (Table).

Age strongly influenced survival rate; the oldest patients had the poorest survival rates ($p<0.001$; Figure 1). Most deaths in patients <45 years of age (73/104 [70%]) occurred within the first 2 days, whereas deaths in older age groups were more dispersed over time. Other patient factors were independently associated with risk for death (online Appendix Table, available from http://www.cdc.gov/EID/content/15/8/1304-appT.htm). Of patients without any identified concurrent illnesses, 87/518 (17%) died within 7 days.

Case-fatality rates paralleled seasonal incidence (online Appendix Figure, available from http://www.cdc.gov/EID/content/15/8/1304-appF.htm), and were highest from December to April (17%–21%), gradually falling through the summer to their lowest point in October (6%). After adjustment for other significant factors, patients whose infection was diagnosed in October were 82% less likely to die than were those whose infection was diagnosed in January.

Patients identified with necrotizing fasciitis had the highest risk for death within 7 days (34%), >2 times higher than patients with other clinical manifestations, after adjustment for other significant factors. Patients who reported gastrointestinal symptoms were 2 times as likely as those who did not to die within the first 7 days ($p = 0.02$). Only 9/76 (12%) patients who had gastrointestinal symptoms met the case definition for STSS. Cellulitis, the most common clinical symptom, was associated with more deaths (130/438 [30%] patients) than was any other condition. Survival probability in the 30 days after a culture-positive specimen was significantly reduced among patients in

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Table. Conditions selected as cause of death within 7 days after diagnosis of severe *Streptococcus pyogenes* infection, England and Wales, 2003–2004

Condition classification (International Classification of Diseases, 10th Revision, code), N = 557*	Underlying cause, no. (%)	All mentions,† no. (%)
Infectious and parasitic diseases (A00–B99)	67 (12)	303 (54)
Neoplasms (C00–D48)	52 (9)	80 (14)
Diseases of the blood and blood forming organs and certain disorders involving the immune mechanism (D50–D89)	0 (0)	9 (2)
Endocrine system (E00–E90)	12 (2)	50 (9)
Mental and behavioral disorders (F00–F99)	19 (3)	32 (6)
Diseases of the nervous system (G00–G99)	7 (1)	12 (2)
Diseases of the eye and adnexa (H00–H59)	4 (1)	5 (1)
Diseases of the ear and mastoid process (H60–H95)	2 (<1)	2 (<1)
Diseases of the circulatory system (I00–I99)	108 (19)	297 (53)
Diseases of the respiratory system (J00–J99)	99 (18)	223 (40)
Diseases of the digestive system (K00–K93)	32 (6)	66 (12)
Diseases of the skin and subcutaneous tissue (L00–L99)	67 (12)	102 (18)
Diseases of the musculoskeletal system and connective tissue (M00–M99)	39 (7)	69 (12)
Diseases of the genitourinary system (N00–N99)	17 (3)	65 (12)
Complications of pregnancy, childbirth, and the puerperium (O00–O99)	1 (<1)	1 (<1)
Certain conditions originating in the perinatal period (P00–P96)	1 (<1)	3 (1)
Congenital abnormalities (Q00–Q99)	2 (<1)	7 (1)
Symptoms, signs, and abnormal clinical and laboratory findings not elsewhere classified (R00–R99)	7 (1)	49 (9)
Injury, poisoning, and certain other consequences of external causes (S00–T98)	0 (0)	49 (9)
External causes of mortality (V01–Y98)	21 (4)	61 (11)

*Percentages based on 557 deaths (cause of death was not available for 2 patients).
†Conditions noted on the death certificate as the underlying or contributory cause of death.

whom STSS developed (p<0.001; Figure 2); 47/178 (26%) of these patients died within a day of specimen collection.

Risk for death varied according to the *emm*/M-type responsible for the infection. The highest risk was associated with *emm*/M3 (33%) or *emm*/M1 (28%), with *emm*/M3 being borderline significant after adjustment for other significant factors.

Conclusions

This analysis highlighted the scale and rapidity of deaths in patients with severe *S. pyogenes* infection, reemphasizing the importance of early recognition of invasive disease and prompt initiation of antimicrobial drug and supportive therapy. A number of factors conferred a heightened risk for death, which other studies also have found: increasing age, diagnosis of necrotizing fasciitis or pneumonia, and underlying malignancy (*1,10,11*). Although necrotizing fasciitis carried the highest risk for all-cause mortality, it is a relatively rare condition, accounting for only 10% of all deaths, compared with the more common and typically less severe cellulitis (30% of deaths). Some evidence indicated that *emm*/M3 was more commonly associated with death than were other *emm*/M-types, also found elsewhere (*1,11*). Development of STSS was a strong predictor of poor outcome, although whether this syndrome is independently related to death is unclear, given that several of its constituent markers effectively denote the progressive failure of organ systems. For this reason, STSS was not included in the multivariable analysis. Notably, patients with gastrointestinal symptoms had a poorer outcome than others; presumably this is a sign of overwhelming sepsis possibly linked to toxin production. Although relatively uncommon (3% of case-patients), gastrointestinal symptoms clearly are of diagnostic importance and should be included in the severity assessment of patients with other signs and symptoms of *S. pyogenes* infection.

An intriguing and novel finding from this analysis is that risk for death mirrors seasonal changes in incidence (highest in the winter/spring months and falling substantially to a nadir in October). An unadjusted-for confounder could explain this pattern, such as a preceding viral infection, but no candidates with a suitable seasonal pattern

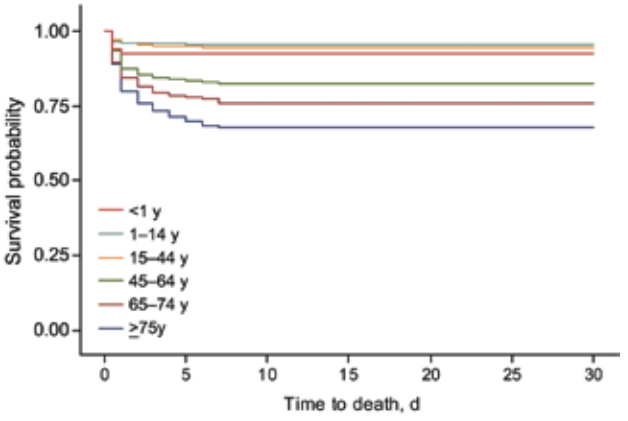

Figure 1. Kaplan-Meier analysis of time to death after diagnosis of severe *Streptococcus pyogenes* infection, by age, England and Wales, 2003–2004.

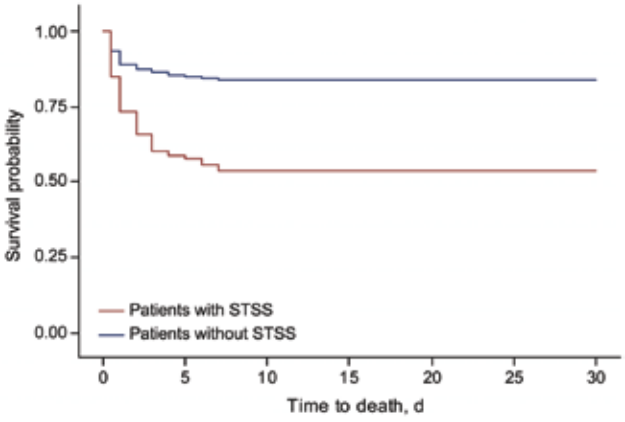

Figure 2. Kaplan-Meier analysis of time to death after diagnosis of severe *Streptococcus pyogenes* infection, by development of streptococcal toxic shock syndrome (STSS), England and Wales, 2003–2004.

are apparent. Although *emm*/M-type was included in the model, differences could exist in the circulation of specific subtypes with particular virulence profiles throughout the year, again not accounted for in this analysis. Seasonal differences in case-fatality rates may yield some important clues about the drivers behind the seasonal incidence pattern of *S. pyogenes* infections. An explanation centered wholly around transmission dynamics seems less favored because it would not explain the changing risk for death. An immunologically focused explanation would fit better because it could explain both the changing incidence rate and the changing case-fatality rate. The length of daylight in a given day can affect the production of vitamin D and melatonin. Both vitamin D and melatonin are known to impact immune function (*12–14*). Although neither appear to be consistent with an autumnal boost to host immunity, if the beneficial effect of vitamin D production during the summer has a cumulative maximal impact in the autumn (*15*), then this theory could offer an explanation.

Because *S. pyogenes* is a largely sporadic infection occurring diffusely throughout the population, opportunities for control of severe infections remain limited. The 26 serotypes included in a multivalent vaccine currently under phase II clinical trials represent 67% of all isolates collected in this study, and 80% of all deaths occurring within 7 days after diagnosis (*16*). The impact of any vaccine will clearly depend on many factors, with the possibility of serotype replacement undermining any such efforts. The challenge to provide a lasting control measure for these devastating infections will continue long into the future.

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Human Rabies and Rabies in Vampire and Nonvampire Bat Species, Southeastern Peru, 2007

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After a human rabies outbreak in southeastern Peru, we collected bats to estimate the prevalence of rabies in various species. Among 165 bats from 6 genera and 10 species, 10.3% were antibody positive; antibody prevalence was similar in vampire and nonvampire bats. Thus, nonvampire bats may also be a source for human rabies in Peru.

Rabies is one of the better known encephalitis viruses of the family *Rhabdoviridae* and genus *Lyssavirus*. In the sylvatic cycle, this infection is maintained as an enzootic disease in several species, such as foxes, raccoons, and bats. The hematophagous bat, *Desmodus rotundus,* is the main vector and reservoir for sylvatic human rabies in South America (*1*), in contrast with other areas in the world where dogs serve as the main source of infection for humans in the urban cycle.

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The Study

During December 2006–March 2007, a total of 23 human sylvatic rabies cases occurred in Puno (n = 6) and Madre de Dios (n = 17), Peru. The affected population consisted mainly of gold miners and their families who relocate to the area during the rainy season for small-scale mining before returning to their original towns for agricultural activities during the rest of the year. A vampire bat (*D. rotundus*) rabies virus variant was identified from clinical samples of deceased patients (*2*).

After the rainy season ended, a team from the US Naval Medical Research Center Detachment, the Ministerio de Salud (Peruvian Ministry of Health), the Servicio Nacional de Sanidad Agraria (Agricultural Health Service), and the Museo de Historia Natural (Natural History Museum) from the Universidad Nacional Mayor de San Marcos (San Marcos University) traveled to the area to conduct bat collections. The objective of the survey was to identify the prevalence of rabies infection among hematophagous (vampire) and nonhematophagous bats and to assess the distribution of bat genera within the outbreak area.

We sampled 2 study sites (A and B) for this survey. The first collection was conducted in the region of Madre de Dios (location A, 13° 7′53.53″S, 70°24′28.27″W) from May 2 through May 4, 2007. The second site was located in the region of Puno (location B, 13°15′29.57″S, 70°19′39.14″W) and sampled from May 6 through May 10, 2007 (Figure). Both trapping sites were located within 1 km of reported rabies cases in humans.

Bats were collected by using mist nets. These nets were situated along the river banks, in secondary forests, and in manioc and banana plantations. In addition to the above habitats, bats in location B were collected in cattle grazing areas and in 2 caves. Mist nets were set out every afternoon before dusk, checked every 1.5 hours, and closed at midnight, after 7 hours.

Bats were removed from mist nets by using protective leather gloves. Each animal was placed inside a canvas bag, transported to a processing location, and kept until the following morning when they were processed as previously described (*3*). Brain tissues and blood samples collected on necropsy were kept in a liquid nitrogen container for storage and shipping to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA. Carcasses were tagged and stored in formalin for shipment to the Museo de Historia Natural in Lima for positive species identification.

Blood samples processed at CDC were tested for rabies virus–specific antibodies by using a rapid fluorescent focus inhibition test (RFFIT). Brain stems were tested by direct fluorescence antibody (DFA) for evidence of active disease. Antibody prevalence was calculated by using the binomial exact method; antibody prevalence rates between

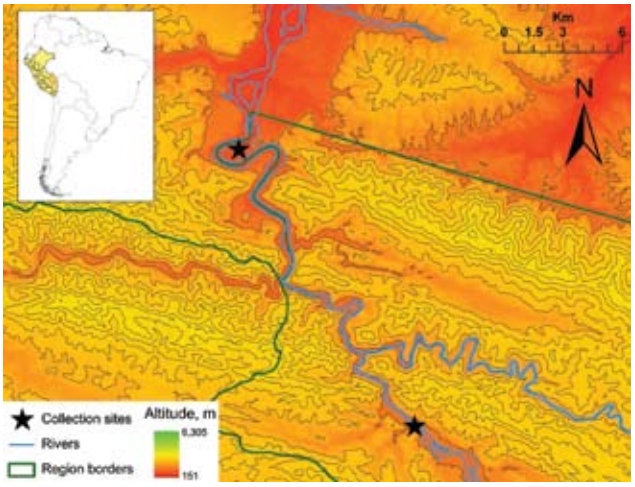

Figure. Bat sampling areas, southeastern Peru, 2007.

bat species, location, and genera were compared by using the χ^2 and Fisher exact tests. All analyses were performed with Stata 10.0 (StataCorp, College Station, TX, USA).

A total of 195 bats were captured. All brain tissues were negative for rabies infection by DFA. Sufficient quantity of serum for RFFIT was available from only 165 (85%) of the sampled animals, which were included in this study. The bats that were collected represented 6 genera, including 10 species; 103 (62%) were females; 62 (38%), males; 25 (15%), juveniles; 140 (85%, adults; and 125 (76%) were *Carollia* spp. (Table 1). One hundred thirty-seven animals (83%) were collected from community B. All vampire bats (n = 7) were collected in this location as well as other non-*Carollia* insectivorous and frugivorous bats (p = 0.001).

Bats from the genus *Carollia* were collected more frequently from natural, non-disturbed refuges (e.g., creeks, caves), while other insectivorous, frugivorous, and vampire bats were found in more visibly disturbed or modified foraging areas (e.g., plantations, cattle farms) (p<0.001). Seventeen bats were antibody positive to rabies virus (cut-off value 0.5 IU), for an antibody prevalence of 10.3% (95% confidence interval 6.1–16.0). Antibody prevalence was similar (p = 1.000) among vampire bats (1/7, 14%), *Carollia* spp. (12/125, 10%), and other nonvampire bat genera (*Uroderma*, *Sturnira*, *Platyrhinus,* and *Artibeus*) (4/33, 12%) (Table 2). No statistical differences were found between antibody prevalence and sex (p = 0.111), age (p = 0.078), habitat (p = 1.000), or collection site (p = 1.000) using Fisher exact test.

Conclusions

In recent years, cases of rabies among humans in urban areas (transmitted by domestic animals) have declined considerably in the Americas. This is likely the result of an aggressive initiative by the member states of the Pan American Health Organization (PAHO) to eliminate urban rabies in the Americas (*4*). Consequently, rabies infections acquired by humans from wild animals, or sylvatic rabies, now represents the primary source for human infection in the region (*1*), with a similar trend for Peru (*5*).

Haematophagous bats, including *D. rotundus*, are usually the species associated with sylvatic bat rabies outbreaks in South America, but little is known about the role of nonhematophagous bats. An investigation in Chile found that nonvampire bats may in fact serve as adequate vectors of sylvatic rabies and even confirmed a single human infection of nonvampire-bat variant rabies linked to a nonhematophagous bat (*6*,*7*). Likewise, PAHO reported 3 cases of sylvatic rabies transmitted by nonhematophagous bats in 2004 (*4*). This may suggest that insectivorous and frugivorous bats have a more specific role in the transmission of rabies virus in South America.

The antibody prevalence of 10.3% found in our study is concordant with the 37% antibody rates found in nonhematophagous bats in Colima, Mexico (*8*) and 7.6% and 12.8% antibody prevalence among vampire and nonvampire bats in Grenada and Trinidad, respectively (*9*). Although the transmission of rabies virus seems to occur very early in life (*10*,*11*), our study did not demonstrate evidence of antibodies to the virus among juvenile bats. Likewise, the distribution of gender suggested higher antibody prevalence among females, although the finding was not significant, perhaps due to small sample size.

The different distribution of bat species may be related to food availability, which would explain why *D. rotundus* bats were found near cattle farms and the more ubiquitous distribution of *Carollia perspicillata* bats, which feed primarily on fruit in addition to pollen and insects. The bat species collected in our study have been previously found in areas at similar altitudes in Peru; therefore, their distribution in this area follows a regular pattern (*12*,*13*). Additionally, the lack of surveillance for bat populations in the area prevents further inference about a possible source of infection among these bat populations. Although none of

Table 1. Bat species collected for rabies testing, southeastern Peru, 2007

Species	Frequency (%)
Carollia perspicillata	95 (57.58)
Artibeus lituratus	18 (10.91)
Carollia brevicauda	17 (10.30)
Carollia benkeithi	13 (7.88)
Desmodus rotundus	7 (4.24)
Anoura caudifera	6 (3.64)
Choeroniscus minor	4 (2.42)
Artibeus obscurus	2 (1.21)
Dermanura anderseni	2 (1.21)
Artibeus planirostris	1 (0.61)
Total	165 (100)

Table 2. Rabies antibody prevalence, vampire and nonvampire bats, southeastern Peru, 2007*

Variables	No. positive/ no. sampled	p value
Sex		0.111
F	14/103	
M	3/62	
Age		0.078
Subadult	0/25	
Adult	17/140	
Genera		0.685
Desmodus sp.	1/7	
Carollia sp.	12/125	
Others	4/33	
Isolation location		1.000
A	3/28	
B	14/137	
Refuges and foraging areas†		1.000
Natural	7/77	
Disturbed	8/79	

*n = 165. Overall prevalence was 10.3 (95% confidence interval 6.1–16.0).
†Information on refuges and foraging areas is missing for 9 individuals, including 2 positive *Carollia perspicillata* bats.

the bats tested in our investigation had active rabies infections, both vampire and nonvampire bats had evidence of antibodies to rabies virus, which perhaps suggests ongoing cross-species transmission (spillover) among multiple bat species.

Peru and other South American countries should enforce the comprehensive, more aggressive preventive measures suggested at the XI Reunion de Directores de los Programas Nacionales de Control de Rabia en America Latina (Meeting of the Directors of National Rabies Control Programs in Latin America) for certain prioritized areas. These activities include preexposure prophylaxis, surveillance, closer coordination with local animal health authorities, and community education.

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (14). All specimens were collected with the authorization of the Instituto Nacional de Recursos Naturales of Peru (permit no. 068-2007-INRENA-IFFS-DCB).

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Avian Influenza Virus A (H5N1), Detected through Routine Surveillance, in Child, Bangladesh

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We identified avian influenza virus A (H5N1) infection in a child in Bangladesh in 2008 by routine influenza surveillance. The virus was of the same clade and phylogenetic subgroup as that circulating among poultry during the period. This case illustrates the value of routine surveillance for detection of novel influenza virus.

Human infection with highly pathogenic avian influenza (HPAI) virus A (H5N1) has been associated with severe disease (pneumonia, multiorgan failure) and often with death (*1*). However, a wider spectrum of subtype H5N1 illness has been reported (*2*). The panzootic subtype H5N1 virus strains circulating among poultry and wild birds are derived from the Asian influenza (H5N1) lineage first identified in the People's Republic of China in 1996 (*3*). HPAI (H5N1) virus was first documented in poultry in Bangladesh during March 2007; by April 2008, the virus had spread to 47 of 64 districts in Bangladesh (*4*,*5*). We report detection of a case of subtype H5N1 infection in a human in Bangladesh, which was discovered through routine outpatient influenza surveillance.

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The Study

In partnership with the government of Bangladesh, the US Centers for Disease Control and Prevention (CDC) and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) established nationwide hospital-based surveillance to identify clusters of persons with life-threatening influenza virus infections (*6*). Additionally, ICDDR,B has had an urban field site in Dhaka (Kamalapur), where population-based respiratory disease surveillance was begun in 1998. In April 2004, influenza surveillance was initiated there in collaboration with CDC (*7*). The site and its surveillance system have been described (*8*). As part of this surveillance, a blood culture for bacterial pathogens was collected from every child with acute respiratory or febrile illness, and from 1 in 5 (20% sampling frame) a nasopharyngeal wash specimen was collected for influenza virus culture in MDCK cells, and acute-phase and convalescent-phase serum samples were collected for detecting other respiratory viruses.

On January 29, 2008, a 16-month-old boy was brought to the Kamalapur clinic with a history of 7 days of fever, 5 days of cough and rhinorrhea, and 3 days of difficult/fast breathing and loss of appetite (Table). The child's mother described his activity and alertness status as normal. She said he had not exhibited diarrhea, convulsions, or other

Table. History of illness in 16-month-old boy with influenza virus A (H5N1) infection, Bangladesh, 2008

Manifestation	Finding/duration, d
Initial examination	
Fever*	Yes, 7
Rhinorrhea	Yes, 5
Cough	Yes, 5
Difficult/fast breathing	Yes, 3
Nausea/vomiting	No
Anorexia	Yes, 4
Loose/watery/mucoid/bloody stool	No
Mental status/activity changes	No
Convulsions	No
Antimicrobial drugs or other medications prior to clinic visit	No
Clinical findings (day 7)	
Temperature,°C	38.1
Pulse, beats/min	124
Blood pressure, systolic/diastolic	90/50
Respiratory rate, breaths/min	40
Head, eyes, ears, nose, throat	Rhinorrhea (clear)
Chest auscultation	Clear bilaterally
Neurologic	Alert, no distress, nonfocal
Weight, kg	8.7
Weight-for-age, %†	78.3
Chest radiograph	Bilateral perihilar alveolar infiltrates (air bronchograms)

*Chief complaint and first symptom.
†Based on National Center for Health Statistics reference data.

signs of illness. The child had been hospitalized 1 year earlier for acute watery diarrhea that resolved uneventfully. No underlying illnesses had been diagnosed, and he had not received any medications before this clinic visit. His routine vaccinations were up to date.

Examination showed that the child had a fever, a mildly elevated respiratory rate, and a clear nasal discharge (Table). He showed no evidence of respiratory distress, and the lungs sounded clear. Oral thrush was noted. The child weighed 8.7 kg (78th percentile for age). Blood for bacterial culture and viral serologic testing and a nasopharyngeal wash sample were collected according to accepted routine. A chest radiograph showed scattered bilateral alveolar infiltrates. Because of the duration of his fever, he received a diagnosis of suspected typhoid fever, which is endemic to this community (*9*), and was given oral amoxicillin and nystatin for oral thrush.

At a follow-up visit to the clinic on January 31, the patient was afebrile; he was in no distress and had a lower respiratory rate (36 breaths/min). His lungs sounded clear. The patient again visited the clinic on February 5 and February 10 and was afebrile on both occasions with a mildly elevated respiratory rate (38 breaths/min) and clear lungs. The mother reported that the child had had loose stools (possibly associated with amoxicillin) on February 10, but she denied that he had diarrhea. The child was never hospitalized; he completed a 14-day course of amoxicillin for suspected enteric fever and recovered fully. His blood culture was negative for any organism, and his final diagnosis was upper respiratory tract infection. On February 13, a convalescent-phase serum sample was collected as part of routine surveillance.

Culture of the child's nasopharyngeal wash sample showed cytopathic effects consistent with influenza virus infection; an aliquot reacted to influenza virus A antiserum but not to antiserum to subtypes H1 or H3. An aliquot of the viral culture material was shipped frozen on April 22, 2008, to CDC, where the isolate was identified as HPAI (H5N1) virus by real-time reverse transcription–PCR. The clinical specimen was recultured on embryonated chicken eggs. The complete genome was sequenced (GenBank accession nos. FJ573465–FJ573472). The virus was identified as an H5N1 clade 2.2 strain on the basis of the hemagglutinin sequence (Figure). After identification of the influenza virus isolate as HPAI (H5N1) in May 2008, an epidemiologic investigation was initiated, and serum samples were collected from the child and 3 family members. The child lived with both parents and a sister in a 1-room residence. In late January 2008, the father had brought home a well-appearing live chicken from a local market located 50 m from the house. The chicken was kept on a veranda outside the child's room. The mother slaughtered the chicken inside the bathroom while the child slept; she did not report having washed her hands before she handled the child. The waste materials from the slaughter were then stored in a tied polyethylene bag near the house entrance for 2 hours before disposal. Microneutralization assay, using the child's subtype H5N1 isolate at CDC, demonstrated a 4-fold rise in subtype H5N1 neutralizing antibodies between the child's January 2008 serum specimens (titer <20/20) and May 2008 (titer 160/80) serum specimens. Serum specimens from the child's family members tested seronegative for subtype H5N1 neutralizing antibodies (the sister was away from the household in January).

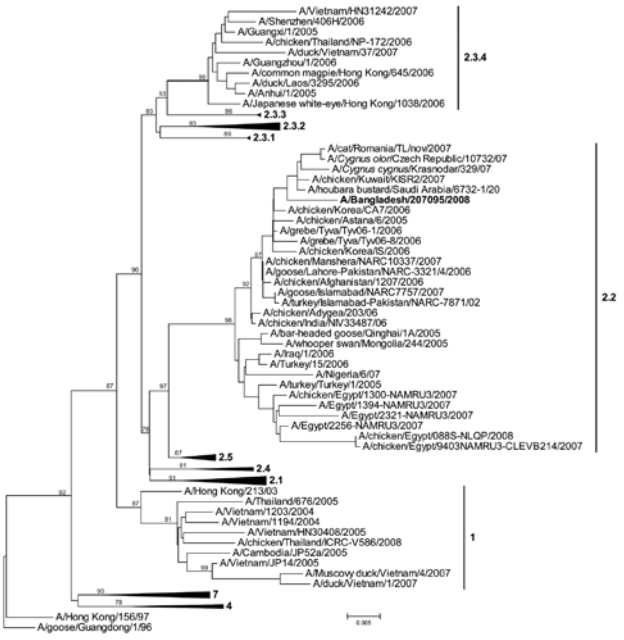

Figure. Phylogenetic tree of virus hemagglutinin sequences generated by neighbor-joining analysis. Bootstrap values at each node represent 1,000 replicates. Scale bar represents 0.005 nt substitutions. The virus found in the child, A/Bangladesh/207095/2008, is indicated in **boldface**.

Conclusions

We report HPAI (H5N1) in a child in Bangladesh; the infection was confirmed by virus isolation from an upper respiratory specimen and by serologic testing. The source of the child's subtype H5N1 infection is uncertain. One potential exposure was the healthy-appearing chicken that was brought inside the home. The child did not have direct contact with the chicken, although indirect contact was suggested because his mother handled him after slaughtering the chicken. The owner of the poultry shop where the chicken was purchased reported that 5%–10% of chickens had died each day during January 2008 (*10*). Because the shop was located 50 m from the home, environmental subtype H5N1 exposures cannot be ruled out. This virus

was of the same clade and phylogenetic subgroup reported in poultry in Bangladesh during this period (*5*). No other household members or neighbors reported illness, and no other family members had serologic evidence of subtype H5N1 infection. In nearly 25% of reported subtype H5N1 cases worldwide, the exposure source is unclear (*2*).

In Bangladesh and other countries with influenza (H5N1) outbreaks among poultry, surveillance for human subtype H5N1 cases is focused on hospital-based case finding for febrile patients with severe acute respiratory illness. This child was not suspected of having subtype H5N1 infection and had had no known poultry contact; his illness would not have met standard criteria for subtype H5N1 testing (*11*). Instead, an upper respiratory tract specimen was collected from the child as part of routine influenza surveillance among pediatric outpatients. Similar clinically mild cases of subtype H5N1 infection in children have been identified in Turkey (*12*), Indonesia (*13*), and Egypt (*2*).

The 1 in 5 sampling frame, a major limitation of this study, raises the possibility that undetected mild cases of subtype H5N1 infection have occurred in children in this population. Other limitations include the elapsed time between illness onset and investigation and the identification of only 1 case.

The public health value of identifying the cause of severe acute respiratory illness clusters with pandemic potential is clear. This case highlights the value of routine outpatient surveillance for detecting both seasonal and novel influenza A viruses, particularly in settings in which subtype H5N1 strains circulate among poultry. Because exposure of subtype H5N1 to humans increases its opportunities for genetic mutation or reassortment, or both, with human influenza A viruses (*3*), other surveillance strategies, including cross-sectional and longitudinal serosurveys among potentially exposed persons, can help inform the extent of, and risk for, asymptomatic and clinically mild subtype H5N1 infection.

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Pigs as Source for Toxigenic *Corynebacterium ulcerans*

To the Editor: Toxigenic *Corynebacterium ulcerans* may cause a zoonotic infection similar to diphtheria caused by *C. diphtheriae*. Previously, dairy cattle were considered to be the main reservoir for *C. ulcerans* (*1*), but recent publications suggest pet dogs and pet cats as carriers (cats often show bilateral nasal discharge) (*2*). We report a case of severe *C. ulcerans* diphtheria-like disease in a person who had had contact with pigs.

In December 2007, a previously healthy 56-year-old female farmer was admitted to the Ear, Nose and Throat Department of the University Hospital Erlangen with a 1-week history of sore throat and progressive dysphagia. She did not report fever and had not received prior treatment with antimicrobial drugs. She had thick, whitish pseudomembranes on her uvula, pharynx, and both tonsils. Endoscopic examination of her larynx and hypopharynx showed that both vocal cords were mobile and the mucosa was erythematous. Enlarged cervical lymph nodes were palpable on both sides of her neck. She had no signs of cranial nerve palsies. Her temperature was 36.5°C. Because of the extensive oropharyngeal pseudomembranes, diphtheria was suspected and diphtheria antitoxin (30,000 IU) was administered intramuscularly. The patient was isolated and received intravenous penicillin (5 million units 4×/day).

A pharyngeal swab obtained from below the whitish pseudomembranes grew toxigenic *C. ulcerans*. Species identification was achieved by biochemical differentiation (API Coryne code 0111326), *rpoB* sequencing (*3*), and MALDI-TOF analysis (Microflex LT and Biotyper 2.0 Software; Bruker Daltonics, Bremen, Germany). Toxigenicity of the strain, named KL126, was verified by using a *C. diphtheriae tox*–PCR (*4*–*6*), a *C. ulcerans tox*–specific PCR (*4*), and the Elek test as described previously (*4*,*5*). The *tox* sequence (GenBank accession no. FJ858272) differs from 2 other published *C. ulcerans tox* sequences (AB304279.1 and AY703827.1) at only 3 bp.

The patient recovered quickly, and the pseudomembranes vanished within 2 days. However, because an allergic rash had developed after her third day of treatment with penicillin, antimicrobial drug treatment was switched to intravenous erythromycin (500 mg 4×/d). When 1 day later the standardized antibiogram showed resistance to erythromycin, the patient received intravenous ceftriaxone (2 g 1×/d) for 12 days. Seven days after initiation of antimicrobial drug therapy, pharyngeal swabs were taken on 3 consecutive days. Because *C. ulcerans* no longer grew on culture, the patient was discharged from the hospital. However, 2 days later she was readmitted to hospital for severe polyneuropathy with neuralgia and weakness of both arms, acute difficulty swallowing, and hoarseness. Signs of cardiomyopathy, including sinus bradycardia and grade I atrioventricular block, were present. The patient recovered after symptomatic treatment and returned home after 2 weeks. According to her records, the patient had received a basic vaccination against diphtheria in 1960 and a booster in 1998.

The literature describes the classic animal sources for toxigenic *C. ulcerans* as dairy cattle with mastitis (*1*). Since 2005, toxigenic *C. ulcerans* carriage in companion animals, e.g., pet cats and dogs, has been reported (*2*). Two cases of transmission of a toxigenic *C. ulcerans* strain from pet dogs to their immunocompromised female owners have been documented in France (*7*,*8*). In 2008, toxigenic *C. ulcerans* in 2 dead killer whales from a Japanese zoo was reported (*9*).

To determine the source of our patient's illness, an outbreak investigation involving her family and their farm animals was conducted. Their medium-sized pig-breeding farm was located in a remote rural village surrounded by woods; they raised ≈500 pigs in a nonindustrialized manner, and no piglets were purchased from outside the farm. Pharyngeal swabs of 3 family members, 19 pigs, and the farm dog were analyzed for *C. ulcerans*. Although all family members and the dog were negative for *C. ulcerans*, 1 of the 19 asymptomatic pigs harbored a toxigenic strain of *C. ulcerans*. Sequencing of *rpoB* and *tox* showed 100% homology between the human and the pig strains. Ribotyping (*10*) confirmed this result, suggesting the identity of both strains; the obtained ribotype is similar to the reported U1 ribotype profile found in humans and cats (*2*).

We report proven transmission of a toxigenic *C. ulcerans* strain between a livestock animal and a human, as well as harboring of toxigenic *C. ulcerans* in pigs. Introduction of *C. ulcerans* from wild animals seems unlikely because the barn doors were reportedly closed at all times. Because handling of *C. ulcerans*–infected pigs may lead to diphtheria-like illnesses, studies of toxigenic *C. ulcerans* carriage among pigs are needed. Similar to our case, diphtheria-like disease caused by an erythromycin- and clindamycin-resistant toxigenic *C. ulcerans* strain in a US patient has been recently (in 2008) reported (*1*). Because current recommendations based on *C. diphtheriae*–caused disease consider erythromycin as the second-line option for treatment or postexposure prophylaxis, these findings highlight the importance of antimicrobial-drug susceptibility testing of toxigenic *C. ulcerans* strains.

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Campylobacter jejuni HS:23 and Guillain-Barré Syndrome, Bangladesh

To the Editor: Guillain-Barré syndrome (GBS) is an acute peripheral neuropathy triggered by a preceding infectious illness. Gastroenteritis caused by *Campylobacter jejuni* is the most frequently reported antecedent event (*1*). In Japan, South Africa, China, and Mexico, *Campylobacter* strains with certain Penner heat-stable (HS) serotypes, including HS:19 and HS:41, are overrepresented among isolates from GBS case-patients, compared with isolates from enteritis case-patients (*2*,*3*). Several studies indicate that *C. jejuni* HS:19 and HS:41 have a clonal population structure and suggest that these serotypes might have unique virulence properties that are intricately linked to development of GBS (*4*). However, data from the United Kingdom and the Netherlands suggest that such virulence properties may not be restricted to specific HS serotypes because many other serotypes can be cultured from patients with GBS (*5*). We report a non-HS:19 and non-HS:41 *C. jejuni* serotype and sequence type (ST)–3219 that are overrepresented among isolates from GBS patients in Bangladesh.

We conducted a prospective case-control study of the serotype and genotype of *C. jejuni* associated with GBS in Bangladesh. Case-patients were 97 persons with GBS admitted to Dhaka Medical College Hospital, Bangabandhu Sheikh Mujib Medical University, and Dhaka Central Hospital during July 2006–June 2007. All fulfilled the diagnostic criteria for GBS of the National Institute of Neurological Disorders and Stroke of the US National Institutes of Health (Bethesda, MD, USA) (*6*). The control group comprised 97 patients with other neurologic diseases, matched with

case-patients by sex, age, and date of admission to the hospital. A second control group comprised 97 healthy family members of case-patients. Up to 3 stool samples were cultured from each case-patient and control.

Campylobacter strains were presumptively identified with Gram stain, oxidase, and hippurate hydrolysis tests and confirmed with a *C. jejuni* species-specific PCR. Serotyping was performed at the National Laboratory for Enteric Pathogens, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada. All strains were serotyped according to the HS serotyping schemes of Penner et al. (*7*). To determine the class of lipooligosaccharides (LOS) locus in each of the *C. jejuni* strains, genomic DNA was isolated by using the DNeasy tissue kit (QIAGEN, Venlo, the Netherlands). PCR analysis was performed with primer sets specific for classes A, B, C, D, and E (*8*).

We isolated *C. jejuni* from fecal samples of 10 case-patients. *Campylobacter* strains were not isolated from the control groups ($p<0.001$). Serotyping of the 10 GBS-related strains showed 4 different HS serotypes. *C. jejuni* HS:23 was found in 5 (50%) strains; HS:19, in 2 (20%); HS:55 and HS:21, in 1 strain each. One strain was untypeable according to the HS typing scheme. In a collection of clinical *C. jejuni* isolated during the same period from patients with enteritis, HS:23 was encountered in 9 (28%) of 32 patients. Serotypes previously associated with GBS were HS:1, HS:2, HS:4, HS:4/50, HS:5, HS:10, HS:13/65, HS:16, HS:19, HS:23, HS:35, HS:37, HS:41, HS:44, and HS:64 (*5,9*).

Nine (90%) of the *C. jejuni* isolates from the case-patients had the class A or class B LOS, which are highly associated with the presence of ganglioside-mimicking structures in LOS (*10*). Godschalk et al. found that 14 (82%) of 17 GBS-associated isolates possessed a class A/B/C locus (*8*). Parker et al. (*10*) found that all GBS-related strains and 64% of the other clinical and environmental isolates belonged to LOS class A/B/C loci. The expression of ganglioside-mimicking structures in *Campylobacter,* LOS is considered essential for the induction of autoantibodies that lead to GBS. Godschalk et al. (*8*) demonstrated that specific genes involved in *C. jejuni* LOS biosynthesis are crucial for the induction of antiganglioside antibodies that lead to GBS.

We performed multilocus sequence typing to examine the overall genomic variation among 10 GBS-related *C. jejuni* strains. We identified 6 different STs among the GBS-related *C. jejuni* strains (Table). However, ST-3219 has a new combination of alleles and was identified in 4 strains. Concordantly, the analysis demonstrated that *C. jejuni* isolates with serotype HS:23 were all ST-3219. Of particular interest, ST-985 (BD-67) shared 5 alleles (*aspA, uncA, glnA, glyA, pgm*) with ST-3219 (Table).

Our findings of a *C. jejuni* HS:23 serotype and ST-3219 that is highly prevalent among GBS-related *C. jejuni* strains from Bangladesh are consistent with previous observations that specific LOS types and serotypes are overrepresented among GBS-related *C. jejuni* strains. These observations support the hypothesis that, although a great variety of *C. jejuni* serotypes can be isolated from GBS patients in some geographic areas, specific clonal serotypes and multilocus types are prevalent in GBS patients in other places. The association of GBS with *C. jejuni* LOS class A/B/C is the only consistent finding when universal collections of GBS-associated strains are considered.

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Table. Serotyping and multilocus sequence typing analysis of *Campylobacter jejuni* strains associated with Guillain-Barré syndrome, Bangladesh*

Strain	Year	Disease	LOS class	Penner type(s)†	ST	Allele, no.						
						aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
BD-07	2006	GBS	A	HS:19	22	1	3	6	4	3	3	3
BD-10	2006	GBS/MFS	B	HS:23	3219	10	27	33	19	10	5	7
BD-22	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7
BD-27	2006	GBS	A	UT	587	1	2	42	4	90	25	8
BD-34	2006	GBS	B	HS:23	3219	10	27	33	19	10	5	7
BD-39	2006	GBS	A	HS:19	660	1	3	6	4	54	91	3
BD-67	2007	GBS/MFS	B	HS:23	985	10	27	89	19	10	132	7
BD-74	2007	GBS/MFS	B	HS:23	3219	10	27	33	19	10	5	7
BD-75	2007	GBS	A	HS:55	587	1	2	42	4	90	25	8
BD-94	2007	GBS	E	HS:21	2109	4	7	10	4	10	7	1

*LOS, lipooligosaccharides; ST, sequence type; GBS, Guillain-Barré syndrome; HS, heat stable; MFS, Miller-Fisher syndrome; UT, untypeable.
†Penner HS serotypes.

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Enzootic Sparganosis in Guangdong, People's Republic of China

To the Editor: Sparganosis is a worldwide parasitic zoonosis caused by infection with spargana, the plerocercoid larvae of various diphyllobothroid tapeworms belonging to the genus *Spirometra* (*1–3*). Sparganosis poses a serious threat to human health; the spargana invade mainly the brain, eye, abdominal cavity, spinal cord, and subcutaneous tissues; can damage local tissues; and can cause blindness, paralysis, and even death (*4*,*5*).

In the People's Republic of China, sparganosis has emerged as an important foodborne parasitic disease, with ≈1,000 human cases reported in 22 provinces during 1927–2007. Guangdong Province has the most cases (*6*). Persons in Guangdong Province eat frog meat and place frog poultices made from raw frog meat on open wounds and lesions, which facilitates human infection with spargana. To assess the risk for human infection with sparganosis in this province and to strengthen public food safety awareness, we conducted a comprehensive investigation of spargana infection in frogs, the second intermediate host of *Spirometra*.

By necropsy we examined for spargana 544 frogs (446 *Rana nigromaculata* and 98 *R. tigrina*) from Yunfu, Maoming, and Zhanjiang in western Guangdong Province during October 2007–October 2008 (*7*). Of these 544 frogs, 455 were wild, and 89 were aquacultured. Spargana were found in 27.3% (124/455) of examined wild frogs; of these, 30.0% (107/357) were *R. nigromaculata*, significantly more ($p<0.05$) than the 17.3% (17/98) that were *R. tigrina*. This finding suggests that *R. nigromaculata* is the main intermediate host of *Spirometra* in western Guangdong Province.

We found 719 spargana in infected wild frogs. The number of worms per frog ranged from 1 to 41, with an average of 5.8 worms per infected frog. No spargana were found in 89 aquacultured *R. nigromaculata* frogs.

The examined wild frogs looked normal and healthy and had no obvious symptoms. During necropsy, we detected local edema, muscle bleeding, and fragile tissues in the tissues invaded by sparganа. We also found cysts in some tissues that contained 1 or a few worms. Spargana dissected from host tissue were flat, white worms, which continuously crept in the normal saline. These worms ranged from 2 mm to 115 mm long and from 1 mm to 2 mm wide.

Frogs are the second intermediate hosts of *Spirometra* spp.; pigs, mice, and humans become infected as paratenic hosts by ingesting *Spirometra* larvae in cyclops or frogs (*8*,*9*). Because persons in Guangdong

Province enjoy eating frog meat, particularly from wild frogs, many frogs have been sold in the market, including a substantial number of wild frogs. The results of our survey show that infection of wild frogs with spargana reached 27.3% in western Guangdong Province; hence, consumption of wild frogs (and use as poultices) poses a high risk for sparganum infection. Therefore, public health officials, epidemiologists, medical practitioners, parasitologists, veterinarians, and the general public should be aware of such risks and should implement strategies to reduce or eliminate them.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Human Rhinovirus Group C in Hospitalized Children, Singapore

To the Editor: Human rhinovirus (HRV) is a common etiologic agent of upper respiratory tract infections and is associated with symptoms such as asthma and wheezing. HRV has >100 serotypes, and recently, several groups reported a new HRV group C (HRV-C) in children that is associated with more severe respiratory infections (*1*–*5*). We examined the incidence of respiratory viruses in children hospitalized in Kandang Kerbau Women's and Children's Hospital, Singapore (*6,7*). These studies also identified human metapneumovirus and human bocavirus (HBoV) among children in Singapore. We recently performed a retrospective study by using PCR-based testing (*8*) to identify HRV, in particular HRV-C, in these patients. From October 2005 through March 2007, a total of 500 nasopharyngeal swab specimens from pediatric patients (age range 1 month through 12 years) were collected and tested for HRVs.

PCR-based testing identified HRV with an incidence rate of 12.8% (64/500), the highest incidence rate in Singapore, compared with incidence rates of other respiratory viruses reported in the same study (*7*). Of the HRV-positive patients, 31 (48.4%) of 64 had symptoms of lower respiratory tract infections (LRTIs) and 16 (25%) of 64 had symptoms of upper respiratory tract infections. Ten patients infected with HRV were co-infected with a second respiratory virus, HBoV (8/10) or respiratory syncytial virus (RSV) (2/10).

HRV-C was detected by molecular serotyping as described (*3*). Briefly, the first PCR was performed with the forward primer P1–1 (5′-CAA GCA CTT CTG TYW CCC C-3′) and the reverse primer P3–1 (5′-ACG GAC ACC CAA AGT AG-3′). A second

heminested PCR was performed with forward primer P1–1 but with 3 different reverse primers, P2–1 (5′-TTA GCC ACA TTC AGG GGC-3′), P2–2 (5′-TTA GCC ACA TTC AGG AGC C-3′), and P2–3 (5′-TTA GCC GCA TTC AGG GG-3′). PCR amplicons were sequenced by using the P1–1 primer. DNA sequences were blasted by using the National Center for Biotechnology Information database (Bethesda, MD, USA) and aligned with available sequences by using Clustal X version 1.83 software (www.bips.u-strasbg.fr/fr/documentation/clustalx). All protocols are available on request.

A phylogenetic tree (GenBank accession nos. FJ645828–FJ645771) was constructed by using neighbor-joining method with 1,000 bootstrap replicates and MEGA version 4 software (*9*). The tree showed similar branching of known HRVs into serogroups (HRV-A, HRV-B, and HRV-C) as described (*3*). Forty-seven (73%) of the 64 HRV specimens from Singapore were grouped into HRV-A, 9 (14%) into HRV-B, and 2 (3%) into HRV-C. We also found a cluster of 10 HRV-A strains (Figure) diverging from the reference HRV-A strains. This finding suggests that these strains could be new strains of the HRV-A, as reported (*3*). We could not determine virus subtype for 6 specimens, possibly because of low virus load.

Our results confirm that HRV infections in Singapore are caused mainly by HRV-A. An increase in HRV-C infections with the onset of winter has been reported in the People's Republic of China (26%) (*5*) and the Hong Kong Special Administrative Region of China (80%) (*2*). These findings indicate that the incidence of HRV-C infections is seasonal, which may account for the apparent low rates of HRV infection in Singapore. However, the incidence rate for HRV-C infections in Singapore was higher than that for HRV-C infections in Australia (1.4%) (*4*), which has a clearly defined winter season.

The 2 patients in which HRV-C was detected had asthma (virus strain SING-06–263) and bronchiolitis (virus strain SING-06–291). These observations are consistent with reports of HRV-C in patients with severe wheezing (*2,4,10*). We also detected co-infection with another virus in 10 patients infected with HRV. Of these 10 co-infections, HRV-A was detect-

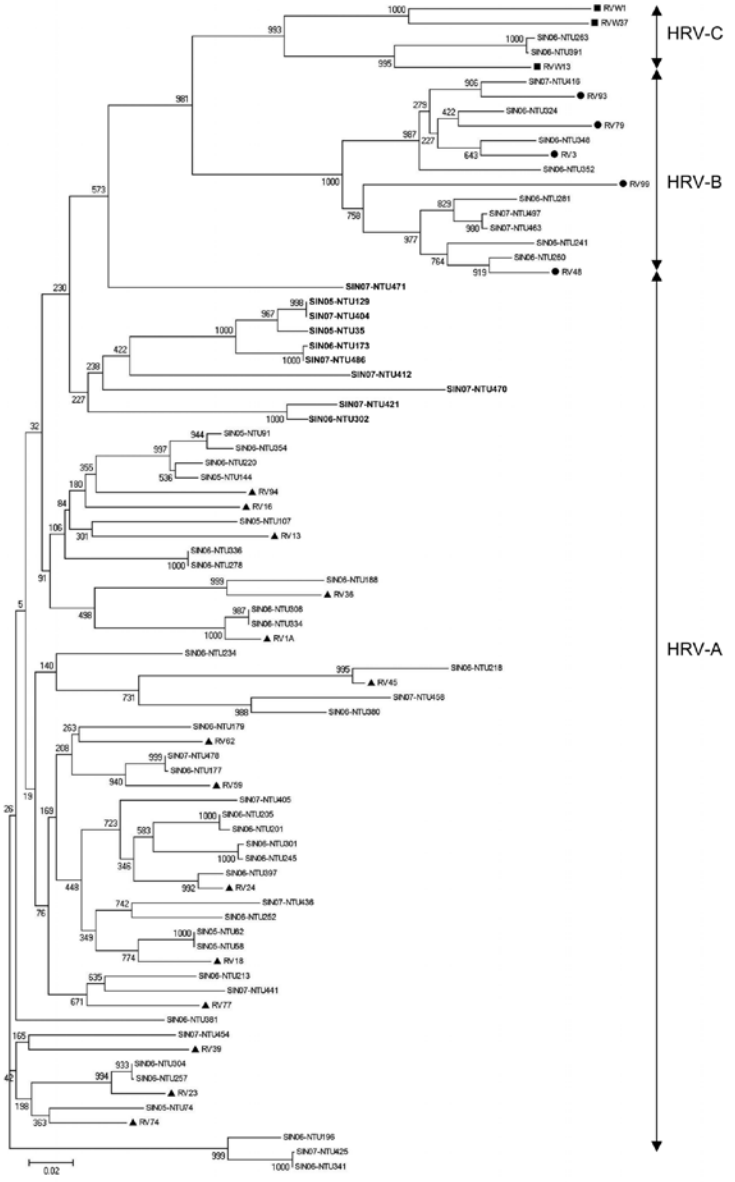

Figure. Phylogenetic analysis of human rhinoviruses (HRVs) from Singapore based on nucleotide sequences of the 5′ noncoding region. The tree was constructed by using the neighbor-joining method with 1,000 bootstrapped replicates generated by MEGA version 4 software (*9*). Sequences (GenBank accession nos. FJ645828–FJ645771) of viruses from Singapore (SIN) are indicated, where the 2 numbers represent the year the specimen was collected, and NTU (Nanyang Technological University) followed by 3 numbers represents the specimen number. Representative strains of HRV-C are indicated by squares, HRV-B by circles, and HRV-A by triangles. RV indicates rhinovirus strains, followed by the serotype no. These sequences were obtained from the report by Lee et al. (*3*). **Boldface** indicates a cluster of 10 HRV-A strains that diverged from reference HRV-A strains. Scale bar indicates nucleotide substitutions per site.

ed in 7 patients; 5 were co-infected with HBoV (2 patients had LRTIs, 2 had upper respiratory tract infections, and 1 had undefined symptoms), and 2 were co-infected with RSV (both patients had symptoms of LRTIs). Of the other 3 patients co-infected with HRV and HBoV, 1 was infected with HRV-B (had LRTI), 1 with HRV-C (had LRTI), and 1 with an untypeable HRV (had undefined symptoms). Co-infections with HRV and RSV (*4,5*) and HRV and HBoV (*4*) have been reported.

Although the clinical role of these co-infections needs to be clarified, these studies suggest that co-infections may result in more severe disease symptoms. The role of HRV-C in causing illness among the children of Singapore will require further study.

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Nondominant Hemisphere Encephalitis in Patient with Signs of Viral Meningitis, New York, USA

To the Editor: Herpes simplex virus (HSV) is the most common cause of sporadic fatal encephalitis across the globe and for all ages. HSV is the etiologic agent of 10%–20% of the 20,000 cases of encephalitis per year in the United States (*1*); >50% of untreated cases are fatal. Of the 2 types of HSV, HSV-1 and HSV-2, HSV-1 most commonly affects persons 20–40 years of age, whereas HSV-2 commonly affects neonates. This rapidly progressive disease is a common cause of fatal encephalitis in the United States. Signs and symptoms include fever and headache for a few days, followed by confusion, focal deficits, seizures or hemiparesis, hallucinations, and altered levels of consciousness (*2*). One third of all HSV encephalitis cases afflict children and adolescents. Lumbar puncture typically shows lymphocytic pleocytosis, increased erythrocytes, and elevated protein (*2*); glucose level is typically within normal limits. Serologic assays often show prior infection. Brain imaging frequently indicates unilateral frontal or temporal lobe abnormalities with edema or hematoma (*3,4*). The involvement of the nondominant brain hemisphere is associated with atypical signs and symptoms (*5*). Diagnosis is usually made by using PCR to examine viral DNA in cerebrospinal fluid (CSF) (*6*). This method of finding DNA in CSF is highly sensitive (98%) and specific (94%–100%). Without therapy, 70% of patients die; with therapy, 20%–30% die (*6*). Illness includes behavioral sequelae.

A 43-year-old female immigrant from China was admitted to Flushing

Hospital Medical Center in Flushing, New York, with complaints of headache, fever, and vomiting, which she had experienced for ≈1 week. She had no photophobia, confusion, or rash; neurologic examination found no abnormalities. CSF contained 81 leukocytes with 82% lymphocytes, 3 erythrocytes, protein at 194 mg/dL, and glucose at 67 mg/dL. CSF was positive for HSV-1 viral DNA by PCR. A computed tomography (CT) scan of the head showed unilateral temporal lobe edema. Intravenous acyclovir 10 mg/kg every 8 hours was administered. HIV test was negative. On day 5, a repeat CT scan showed worsening edema and hemorrhage, despite clinical improvement (Figure). CSF contained 490 leukocytes with 99% lymphocytes and protein at 336 mg/dL. After continued treatment with parenteral acyclovir, the patient's symptoms resolved. On day 12, the patient was discharged after a final CT scan showed resolution of hemorrhage and edema and CSF contained decreased leukocytes and protein.

Although this patient had classic signs of meningitis without encephalitis, the CT scan of the head showed cerebral involvement. These factors can be explained by the location of cerebral inflammation in the nondominant lobe of the brain, thereby masking signs of encephalitis. The classic teaching that viral meningitis may not need treatment may miss the occasional viral encephalitis if brain imaging and CSF PCR are not performed. Failure to perform these tests may lead to illness and death from HSV encephalitits if this disease is not considered as a possible diagnosis.

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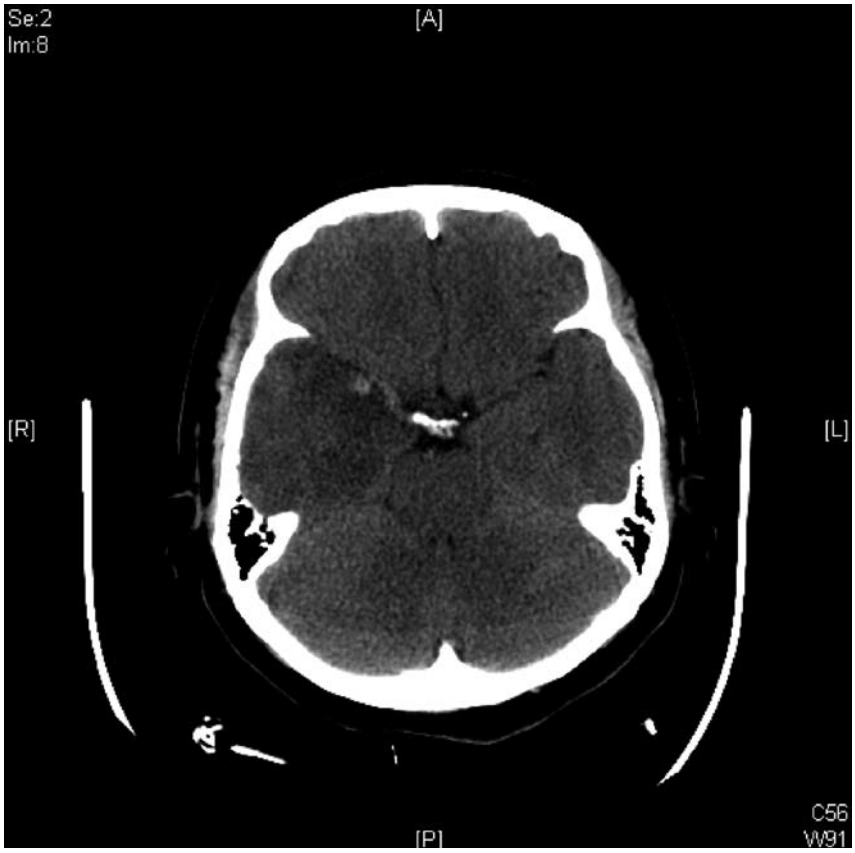

Figure. Computed tomograph scan showing hemorrhage in edematous part of brain of patient with herpes simplex virus encephalitis, day 5 of hospitalization.

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Tick-Borne Rickettsiosis in Traveler Returning from Honduras

To the Editor: Although tick-borne rickettsioses are widespread globally, few reports document their presence in Central America (*1*). Serosurveys detected rickettsial antibodies in humans in Central America in 1971 in Costa Rica, Honduras, Nicaragua, and Panama (*2,3*). An outbreak of rickettsial illness was reported to have occurred in Costa Rica in 1974, where 2 case clusters affected 6 of 15 family members (*4*). A rickettsial organism was isolated from a patient who died in Panama in 1950 (*5*), and more recently *Rickettsia rickettsii* was con-

firmed in a fatal case in Panama (*6*). We report a patient with serologic evidence of rickettsiosis after a tick bite sustained during travel in Honduras.

A 51-year-old man sought medical evaluation after returning from travel to Roatan, Honduras, where he was bitten by a tick in the lower abdomen. He reported erythema and induration at the site of the tick bite with associated central necrosis. He described an illness with headache, fever, weakness, dizziness, abdominal discomfort, diarrhea, flu-like symptoms, and respiratory symptoms affecting him 1–2 weeks after the tick bite. He was evaluated while still traveling and received multiple diagnoses, including malaria, respiratory infection, and parasites, and was given chloroquine, primaquine, penicillin, and mebendazole. His condition improved. He returned to the United States 2 months after the tick bite.

His travel history included Thailand, Jamaica, Aruba, the Bahamas, Belize, Germany, Spain, Hungary, the Netherlands, and New Zealand. The patient recalled removing ticks from his body as a child in Maryland but had no associated illness. He denied any recent tick bite other than the one in Honduras, where he had close contact with a horse and dogs and was frequently outdoors.

Results of his physical examination were unremarkable; routine laboratory studies showed values within reference ranges. No antibodies to *Borrelia burgdorferi* or *Plasmodium* spp. were detected.

Serologic analysis for rickettsia, performed by a commercial laboratory (Focus Diagnostics, Cypress, CA, USA) and the Centers for Disease Control and Prevention (Atlanta, GA, USA), showed elevated titers (Table). The patient took doxycycline (100 mg 2×/d) for 10 days and subjectively improved.

On the basis of infections documented in the Americas, *R. rickettsii*, *R. africae*, and other less well-known rickettsial pathogens such as *R. parkeri* or *R. massiliae* (*1*) are possible etiologic agents in our case. The most common human-biting tick in Central America is *Amblyomma cajennense*, which is a known vector of *R. rickettsii*. Serosurveys have suggested the presence of *R. rickettsii* infection in the Yucatan (*7*), and PCR has confirmed a case of *R. rickettsii* in that region (*8*). *Rhipicephalus sanguineus,* the vector of *R. conorii* (boutonneuse fever) in the Mediterranean, is also found in Mexico, but to date no transmission of *R. conorii* has been documented in the Americas. *R. sanguineus* was implicated in a cluster of human Rocky Mountain spotted fever cases in the United States in 2002–2004 (*9*).

Our patient had not traveled to the areas of the Caribbean where transmission of *R. africae* has been documented, but he had lived in areas of the United States with potential transmission of rickettsial infections. *R. parkeri* is a newly recognized pathogen in the Americas (*10*). Its vectors (*A. maculatum* and *A. triste*) are found in parts of North, Central, and South America. The illness caused by *R. parkeri* appears to be less severe than Rocky Mountain spotted fever and could be consistent with our patient's illness. Because antibodies against *R. parkeri* and *R. rickettsii* cross-react, serologic analysis is of little use for differentiating these 2 organisms.

The history and serologic findings for our patient suggest a recent tick-borne rickettsiosis, most likely acquired in Honduras. However, we can neither confirm that infection was recent nor confirm the species. The history of a tick bite and description of the skin lesion are consistent with an eschar, but no physical evidence of the tick or eschar remained at the time of evaluation. Diagnosis of rickettsial infection can be confirmed by demonstrating at least a 4-fold increase in titers between acute-phase and convalescent-phase serum samples, by identification of rickettsiae in an acute-phase serum or tissue sample, or by culture. Use of an immunofluorescent antibody assay alone does not identify the specific agent causing spotted fever.

Because our patient was examined 2 months after the exposure, options for making a diagnosis were limited. Extensive serologic cross-reactivity exists among the rickettsial species, which precludes the determination of species in our case. Although antibodies to rickettsiae can be long-lived, the extremely high levels of immunoglobulin (Ig) G and IgM suggest a recent rickettsial infection in our patient. Testing was unavailable for other rickettsiae (e.g., *R. parkeri*, *R. massiliae*).

Table. *Rickettsia* spp. serologic titers for a man who returned from Honduras, 2005*

Test	Titers on specific date				
	May 26	Jun 10	Jul 14	Aug 25	Nov 22
RMSF IgG†	Positive, ≥1,024		Positive, ≥1,024	Positive, ≥1,024	Positive, ≥1,024
RMSF IgM†	Positive, 512		Positive, 256	Positive, 128	Positive, 256
R. conorii IgG†			≥1,024	512	512
R. conorii IgM†			64	64	64
R. africae IgG‡		2,048		4,096	
R. africae IgM‡		512			
R. conorii IgG‡		2,048		4,096	
R. conorii IgM‡		512			
R. rickettsii IgG‡		1,024			
R. rickettsii IgM‡		256			

*Reference titer for negative/normal result is <64. RMSF, Rocky Mountain spotted fever; Ig, immunoglobulin.
†Testing done by Focus Diagnostics, Cypress, CA, USA.
‡Testing done by Centers for Disease Control and Prevention, Atlanta, GA, USA.

Our patient likely had rickettsial infection acquired in Honduras. We present this case to alert clinicians to consider the diagnosis of rickettsial infections in the Americas, even if infections have not been previously documented in a specific country or region. Because rickettsial infections can be severe and are treatable, the clinician should consider rickettsial infections in returned travelers with compatible clinical findings. Our case also demonstrates the potential role of travelers as sentinels of emerging infectious diseases.

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KI and WU Polyomaviruses in Patients Infected with HIV-1, Italy

To the Editor: Before 2007, two human polyomaviruses were known to infect humans: BK virus and JC virus (*1*,*2*). Recently, 2 novel polyomaviruses, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), were identified in the respiratory secretions of children with signs of acute respiratory signs (*3*,*4*); little evidence exists to suggest that these viruses are causative agents of respiratory tract disease (*5*). To determine the prevalence of WUPyV and KIPyV in the plasma of HIV-1–infected patients, we screened 62 persons who were HIV-1 positive by using PCR to detect the 2 viruses. We also conducted phylogenetic analysis of the identified strains.

Plasma specimens were collected at Istituto di Ricovero e Cura a Carattere Scientifico Instituto Fisioterapico Ospetaliere–San Gallicano Institute and Tor Vergata University Hospital, Rome, Italy, from April 2005 through September 2008. Patients were adults (37–54 years of age, median age 45.5 years) and were being treated with antiretroviral drugs. HIV-1 viral load determination, CD4+ counts, and HIV-1 genotyping were performed as part of the routine investigation. Plasma viremia levels ranged from <50 to 2,877,764 copies/mL, and CD4+ counts ranged from 150 to 1,218. Most patients (64.5%) were infected by HIV-1 subtype B. Other subtypes found were F, G, and C.

Total DNA was extracted from 0.2 mL of plasma by using the QIAamp DNA Mini Kit according to the manufacturer's instruction (QIAGEN S.p.A., Milan, Italy) and then stored at –80ºC until analysis. KIPyV and WUPyV PCR screening was carried out as described (*3*,*4*). Positive isolates were reamplified with primers encompassing the N-terminal part of the large T antigen (*T-Ag*) and almost the entire small t antigen (*t-Ag*) genes. KIPyV was amplified as described (*6*), and, for WUPyV, the primers were FWUV4460 5′-ACTGAGACCACCAGTAATCCCAG-3′ (4460–4482 nt) and RWUV5200 5′-AAGCAGAGGGCCTTGCTGAGGCG-3′ (5200–5178 nt). The thermal cycling profile was 1 cycle at 94ºC for 10 min and then 40 cycles at 94ºC for 30 s, at 65ºC for 30 s, and at 72ºC for 60 s. The amplified *t-Ag* fragments were sequenced as described (*6*). The obtained sequences (KIV-RM21, KIV-RM22, and WU-IT3) were submitted to GenBank (accession nos. FJ842112–FJ842114) and matched against all deposited sequences (www.ncbi.nlm.nih.gov/BLAST). ClustalX software (http://bips.u-strasbg.fr/fr/documentation/

clustalx/#g) was used to obtain alignment with a set of KIPyV and WUPyV isolates from Italy (*6*,*7*) (accession nos. FJ389513–FJ389516, FJ594120–FJ594126, FJ594118, FJ594119, FJ804123, FJ811519–FJ811524, FJ824854, and FJ821706) and prototype strains for KIPyV (EF127906, EF127908, and EF520288) and for WUPyV (EF444549–EF444554, EU711054–EU711058, EU296475, EU358768, and EU358769). Alignment was manually edited with the Bioedit software (www.mbio.ncsu.edu/BioEdit/bioedit.html) (*6*). Positions containing gaps were removed from the final alignment. For our dataset, the best fitting nucleotide substitution model was tested with a hierarchical likelihood ratio test following the strategy described (*6*),which used a neighbor-joining (NJ) base tree with LogDet-corrected distances (http://paup.csit.fsu.edu/about.html). Maximum-likelihood (ML) trees were then inferred with the selected model and ML-estimated substitution parameters. The heuristic search for the ML tree was performed by using an NJ tree as starting tree and the tree–bisection-reconnection branch-swapping algorithm. NJ trees were also estimated by using pairwise distances inferred by ML with the best fitting nucleotide substitution model. Calculations were performed by using PAUP* 4.0b10 (http://paup.csit.fsu.edu/about.html) (*6*). Statistical support for internal branches in the NJ trees was obtained by bootstrapping (1,000 replicates) and with the ML-based, zero–branch-length test for the ML trees (*6*).

Of 62 plasma specimens screened, PCR detected KIPyV in 2 (3.2%) and WUPyV in 1 (1.6%). All 3 patients were infected by HIV-1 subtype B. Phylogenetic analysis of the *t-Ag* of the 3 isolates showed that KIV-RM21 and KIV-RM22 are not closely related to the KIPyVs isolated in Italy from feces (KIV-RM5 to KIV-RM11), respiratory tract (KIV-RM1 to KIV-RM4), and tonsils (KIV-RM12 to KIV-RM20), nor are they related to those previously identified (*6,7*). Similarly, WUV-IT3 was not related to WUV-IT1 or WUV-IT2 nor to WUPyVs identified in stool and respiratory tract secretions (Figure).

To date, KIPyV and WUPyV have been detected in respiratory secretions and stool and serum specimens from pediatric patients with acute respiratory symptoms and have been found in respiratory tissue of adults and children (*3*,*4*,*6*,*8*). Few data are available on the detection and reactivation of these novel polyomaviruses in immunocompromised patients (*9*,*10*). In this study, KIPyV and WUPyV sequences were found in 3.2% and 1.6% of HIV-1–infected patients, respectively. None of the patients had respiratory symptoms, so the presence of the 2 viruses in plasma raises the question of whether they play a pathogenic role in immunocompromised patients.

Molecular analysis of the KIPyV and WUPyV identified in plasma showed that these polyomaviruses were not closely related to strains identified previously in other countries nor to the KIPyVs and WUPyVs identified in Italy in stool, respiratory tract tissue, and tonsils. Whether this difference reflects a tropism of some strains for a particular tissue or organ remains to be established. Further studies are needed to clarify the possible patho-

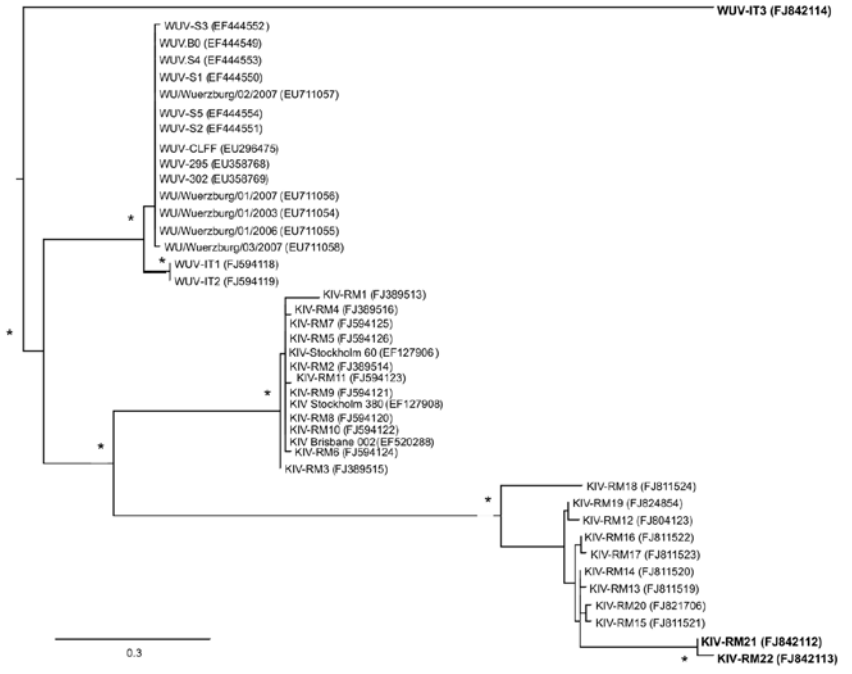

Figure. Unrooted phylogenetic tree showing analysis of KI (KIV-RM21, KIV-RM22) and WU (WUV-IT3) polyomaviruses (KIPyVs, WUPyVs, respectively) identified in the plasma of HIV-1–positive patients. The identified strains are indicated in **boldface**, and the phylogenetic analysis refers to the small t region. The other polyomaviruses shown in the figure are the KIPyVs (KIV-RM1 to KIV-RM20) and WUPyVs (WUV-IT1 and WUV-IT2) identified in Italy in previous studies (*6,7*) and the prototype strains for KIPyV (GenBank accession nos. EF127906, EF127908, EF520288) and WUPyV (GenBank accession nos. EF444549–EF444554, EU711054–EU711058, EU296475, EU358768, and EU358769). GenBank accession numbers for all virus strains are shown in parentheses. Multiple nucleotide sequence alignments were performed by using ClustalX software (http://bips.u-strasbg.fr/fr/documentation/clustalx/#g), and the phylogenetic tree was constructed by using the neighbor-joining algorithm with LogDet-corrected distances (http://paup.csit.fsu.edu/about.html) (*8*). An asterisk (*) beside a branch represents significant statistical support for the clade subtending that branch ($p<0.001$ in the zero-branch–length test) and bootstrap support >75%. Scale bar indicates nucleotide substitutions per site.

genic role of KIPyV and WUPyV in immunocompromised patients.

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Extreme Drug Resistance in *Acinetobacter baumannii* Infections in Intensive Care Units, South Korea

To the Editor: *Acinetobacter* spp. have emerged as a cause of nosocomial infections, especially in intensive care units (ICUs). In South Korea, *Acinetobacter* spp. was ranked as the third most frequently found pathogen in ICUs (*1*). With the emergence of multidrug-resistant (MDR) or pandrug-resistant (PDR) isolates, few drugs are now available to treat MDR or PDR *Acinetobacter* infections; polymyxins are the only therapeutic option in many cases (*2*). Current polymyxin resistance rates among *Acinetobacter* isolates are low worldwide (*3*). We report the emergence of extreme drug resistance (XDR) in *A. baumannii* isolates from patients in ICUs of Samsung Medical Center in Seoul, South Korea. These isolates were resistant to all tested antimicrobial drugs, including polymyxin B and colistin, to which PDR isolates are normally susceptible.

Sixty-three nonduplicate *Acinetobacter* spp. isolates were collected from the ICUs from April through November 2007. Species identification was performed based on partial RNA polymerase β-subunit gene sequences, amplified rDNA restriction analysis, and the gyrase B gene–based multiplex PCR method (*3*). Forty-four isolates were identified as *A. baumannii*: 9 as genomic species 3, six as genomic species 13TU, 2 as *A. baumannii*-like species, and 1 each as *A. junnii* and genomic species 10.

In vitro susceptibility testing was performed and interpreted by using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (*4*). Colistin and polymyxin B resistances were defined as MIC ≥4 mg/L (*4*). MDR was defined as characterized by resistance to ≥3 classes of antimicrobial drugs, and PDR was defined as characterized by resistance to all antimicrobial drugs, regardless of colistin and polymyxin B susceptibility. XDR was defined as resistance to all antimicrobial drugs. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed for all PDR isolates according to previously described methods (*5,6*). Genes encoding oxacillinases, such as those classified as OXA-23-like, OXA-24/40-like, OXA-51-like, and OXA-58-like, were detected as previously described (*7*). PCR and sequence analyses were performed to detect and characterize the other antimicrobial resistance genes, according to methods reported (*8*).

Of 63 *Acinetobacter* isolates, 31.7% and 34.9% were resistant to imipenem and meropenem, respectively. Of the 63 isolates, 27.0% and 30.2% were resistant to polymyxin B and colistin, respectively. For the other antimicrobial drugs, *Acinetobacter* spp. isolates showed antimicrobial resistance rates >50%. Nineteen isolates (30.2%), all belonging to *A. baumannii*, were PDR. Most of these PDR isolates (16/19, 84.2%) were collected from endotracheal aspirate, and others were from peritoneal fluid and sputum. When characterized by PFGE and MLST, all PDR isolates belonged to a single clone, ST22, and all contained the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$ genes. IS*Aba1* was detected upstream of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$ in all PDR isolates. In addition, most PDR isolates contained $bla_{\text{TEM-116}}$, $bla_{\text{PER-1}}$, and $bla_{\text{ADC-29}}$ genes. TEM-116 is a point mutant derivative of TEM-1, Val84→Ile. All β-lactamase genes were located on a plasmid. Also, IS*Aba1* was located at the upstream of all the bla_{ADC}, which was shown by PCR. However, none of the isolates had $bla_{\text{CTX-M}}$, bla_{VEB}, bla_{IMP}, bla_{VIM}, or bla_{GIM}.

Of the PDR isolates, 8 were resistant even to colistin and polymyxin B. These 8 isolates also showed resistance to tigecycline (MICs 4 mg/L). Thus, they were resistant to all antimicrobial drugs tested in this study and were considered to have XDR. The underlying diseases of the patients whose isolates were examined varied (Table). Although 2 isolates with XDR were colonizers, 6 caused infections. All but 1 patient was treated with mechanical ventilation before isolation of the pathogen. Number of hospital days before isolation of *A. baumannii* was 13–256 days, and the number of ICU days before isolation was 2–38 days. Four patients were immunocompromised, and 3 had bacteremia. Among the patients with infections characterized by XDR, the overall 30-day mortality rate was 66.7%, and the infection-related 30-day mortality rate was 50.0%. All 8 isolates with XDR showed common characteristics: ST22 containing OXA-23, OXA-66, TEM-116, PER-1, and ADC-29.

We report the emergence of XDR in PDR *A. baumannii* isolates in South Korea. Characteristics of PDR *A. baumannii* isolates suggest that they spread from a single clone. A single *A. baumannii* strain with XDR might evolve from the prevailing PDR *A. baumannii* and could disseminate in the ICU, probably after contamination of the hospital environment and by nosocomial transmission. In South Korea, a high resistance rate to imipenem and meropenem in *Acinetobacter* spp. isolates may lead to extensive use of polymyxins (*3*). Thus, we can hypothesize that the most prevalent carbapenem-resistant, or MDR *A. baumannii* clone, became PDR and then evolved into clones with XDR by acquisition of polymyxin resistance caused by antimicrobial pressure. Our investigation showed a simultaneous emergence of resistance to all antimicrobial agents available, including colistin, polymyxin B, and tigecycline. XDR poses serious problems in the treatment of patients with *A. baumannii* infections, especially given the slow development of new antimicrobial agents.

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Table. Clinical characteristics of 8 patients infected with extremely drug-resistant *Acinetobacter baumannii* isolates, South Korea*

Strain no.	Patient age/sex	Underlying disease	Infection†	Days before isolation: Hospitalized	Days before isolation: In ICU	Concurrent bacteremia	30-d outcome	Infection-related death
07AC–052	60 y/F	Acute myeloid leukemia	Pneumonia	15	8	No	Died	Yes
07AC–159	79 y/M	Lymphoma	Pneumonia	35	9	No	Died	No
07AC–192	50 y/M	Status postliver transplantation	Pneumonia	256	2	Yes	Survived	NA
07AC–204	55 y/F	Steven-Johnson syndrome	Pneumonia	13	13	Yes	Survived	NA
07AC–336	16 mo/M	Medulloblastoma	Pneumonia	32	13	Yes	Died	Yes
07AC–347	17 mo/M	Hepatoblastoma	Pneumonia	135	28	No	Died	Yes
07AC–329	1 mo/F	Edward syndrome	Colonization‡	33	33	NA	NA	NA
07AC–063	56 y/M	Lung cancer	Colonization‡	26	21	NA	NA	NA

*ICU, intensive care unit; NA, not applicable. All but 1 patient (with strain 07AC–192) had mechanical ventilators. Four patients (with strains 07AC–159, 07AC–192, 07AC–336, and 07AC–063) were immunocompromised hosts who had daily administration of corticosteroid (>20 mg/d of prednisolone or an equivalent drug) during >2 wk and treatment with chemotherapy for an underlying malignancy during 1 month before hospital admission.
†Infection is defined as invasion of the body tissues by microorganisms resulting in disease.
‡Colonization occurs when an agent's presence in a host does not cause a specific immune response or infection.

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More Diseases Tracked by Using Google Trends

To the Editor: The idea that populations provide data on their influenza status through information-seeking behavior on the Web has been explored in the United States in recent years (*1*,*2*). Two reports showed that queries to the Internet search engines Yahoo and Google could be informative for influenza surveillance (*2*,*3*). Ginsberg et al. scanned the Google database and found that the sum of the results of 45 queries that most correlated with influenza incidences provided the best predictor of influenza trends (*3*). On the basis of trends of Google queries, these authors put their results into practice by creating a Web page dedicated to influenza surveillance. However, they did not develop the same approach for other diseases. To date, no studies have been published about the relationship of search engine query data with other diseases or in languages other than English.

We compared search trends based on a list of Google queries related to 3 infectious diseases (influenza-like illness, gastroenteritis, and chickenpox) with clinical surveillance data from the French Sentinel Network (*4*). Queries were constructed through team brainstorming. Each participant listed queries likely to be used for searching information about these diseases on the Web. The query time series from January 2004 through February 2009 for France were downloaded from Google Insights for Search, 1 of the 2 websites with Google Trends that enables downloading search trends from the Google database (*5*). Correlations with weekly incidence rates (no. cases/100,000 inhabitants) of the 3 diseases provided by the Sentinel Network were calculated for different lag periods (Pearson coefficient ρ).

The highest correlation with influenza-like illness was obtained with the query grippe –aviaire –vaccin, the French words for influenza, avian, and vaccine respectively ($\rho = 0.82$, $p<0.001$). The minus sign removed queries that contained the terms avian or vaccine. Use of the query word grippe alone resulted in a lower correlation ($\rho = 0.34$, $p<0.001$). The high double peak in 2005–2006 and the smaller peaks preceding annual epidemics observed with the query word grippe alone were decreased by this specification. However, the unusual double-peak shape of the 2005–2006 epidemic remained (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/15/8/1327-appF.htm).

The highest correlation with acute diarrhea was obtained when we searched for the French word for gastroenteritis ($\rho = 0.90$, $p<0.001$). Various spellings were used to account for the presence/absence of an accent or a hyphen. The Google database was searched for gastro-enterite + gastro-entérite + gastroentérite + gastroenterite + (gastro enterite) + (gastro entérite). The + sign coded for or, enabling searches for queries containing ≥ 1 of the terms. The second highest correlation was obtained when the keyword gastro ($\rho = 0.88$, $p<0.001$) (online Appendix Figure, panel B) was used. The highest correlation with chickenpox was obtained with the French word for chickenpox (varicelle) ($\rho = 0.78$, $p<0.001$) (online Appendix Figure, panel C).

A time lag of 0 weeks gave the highest correlations between the best

queries for influenza-like illness and acute diarrhea and the incidences of these diseases; the peak of the time series of Google queries occurred at the same time as that of the disease incidences. The best query for chickenpox had a 1-week lag, i.e., was 1 week behind the incidence time series.

In conclusion, for each of 3 infectious diseases, 1 well-chosen query was sufficient to provide time series of searches highly correlated with incidence. We have shown the utility of an Internet search engine query data for surveillance of acute diarrhea and chickenpox in a non–English-speaking country. Thus, the ability of Internet search-engine query data to predict influenza in the United States presented by Ginsberg et al. (*3*) appears to have a broader application for surveillance of other infectious diseases in other countries.

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Human-to-Dog Transmission of Methicillin-Resistant *Staphylococcus aureus*

To the Editor: In November 2007, a 76-year-old man with diabetes mellitus, chronic lymphocytic leukemia, and chronic obstructive pulmonary disease, who was being treated with prolonged corticosteroid therapy, received a diagnosis of invasive pulmonary aspergillosis. After 4 weeks of voriconazole therapy, cellulitis with substantial erythema, induration, and tenderness developed in his right bicep muscle. Bacterial cultures from a skin biopsy sample yielded methicillin-resistant *Staphylococcus aureus* (MRSA), resistant to trimethoprim/sulfamethoxazole, clindamycin, erythromycin, tetracycline, and ciprofloxacin. The patient received intravenous vancomycin for 3 weeks. After prolonged hospitalization, he was discharged but again hospitalized in February 2008 for cellulitis in the right ankle. Cultures of drainage around the ankle grew MRSA with a susceptibility pattern identical to that of the previous isolate. In April 2008, after the patient had received vancomycin for 1 week and the infection had resolved, a nasal swab showed carriage of MRSA with a susceptibility pattern identical to that of the previous isolates.

In late February 2008, the man's 8-year-old spayed female Labrador retriever was examined for cellulitis and generalized abscessation of the neck area, which had not responded to empirical treatment with oral cephalexin. In December 2007, she had undergone surgery for a ruptured cranial cruciate ligament (right tibial plateau-leveling osteotomy). She had chewed some sutures out after surgery, and cultures of a purulent discharge from the incision grew *Pseudomonas aeruginosa*;

this infection was successfully treated with enrofloxacin. As a result of implant failure, surgery was repeated in early February 2008. Cultures of the joint fluid and implants at this time were negative.

Physical examination in late February showed a large, firm area of extensive swelling on the ventral aspect of the dog's neck and purulent discharge from ulcerations (Figure). She had dried discharge around her right stifle and was moderately lame on that leg. Cultures of blood, tissue samples from her neck, and fluid draining from the right stifle joint all grew MRSA (also resistant to trimethoprim/sulfamethoxazole, clindamycin, erythromycin, tetracycline, enrofloxacin, marbofloxacin, and orbifloxacin). A biopsy sample of the neck showed severe, acute, multifocal, neutrophilic vasculitis with numerous fibrin thrombi and moderate to severe, superficial to deep, perivascular to periadnexal, suppurative lymphohistiocytic dermatitis. The dog became increasingly lethargic; systemic inflammatory response syndrome developed, and the neck became severely ulcerated and necrotic. Aggressive therapy with intravenous hydration and antimicrobial drugs (clindamycin and cefazolin, given before culture results were known) produced little clinical response. Extensive regions of skin sloughed, the face and neck became edematous, and focal masses developed within the lips. Signs of septic shock developed, and the dog was humanely euthanized 48 hours after this admission for skin lesions.

Pulsed-field gel electrophoresis was performed on isolates from the man and the dog. The isolates were indistinguishable and not consistent with recognized USA epidemic clones. *Spa* typing was also performed, and all isolates were *spa* type 3, also known as t037 according to the Ridom classification. Genes encoding for production of the Panton-Valentine leukocidin gene were not detected by real-time PCR (*1*).

Prevalence of MRSA in humans is increasing in most of the world. Similarly, MRSA colonization and infections in pets have increased in the past few years (*1*–*5*). MRSA can be transmitted between persons and their pets (*1*–*4*,*6*), although the route of transmission, risk factors for transmission, and incidence of interspecies transmission are not well understood. We describe a case of human-to-dog transmission of MRSA, which led to euthanasia of the dog.

Given the degree of antimicrobial-drug resistance in the MRSA isolates and the close ongoing contact of the human with the healthcare system, we suspect that the source of the MRSA infection was the human healthcare system. The dog likely acquired MRSA through close contact with her owner in that she had an open wound from complications of her orthopedic surgery. Most cases of MRSA in dogs have been associated with colonization, skin and soft tissue infections, or surgical site infections (*1*,*7*,*8*). Human-to-dog transmission is also supported by the temporal association of both infections and the dog's negative bacterial cultures in early February.

Studies of MRSA infection and colonization in household pets show that pets tend to be infected or colonized with MRSA strain types from the local human population (*3*,*7*). We, along with others, believe that MRSA in pets is closely linked to MRAS in humans and that infected or colonized humans may often be the source of MRSA in household animals (*5*). For some MRSA cases, infection reportedly resolved after the reservoir (either humans or animals in the household) was identified and treated appropriately (*4*–*6*). With MRSA infections reaching epidemic proportions, physicians and veterinarians must be aware of MRSA and the risk for cross-infection between species. To help develop infection control and treatment strategies to reduce the risk for infection within a household, further study is needed to clarify the epidemiology of interspecies transmission of MRSA.

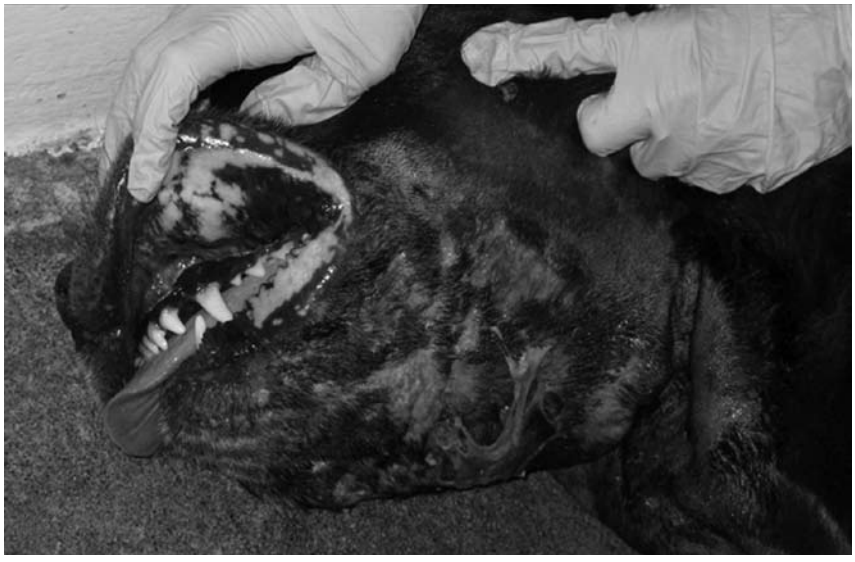

Figure. Extensive neck swelling with ulcerations and purulent discharge on 8-year-old spayed female Labrador retriever. Culture of the exudate and a macerated skin biopsy specimen grew methicillin-resistant *Staphylococcus aureus*. A color version of this figure is available online (www.cdc.gov/EID/content/15/8/1329-F.htm).

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Potential Malaria Reemergence, Northeastern Thailand

To the Editor: The emergence and reemergence of infectious diseases are major problems for healthcare systems worldwide. Unfortunately, because accurate prediction of the occurrence of such diseases is difficult, if not impossible, surveillance and control can be carried out only after the outbreak has occurred. Predicting the likelihood of a disease outbreak should make it possible to start surveillance programs before outbreaks occur and to initiate control programs before the population has become seriously affected. We used data on changes in land use patterns to predict the likelihood of malaria reemergence in northeastern Thailand.

Because natural rubber is of major economic importance and cannot be replaced by synthetic alternatives, the demand for and production of this commodity has consistently increased (*1*). This situation has led to changes in agricultural practices in various countries in Southeast Asia; rubber production has increased in Myanmar, Laos, Thailand, and Vietnam (*1*,*2*).

Northeastern Thailand (Isaan) is a relatively poor area, and most rubber plantations belong to smallholders and provide them with a large source of income (*3*). In 1993, ≈284 km^2 of northeastern Thailand were covered by rubber plantations; this area increased to 422 km^2 in 1998 and to 948 km^2 by 2003 (*3*). Since then, planting has increased exponentially so that, by 2006, the total area planted with rubber was >2,463 km^2; new plantings expanded another ≈1,345 km^2 from 2004 to 2006 and increased to a total of 5,029 km^2 in 2007 (*3*). The plants mature ≈6 years after planting; at that stage, the trees can reach 10–12 m in height, although the growth rate depends on the physical and biotic environment (*4*).

Deforestation in northeastern Thailand early in the last century led to an extreme reduction in the incidence of malaria (*5*) because the main vector mosquito in this area, *Anopheles dirus* sensu stricto, is forest dwelling and requires a shaded environment for its survival and reproduction (*6*). Currently, the northeastern part of the country is relatively free of autochthonous malaria cases except for 3 provinces that border Cambodia and Laos (*5*), Srisaket, Ubon Ratchathani, and Surin. In Srisaket and Ubon Ratchathani, 25% and 31%, respectively, of malaria cases are imported, particularly from Cambodia (*7*).

Mosquitoes are sensitive to changes in environmental conditions, such as shade, temperature, and humidity. These conditions are often influenced by land use change, such as conversion of rice paddies to rubber plantations (*8*). In addition to providing economic benefits for the population, rubber plantations also provide suitable habitats for *A. dirus* s.s., perhaps even better habitats than those found in the original rain forest; new plantations lead to increased mosquito density and disease incidence (*8*). Thus, planting large tracts of rubber potentially increases the likelihood of the reemergence of malaria in northeastern Thailand, although a malaria vector such as *A. dirus* s.s. could return without reemergence of the disease (*9*).

Should malaria return, the greatly reduced contact between the local Isaan population and *Plasmodium* spp. over the past ≈50 years suggests that malaria would enter a highly susceptible population, potentially leading to major health problems at the individual and regional levels. This possibility is of particular concern because several strains of *Plasmodium* in Thailand and surrounding countries are multidrug resistant, which leads to treatment difficulties (*5*).

Each land use change creates different microclimatic conditions,

which directly and indirectly affect the occurrence and distribution of malaria (*10*). Whether malaria will return as a major health threat likely depends on the size and fragmentation of the individual plantation areas. The required size of a plantation for the survival of the vector population is unclear, but large areas of plantation tend to offer dense vegetation and, therefore, high humidity and shade, which provide suitable environmental conditions for larval habitats, even during the dry season (*8*). Conversely, during the rainy season, conditions at the edges of fragmented forests, where human settlements are often located, become favorable for larval habitats, rendering villagers susceptible to the disease (*6*). In addition to changes in habitat and microclimate, social or political changes in the region may affect the transborder movement of malaria into Thailand with consequences for potential reemergence (*7*).

Although the association between rubber plantations and malaria is well known in Southeast Asia, the potential for reemergence should receive substantially more attention from economic, agricultural, and environmental planning bodies. Changes in land use and land cover have the potential to facilitate the transmission of disease to humans. Understanding the influence of land use change on malaria occurrence is critical for shaping future surveillance and control strategies.

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Fatal Borreliosis in Bat Caused by Relapsing Fever Spirochete, United Kingdom

To the Editor: Tick-borne relapsing fevers caused by members of the genus *Borrelia* have been encountered throughout Africa, Asia, the Americas and, rarely, in southern Europe (*1*). The *Borrelia* species associated with relapsing fevers form a monophyletic group within the genus, although not all members of this group have yet been implicated as agents of human disease. For example, a novel spirochete that is closely related to the relapsing fever agent *Borrelia turicatae* has recently been detected in *Carios kelleyi*, an argasid bat tick (*2*,*3*). We report the discovery of a spirochete causing fatal borreliosis in a bat in the United Kingdom.

The infected bat was a juvenile female *Pipistrellus* species that was found alive but on the ground near the town of Mevagissy in southwestern England in August 2008; despite rehabilitation efforts, it died a few days later. A postmortem examination showed pale skeletal muscles, anemia, excess blood-tinged pleural fluid, a healthy thymus, but enlarged cranial thoracic lymph nodes. The liver was greatly enlarged and mottled, the spleen was also large and unusually dark, and the adrenal glands were enlarged and pale with areas of hemorrhage. The kidneys were pale with a fine speckling

pattern over the cortex. Histopathologic examination of the liver showed multifocal necrosis and vacuolation of hepatocytes and infiltration by macrophages. The lungs were congested and infiltrated by inflammatory cells, and large numbers of granulocytes were found in the blood vessels. The spleen showed marked extramedullary hemopoiesis. Tissue sections stained by the Warthin-Starry technique exhibited numerous long, undulating, argophilic bacilli. These organisms were present in large numbers in the liver lesions (Figure), but were also found in the parenchyma of lung and spleen and in blood vessels.

On the basis of these observations, a diagnosis of fatal hepatitis and septicemia caused by a spirochete was made. DNA from the bat's liver was extracted and analyzed by using a PCR specific for an almost complete fragment of the 16S rRNA-encoding gene, as previously described (*4*), but with an annealing temperature of 45°C. This DNA extract was also incorporated into PCR assays targeting *glpQ* and *flaB* gene fragments (*5*). The products of these reactions were sequenced, and sequence data were assembled and analyzed by using Staden (*6*) and MEGA (*7*).

We obtained unambiguous sequence data for all 3 loci, comprising of 1,364 bp of the 16S rRNA-encoding gene (GenBank accession no. FJ868583), 1,239 bp of *flaB* and flanking regions (GenBank accession no. FJ868584), and 480 bp of *glpQ* (GenBank accession no. FJ868585). Each of these was aligned with homologous sequences available for other *Borrelia* species and used for phylogenetic analyses. Inferences made by using all loci were congruent, with the UK bat–associated spirochete lying close to, but distinct from, a cluster containing *B. recurrentis, B. duttonii,* and *B. crocidurae* (Figure; data not shown).

These 3 species are associated with relapsing fevers in Africa and Asia. The UK bat–associated spirochete bore no specific evolutionary relatedness to *B. johnsonii*, the newly characterized member of the relapsing fever group of *Borrelia* species associated with *C. kellyi* in the United States (Figure) (*3*). An *Argas vespertilionis* larval tick was found attached to the infected bat and may have been the source of its infection. PCR was not performed on the tick because it was near-replete with blood that was intensely infected with spirochetes. *A. vespertilionis*, commonly known as the short-legged bat tick, is widely distributed, parasitizing numerous bat species across Europe, southern Asia, and North Africa (*8*).

Given the close relationship between the novel spirochete we encountered and known pathogens, the reported propensity of *A. vespertilionis* to bite humans (*9*), and the wide geographic range of this tick, our findings have repercussions for public health in many parts of the Old World. Furthermore, although bats are likely the reservoir host for this organism, our study also identifies it as a pathogen, and as such its discovery has implications for the conservation of numerous threatened bat species across Europe and throughout the world.

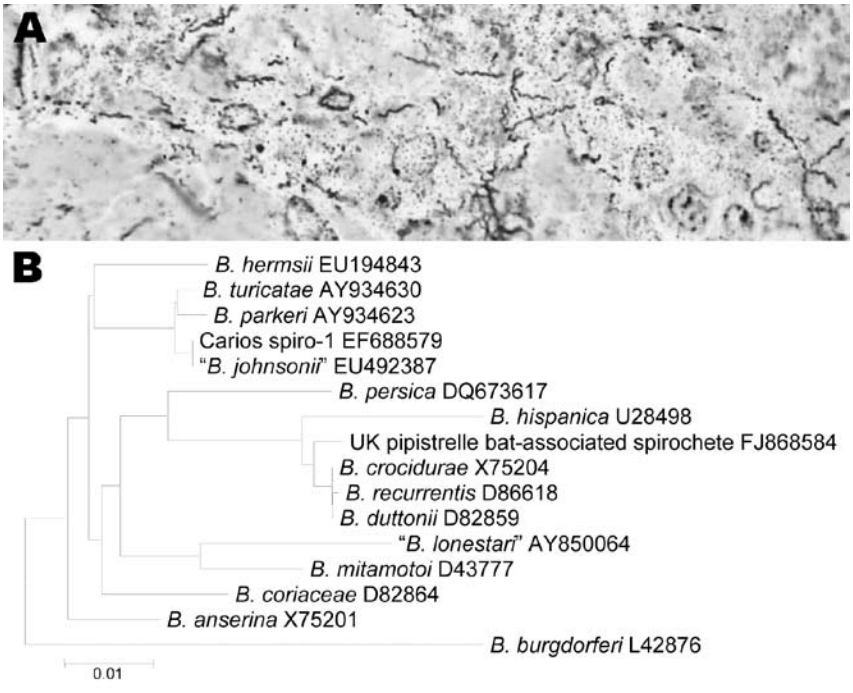

Figure. A) Warthin-Starry–stained section of bat liver showing numerous spirochetes. B) Phylogram inferred from 776-bp alignment of *flaB* fragments obtained from infected bat liver tissue and for other members of the relapsing fever group of *Borrelia* species for which sequence data were available. *B. burgdorferi* is included as an outgroup. The numbers appearing after the names of the *Borrelia* species are the relevant GenBank accession numbers. Scale bar indicates nucleotides substitutions per site.

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Past, Present, and Future of Japanese Encephalitis

To the Editor: We are writing in response to the perspective on Japanese encephalitis (JE) by Erlanger et al. (*1*). Growing awareness is encouraging, yet because JE is a largely neglected disease, information is often contradictory or not readily available. We would like to supplement the authors' review with clarification on available vaccines and actions countries are taking to evaluate and control JE.

There is room for improvement or expansion on collecting and reporting JE surveillance data. However, as vaccine availability increases, many countries are eager to determine the impact of JE and to make informed decisions on immunization programs. For example, surveillance in Indonesia from 2005 through 2006 confirmed human cases throughout the country (*2*). In Cambodia, JE surveillance commenced in 2006, and an immunization program is being planned (*2*). Regional JE laboratory networks established by the World Health Organization are also helping countries gather this information by strengthening diagnostic capacity.

Cambodia plans to introduce the live, attenuated SA 14-14-2 vaccine from China's Chengdu Institute of Biological Products. This vaccine has recently become internationally available and is increasingly replacing the inactivated, mouse brain–derived vaccine in Asia. A single dose of the SA 14-14-2 vaccine demonstrated 96% efficacy after 5 years (*3*), and the Institute's commitment to an affordable price for developing countries has broadened accessibility (*4*). The government of India introduced the SA 14-14-2 vaccine in 2006, and nearly 50 million children 1–15 years of age have been reached through vaccination campaigns and routine immunization. The vaccine also is available through public programs or private markets in China, Nepal, South Korea, Sri Lanka, and Thailand.

JE vaccine candidates in late-stage development for children include a live, attenuated chimeric virus vaccine and an inactivated, Vero cell–derived vaccine, each based on the SA 14-14-2 virus strain. Additionally, 2 inactivated, Vero–cell derived vaccines based on the Beijing-1 strain are being developed in Japan (*5*).

New vaccine development, along with progress in surveillance and immunization, offers promise for sustainable control of clinical JE. To achieve this, global partners are working together to develop a strategic plan for JE control by 2015 (*6*).

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Avian Influenza

David Swayne, editor

Wiley-Blackwell Publishing, Hoboken, NJ, USA, 2008

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ISBN-13: 978-0813820477
Pages: 628; Price: US $120.66

David Swayne has recruited top experts worldwide to contribute to his new book, Avian Influenza. Each of the 25 chapters focuses on a specific area of expertise, and together they offer a variety of perspectives on this disease from the technical to the historical to the strategic.

The history of avian influenza starts with a fascinating look at the domestication of poultry and moves to a description of the first known fowl plague outbreaks in Europe in the late 1800s and a description of the initial determination of the pathogenic agent. Epidemiologic details of outbreaks caused by high and low pathogenicity influenza viruses since that time are given for the different geographic regions. The current panzootic of highly pathogenic avian influenza virus (H5N1) is described event by event since 1996, and the phylogenetic relationships among influenza viruses (H5N1) and potential methods of spread are discussed. This valuable historical and technical background offers context and perspective to ongoing thinking about current (and future) influenza-related events.

Technical chapters cover the epidemiologic and virologic aspects of avian influenza, such as viral determinants of pathogenicity and pathophysiology of different avian influenza viruses in various species of birds and mammals. Also covered are disease control topics such as poultry culling/depopulation, carcass disposal, virus inactivation, biosecurity, vaccinology, and diagnostic testing. Disease impacts on trade and economics, including livelihoods, are also discussed, and a description of the animal health sector strategy for global control of highly pathogenic avian influenza is presented in conclusion. Although much of the recently available research and experience is focused on the H5N1 subtype, Dr Swayne and his collaborators have methodically extended their discussion to include other avian influenza subtypes and both high and low pathogenicity viruses wherever information exists.

The focus of the book is clearly on avian influenza in birds. However, information on public health aspects of the disease is provided in a chapter devoted to this topic as well as in other chapter contexts as appropriate. The relevant aspects of human seasonal influenza and pandemic influenza are reviewed, and the occurrence and impact of zoonotic infections with avian influenza virus (H5N1) and other high and low pathogenicity avian influenza viruses are detailed. In this chapter, the authors emphasize that surveillance and monitoring for avian influenza worldwide should be increased and made more sustainable and that collaborative efforts between animal health and public heath authorities should continue to be strengthened.

The authoritative and comprehensive nature of the information provided, as well as extensive reference lists and a volume-wide index, make this book a valuable reference asset for the scientific community, researchers, veterinary practitioners, governmental animal health agencies, those in the poultry industry, and others working to control and prevent avian influenza globally, including those working in public health. As an added benefit, all book proceeds will go to the American Association of Avian Pathologists to further educational programs on avian diseases worldwide.

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Erratum

Vol. 14 No. 8

The author list was incorrect in the article Imported Dengue Hemorrhagic Fever, Europe (M.J. Pinazo et al.). The authors were María Jesús Pinazo, José Muñoz, Ljiljana Betica, Tomislav Maretic, Sime Zekan, Tatjana Avsic-Zupanc, Ethel Sequeira, Antoni Trilla, and Joaquim Gascon. The article has been corrected online (www.cdc.gov/eid/content/14/8/1329.htm).

Erratum

Vol. 15 No. 3

The name of Oliver Donoso Mantke was incorrect in the author list for the article Coordinated Implementation of Chikungunya Virus Reverse Transcription–PCR (M. Panning et al.). The article has been corrected online (www.cdc.gov/eid/content/15/3/469.htm).

George Martin Baer (1936–2009)

George Martin Baer, DVM, MPH, who for many years was chief of the Rabies Laboratory in the Division of Viral and Rickettsial Diseases at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, died suddenly at his home in Mexico City on June 2, 2009, at the age of 73. He was an eminent veterinary virologist whose book, The Natural History of Rabies, the second edition of which was published in 1991, is the major reference work in the field.

He has been widely acclaimed as "the father of oral rabies vaccination" as a result of his discovery of a strain of rabies virus (SAD strain) that immunizes foxes when given into the mouth cavity. His intensive work on the development of baits for vaccine administration to canines paved the way for successful mass fox vaccination campaigns conducted in Switzerland, several other European countries, and Canada.

Dr Baer was born in London in 1936, but after the outbreak of World War II his family moved to the United States, and he was brought up in New York. He attended Cornell University in Ithaca, New York, and received a bachelor of science degree in agricultural sciences in 1954 and a Doctor of Veterinary Medicine degree in 1959. In 1961, he earned a Master of Public Health degree from the University of Michigan at Ann Arbor. He then came to CDC as an Epidemic Intelligence Service Officer, initially assigned to the New York State Health Department in Albany, where he first became interested in rabies. He subsequently joined the CDC Southwest Rabies Investigations Laboratory in Las Cruces, New Mexico, and from 1966 through 1969 was a consultant for the Pan American Health Organization in Mexico. He returned to CDC in Atlanta in 1969 to become chief of the Rabies Laboratory and served until his retirement in August 1991. He and his family then moved to Mexico City, where he founded a diagnostic laboratory and continued to use his expertise on rabies as a member of the Mexican International Steering Committee for the Rabies in the Americas Conference.

Dr Baer was a valued supporter of and reviewer for Emerging Infectious Diseases and will be greatly missed. We offer our condolences to his wife, Maria Olga, and daughters, Katherine, Alexandra, and Isabella, as well as to his 4 granddaughters.

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Torii Kiyomasu I (active 1696–1722) Kintoki Wrestling with a Black Bear (c. 1700) Color woodblock print (55.2 cm × 32.1 cm) Courtesy of the Honolulu Academy of Arts, Hawaii, USA. Gift of James A. Michener, 1975 (16,576)

For the world does not yet censure Those who tread the paths of dreams

—Ono no Komachi

Polyxeni Potter

"Gourd-shaped legs and wriggling worm lines" is how a Japanese critic described the style of early Torii artists of the Edo period (1615–1868). Named after the bustling city that would become present-day Tokyo, this period was marked by peace and a thriving urban culture. The most characteristic expression was *ukiyo-e* or the floating world, which concerned itself with the fleeting activities of everyday life. The genre produced poetry and drama but most of all, woodblock prints that inspired the global art scene of succeeding generations.

Fodder for *ukiyo-e* came from Edo's entertainment districts, with their teahouses and other establishments frequented by actors, courtesans, and throngs of spectators. A major source was *Kabuki,* a form of classical Japanese theater that featured both heroic and ordinary figures in plays filled with spectacular costumes and scenery. Another was woodblock prints, depicting lively scenes from these plays in posters, play bills, and storybooks. These became a source of pride for printmakers, who would sign them as "An artist of Japan." A group known as the Torii School achieved success and popularity with highly skilled designs that captured the boisterous energy of *Kabuki* acting and the authentic Japanese spirit that fueled the period. Kiyomasu I was member of that school and, as often the custom, adopted its name.

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Torii Kiyomasu I was one of the earliest masters of floating-world scenes. Details of his life and work are sketchy and intertwined with those of other founders of the Torii School, but his surviving work is considered more accomplished than theirs. Unlike most designs cut in blocks of wood during this period, his were marked by fluid calligraphic lines and contained areas of orange lead pigment applied by hand with stencils. His theatrical characters burst out the edges of the paper. Caught as they were in the middle of intense activity, they exuded a thick physicality, their muscles quivering, twisted, or coiled; their gestures slicing through the air.

Kintoki Wrestling with a Black Bear, on this month's cover, showcases Kiyomasu's bold design and his preoccupation with movement, as well as the period's inclination to blend art with legend, image with words, often adding contemplative verses to expand a visual theme. Kintoki was a mythical character. Known as Kintarō in his younger years, he was a child hero, a larger than life latter-day Hercules, who dazzled with his strength and ability to overcome obstacles. Kintoki dreamed big, fought monsters, spoke the language of animals, wrestled with bears, and always won.

Kintoki's struggle with the black bear seems emblematic on several levels. Here is a Japanese artist, Kiyomasu I, in the throes of a struggle for independence and identity, breaking away from traditional ways, many of them borrowed from China, and venturing into new, more authentic expression. Then there is Kintoki, the national hero, in a steamy scene towering over a wild beast, his muscles

straining, eyes bulging, gourd legs curling, garments flying through the air. If he did speak the bear's language, what was he saying through clenched teeth? On yet another level, Kiyomasu's print is a symbol of the human struggle against the wild, against brute strength requiring heroic intervention and against infections challenging the fringes of human purview.

Many of these are infections spreading from animals to humans. Rabies is one of them, a disease known since antiquity. Homer mentioned "raging dogs" in the Iliad and wrote that Sirius, the dog star of the Orion, exerted a malignant influence on human health. In Japan, rabies existed since the 10th century and was part of life during the Edo period. Wildlife rabies was also known. George Fleming, in his book Rabies and Hydrophobia (1872), wrote of a rabid bear in the French city of Lyon causing havoc in the year 900. But for Kintoki, overcoming the bear was a matter of power and skill. The match was fair. The strongest contestant won. No virus was involved, even if rabies had not yet been eliminated in Japan, as it would be in the 1950s.

In North America, dog rabies was introduced by European settlers in the 1700s. Compulsory vaccination programs after World War II controlled the disease, and in 2007, dog rabies was declared eliminated in the United States. All the same, wildlife rabies increased. The disease source shifted from domesticated animals to mainly raccoons, skunks, foxes, and bats, and rabies reemerged as a major zoonosis.

"The only remedy is to throw the patient unexpectedly into a pond, and if he has not a knowledge of swimming to allow him to sink, in order that he may drink, and to raise and again depress him, so that though unwillingly, he may be satisfied with water; for thus at the same time both the thirst and dread of water is removed." This remedy, described by Celsus, a physician and naturalist of antiquity, was one of many outlandish approaches against rabies. Chinese physicians offered, among other "cures," a mixture of musk and cinnabar suspended in rice spirits. But it was vaccination programs that finally controlled the disease in many areas of the world and even eliminated it in a few.

Kintoki's struggle with the black bear brings back the specter of *lyssa*. With increased disease in free-ranging animals, public health workers have to reach deeper in their bag of tricks for yet more safe and cost-effective solutions. And like the Japanese strongman, they must imagine that they can overcome all obstacles and eliminate all scourges, even rabies. Ongoing wildlife epizootics could reintroduce the disease in unvaccinated domesticated animals, perpetuating the problem for all of us. If rabies exists somewhere, it exists everywhere. For as the poet put it, "… The moon shining/over Niho Bay/is the very same/as at Suma and Akashi."

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Using Environmental and Global Changes to Predict Infectious Disease Outbreaks

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Zika Virus Outside of Africa

Chicken Consumption and Use of Acid-Suppressing Medications as Risk Factors for *Campylobacter* Enteritis, England

Characteristics of Infections with Novel Influenza Virus A (H1N1), China, 2009

Pathogenesis of Feline Infectious Peritonitis Virus

Genetic Characterization of Foot-and-Mouth Disease Viruses, Ethiopia, 1981–2007

Increasing Incidence of Zygomycosis (Mucormycosis), France, 1997–2006

Diagnosis of Bocavirus-induced Wheezing in Children, Finland

Bat Coronaviruses, Ghana

Recent Ancestry of Kyasanur Forest Disease Virus

Etiology of Encephalitis in Australia, 1990–2007

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Interactions Relevant for Infectious Disease Transmission in Nurses

Predicting Phenotype and Emerging Strains among *Chlamydia trachomatis* Infections

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Program to Eradicate Malaria in Sardinia, 1946–1950

Highly Pathogenic Avian Influenza Virus (H7N3) in Domestic Poultry, Canada

Merkel Cell Polyomavirus DNA in Persons without Merkel Cell Carcinoma

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

Upcoming Infectious Disease Activities

August 3–4, 2009
UC Berkeley CIDER SUMMIT 2009: Advances in the Control and Epidemiology of Emerging Infectious Diseases
Sheraton Gateway Hotel
Burlingame, CA, USA
http://www.idready.org/CIDERSummit2009.html

August 10–21, 2009
11th International Dengue Course
http://www.ipk.sld.cu/cursos/dengue2009/indexen.htm

August 29–September 2, 2009
Infectious Disease 2009
Board Review Course
14th Annual Comprehensive Review for Board Preparation
McLean, VA, USA
http://www.cbcbiomed.com

October 29–November 1, 2009
47th Annual Meeting of IDSA and HIVMA
Philadelphia, PA, USA
http://www.idsociety.org/Content.aspx?id=12006

November 7–11, 2009
American Public Health Association's 137th Annual Meeting and Exposition
Philadelphia, PA, USA
http://www.apha.org/meetings

November 18–22, 2009
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Marriott Wardman Park
Washington, DC, USA
http://www.astmh.org/meetings/index.cfm

Announcements

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Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

National Outbreak of *Acanthamoeba* Keratitis Associated with Use of Contact Lens Solution, United States

CME Questions

1. Which of the following most accurately describes the incidence and characteristics of Acanthamoeba keratitis (AK) in the United States?

A. Occurs primarily among hard contact lens users
B. Annual incidence is 1–2 cases per 1 million
C. A painless corneal infection
D. Has a benign course

2. Which of the following is least likely to describe the characteristics of AK associated with the cases reported in this article?

A. Median patient age was 40 years
B. Most used a contact lens cleaning solution
C. Most used soft contact lenses
D. Median time to treatment was 49 days

3.Which of the following is most likely to be an independent predictor of AK in contact lens users?

A. Swimming in lakes while wearing lenses
B. Hispanic ethnicity
C. Topping off lens solutions
D. Lack of handwashing before lens insertion

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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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 www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, 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. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

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

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

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

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.