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Foodborne Disease

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On the Cover

Diego Velázquez (1599-1660)
An Old Woman Cooking Eggs (1618)
Oil on canvas (100.5 cm x 119.5 cm)
The National Gallery of Scotland, Edinburgh

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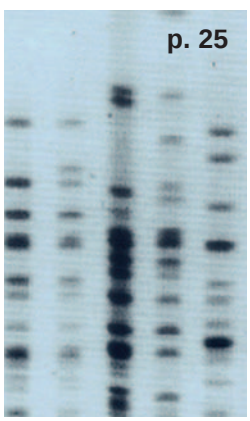

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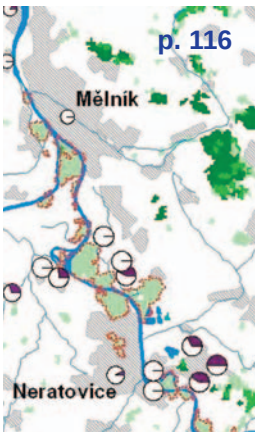

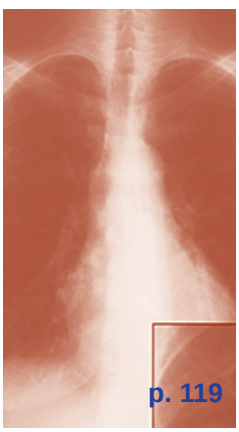

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Hedgehog Zoonoses

Patricia Y. Riley* and Bruno B. Chomel*

Exotic pets, including hedgehogs, have become popular in recent years among pet owners, especially in North America. Such animals can carry and introduce zoonotic agents, a fact well illustrated by the recent outbreak of monkeypox in pet prairie dogs. We reviewed known and potential zoonotic diseases that could be carried and transmitted by pet hedgehogs or by wild-caught hedgehogs that have been rescued.

Pets play an important role in societies throughout the world (1). They are important companions in many households, contributing to the physical, social and emotional development of children and the well-being of their owners, especially the elderly (1). Although pets offer significant benefits, potential hazards are associated with pet ownership (1). Exotic animals are increasingly being invited into homes as pets (2). However, neither pet owners nor nonveterinary healthcare providers are sufficiently knowledgeable about the potential of many of these animals to transmit zoonotic diseases (2).

Hedgehogs are small, nocturnal, spiny-coated insectivores that have been gaining popularity as exotic pets (3). These animals are considered to be unique, low-maintenance pets (4), and an estimated 40,000 households in the United States now own them (5). These animals originally arrived from Europe, Asia, and Africa, and although several species exist, 2 in particular are commonly seen as pets (3): the European hedgehog, *Erinaceus europaeus*, and the smaller African pygmy hedgehog, *Atelerix albiventris* (3). The importation of these pets from Africa to the United States has been prohibited since 1991 (Title 9 Code of Federal Regulations Section 93.701) due to their potential to carry foot-and-mouth disease, a foreign animal disease of serious economic concern to the livestock industry (6). In the United States, persons who sell hedgehogs are required to have a U.S. Department of Agriculture (USDA) license (http://www.nal.usda.gov/awic/newsletters/v9n1/9n1aphis.htm). In some states, such as Arizona, California (http://www.dfg.ca.gov/licensing/pdffiles/fg1518.pdf), Georgia, Hawaii, Maine, Pennsylvania, Vermont, and Washington D.C., owning a hedgehog as a pet is illegal (www.hedgehogwelfare.org), as is the case in some of New York City boroughs (Manhattan, Brooklyn, Queens, Bronx, Staten Island) (source: http://www.petfinder.org/shelters/CT171.html).

Hedgehogs live in a variety of habitats where they dig their own burrows, spend most of the daylight hours asleep, and emerge at night to forage (3). Hedgehogs are characterized by short, grooved spines covering the entire dorsum of the body (3). When frightened by an unfamiliar sound or movement, the animal rolls into a tight ball (3). In this defensive posture, the hedgehog brings its snout and limbs close under its body, causing the spines to become erect (3). The spines, modified hairs having a spongy matrix and outer keratinous shaft (7), are not barbed (8). The spines rarely cause serious injury to handlers (8) but can readily penetrate the skin (7). However, 1 report described 3 patients in whom an acute, transient, urticarial reaction developed after contact with the extended spines of pet hedgehogs (7).

Hedgehogs display an unusual behavior called "anting" or "anointing" (7). When first encountering a new or interesting object or food, the animal will lick the substance repeatedly until a frothy saliva forms in its mouth (3). The animal then rubs the excess saliva and froth onto its skin and spines (3). This behavior may cause saliva to accumulate on the spines, making the hedgehog less palatable to predators (7).

In addition to the contact urticaria that has been reported in some hedgehog handlers (7), hedgehogs pose a risk for a number of potential zoonotic diseases (2). Major microbial infections associated with hedgehogs include bacteria such as *Salmonella* and *Mycobacteria*, as well as some fungal and viral diseases (2). Many disease conditions can cause immunodeficiency in humans; the most notable is AIDS (9). Similarly, immunosuppressive strategies employed to prevent rejection of bone marrow or solid organ transplants render such patients extremely susceptible to viral and mycobacterial infections (9). An increasing percentage of the population is becoming susceptible to severe diseases associated with exotic pet ownership, as illustrated by the recent monkeypox outbreak in pet prairie

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dogs (10). Immunocompromised persons may be at increased risk for infections from hedgehogs and should be particularly careful.

The following review focuses on the zoonotic or potentially zoonotic agents carried by hedgehogs (Table). The risks are particularly of concern for people rescuing wild-caught hedgehogs and adopting them as pets. We distinguished major established zoonotic infections, such as salmonellosis or ringworm, from other less common or potential zoonoses carried by hedgehogs.

Bacterial Zoonoses

Salmonellosis is the main zoonotic disease associated with hedgehogs, as well as several other exotic pet species (4). Although affected hedgehogs can display anorexia, diarrhea, and weight loss, ≈28% of hedgehogs are asymptomatic carriers (11). Several recent reports have shown that hedgehogs play a major role in the transmission of *Salmonella* Tilene, a rarely encountered serotype of humans (4,6,12). In 1994, *Salmonella* serotype Tilene infection was diagnosed in a 10-month-old girl in the state of Washington (6). The source of the organism was traced back to African pygmy hedgehogs raised by the child's family. Although the infant had no direct contact with the hedgehogs, the animals were handled frequently by 1 member of the family. Cultures from the child's asymptomatic parents were negative, and the breeding herd of 80 hedgehogs was apparently healthy, but a stool sample from 1 of 3 hedgehogs yielded *S.* Tilene. A second *S.* Tilene case was reported later that year from Texas (6). That patient's family also owned a hedgehog.

From 1995 to 1997, 10 laboratory-confirmed cases of *S.* Tilene infection (12) were reported in Canada. With 1 exception, all cases occurred in children, with 5 cases in children <3 years. All but 1 patient came from or had contact with families owning African pygmy hedgehogs. The adult and older children cases were either directly responsible for the care of the hedgehogs or had other direct contact with the animals. Four of the cases were associated with breeding herds of hedgehogs.

The risk for such infections can be reduced by hand washing after handling of pets, especially before eating or handling food and by avoiding contact with pets' feces (6). Similarly, pets should be fed, housed, and handled properly, and all pets should be carefully watched for signs of illness and treated appropriately when ill (12).

Two human outbreaks of salmonellosis caused by *S.* Typhymurium 4,5,12:I:1,2 were reported in Norway; *Salmonella*-infected hedgehog populations most likely constituted the primary source of human infection, as ≈40% of the animals tested carried the same strain (13). Furthermore, the pulsed-field gel electrophoresis profiles of isolates from hedgehogs and human beings were identical within each of the 2 outbreak areas. In an evaluation of mortality of hedgehogs in East Anglia and presence of possible zoonotic diseases, salmonellosis was found to be the most common zoonotic infection (14). *S.* Enteritidis PT 11 was found in 13 hedgehogs, which suggests a special association between this phage type of *S.* Enteritidis and hedgehogs in East Anglia. This phage type has also been isolated from human beings. *S.* Typhymurium PT 104 was also isolated from 1 animal in the same study. Overall, the incidence of *Salmonella* was 18.9% of 74 hedgehogs (14).

These authors also reported the first isolation of *Yersinia pseudotuberculosis* in the United Kingdom from 2 hedgehogs that died at a rehabilitation center in Berkshire (14). The organism primarily causes in humans a gastroenteritis characterized by a self-limited mesenteric lymphadenitis, which mimics appendicitis. Postinfectious complications include erythema nodosum and reactive arthritis.

Another condition of zoonotic concern reported in hedgehogs is systemic mycobacteriosis caused by *Mycobacterium marinum* (15). A European hedgehog was brought for treatment with nonsuppurative masses in the subcutis of the ventral cervical region (15). The animal worsened and died. On necropsy, multiple granulomatous lesions found in lymph nodes, lungs, spleen, liver, and heart were positive for *M. marinum* in culture. In the reported case, the hedgehog apparently acquired *M. marinum* from the fish tank in which the animal was housed at

Table. Zoonotic and potentially zoonotic viral, bacterial, protozoal, and mycotic zoonoses of hedgehogs

	Bacterial	Viral	Protozoal	Mycotic
I. Confirmed zoonotic diseases carried by hedgehogs:	*Salmonella* spp.* *Yersinia pseudotuberculosis*† *Mycobacterium marinum*	Rabies Herpesvirus, including human herpes simplex		*Trychophyton mentagrophytes var. erinacei** *Microsporum* spp.†
II. Potential zoonotic diseases carried by hedgehogs:	*Chlamydia psittaci* *Coxiella burnetii* *Yersinia pestis*	Arboviruses Tickborne encephalitis Crimean-Congo hemorrhagic fever Tahyna virus Bhanja virus Paramyxovirus	*Cryptosporidium* *Toxoplasma gondii*	*Candida albicans*

*Most common zoonoses.
†Common zoonoses.

a pet store (15). In humans, the organism is associated with a cutaneous disease called "fish-tank granuloma," which is frequently contracted from contact with aquariums (16). The organism typically gains entry through some wound or abrasion in the skin, such as may be produced by hedgehog spines, and may spread systemically along the lymphatic system. The resulting disease produces lesions resembling those of sporotrichosis, tularemia, nocardiosis, and blastomycosis.

In Madagascar, *Y. pestis* was isolated from an endemic hedgehog (*Hemicentetes nigriceps*), and plague antibodies were detected in another endemic hedgehog species (*Tenrec ecaudatus*) (17). However, no human infection directly related to hedgehog exposure has been documented.

In a serosurvey of European hedgehogs in Styria, Austria, antibodies against *Coxiella burnetii* (Q fever), *Chlamydia* (ornithosis), and *Toxoplasma gondii* (toxoplasmosis) were detected among 64 animal tested (18). Potential infection of persons who are rehabilitating or caring for rescued hedgehogs by these infectious agents should therefore be considered.

Mycotic Zoonoses

Dermatophytosis, or ringworm, has been described in the hedgehog (8). Lesions in the animal are similar to those found in other species and include nonpruritic, dry, scaly skin with bald patches and spine loss (8). *Trichophyton mentagrophytes* var. *erinacei* is the dermatophyte most commonly isolated from the quills and underbelly of hedgehogs, although *Microsporum* spp. have also been reported (8). Hedgehogs can be asymptomatic carriers of these fungi (8), and herein lies their potential for zoonotic transmission.

Several reports demonstrate the ability of the hedgehog to transmit dermatophytes to humans (19–21). *Trichophyton mentagrophytes* var. *erinacei* causes an extremely inflammatory and pruritic eruption, which frequently resolves spontaneously 2–3 weeks after onset (8). The disease may present as a localized rash with pustules at the edges and a thickened and intensely irritating area in the center of the lesion (19).

In 1 report of 3 human ringworm cases, 1 of the patients merely handled the hedgehog in a pet store for 1 to 2 minutes (21). A second patient observed multiple annular, erythematous, bullous lesions on her legs, arms, and abdomen within 1 week of purchasing an African pygmy hedgehog from a pet store.

In a second report of human ringworm associated with handling of European hedgehogs, a patient had raised erythematous lesions on her right wrist and 2 fingers of the left hand (20). Because the patient was pregnant at the time, she was treated with topical antifungals only. After 1 month, the skin eruption had spread, with resultant destruction of the nails on both affected fingers. When the patient was able to be treated systemically, the infection cleared within 8 weeks.

In addition to the dermatophytes, hedgehogs have been reported to be infected with *Candida albicans* (22,23). Intestinal candidiasis was reported in an immunocompromised animal (22). Infection of the footpads with *C. albicans* has also been reported in an African pygmy hedgehog (23). The zoonotic potential of hedgehogs transmitting this infection to humans is therefore possible.

Viruses

Several arboviral encephalitis viruses have been studied in the hedgehog. One study showed that hedgehogs are susceptible to even small amounts of Tahyna virus of the family *Bunyaviridae* and may produce a viremia level sufficient to ensure infection of mosquitoes (24). Hibernating hedgehogs may play a role as potential long-term reservoirs of the virus (24). Bhanja virus, also a member of the *Bunyaviridae*, has also been studied in European hedgehogs (25,26). Hedgehogs did not generate viremia titers, which indicates that the animals are likely not maintenance hosts for the virus (25). However, Hubalek et al. (26) suggested that the low level of antibodies may indicate that hedgehogs are passive distributors of infected ticks. The potential role of hedgehogs as natural hosts of these viruses and the risk that they may introduce these arboviruses in nondisease-endemic areas still need to be documented.

Tickborne encephalitis virus has been found in European hedgehogs (27). In at least 1 naturally occurring focus of the virus, the frequency of antibodies was considerably higher in hedgehogs than in small rodents, possibly related to the longer life cycle of hedgehogs as opposed to rodents, thus increasing the probability of reinfection (27). This study clearly demonstrated the importance of hedgehogs as hosts of ticks and reservoirs of the virus (27). Kozuch et al. (28) also demonstrated that alimentary infection of hedgehogs was possible. In addition, hedgehogs can maintain the virus during hibernation and may act as reservoirs during epidemic and interepidemic periods (29).

Crimean-Congo hemorrhagic fever (CCHF) is an important zoonotic disease of humans in the Middle East, as well as in Eastern Europe and Asia (30). The infection, which is often lethal, may be acquired through tick bite, contact with blood or tissues from infected livestock, or by treating CCHF-infected patients (medical personnel) (30). Studies in both European and African hedgehogs have demonstrated infection with CCHF virus (31,32). In European hedgehogs, viremia of sufficient intensity to infect feeding ticks does not develop (33). African pygmy hedgehogs appear susceptible to CCHF infection, but their role as amplifying hosts is yet undetermined; the South African hedgehog (*Atelerix frontalis*) does not appear to be

an amplifying host (32). Although the response of individual species to CCHF virus varies markedly, hedgehogs act as important hosts to the immature stages of many of the tick species from which the virus has been isolated and therefore may be a potential source of infection (32).

Vizoso and Thomas (33) reported that a paramyxovirus of the Morbillivirus group had been isolated from 1 sick and several apparently healthy European hedgehogs. The sick animal displayed neurologic signs, ulcerated lesions on the soles of the feet, and histologic changes resembling canine distemper, another virus of the Morbillivirus group. Paramyxoviruses are commonly present in hedgehogs, and the possibility exists that the viruses cross species barriers, giving rise to infections in humans (33).

Herpesvirus infection has been reported in both European and African pygmy hedgehogs (34–36). In both species, the primary site of infection has been the liver. In the first report, a feral European hedgehog had severe hepatic necrosis with extensive parenchymal hemorrhage and hyperemia (34). Herpesvirus particles were demonstrated by transmission electron microscopy. In a second report, involving a litter of orphaned European hedgehogs, 1 animal was necropsied and found to have a pale yellowish, friable liver with severely distorted general architecture (35). Culture of liver homogenate yielded herpeslike particles, which cause a cytopathic effect characteristic of alpha herpesvirus. A more recent report involved an African pygmy hedgehog owned by a private breeder (36). That animal was initially brought for treatment of acute posterior ataxia, which rapidly progressed to paresis. The hedgehog was treated for a prolapsed intervertebral disc and at first improved dramatically, but died 2 weeks after initial evaluation. On necropsy, the liver showed randomly distributed foci of necrosis and extensive inflammatory reaction. No bacteria were cultured, but viral culture produced an isolate that was subsequently identified as human herpes simplex virus. The source of the infection is uncertain, but the owner reported that family members occasionally suffered from cold sores (36).

A case of rabies in a hedgehog has been reported once (37). A family in inner-city Budapest found a hedgehog and, while playing with it, was exposed to typical salivary secretions. The animal later died and was confirmed to be positive for rabies. All family members were given postexposure prophylaxis to prevent infection.

Both European and African hedgehogs are susceptible to foot and mouth disease (FMD) virus. Although not technically a zoonosis, FMD could have a devastating impact on susceptible livestock. The earliest record of infection in hedgehogs suggested that the animals could have played a role in local spread of the disease (38). Typical vesicular lesions can develop on the hedgehog's tongue, snout, and feet (38). Virus isolated from several hedgehogs trapped on or near infected premises during an outbreak of FMD in Britain was the same as that isolated from cattle in that outbreak (38). The full cycle of infection between cattle and hedgehogs was also successfully demonstrated with African hedgehogs (39).

Protozoa

Intestinal cryptosporidiosis was the cause of death in a captive juvenile African pygmy hedgehog (40). Large numbers of *Cryptosporidium* spp. developmental stages were present throughout much of the affected animal's intestine. *Cryptosporidium parvum* is infectious to humans and represents a public health threat because of its waterborne transmission. The protozoan in this report was not identified to species, but zoonotic potential exists and appropriate precautions should be taken to prevent transmission to humans (40). Thorough hand washing with soap and water after handling hedgehogs is an important precaution to avoid contamination. Similarly, if one should clean up fecal materials from a pet, the use of disposable gloves and washing the hands afterwards is highly recommended.

Conclusion

All pets have a flora of parasites and microbes, some of which are potentially zoonotic (12). Ownership of exotic pets may lead to increased exposure to various infectious agents, including exotic ones. However, the risk for exposure to zoonotic agents is substantially increased for people rescuing sick hedgehogs or rehabilitating them. Preventing illness depends on good hygiene and increased awareness, especially when young, elderly, or immunocompromised persons are involved (12). Pet hedgehog owners should systematically wash their hands after contact with their pets and make sure that any person who handled their pet will do so. Similarly, such pets should not be housed or handled in food preparation areas, as is recommended also for pet reptiles (41). Control of zoonotic diseases is dependent on breaking the cycle of transmission, and education is the key to control (1). Veterinarians should be the optimal source of providing correct information to clients making choices about owning exotic pets (1). Overall, ownership of exotic pets should not be encouraged because exotic animals and wildlife do not usually make good pets and can transmit zoonotic agents, as stated by the American Veterinary Medical Association and the National Association of State Public Health Veterinarians. In view of the popularity of hedgehogs and growing numbers of at-risk persons, veterinarians and other medical personnel must increase their understanding of the animal's zoonotic potential.

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Border Screening for SARS

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With the rapid international spread of severe acute respiratory syndrome (SARS) from March through May 2003, Canada introduced various measures to screen airplane passengers at selected airports for symptoms and signs of SARS. The World Health Organization requested that all affected areas screen departing passengers for SARS symptoms. In spite of intensive screening, no SARS cases were detected. SARS has an extremely low prevalence, and the positive predictive value of screening is essentially zero. Canadian screening results raise questions about the effectiveness of available screening measures for SARS at international borders.

The first cases of severe acute respiratory syndrome (SARS) in Canada were recognized almost simultaneously in Vancouver and Toronto. In Toronto, the index case was diagnosed on March 13, 2003, when a cluster of SARS cases was identified and traced back to a traveler from Hong Kong, who arrived in Canada on February 23, 2003 (1). Two epidemic waves of SARS occurred in Toronto (2), which resulted in a national total of 251 probable cases with 43 deaths.

In the period that followed the initial reports of this new syndrome from Hong Kong and Vietnam, the disease spread rapidly to other countries by international airline travelers. On March 12, 2003, the World Health Organization (WHO) issued a global health alert (3) in response to the clusters of SARS in the Hong Kong Special Administrative Region, China, Vietnam (Hanoi City), and Singapore. WHO recommended increased national and international vigilance to recognize and report suspected cases of SARS. Subsequently, on March 15, 2003, WHO issued the first of several international travel advisories that identified major locations where SARS transmission was substantial and ongoing and advised international travelers about travel to affected areas. On March 27, 2003, WHO recommended that affected areas begin screening departing airline passengers for symptoms suggestive of SARS.

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Health Canada monitored the spread of this new syndrome through the WHO-Health Canada Global Public Health Intelligence Network and regular communications with other international and Canadian provincial and territorial public health agencies. As soon as the rapid, international spread of SARS became evident and after SARS was imported into Canada, Health Canada undertook a variety of measures designed to limit importation and exportation of disease and the spread of the disease within Canada. We describe the measures taken to mitigate the spread of SARS and provide data on the effectiveness of these measures.

Methods

Health Canada used a graduated, phased response to additional imported SARS cases. The response consisted of an information phase (March 18–May 14, 2003), a screening phase (May 14–July 5, 2003), and a special measures phase (March 13–July 5, 2003).

Information Phase

To mitigate the risk of importing SARS cases from other internationally affected areas, Health Canada distributed passenger health alert notices (HANs) for incoming passengers from affected areas in Southeast Asia on March 18, 2003. On arrival, posters directed passengers to pick up health information about symptoms and signs of SARS and advised them to consult a physician if a SARS-like illness developed after their arrival in Canada. This information was printed in several languages on conspicuous, yellow, 8 ½" x 11" paper (referred to as "yellow cards") and contained key telephone numbers.

The initial posters and yellow HANs were placed at arrival sites in the Vancouver International Airport and Toronto's Pearson International Airport. They were quickly made available in 12 other airports that received international passengers who might have traveled from the Far East. HANs were provided to inbound passengers at 18 land border crossings between the United States and Canada. No record was kept of how many passengers picked up HANs.

With the advent of SARS transmission in Toronto, Health Canada implemented similar HANs in a different color (cherry) to mitigate the risk of exporting SARS cases. The cherry-colored HANs were distributed to persons departing for international destinations from Toronto's Pearson International Airport. Passengers with symptoms or signs of SARS were asked to self-defer their travel. In these instances, Health Canada requested airlines to waive their policies on nonrefundable tickets, and while many did so, the refund and rescheduling policies and conditions were not uniform.

Screening Phase

Because of the continuing outbreak in Toronto, domestic spread in other affected countries in Southeast Asia, and international spread to other countries, Health Canada intensified its initial response by instituting both inbound and outbound passenger screening to identify persons with symptoms or signs compatible with SARS. All passengers were now required to obtain, read, and respond to questions on yellow or cherry HANs. Three questions were added to both HANs: Do you have a fever? Do you have one or more of the following symptoms: cough, shortness of breath, difficulty breathing? Have you been in contact with a SARS-affected person in the last 10 days? All passengers were required to circle "yes" or "no" responses. Their responses were verified either by customs officials (for inbound passengers) or by airline check-in agents (for departing passengers from Toronto Pearson Airport). Quality control checks (random sampling and spot checks of prescribed procedures) were instituted to ensure compliance by those responsible for verifying passenger responses. For example, during a 1-week period, 82% of departing passengers received a cherry card at check in, and 73% were questioned about their responses by the check-in ticket agent.

Secondary screening procedures were established for all passengers who answered yes to any of the questions. It was mandatory for any such passenger to be referred to a screening nurse who administered a standard in-depth questionnaire and protocol. The secondary screening protocol included reasons for assessment, symptoms present at time of assessment, oral temperature, and defined criteria for disposition. On the basis of the responses elicited in the protocol, a passenger was released or referred to a predetermined hospital for an in-depth medical evaluation.

In parallel to these measures, Health Canada initiated a pilot study on May 8, 2003, on the use of infrared thermal scanning machines to detect temperatures >38°C in selected international arriving and departing passengers at Vancouver's International and Toronto's Pearson International airports. Thermal scanning complemented other measures in the overall screening process by helping to triage the large volume of passengers who transit airports. Any passenger with an elevated temperature reading was referred to the screening nurse for confirmation, completion of the screening protocol, and referral to hospital, if necessary.

Special Measures

Passenger Contact Tracing

With previous documentation of transmission of tuberculosis on long flights (4,5), Health Canada initiated passenger contact tracing to identify any secondary transmission associated with air travel. Health Canada's protocols for airplane passenger contact tracing evolved throughout the SARS outbreak and were updated as new information became available. From March 13 to March 21, 2003, contact tracing of passengers included follow-up of passengers seated in the same row, 2 rows in front, and 2 rows behind someone with a probable case who was symptomatic while in flight. As of March 22, airplane passenger contact tracing was expanded to include persons with suspected cases who were symptomatic while in flight. As of March 31, contact tracing was expanded again to include all passengers on a given flight with a probable or suspected case who were symptomatic while in flight (6).

Because of the lack of internationally accepted standards for developing and retaining passenger manifests, Health Canada personnel encountered excessive delays in obtaining the manifests from various airlines. In response, Health Canada initiated a traveler contact information form that collected location information and that all inbound passengers were required to complete before arrival. Upon landing, all forms were collected from passengers by Health Canada personnel and retained for possible contact tracing if a case was subsequently identified. The traveler contact information form reduced the time for securing the manifest from weeks to 2 days.

All screening measures (HANs, thermal screening, and traveler contact information form) continued after July 5, 2003, when WHO declared that SARS outbreaks had been contained worldwide. This report only includes data up to that date, when international movement of SARS was a real possibility.

Results

No attempt was made to evaluate the initial information phase. Data were collected for the screening phase. Table 1 summarizes the screening results for inbound and outbound HAN screening measures. As of July 5, 2003, a total of 1,172,986 persons received either yellow or cherry HANs. A total of 2,889 persons answered yes to at least 1 screening question on the HAN and were referred to

Table 1. Air passenger screening results for HAN* given to passengers on outbound and inbound flights, May 14–July 5, 2003

Measure	Location	Cumulative results			Comment
		Persons given HAN (n)	Persons referred (n)	Final disposition	
HAN outbound	Toronto	495,492	411	All cleared	All international flights departing from Toronto required to use cherry-colored HAN
HAN inbound	Toronto	349,754	1,264	All cleared	All international flights arriving in Toronto (70+ airlines) and Vancouver (100+ airlines) required to use yellow-colored HAN
	Vancouver	115,227	669	All cleared	
	Other	212,513	545	All cleared	
Total		1,172,986	2,889	All cleared	

*HAN, health alert notice.

secondary screening according to protocol. None of the 411 outbound passengers who were referred for secondary screening in Toronto were asked to defer their travel.

All persons were cleared, and none were referred for additional medical examination. In addition, 763,082 persons (467,870 inbound and 295,212 outbound) were screened by the thermal scanners (Table 2). Only 191 persons had an initial temperature reading >38°C and were referred for secondary evaluation. No data were collected systematically to correlate thermal scanner results with results of temperature taking by secondary screening nurses. Some of the persons arriving or departing Toronto and Vancouver airports were screened by both HAN and thermal scanning measures.

During this period, no screening measure put in place by Health Canada detected any cases of SARS at border entry points. Careful analysis of the travel histories of suspected and probable SARS patients who traveled to Canada showed that persons became ill after arrival and would not have been detected by airport screening measures.

Table 3 summarizes the travel histories of persons departing Canada whose ilnesses were subsequently diagnosed as SARS-like illness. Health Canada collaborated with many international public health authorities to document travel and illness histories of possible SARS patients who departed Canada and whose illneses were diagnosed and reported internationally (7–9). Health Canada investigated >40 such reports, of which 11 are now attributed to Canada (10). In all but 2 cases (cases 2 and 11), onset of illness occurred after departure from Canada. Of these 11 persons who traveled from Canada, all met the WHO probable SARS case definition. Only 3 of these case-patients met the Canadian probable case definition. Another 3 case-patients would meet the Canadian geo-linked case definition; 1 case met the Canadian "person under investigation" category; and 4 case-patients did not meet any Canadian SARS case definition. Of the 3 case-patients who did meet the Canadian definition, none would have been detected by exit screening. Only 2 (patients 2 and 11) of the 11 persons had symptoms at the time of travel, but both would have been cleared by the criteria established in the secondary screening protocol.

We identified 18 symptomatic probable or suspected SARS patients on 29 flights (10 patients traveled on ≥2 flights). No documented transmission was identified. Detailed results of Canada's airplane passenger contact tracing can be found elsewhere (6).

Discussion

Patterns of international travel continue to increase in complexity and volume. In Canada, >18 million persons enter annually by air; 91% arrive at 6 international airports. Similarly, a large number depart from several international airports. Additionally, because of an open land border with the United States, ≈100 million persons cross the land border in both directions annually.

With travel to Canada from anywhere in the world taking <24 hours, the possibility of detecting a dangerous infectious disease at border points of entry is challenging. Given the relatively short travel time, detecting persons at the border who are incubating any of the known infectious disease pathogens is unlikely. The absence of symptoms or signs of infection and a corresponding lack of specific,

Table 2. Air passenger screening results for thermal imaging scanners, May 16–July 5, 2003

Location	Measure	Cumulative results		
		Persons scanned (n)	Persons referred (n)	Final disposition
Toronto	Inbound	355,532	83	All cleared
	Outbound	281,959	94	All cleared
	Subtotal	637,491	177	All cleared
Vancouver	Inbound	112,338	12	All cleared
	Outbound	13,253	2	All cleared
	Subtotal	125,591	14	All cleared
Total		763,082	191	All cleared

Table 3. Travel histories of persons departing from Canada in whom a SARS-like illness was subsequently diagnosed*

Case no.	Age	Sex	Depart date, Toronto	Onset of illness	Probable SARS†	Link‡	Remarks
1	3	F	March 28	March 31	Yes	No	PCR negative for SARS-CoV
2	5	M	March 28	March 25	Yes	No	No evidence of pneumonia, PCR negative for SARS-CoV
3	1	M	March 28	April 1	Yes	No	No evidence of pneumonia, PCR negative for SARS-CoV
4	26	F	March 31	April 3	Yes	No	PCR and serologic test results negative for SAR-CoV
5	52	M	April 1	April 3	Yes	Yes	Traveled by car, PCR and serologic test results positive for SARS-CoV
6	46	F	April 3	April 6	Yes	Yes	Fatal SARS case
7	24	M	April 28	April 30	Yes	No	Serologic and PCR test results negative for SARS-CoV
8	28	F	April 24	May 3	Yes	No	No laboratory results available
9	29	M	May 10	May 13	Yes	No	Traveled by car, PCR negative and acute serologic test results negative for SARS-CoV (convalescent-phase serologic test results not available)
10	47	M	May 17	May 24	Yes	Yes	Acute–phase serologic test results positive for SARS-CoV
11	25	M	July 14	July 7	Yes	No	Laboratory results unavailable

*SARS, severe acute respiratory syndrome; F, female; M. male; SARS-CoV, SARS-associated coronavirus; PCR, polymerase chain reaction.
†Met the World Health Organization probable SARS case definition at time of illness.
‡Is an epidemiologic link to another SARS case established?

extremely rapid, easy-to-use diagnostic tests make border detection of infectious diseases unlikely.

The effectiveness of screening measures for detecting SARS cases at border points of entry was limited by 2 factors. First, screening measures themselves, i.e., HAN questionnaires and thermal scanning machines, were nonspecific for SARS. Second, the prevalence of SARS among international passengers arriving or departing from Canada was low. For example, 5 SARS patients entered Canada from March through May. None of these patients had signs or symptoms during transit through airports. If the same rate of entry were to continue for 1 year, then 20 cases might be expected among the 18 million persons entering the country annually, for a prevalence of ≈1.1 SARS cases per 1 million passengers. For such a rare disease, the positive predictive value of a positive screening result is essentially zero. The results demonstrate that available screening measures are not effective for detecting SARS. Despite extending screening measures to all arriving air passengers, no SARS cases were identified. These findings raise questions about whether such measures are effective tools for detecting and controlling the spread of SARS, and whether, from a public health point of view, other, more effective, strategies might exist.

Instituting infectious disease screening procedures at border points of entry could have advantages. For example, easily visible measures, such as thermal scanning machines, may generate a sense of confidence or reassurance that disease will be detected and prevented from entering the country. No data are available to assess whether or not the measures implemented at the airports actually generated confidence or reassurance in the public. Given the poor positive predictive value of available SARS screening measures, any sense of reassurance might be quickly dispelled when the first case is detected in spite of screening measures.

We conclude that available screening measures for SARS were limited in their effectiveness in detecting SARS among inbound or outbound passengers from SARS-affected areas. We suggest that in-country, acute-care facilities (hospitals, clinics, and physicians' offices) are the de facto point of entry into the healthcare system for travelers with serious infectious diseases. If a visitor or returning citizen becomes ill after arriving in Canada, he or she will likely seek medical care in clinics or emergency rooms. Acute-care facilities must consider travel histories of all patients with suspected infectious diseases and implement standard precautions and infection control measures.

An estimated Can$7.55 million was invested in airport screening measures from March 18 to July 5. Rather than investing in airport screening measures to detect rare infectious diseases, investments should be used to strengthen screening and infection control capacities at points of entry into the healthcare system. Additional useful measures could focus on public education about infectious disease prevention and care.

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Capacity of State and Territorial Health Agencies To Prevent Foodborne Illness

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The capacity of state and territorial health departments to investigate foodborne diseases was assessed by the Council of State and Territorial Epidemiologists from 2001 to 2002 with a self-administered, Web-based survey. Forty-eight health departments responded (47 states and 1 territory). The primary reason for not conducting more active case surveillance of enteric disease is lack of staff, while the primary reasons for not investigating foodborne disease outbreaks are limited staff and delayed notification of the outbreak. Sixty-four percent of respondents have the capacity to conduct analytic epidemiologic investigations. States receiving Emerging Infections Program (EIP) funding from the Centers for Disease Control and Prevention more often reported having a dedicated foodborne disease epidemiologist and the capability to perform analytic studies than non-EIP states. We conclude that by addressing shortages in the number of dedicated personnel and reducing delays in reporting, the capacity of state health departments to respond to foodborne disease can be improved.

Foodborne illnesses are common. Each year an estimated 76 million foodborne illnesses occur, with 325,000 hospitalizations and 5,000 deaths (1), and a recent estimate of annual costs for medical treatment, productivity loss, and premature deaths resulting from these illnesses is $6.5 billion (2). The National Food Safety Initiative (NFSI) was started in 1997 as an effort to decrease the incidence and risk for foodborne illness (3). The NFSI ended in 2001, but at the Centers for Disease Control and Prevention (CDC), the former NFSI funding and activities have been institutionalized as an ongoing food safety program. Continued progress on the part of regulators and industry to improve food safety are dependent on local, state, and federal agencies' ability to conduct epidemiologic and laboratory investigations that identify the offending agents and link them with specific foods.

Improvements in detecting and investigating foodborne illnesses were made during the 1990s when CDC implemented the Foodborne Diseases Active Surveillance Network (FoodNet), a component of the Emerging Infections Programs (EIP), and PulseNet (4,5). EIP is a network of epidemiology programs in state health departments that is funded and coordinated by CDC. It is intended to be a national resource for surveillance and epidemiologic research that goes beyond the routine public health department functions. Active, laboratory-based surveillance is the foundation of 2 core EIP projects conducted at all sites: Active Bacterial Core Surveillance and Foodborne Disease Active Surveillance. Ten states currently receive EIP support from CDC. PulseNet, unlike EIP, is intended to be a national molecular subtyping network for foodborne disease surveillance. It was established by the CDC in 1996 to facilitate subtyping bacterial foodborne pathogens by state health department laboratories. Even after implementing FoodNet and PulseNet, much work remains to improve the state and local public health agencies' capacity to detect and investigate foodborne disease.

In 1999, CDC provided funding to both Council of State and Territorial Epidemiologists (CSTE) and the Association of Public Health Laboratories (APHL) to conduct assessments of states' foodborne disease investigation capacity. The purpose of both assessments was to determine priorities for improving food safety program support. The CSTE assessment was intended to concentrate

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primarily on state and territory health departments' capacity to monitor and investigate foodborne illness. This report presents the results of the CSTE survey, which was conducted from October 2001 to March 2002, of 48 state and territorial health agencies,

An expert CSTE committee, composed of state and local epidemiologists from Colorado; Philadelphia; and Los Angeles County, California; an environmentalist from DeKalb County, Georgia; a state laboratorian from Rhode Island; staff from the CDC's National Center for Infectious Disease, Division of Bacterial and Mycotic Diseases-Food Safety Office; and CSTE staff from its national office developed a survey instrument that was pilot-tested in 6 states and subsequently revised. The final instrument consisted of 106 questions. We present analyses of selected questions; a complete tabulation of all results and display of the questionnaire are available from the CSTE website (6). The data can be used as a baseline reference for future surveys of state and territorial capacity to investigate foodborne disease.

Methods

The assessment instrument was a self-administered, Web-based survey. Respondents were state and territorial epidemiologists with knowledge in the area of foodborne diseases. The assessment was conducted from October 2001 through February 2002, and during the 5-month survey period, reminder telephone calls and emails were made from the CSTE national office to health agencies that had not yet responded.

The instrument's 106 questions covered background information about the responding agency, epidemiologic surveillance capacity to identify sporadic and outbreak-related illnesses; capacity to investigate and respond to outbreaks; public health infrastructure to support food safety activities, defined as staffing, facilities, equipment, supplies, information, communication between epidemiology and laboratory units, and education and training of staff; and legal authority of the agency. We restricted results in this article to questions pertaining to agency capacity and operations, barriers to the investigation of foodborne diseases, and staffing of the epidemiology program (a subset of "barriers").

Forty-eight health departments responded (47 states [response rate = 94%] and 1 territory [Guam]); Pennsylvania, Illinois, Nevada, and Puerto Rico did not submit responses. Some questions did not elicit 48 responses. Responses reflect the perspective of the epidemiology program in the agency. The frequency and percentage for each response were calculated on the basis of the total number of responses to that question. Percentages are rounded to the nearest integer. The phrasing of questions in tables in the Results section has, in some instances, been shortened from the exact words used in the questionnaire.

We also examined responses by whether the responding agency received EIP funding from CDC (8 of 9 EIP sites that were funded at the time responded to the survey: Colorado, Connecticut, Georgia, New York, Minnesota, Oregon, Tennessee, and California/San Francisco Bay) and whether the responding agency was a jurisdiction with large population (10 largest population states in 2000 U.S. census; number of respondents = 8; population range 33,871,648–8,186,453), medium population (states ranked 11th to 20th in population in 2000 census; number of respondents = 10; population range 8,049,313–5,130,632), or small population (the remaining states and 1 territory; number of respondents = 30; population range 4,919,479– 154,805). The term "small population states" includes 29 states and 1 territory (Guam). The 8 responding EIP sites included 3 large, 1 medium, and 4 small population states.

Results

Forty percent of the states receive laboratory reports electronically. The primary reason reported for not conducting more active case surveillance is lack of staff. The primary reasons reported for not investigating foodborne disease outbreaks are limited staff and delayed reporting of the outbreak. Sixty-four percent of respondents have the capacity to conduct analytic epidemiologic investigations. Thirty-five percent of respondents have a protocol to guarantee chain of custody for food specimens. Eighty-one percent of respondents can obtain public health laboratory, environmental health, and sanitation support 24 hours per day. Fifty-four percent of respondents have broadcast fax or email capability to hospital emergency rooms and to physicians (Tables 1–3).

We did not find that EIP sites always reported more capacity and more advanced operations than non-EIP sites. A greater percentage of EIP sites than non-EIP sites reported adequate capacity to conduct analytic epidemiologic studies (88% vs. 59%) and having a regulation or statute specifically requiring the submission of certain enteric isolates to the public health laboratory (75% vs. 50%). On the other hand, a smaller percentage of EIP sites than non-EIP sites reported having the capacity to broadcast faxes to hospital emergency departments (50% vs. 55%) and to conduct syndromic surveillance for diarrheal disease (0% vs. 18%). The percentage of EIP sites having a protocol to guarantee chain of custody for food environmental specimens was nearly the same as for non-EIP sites (38% vs. 36%).

Likewise, we found that large population states did not consistently have more capacity and more advanced operations than medium or small population states or

Table 1. Capacity and operations of epidemiologic, laboratory, and environmental programs in state and territorial health departments

Question	n	% yes	% no	% not sure
Does the Epidemiology Office have the ability to receive electronic laboratory reporting of enteric diseases?	48	40	60	
Do you routinely collect stool samples for testing?	47	98	2	
Do you routinely collect vomitus for testing?	47	38	62	
Do you have broadcast fax or email capability to: (list all that apply)	48			
Other health departments within the state		88		
Hospital infection control specialists		77		
Hospital emergency rooms		54		
Physicians		50		
Other state health departments		40		
Other		19		
Do you conduct syndromic surveillance for diarrheal disease?	48	15	77	8
Do you have adequate capacity to conduct an analytic epidemiologic investigation, i.e., case-control or cohort studies?	47	64	30	6
Does your agency have legal authority to:				
Collect reports of suspected enteric diseases?	48	90	4	6
Perform on-the-spot emergency environmental/sanitation inspections?	48	85	4	10
Exclude sick or infected employees from food handling duties?	48	83	13	4
Share information related to foodborne outbreaks with federal agencies, e.g., USDA, FDA, and CDC?*	47	83	4	13
Close a food service facility?	48	81	15	4
Collect reports of clinical syndromes?	48	71	19	10
Embargo or condemn food?	47	66	11	23
Is there a regulation/statute specifically requiring submission of certain enteric isolates to the public health laboratory?	48	54	38	8
Does the department of health have a protocol to guarantee chain of custody for food environmental specimens?	48	35	48	17

*USDA, United States Department of Agriculture; FDA, Food and Drug Administration; CDC, Centers for Disease Control and Prevention.

territories. Seventy-five percent of large states, 90% of medium states, and 52% of small population states reported adequate capacity to perform analytic epidemiologic studies. Thirty-eight percent of large states, 30% of medium, and 67% of small states reported the capacity to broadcast fax to hospital emergency departments. The differences between state size and having a chain of custody protocol for food specimens were relatively small (50% of large, 40% of medium, and 30% of small population states), while the differences in percentage reporting a legal requirement to submit certain enteric isolates to the public health laboratory were relatively large: 38% of large states, 70% of medium states, and 53% of small states.

As for factors that limit ability to investigate outbreaks, the most common reason given by both EIP and non-EIP sites was "delayed notification" (88% vs. 83%). The percentage of EIP sites and non-EIP sites reporting "limited staff" (63% vs. 68%) and "lack of importance" (50% vs. 45%) were similar. Delayed notification was the most frequent reason given by large (75%), medium (100%), and small (80%) population states for not investigating outbreaks. Seventy percent of small states compared to 70% of medium states and 50% of large states reported limited staff as a reason for not investigating outbreaks.

Seventy-two percent of EIP sites versus 83% of non-EIP sites reported having laboratory support 24 hours per day, whereas 75% of EIP sites compared to 43% of non-EIP sites reported having a dedicated enteric/foodborne epidemiologist. For these same two questions, 73% of small population states versus 100% of large and 90% of medium states had laboratory support 24 hours per day, and 75% of large and 80% of medium states had a dedicated enteric/foodborne disease epidemiologist compared to 30% of small population states. Lastly, during outbreaks, 100% of EIP sites versus 68% of non-EIP sites reported that they had enough people to enter data. For this question, the differences between large, medium, and small population states were relatively small (88%, 70%, 70%, respectively).

Discussion and Conclusion

In the United States, the primary responsibility for foodborne disease surveillance and investigation lies with state, territorial, and local health agencies, with technical backup and funding support from CDC and other federal agencies, including the Food and Drug Administration and the Food Safety and Inspection Service of the U.S. Department of Agriculture. Within a state public health agency, reducing the incidence of foodborne disease requires a sensitive surveillance system, timely epidemiologic investigation of sporadic cases and outbreaks with the most current laboratory technologies, and coordina-

Table 2. Barriers to investigating foodborne disease in state and territorial health departments

Question	n	% yes	% no	% not sure
Of the outbreaks that are not investigated, which factors most limit your ability to investigate? (list all that apply)	48			
Delayed notification		83		
Limited staff		67		
Lack of apparent importance		46		
Laboratory capacity		21		
Jurisdictional issues		19		
Political consideration		13		
Expertise		13		
Other		13		
Travel policy constraints		11		
Statistical support		8		
Ability to pay overtime		8		
In outbreaks in which food specimens were not submitted, what were the barriers to laboratory testing?	47			
Leftovers not available		98		
Wrong food collected		32		
Unnecessary		17		
Other		13		
No capability for food testing, i.e., laboratory equipment		11		
Insufficient expertise at laboratory		6		
Too expensive		4		
Do you feel there are barriers for conducting more active case surveillance?	48	88	8	4
If yes, which of the following reasons apply: (list all that apply)	42			
Lack of staff		81		
Too time-consuming		60		
Other		33		
Low priority		29		
Lack of expertise		12		

tion of epidemiology, environmental, and laboratory programs.

This report is the fourth in a series by CSTE to assess epidemiologic capacity in state and territorial health departments. The 3 previously published surveys concerned overall capacity, maternal and child health capacity, and chronic disease capacity (7–10). While infectious disease capacity was addressed in the overall survey conducted from November 2001 through April 2002, this report is the most detailed analysis of states' and territories' foodborne disease capacity to date. The findings in the overall capacity report concerning reasons why outbreaks were not investigated by the state health department are similar to findings in our report: of 42 respondents 40 (95%) reported delayed notification of case reports, 33 (79%) reported limited staff, and 31 (74%) reported competing priorities for use of public health resources (7).

In the areas of foodborne disease surveillance and investigation, our report documents that the aggregate perception of a large sample of epidemiologic leaders in state and territorial health departments is that, as of 2002, more resources were needed. The data are self-reported and do not include responses from a few large states and Puerto Rico. The survey found that lack of staff was the most frequent reason (81% of respondents) for not conducting more active case surveillance, and the most frequent reasons given for not investigating outbreaks were delayed notification (83%) and limited staff (63%) (Table 2).

Our findings are also consistent with a 50-state survey conducted by the General Accounting Office in 2000 to 2001 (2). That survey found, for example, 32 (64%) of 50 states indicated that more trained epidemiologists were needed at the state level to investigate outbreaks, and 44 (88%) of 50 states indicated that more trained epidemiologists were needed at the local level to investigate outbreaks.

If a state or territory had more epidemiologists to conduct surveillance, fewer delays would likely occur in recognition of outbreaks, and more expertise would be available to conduct investigations. Thus, by addressing shortages in the number of dedicated personnel and reducing delays in reporting, the capacity of state health departments to respond to foodborne disease can be improved.

We also performed comparisons of EIP to non-EIP sites and of large, medium, and small population states. Only 8 of 10 possible large population states and 8 of 9 EIP states were included, so the analyses must be interpreted cautiously. Because these comparisons were conceived after the survey data had been collected, we did not perform analytic statistical tests, which could be misinterpreted.

Table 3. Staffing in epidemiology programs for foodborne disease surveillance and investigation

Question	n	% yes	% no	% not sure
For sporadic cases, do you have enough people to:	48			
Compare to standardized case definition		85	15	
Enter data		79	19	2
Review data for consistency and completeness		71	27	2
During outbreaks, do you have enough people to:	48			
Compare to standardized case definition		90	10	
Enter data		73	19	8
Review data for completeness and consistency		71	23	6
In your enteric/foodborne disease epidemiology program, do you have sufficient statistical support?	47	45	47	9
Do you have a dedicated enteric/foodborne disease epidemiologist at your agency?	48	48	50	2
If yes to question above, what is the highest level of education of the epidemiologist?	23			
Masters degree		61		
Doctoral degree		26		
Bachelor degree		13		
During an outbreak investigation, do epidemiologists routinely accompany environmental health/sanitation specialist(s)?	48	44	50	6
Is there a 24-hour on-call response mechanism for foodborne disease issues?	48	96	4	
Can you get public health laboratory support 24/7/365?	48	81	15	4
Can you get environmental health/sanitation support 24 hours per day?	48	60	23	17
Do your epidemiologists receive training in environmental food facility inspections?	48	13	85	2
Do your environmental health/sanitation specialists receive training in epidemiology?	48	63	33	4

Our descriptive findings are presented for interest and generation of hypotheses. We observed that EIP sites more frequently stated they had a dedicated foodborne disease epidemiologist, the capacity to perform analytic epidemiologic studies, and sufficient personnel to enter data during an outbreak than non-EIP sites. These findings would be expected, however, because 1 of the 2 core EIP projects is FoodNet. In other measures of capacity and program structure not specifically funded by the EIP programs, such as on-call laboratory support, not much difference existed between EIP and non-EIP sites.

The findings of this report do not indicate the quantity of resources needed to ensure sufficient capacity to protect the nation, and the survey results do not direct the allocation of new resources. One approach to this issue would be to assess the reported incidence of enteric disease and foodborne outbreaks with respect to self-reported capacity to monitor and investigate foodborne disease. However, the nation's system for identifying, investigating, and reporting foodborne diseases has not produced consistent and reliable data of adequate quality to perform such analyses. For example, in 1997, a total of 27 states and 3 territories reported zero outbreaks (10). More outbreaks must have occurred than were reported. Whatever the various reasons for such underreporting, the existing surveillance data are insufficient for addressing programmatic issues, such as where to invest in the public health system and what improvements in public health may reasonably be expected from such investment. Nevertheless, analyses are not needed to justify that every state and territory needs 24 hours per day epidemiologic, laboratory, and environmental health and sanitation on-call response capacity, as well as the capacity to communicate with public health and medical care providers, policymakers, and the public.

The analyses in this report provide a picture of the status of the nation at a time just before the distribution in 2002 of more than $1 billion to state, territorial, and local health agencies to improve bioterrorism response and preparedness capacity. Several criteria exist for the mitigation of foodborne illness listed in the bioterrorism preparedness cooperative agreement award notice and grant guidelines (Procurement and Grants Office, CDC, Announcement No. 99051). For example, having a formal outbreak investigation team is an illustration of focus area A (preparedness planning and readiness) of the bioterrorism preparedness cooperative agreement criteria; 70% of the respondents reported having this capacity. One of the guidelines in focus area G (education and training) is financial support by the state health agency for enteric disease and foodborne illness continuing education; more than half of the respondents in this survey reported that their agency provides this financial support. Although only 54% of states and territories reported that they could send broadcast faxes of health information to emergency departments, this particular capacity is a high priority for bioterrorism preparedness and is almost certain to have been further improved since the survey was completed.

In addition to the food safety minimum performance and capacity standards for epidemiology and surveillance adopted by CSTE as a position statement in 2003 (11), we recommend that for the short-term, objective measures of foodborne disease surveillance, reporting, and investigation

be developed by local, state, and federal agencies. For example, the intervals from enteric disease onset until the case is reported to CDC may be measured in each state agency. Such measures can be used to indicate areas of need, to document areas of improvement, and to support the appropriation of new funds and the allocation of resources in lieu of enteric disease incidence.

This survey and the surveys of overall, maternal and child health, and chronic disease epidemiologic capacity demonstrate a need for a larger workforce of epidemiologists. In response to the surveys, CSTE convened a workforce summit of leaders from within the CSTE organization, CDC, the Association of State and Territorial Health Officers, the American Public Health Association, and the Association of Schools of Public Health in January 2004 (12). In addition, at its annual meeting held in June 2004, the CSTE membership approved a resolution calling for an annual National Epidemiologist Awareness Day to bring attention to the work of epidemiologists in protecting the nation's health (12). While this report and the mentioned activities of CSTE are specific to disease prevention by states and territories in the United States, similar capacities may be needed by public health agencies in other regions of the world, such as the European Union and the WHO Global Salm-Surv programme. We hope that the survey design and the results will provide guidance and comparisons for readers in other countries.

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Hepatitis C Virus Infection, Linxian, China[1]

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Bloodborne viruses may have spread in rural China during the past 25 years, but population-based prevalence estimates are lacking. We examined the frequency of hepatitis C virus (HCV) and HIV type 1 (HIV-1) among residents of Linxian, a rural community in Henan Province. In 2000, blood was collected from participants (≥55 years of age) who had enrolled in a population-based nutritional intervention trial in 1985. We randomly selected 500 participants for HCV testing and 200 participants for HIV-1 testing. For HCV, 48 (9.6%) of 500 participants were positive by enzyme immunoassay and recombinant immunoblot assay (95% confidence interval, 7.0%–12.2%), and prevalence was lowest in the most geographically isolated participants. Among the HCV-infected participants, 42 had a specimen available from 1985, of which 16 (38.1%) were positive for HCV. For HIV-1, 0/200 participants were positive. We conclude that HCV is now a common infection among older adults in Linxian, China.

Hepatitis C virus (HCV) infection is becoming a global public health problem (1). The overall prevalence of HCV infection is 1% to 2% in most countries that have been studied (2), but the distribution of HCV varies considerably among populations. HCV is most frequently transmitted by percutaneous exposure to infectious blood or blood-derived body fluids, such as through transfusion of contaminated blood or blood products, nonsterile medical injections, or injection drug use. Very high rates of HCV infection are found among persons exposed to HCV through these routes (3). In a nationwide study conducted in 1992, HCV prevalence was reported to be 3.2% in China overall and 3.1% in rural China (4). Other smaller studies have reported HCV prevalence rates of 0% to 3% in rural populations from various Chinese provinces (5–7).

HIV type 1 (HIV-1) infection has been reported among paid blood donors in rural east central China (8–10). Because both HIV-1 and HCV are bloodborne, these reports raise the possibility that HCV may be common in this area. To date, population-based rates of bloodborne viral infections in this region are lacking. To provide such estimates, we used specimens collected as part of a population-based study in Linxian, Henan Province, a rural county in central China where farming is the predominant occupation (Figure).

In 2000, surviving participants of a population-based nutritional intervention trial were resurveyed. The study had been initiated to study the effect of dietary supplements on the risk of esophageal and gastric cancer, which occur at very high rates in this region. Participants were healthy adults from 4 Linxian communes who were 40–69 years of age when enrolled in 1985. We used specimens collected in this study to estimate the prevalence of hepatitis B virus (HBV), HCV, and HIV-1 among older adults in Linxian in 2000.

Methods

Participants and Samples

In 1985, all healthy adults aged 40–69 years from 4 Linxian communes (Yaocun, Rencun, Donggang, and Hengshui) were invited to participate in the Linxian Nutritional Intervention Trial (NIT). NIT is a population-based trial designed to prevent esophageal and gastric cancer, the most common cancers in the Linxian region. Of ≈53,000 potentially eligible participants, 16% refused participation, 12% were not living in Linxian at the time due to temporary employment, 4% were excluded for medical reasons, and 8% did not join the trial for other reasons (11). The remaining 32,902 members of the Linxian population participated in the trial. In 1985, 1 year before the start of

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Figure. Eastern part of China showing the locations of Linxian and Henan Provinces. Adapted from www.Expedia.com.

the intervention, each participant was interviewed, was given a brief physical examination, and had blood drawn.

In 2000, all 23,910 surviving participants were invited to take part in a follow-up survey; 16,488 participants were then resurveyed and had blood samples collected. Analysis of the cause of death from 1985 to 2001 indicated that most deaths were due to esophageal or gastric cancer (28%), heart disease (21%), or stroke (31%). About 2% of deaths were due to cirrhosis and another 2% were due to liver cancer. Higher death rates among men and older participants shifted the demographic pattern among survivors compared to original enrollees. For example, men comprised 40% of participants in 2000 and 44% in 1985. No difference in the distribution of commune of residence was found between survivors and all enrollees.

Ten milliliters of blood was collected in vacutainers that contained sodium heparin; the samples were stored at room temperature and transferred within 3 hours to a field station for processing. Aliquots of plasma were stored at –85°C until they were shipped on dry ice to a biospecimen repository in Frederick, Maryland. Participants in the 2000 blood collection survey received the results of their blood samples, including hematocrit and liver function tests. The study protocol was approved by institutional review boards at the U.S. National Cancer Institute and the Cancer Institute of the Chinese Academy of Medical Sciences.

For HBV and HCV testing, we selected at random 500 NIT participants who were resurveyed in 2000. In addition, we tested available specimens that had been drawn in 1985 among participants who we found to be positive for anti-HCV by recombinant immunoblot assay (RIBA) in 2000. We also randomly selected another 200 NIT participants from participants resurveyed in 2000 for HIV-1 antibody testing. Because HIV-1 testing was not within the scope of the original study, we removed identifying information from these 200 samples and tested them anonymously.

Laboratory Assays

We tested the plasma specimens for HCV antibody by HCV Version 3.0 enzyme immunoassay (EIA) (Ortho Diagnostics, Raritan, NJ) according to the manufacturer's instructions. Samples that were positive by HCV enzyme immunoassay EIA were confirmed by HCV Version 3.0 RIBA (Ortho Diagnostics). Because the specimens had been collected in sodium heparin, which inhibits the polymerase chain reaction (PCR), we could not confirm the presence of HCV by PCR-based assays. As an alternative, we tested specimens for HCV core antigen (Trak-C, Ortho Diagnostics). HCV core antigen is a marker of chronic infection and the analytic sensitivity of the Trak C assay (Ortho Diagnostics) is similar to that of PCR-based assays for HCV RNA (12). The 500 samples were also tested for antibody to HBV core antigen (anti-HBc) by an HBc ELISA (Ortho Diagnostics). Samples that were positive for anti-HBc were tested for HBV surface antigen (HBsAg) by EIA (Bio-Rad, Redmond, WA). A separate group of 200 samples was tested for HIV-1 antibody by EIA (Genetic Systems rLAV EIA, Bio-Rad).

Statistical Methods

Chi-square analysis was used to compare the prevalence of viral infections among participants with different demographic characteristics. The 95% confidence interval (95% CI) of prevalence was calculated based on normal approximation to binomial distribution when that approximation holds. For rare events, the 95% CI was based on the Poisson approximation to the binomial distribution. All statistical analyses were done with the Statistical Analysis System version 8.0 (SAS, Cary, NC).

Results

Demographic characteristics of the 500 participants tested for HCV are summarized in the Table. In 2000, more than half of the participants were 55–64 years of age (54.2%), 34.4% were 65–74 years, and 11.4% were 75–84 years. This age distribution reflects that participants were aged 40–69 years when enrolled in 1985. Our study included 200 (40.0%) men and 300 (60.0%) women. One hundred seventy (34.0%) participants were from Yaocun, 95 (19.0%) from Rencun, 116 (23.2%) from Donggang, and 119 (23.8%) from Hengshui.

Of the 500 participants studied, 63 (12.6%) were positive for HCV antibody by EIA. When the specimens from these 63 participants were tested by HCV RIBA, 48 were positive, 7 were indeterminate, and 8 were negative. Therefore, the estimated prevalence of HCV infection (defined as positive by both HCV EIA and HCV RIBA) in

Table. Prevalence of anti-HCV (by RIBA), HBsAg, and HBcAb in Linxian in 2000*

	N (%)	Anti-HCV % (95% CI)	p	HBcAb % (95% CI)	p	HBcAb and HBsAg % (95% CI)	p
Overall		9.6 (7.0–12.2)		54.6 (50.2-59.0)		6.4 (4.3-8.5)	
Age in 2000 (y)							
≤59	166 (33.2)	8.4 (4.2–12.7)		53.6 (46.0–61.2)		5.4 (2.0–8.9)	
60–64	105 (21.0)	13.3 (6.8–19.8)		50.5 (40.9–60.0)		6.7 (1.9–11.4)	
65–69	90 (18.0)	11.1 (4.6–17.6)		50.0 (39.7–60.3)		4.4 (0.2–8.7)	
70–74	82 (16.4)	6.1 (0.9–11.3)		62.2 (51.7–72.7)		8.5 (2.5–14.6)	
≥75	57 (11.4)	8.8 (1.4–16.1)	0.50	61.4 (48.8–74.0)	0.34	8.8 (1.4–16.1)	0.73
Sex							
Male	200 (40.0)	9.5 (5.4–13.6)		58.0 (51.2–64.8)		5.0 (2.0–8.0)	
Female	300 (60.0)	9.7 (6.3–13.0)	0.95	52.3 (46.7–58.0)	0.21	7.3 (4.4–10.3)	0.18
Commune							
Yaocun	170 (34.0)	11.8 (6.9–16.6)		53.5 (46.0–61.0)		6.5 (2.8–10.2)	
Rencun	95 (19.0)	14.7 (7.6–21.9)		64.2 (54.6–73.8)		6.3 (1.4–11.2)	
Donggang	116 (23.2)	2.6 (0.0–5.5)		53.5 (44.4–62.5)		6.0 (1.7–10.4)	
Hengshui	119 (23.8)	9.2 (4.0–14.4)	0.02	49.6 (40.6–58.6)	0.18	6.7 (2.2–11.2)	0.50

*HCV, hepatitis C virus; RIBA, recombinant immunoblot assay; HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B core antibody; CI, confidence interval.

this population in 2000 was 9.6% (95% CI 7.0%–12.2%). We tested all 23 samples that were positive for HCV RIBA and had sufficient remaining plasma for HCV core antigen. HCV core antigen was found in 16 (69.6%) of 23 specimens.

The prevalence of HCV infection did not differ meaningfully by age or sex (Table), but varied significantly among the four communes. Donggang, the most geographically isolated commune, had a lower prevalence of HCV infection (2.6%) than Yaocun (11.8%, $p < 0.01$), Rencun (14.7%, $p < 0.01$), or Hengshui (9.2%, $p = 0.03$; $p = 0.02$, overall chi-square test). No other significant differences in HCV prevalence were found between the communes.

Among the 48 participants who were positive by HCV RIBA in 2000, 42 had specimens available from 1985 for HCV antibody testing. Of these 42, 16 (38.1%) were positive by both HCV EIA and HCV RIBA, indicating that most participants who were infected with HCV in 2000 had acquired the virus since 1985.

Antibodies to HBV core antigen (HBc), an indicator of past exposure to HBV, were found in 273 (54.6%) of 500 participants. The prevalence of anti-HBc did not vary significantly by age, sex, or commune (Table). Participants who were infected with HCV had a slightly higher prevalence of anti-HBc than those who were not (64.6% vs. 53.5%, $p = 0.14$). Participants who were positive for both anti-HBc and hepatis B surface antigen (HBsAg), a marker for chronic HBV infection, comprised 6.4% of the population. The prevalence of HBsAg differed relatively little by age, sex, or commune (Table).

A separate group of 200 participants was randomly selected for anonymous testing for antibodies to HIV-1. These participants included 77 men (38.5%) and 123 women (61.5%). Age as of the year 2000 was distributed as follows: ≤59 years, 78 (39.0%); 60–69 years, 76 (38.0%); ≥70 years, 46 (23.0%). Seventy-two participants (36.0%) were from Yaocun, 29 (14.5%) were from Rencun, 48 (24.0%) were from Donggang, and 51 (25.5%) were Hengshui. None tested positive for antibodies to HIV-1. The 95% CI for HIV-1 prevalence in this population was 0%–0.03%.

Discussion

In this population-based study of 55- to 84-year-old persons living in a rural area of Henan Province, HCV prevalence in 2000 was 9.6%. HCV was present in the Linxian population in 1985, but most of the HCV-infected participants in this study likely acquired the virus between 1985 and 2000. In contrast to the high rate of HCV infection, HIV-1, which also is transmitted through infected blood, was not found in this population. Evidence of resolved or chronic HBV infection, which is endemic in China, was found in about 55% of the participants.

The observed prevalence of HCV in Linxian is higher than in most previous population-based studies from China. In the nationwide cross-sectional study conducted in 1992, HCV prevalence was 3.10% in residents of rural China and 3.96% in the group aged 50 to 59 years, the eldest in that study (4). However, because HCV prevalence in the nationwide study was determined by HCV EIA alone, estimates from that study are higher than would have been obtained with confirmation by HCV RIBA. The RIBA-confirmed HCV prevalence in Linxian was, therefore, considerably higher than the national rate.

Around the world, the prevalence of HCV infection appears to be low in most populations (13–15), but areas of high prevalence have been found. Perhaps the most notable example is Egypt, where >15% of the population may be infected with HCV (16,17). Transmission of HCV occurred in Egypt from the 1960s through the 1980s when

a campaign against schistosomiasis involved mass parenteral injections, and unsterilized needles were used (17). Evidence also exists that HCV infection may have been transmitted in Egypt through other types of medical care (18). Iatrogenic transmission may have contributed as well to high rates of HCV infection that have been reported from Taiwan (19), Japan (20), and Italy (21).

We could not determine how HCV spread in Linxian because the nutritional trial did not collect information on potential exposure to contaminated blood. Widespread HCV infection in a population generally results from iatrogenic transmission or sharing of recreational drug injection equipment. HCV spreads rapidly among injection drug users, but, to our knowledge, reports of injection drug use in China are limited to younger age groups in border provinces and large cities (22,23), not older residents of inland provinces. It seems unlikely, therefore, that our participants acquired HCV infection through injection drug use. Transmission of HCV has been linked to paid blood and plasma donations in Central Chinese provinces, including Henan, during the 1980s and 1990s (24,25). Reuse of needles and equipment without proper sterilization and reinfusion of pooled red blood cells from multiple donors reportedly led to outbreaks of HCV (24,25). These reports, along with evidence from our study that many HCV-infected participants acquired the virus between 1985 and 2000, suggest that HCV may have been transmitted among the Linxian population through blood or plasma collection activities. Our finding of the lowest HCV prevalence (2.6%) in the most isolated commune seems consistent with this explanation, since geographic isolation may have limited the opportunity to contract HCV infection through these means.

Outbreaks of HIV-1 have been reported among paid plasma donors in central Chinese provinces, including Henan (8–10), but we found no evidence of HIV-1 infection among 200 randomly selected participants. Our analysis yielded an upper 95% CI of 0.03% for the prevalence of HIV-1 in this population, which indicates that HIV-1 infection is, at worst, extremely rare among older residents of Linxian. The absence of HIV-1 in the presence of a relatively high prevalence of HCV is not surprising because the entry of either virus into a community may depend to some degree on chance.

HBV infection is endemic in China (26), and most transmission occurs during the perinatal period when the risk of chronic infection is much higher than in adulthood. Among our participants, 54.6% had HBc, and 6.4% had both anti-HBc and HBsAg (which indicates chronic infection). Most or all of the participants who were chronically infected with HBV likely became infected early in life. More recent bloodborne transmission may have contributed some participants with resolved HBV infection, as suggested by the trend toward higher anti-HBc prevalence among participants who were also infected with HCV.

The accuracy with which our estimates reflect the prevalence of viral infections among older Linxian residents in the year 2000 depends on 2 factors: the test characteristics of the assays we used and how well our study population represents the target population. The third generation HCV EIA we used has high sensitivity and specificity. With confirmation of HCV EIA-positive samples by a highly specific RIBA, we are unlikely to have overestimated the prevalence of HCV antibodies among our participants. We have similar confidence in the assays that we used for HIV-1 and HBV testing.

For practical reasons, we used specimens that had been collected as part of a population-based research effort that began in 1985. The limitations of our approach for determining the prevalence of viral infections among Linxian residents in 2000 should be considered. First, the age criterion of the original study prevented us from ascertaining infection prevalence among persons <55 years of age. HCV prevalence may be higher or lower among younger residents of Linxian. Second, the participants may not represent older adults residing in Linxian in 2000 with regard to viral prevalence. About 60% of eligible residents enrolled in the trial in 1985 and about 70% of surviving enrollees participated in the 2000 follow-up. If surviving enrollees who were infected with one of the viruses that we studied were less likely to participate in the 2000 survey due to poor health, we may have underestimated the true prevalence of infection. However, usually a long period elapses from infection with these viruses to disease, and our results likely provide a reasonable estimate of viral prevalence among older residents of Linxian.

In summary, HCV is now common in this rural Chinese community, at least among its older residents. In contrast, we found no evidence of HIV-1 infection in the population. The public health impact of the high prevalence of HCV infection in Linxian may be substantial. HCV is an important cause of end-stage liver disease and hepatocellular carcinoma, and it can act synergistically with HBV infection (27), which is endemic in China. Future studies should examine the prevalence of bloodborne viruses in other parts of China, how these viruses are transmitted, and the resulting health effects. Efforts to halt the transmission of HCV and other bloodborne viruses in rural China should be a top public health priority.

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Multidrug-resistant *Acinetobacter baumannii*

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To understand the epidemiology of multidrug-resistant (MDR) *Acinetobacter baumannii* and define individual risk factors for multidrug resistance, we used epidemiologic methods, performed organism typing by pulsed-field gel electrophoresis (PFGE), and conducted a matched case-control retrospective study. We investigated 118 patients, on 27 wards in Israel, in whom MDR *A. baumannii* was isolated from clinical cultures. Each case-patient had a control without MDR *A. baumannii* and was matched for hospital length of stay, ward, and calendar time. The epidemiologic investigation found small clusters of up to 6 patients each with no common identified source. Ten different PFGE clones were found, of which 2 dominated. The PFGE pattern differed within temporospatial clusters, and antimicrobial drug susceptibility patterns varied within and between clones. Multivariate analysis identified the following significant risk factors: male sex, cardiovascular disease, having undergone mechanical ventilation, and having been treated with antimicrobial drugs (particularly metronidazole). Penicillins were protective. The complex epidemiology may explain why the emergence of MDR *A. baumannii* is difficult to control.

Acinetobacter baumannii has emerged as an important nosocomial pathogen (1–5). Hospital outbreaks have been described from various geographic areas (6–9), and this organism has become endemic in some of them. The role of the environmental contamination in the transmission of nosocomial infections in general and in *A. baumannii* infections in particular is well recognized (10,11). *A. baumannii* does not have fastidious growth requirements and is able to grow at various temperatures and pH conditions (12). The versatile organism exploits a variety of both carbon and energy sources. These properties explain the ability of *Acinetobacter* species to persist in either moist or dry conditions in the hospital environment, thereby contributing to transmission (13,14). This hardiness, combined with its intrinsic resistance to many antimicrobial agents, contributes to the organism's fitness and enables it to spread in the hospital setting.

The nosocomial epidemiology of this organism is complex. Villegas and Hartstein reviewed *Acinetobacter* outbreaks occurring from 1977 to 2000 and hypothesized that endemicity, increasing rate, and increasing or new resistance to antimicrobial drugs in a collection of isolates suggest transmission. These authors suggested that transmission should be confirmed by using a discriminatory genotyping test (15). The importance of genotyping tests is illustrated by outbreaks that were shown by classic epidemiologic methods and were thought to be caused by a single isolate transmitted between patients; however, when molecular typing of the organisms was performed, a more complex situation of multiple unrelated strains causing the increasing rates of infections by *A. baumannii* was discovered (16–18).

Almost 25 years ago, researchers observed acquired resistance of *A. baumannii* to antimicrobial drugs commonly used at that time, among them aminopenicillins, ureidopenicillins, first and second-generation cephalosporins, cephamycins, most aminoglycosides, chloramphenicol, and tetracyclines (19). Since then, strains of *A. baumannii* have also gained resistance to newly developed antimicrobial drugs. Although multidrug-resistant (MDR) *A. baumannii* is rarely found in community isolates, it became prevalent in many hospitals (20). MDR *A. baumannii* has recently been established as a leading nosocomial pathogen in several Israeli hospitals, including our institution (21,22). Several locally contained small outbreaks of MDR *A. baumannii* occurred in our institution during the late 1990s. In 1999, however, the incidence of MDR *A. baumannii* isolation had doubled compared to the previous 2 years, and the organism became endemic in many wards (unpub. data).

The likelihood of isolation of *A. baumannii* from a hospitalized patient is related to temporospatial (extrinsic, ecologic characteristics) factors such as colonization

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pressure (23), nurse-to-patient ratio, and other ward characteristics and to individual patient risk factors (characteristics). The current study was designed to examine the occurrence and spread of *A. baumannii* within our institution, as well as to define individual risk factors for isolation of this organism.

Methods

Hospital Setting, Data Collection, and Microbiologic Testing

This study was performed at the Tel-Aviv Sourasky Medical Center, Israel, a 1,200–bed tertiary care teaching hospital with 70,000 admissions annually. Approximately 82,500 clinical microbiologic cultures are processed annually. We designed this as a matched case-control study to identify the individual risk factors for having MDR *A. baumannii*. We also performed epidemiologic investigations and genetic typing of the organisms to clarify the spread of this nosocomial pathogen.

Case-patients were defined as patients from whom MDR *A. baumannii* was isolated from any clinical culture (not surveillance cultures) during a 6-month period, from January 1, 2001, to June 30, 2001. A control patient was matched to each study patient on temporospatial factors as previously described (24). Briefly, controls were randomly chosen from the list of patients who stayed on the same ward in the same calendar month as the matched case-patient and who were hospitalized for at least the same number of days by the day the culture yielded MDR *A. baumannii* in the study patient. Controls were not MDR *A. baumannii* positive (i.e., the patient's samples were cultured, and either non-MDR *A. baumannii* or no *A. baumannii* was isolated, or the patient's samples were never cultured). Random control selection was performed by creating a list of all possible controls, assigning each candidate a random number, and choosing the highest random number (without replacement).

Case-patients and control patients were included only once in the study. Data were collected from the patients' records and from hospital computerized databases into a pre-prepared electronic questionnaire (Microsoft Access, Microsoft Corp., Redmond, WA, USA). The parameters registered for each patient (case-patients and controls) were age, sex, habits of smoking and alcohol consumption before hospitalization, cause and ward of hospitalization, transfer from another institution or ward within our institution, intensive care unit (ICU) stay, underlying disorders, immunosuppressive therapy, severity of illness as defined by the McCabe score (25), functional capacity and neurologic condition at time of isolation of A. *baumannii*, Foley catheter, invasive devices, surgery, mechanical ventilation, dialysis, infection, and antimicrobial drug therapy. Only variables occurring before inclusion in the study (culture day for case-patients and match day for controls) were analyzed as possible risk factors. *A. baumannii* was isolated from clinical specimens submitted to the microbiology laboratory and identified by using the Gram-Negative Identification Panel (Microscan, Dade Behring Inc., Sacramento, CA, USA). This system may not distinguish between closely related genotypic strains of *Acinetobacter*, and thus, some of these organisms may belong to these closely related strains. Susceptibilities were determined by automated microdilution broth testing (Neg/Urine Combo panel, Dade Behring Inc.). Resistance to imipenem and meropenem was confirmed by using Kirby-Bauer disk diffusion, according to the National Council for Clinical Laboratory Standards (NCCLS) guidelines. *A. baumannii* isolates were collected prospectively and stored at –70°C for further work-up.

Analysis of Chromosomal DNA by Pulsed-Field Gel Electrophoresis (PFGE)

Isolates of our patients, when available, were kept frozen at –70ºC and genetically characterized with PFGE. DNA preparation and cleavage with 20 U of *Apa*I endonuclease (New England Biolabs, Beverly, MA, USA) were preformed as previously described (26). Electrophoresis was performed in a 1% agarose gel (BMA products) prepared and run in 0.5 x Tris-borate-EDTA buffer on a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The initial switch time was 5 s, the final switch time was 35 s, and the run time was 23 h at 6 V/cm. Gels were stained in ethidium bromide, destained in distilled water, and photographed by using a Bio-Rad GelDoc 2000 camera. DNA patterns were analyzed visually and by using Diversity software (Bio-Rad). PFGE DNA patterns were compared and interpreted according to the criteria of Tenover et al. (27). The obtained PFGE DNA patterns were used to cluster the clones of the *A. baumannii* clinical isolates that were included in the study.

Definitions

We defined *A. baumannii* as MDR when the organism was resistant to all studied agents (including piperacillin/tazobactam, cefepime, ceftazidime, aztreonam, ciprofloxacin, gentamicin, tobramycin), but we allowed susceptibility to amikacin, ampicillin-sulbactam, imipenem, meropenem, and minocycline. Infection was defined according to the Centers for Disease Control and Prevention guidelines and modified to include community-acquired infections and to exclude asymptomatic bacteriuria (28).

Standard criteria were used to define underlying disorders. Disease was considered to be active if signs of disease were clinically apparent or if the patient received

treatment for the disease. A patient was considered to be receiving immunosuppressive therapy if he had undergone chemotherapy within 3 weeks, if he had been treated with ≥20 mg of prednisone daily for ≥2 weeks before entering the study, or if he had recently received antirejection drugs or other immunosuppressive therapy.

Severity of illness due to comorbidities was defined according to the McCabe score (25). Functional capacity during the index hospitalization was divided into 3 categories: independent, needing help for activities of daily living, and bedridden. Renal failure was defined as a creatinine level >2 mg/dL. Neurologic function was categorized according to 3 conditions: full consciousness, confusional state or dementia, and unconscious.

For each patient included in the study, we noted whether a susceptible *A. baumannii* was isolated in any culture before isolation of the MDR strain. We noted the number of antimicrobial drugs that the patient received between the time of admission until inclusion in the study, and we recorded home antimicrobial drug therapy separately. Recent hospitalization was defined as hospital stay within 3 months of the index hospitalization. We noted any surgical procedure, mechanical ventilation, and invasive procedure that took place 1 month before the patient's inclusion in the study.

Statistical Analysis

Statistics were run in Stata version 7 (Stata Corp., College Station, TX). All analyses were matched to correspond to the study design. All variables were examined by univariate analysis with the McNemar test and paired Student *t* test. Variables with a p value <0.2 in the univariate analysis were included in the multivariate model. Risk factors were examined by using conditional logistic regression. A final model was built that included all the variables with a p value <0.2. Variables that were not retained in the model by this procedure were then tested for confounding by adding them 1 at a time to the model and examining their effects on the β coefficients. Variables which caused substantial confounding (change in β coefficient of >10%) were included in the final model. After constructing the explanatory model, the effect of exposure to antimicrobial agents (i.e., antimicrobial treatment before inclusion in the study) was examined by adding them to the model.

In addition to examining statistical significance and confounding, the effect modification between variables was evaluated by testing appropriate interaction terms for statistical significance. Colinearity was examined by replacing variables with each other and examining the effect on the model. All statistical tests were 2-tailed. A p value ≤0.05 was considered significant.

Results

From January 1, 2001, to June 30, 2001, we identified 133 patients with a clinical culture of MDR *A. baumannii*. Four patients were not hospitalized in our institution (i.e., they were hospitalized elsewhere) and were excluded from the study. Charts were available for 120 case-patients, but no controls could be matched for 2 of them. Thus, 236 patients were included in the study (118 case-patients and their matched controls). Sites from which *A. baumannii* was initially isolated included respiratory tract 38 (32%), wounds 23 (19.5%), urine 22 (19%), blood 19 (16%), and sterile fluids and catheter tips 16 (13.5%).

Epidemiology and PFGE Typing

Among the 118 case-patients, the first MDR *A. baumannii* in 104 (88%) was isolated after more than 72 h of hospitalization (mean 17.5 ± 23.7 days). Among the other 14 case-patients, 12 were admitted from another institution or had been hospitalized recently. No nosocomial origin was documented in 2 cases. *A. baumannii* was initially isolated in 27 different wards. Figure 1 shows the case distribution among them. A higher concentration of patients was clearly evident in 3 wards: the general ICU (ward "I", 16 cases), and two internal medicine wards (ward Q, 10 cases, and ward W, 9 cases).

The time distribution of new cases is presented in Figure 2. We did not find any aggregate of cases within a specific ward at any specific time. The occurrence during the months February, May, and June was lower than during January, March, and April. This circumstance is not explained by differences in infection control measures recommended; during the entire study period this included contact isolation of every patient from whom MDR *A. baumannii* was isolated and cohorting of case-patients if single patient rooms were not available. A statement was added to the culture result: "MDR organism; contact

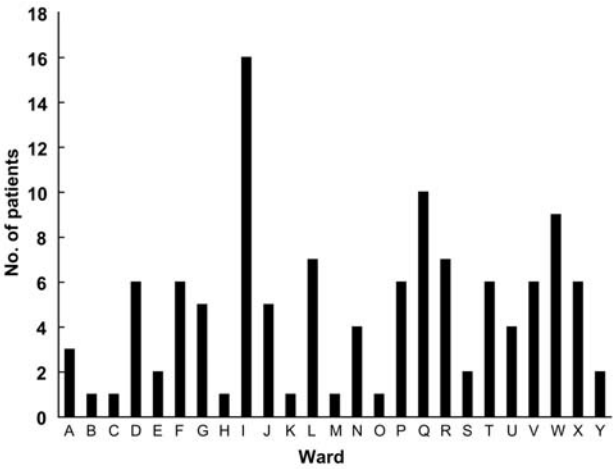

Figure 1. Distribution of case-patients according to ward.

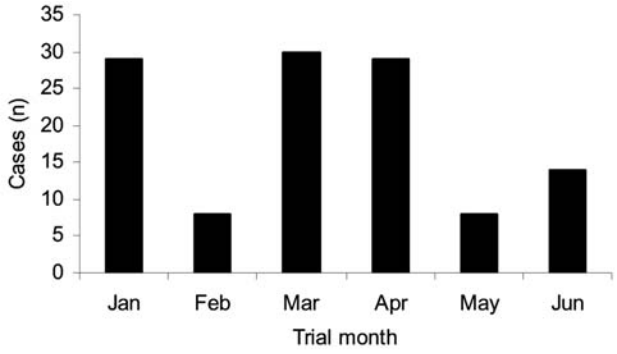

Figure 2. Monthly case distribution, 2001

isolation is required." Epidemiologic nurses checked with the ward to confirm that the patient was isolated. On certain floors, surveillance (nose, forearms, armpits, and perirectal swabs) and environmental cultures (using swabs, contact plates, and direct culturing of fluids) were performed to try to identify a reservoir of organism. In this study, case-patients and controls were matched by ward and calendar time to focus on individual risk factors and not on differences between wards and temporal changes.

A total of 51 unique patient MDR strains were available for further study, and they were analyzed by using PFGE. We identified 10 distinct clones of *A. baumannii*. Figure 3 shows 6 different PFGE-defined clones, each having from 1 to 4 subtypes showing a 1- to 2-band difference. Two of the 10 different clones dominated: 22 case-patients had clone A and 10 case-patients had clone B, although no specific clone dominated in a specific ward but rather each clone was spread among several wards during the entire study period (Table 1). We also found various antimicrobial drug susceptibility phenotypes (all belonging to our definition of MDR) within each PFGE clone, but almost all cases of carbapenem resistance belonged to clone A.

Individual Risk Factors

The study patients' characteristics are displayed in Table 2. Case-patients were similar to their matched control patients with respect to mean age (67.7 vs 64.4 years) and sex distribution (men, n = 71 [60%] vs controls, n = 59 [50%]). The groups were also similar in habits of smoking and alcohol consumption and in the occurrence of coexisting conditions of lung disease, diabetes, kidney, liver disease, malignancy, and posttransplantation condition. The groups differed in the prevalence of ischemic heart disease: study case-patients 69% vs. controls 52% (OR 2.33, p = 0.006).

Hospital events (before study entry) differed between case-patients and controls. Case-patients were more likely to have received mechanical ventilation (OR 2.9, p = 0.001), to be treated with metronidazole (OR 1.9, p = 0.038), and to have a Foley catheter (OR 2.42, p = 0.005). They were less likely to have had another bedside surgical procedure before the isolation of *A. baumannii* (OR 0.53, p = 0.035).

Several variables tended to be more associated with case-patients, but the values did not reach statistical significance: admission from another institute (OR 2.1, p = 0.06), unconsciousness (OR 0.706, p = 0.07), and previous use of third-generation cephalosporin (OR 1.63, p = 0.093) and of macrolides (OR 2.25, p = 0.056). A matched multivariate model, adjusted for the hospital length of stay, was developed by using conditional logistic regression (Table 3). The variables that were identified by this model as being significant risk factors for MDR *A. baumannii* were male sex (OR 3.8, p = 0.002), ischemic heart disease (OR 3.3, p = 0.005), mechanical ventilation (OR 6.2, p < 0.001), and home antimicrobial drug use (OR 4.7, p = 0.018). Two agents used in the hospital were associated with MDR *A. baumannii*: metronidazole was identified as a risk factor (OR 2.3, p = 0.018), and the penicillin group was identified as having a protective effect (OR = 0.38, p = 0.029).

Discussion

We sought to understand the epidemiology of MDR *A. baumannii* and to define the individual risk factors for acquiring this infectious agent. Our findings illustrated its complex epidemiology and delineated individual risk factors. The complex epidemiology may explain the difficulties encountered in controlling the emergence of this nosocomial pathogen.

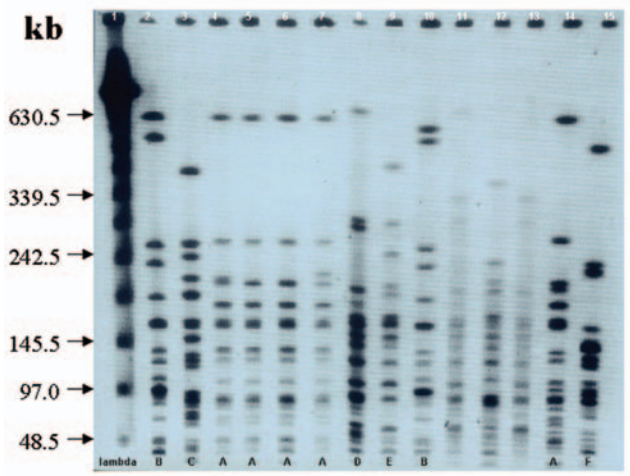

Figure 3. A typical pulsed-field gel electrophoresis analysis of selected isolates of *Adnetobacter baumannii* restricted with *ApaI*. Lane 1 shows λ ladder used as molecular size marker. Lanes 11–13 are of strains not included in the trial. The gel shows 6 different clones of *A. baumannii*: 5 isolates belong to clone A and 2 belong to clone B (the 2 dominant clones). Single isolates belonging to clones C, D, E, and F can be seen.

Table 1. Characteristics of 51 strains available for PFGE typing, 2001*

Clone	No. of isolates	No. of wards	Months	Carbapenem resistance	Sulbactam resistance
A	22	14	January–June	16	1
B	10	8	February–July	1	
C	4	4	January–June	1	2
D	2	2	January, June		
E	2	1	January		
F	3	3	March, April		
I	5	4	February–April		2
Lone†	1	1	January		
Lone†	1	1	January		
Lone†	1	1	April		

*PFGE, pulsed-field gel electrophoresis.
†Lone, unique clone; each had a different ward of isolation.

Almost all cases of MDR *A. baumannii* in our study were hospital acquired: 88% were acquired in our institution during the index hospitalization, and 10% were imported into the hospital by patients with recent exposure to the healthcare system. The MDR *A. baumannii* strains isolated in our institution belonged to multiple PFGE clones: 50% of the isolates that were typed belonged to 2 dominant clones, and the other, nondominant clones caused few cases each.

Clones did not cluster in place (i.e., hospital location) or in time. Moreover, when an increase in incidence was observed in a certain ward, the increase was not associated with a single clone, and up to 4 different clones were present concomitantly in a ward. We also found antimicrobial susceptibility profile variation within clones and similarities between clones, which showed that susceptibility pattern was not a useful marker for clonality. Carbapenem resistance occurred in 75% of the isolates belonging to 1 of the 2 dominant clones (clone A) but was rare among other clones. This finding illustrates well the complexity of the epidemiology of this nosocomial pathogen. Even with molecular typing data, determining the modes of spread of this organism was difficult, partly because we did not have a complete collection of the isolates. Despite our expending extensive effort, we were unable to determine the source of these resistant strains. Although we believe that patient-to-patient transmission through contaminated hands of healthcare workers and fomites is the main route by which these MDR organisms spread, the combined epidemiologic and molecular data did not directly support this hypothesis. The lack of evidence for patient-to-patient spread in our study may be related to transferring patients between wards and the presence of a substantial number of undetected carriers (the "submerged iceberg phenomenon") who spread the bacteria. Alternative explanations, such as repeated entry (import) of the same clone to the hospital ecosystem at various times and locations (e.g., from an disease-endemic institution or contaminated supply or food) must be considered as well.

The individual risk factors for isolation of MDR *A. baumannii* that were identified by the multivariate analysis were male sex, underlying comorbidity of ischemic heart disease, mechanical ventilation, and antimicrobial drug treatment. The finding of male sex and of ischemic heart disease being risk factors for carriage of and infection with resistant gram-negative bacilli had also been observed by our group, as well as by others for carriage of extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* and *Pseudomonas aeruginosa* (29–31). We hypothesize that these associations may be related to the following factors: 1) patient-to-patient transmission within multipatient rooms (patients who are segregated by sex and need for intensive monitoring); 2) use of certain nonantimicrobial medication, such as calcium channel blockers, which may predispose for adherence or invasiveness by affecting the host or the pathogens (32,33); and 3) hormonal or other sex differences which may predispose a person for colonization and infection. These hypotheses are currently being studied in our facilities. The multivariate analysis did not identify admission from another institution as a significant risk factor. This probably relates to the small number (and proportion) of patients admitted from other institutions who were identified to be carriers of MDR *A. baumannii*. These few patients may, however, have played an important role to the entrance of new clones and the spread of the organisms within our institution. Moreover, case-patients that are not detected may still be important in the spread of these organisms. Overall, we believe that the identified risk factors represent both severity of the patient's condition, use of invasive devices, and effect on the normal flora, all of which promote MDR *A. baumannii* colonization, growth, and invasiveness.

The administration of penicillin had a protective effect against isolation of MDR *A. baumannii*. This protective effect was significant after confounding by multivariate analysis was controlled for. Penicillins lack activity against these MDR strains, and the protective effect cannot relate to sulbactam, since a sulbactam combination is seldom used in our institution. To the best of our knowledge, such

Table 2. Group comparison: patients' characterization and possible risk factors for *Acinetobacter baumannii* isolation*†

	Cases, n (%)	Controls, n (%)	OR	95% CI	p value
Demographic parameters					
Average age, y (SD)	67.7 (16.42)	64.4 (19.15)	1.012	0.996–1.03	0.134
Female	47 (40)	59 (50)			
Male	71 (60)	59 (50)	1.48	0.89–2.45	0.13
Smoking	35 (30)	43 (36)	0.72	0.41–1.27	0.26
Alcohol usage	7 (6)	5 (4)	1.5	0.42–5.31	0.53
Admission from home	23 (20)	12 (10)	2.12	0.96–4.76	0.065
Concomitant diseases					
Ischemic heart disease	82 (69)	61 (52)	2.33	1.27–4.27	0.006
Lung disease	59 (50)	50 (42)	1.578	0.82–2.72	0.183
Diabetes	39 (33)	28 (24)	1.578	0.88-2.8	0.119
Liver disease	10 (8)	18 (15)	0.555	0.25–1.2	0.136
Kidney disease	35 (30)	27 (23)	1.388	0.76–2.54	0.288
Posttransplantation	7 (6)	3 (3)	2.333	0.6–9.02	0.22
Malignancy	35 (30)	38 (32)	0.923	0.53–1.6	0.777
Clinical parameters					
Unconsciousness	40 (43)	26 (22)	0.706	0.48–1.02	0.069
Bedridden	88 (75)	79 (76)	1.45	0.82–2.56	0.201
In-house dialysis	11 (9)	4 (3)	2.5	0.78–7.97	0.121
Mechanical ventilation	70 (59)	47 (40)	2.916	1.51–5.61	0.001
Admission in last 3 months	56 (47)	52 (44)	1.148	0.68–1.92	0.6
ICU stay	34 (92)	35 (30)	0.984	0.94–1.02	0.473
Immunosuppression treatment	29 (25)	27 (32)	1.095	0.9–1.97	0.763
Major surgery	33 (28)	33 (28)	1	0.51–1.95	1
Isolation of susceptible *Acinetobacter* before inclusion	6 (5)	6 (5)	1		1
Antimicrobial treatment					
Home antimicrobial treatment	16 (41)	6 (5)	2.666	1.04–6.81	0.04
In-house antimicrobial treatment	104 (88)	96 (81)	1.727	0.82–3.63	0.149
Average number of antimicrobial agents (SD)	3.025 (2)	2.97 (2)	1.015	1	0.847
Penicillin administration‡	42 (63)	54 (46)	0.647	0.37–1.1	0.112
Cephalosporin use (1st, 2nd generation)	31 (26)	34 (92)	0.9	0.476–1.701	0.746
3rd generation cephalosporin use	53 (54)	42 (63)	1.631	0.92–2.88	0.093
4th-generation cephalosporin use	17 (41)	17 (14)	1	0.49–2.04	1
Macrolides	21 (18)	11 (9)	2.25	0.97–5.17	0.056
Metronidazole	48 (14)	35 (30)	1.933	1.03–3.6	0.038
Gentamicin	23 (19)	27 (32)	0.777	0.38–1.56	0.481
Amikacin	12 (10)	19 (16)	0.5	0.2–1.24	0.134
Clindamycin	7 (6)	8 (7)	0.875	0.32–2.41	0.796
Vancomycin	22 (19)	22 (19)	0.944	0.48–1.83	0.866
Carbapenem	10 (8.47)	10 (8.47)	0.875	0.32–2.41	0.796
Invasive procedures					
Central line	69 (58)	70 (59)	0.958	0.54–1.7	0.884
Arterial line	35 (30)	34 (29)	0.933	0.45–1.93	0.85
Foley catheter	96 (81)	76 (64)	2.42	1.3–4.52	0.005
Other bedside procedures§	63 (53)	78 (67)	0.531	0.29–0.95	0.035

*OR, odds ratio; CI, confidence interval; SD, standard deviation; ICU, intensive care unit.
†All variables were recorded up to the time of inclusion in the study.
‡Including semisynthetic penicillin and β-lactamase-containing products.
§Including tracheostomy, bedside débridement, chest tube insertion, and gastrointestinal endoscopy.

an effect has not been observed previously. Specific penicillins may possibly cause specific changes in the microflora that oppose colonization and growth of *Acinetobacter* spp., but the validity of this observation awaits further research. As for many other resistant organisms, metronidazole was a significant risk factor for MDR *A. baumannii*, likely because of its effects on the competitive normal intestinal flora. The observation that carbapenem resistance was much more frequent in the dominant clone could suggest that this phenotype may have contributed to the evolutionary success of the clone.

A previous study clearly demonstrated that the epidemiology and risk factors may vary for different clones (17). This finding may lead to a dilution of effects and

Table 3. Multivariate analysis for risk factors for *Acinetobacter baumannii**†

Parameter‡	OR	95% CI	p value
Male sex	3.84	1.63–8.99	0.002
Ischemic heart disease	3.35	1.44–7.77	0.005
Mechanical ventilation	6.27	2.27–17.33	<0.001
Penicillin use§	0.38	0.16–0.90	0.029
Metronidazole use	2.33	0.98–5.83	0.071
Any home antimicrobial drug treatment	4.74	1.31–17.15	0.018

*OR, odds ratio; CI, confidence interval.
†Adjusted for length of hospital stay prior to entry to the study.
‡All parameters had been present before *A. baumannii* identification.
§Including semisynthetics with or without a β-lactamase inhibitor (never sulbactam).

even to opposing effects by some risk factors. In our study, we did not analyze clone-specific risk factors because we did not believe that we truly had an epidemic clone and because the number of patients affected by each clone was too small to allow a statistically significant comparison.

Temporospatial factors, although they undoubtedly have an important role in the spread of resistant organisms, were not within the scope of this study. We controlled for these factors by the study design, i.e., matching by hospital location, length of stay before inclusion in the study, and calendar time. Confounding may, however, have been introduced to our study by factors for which we did not control, such as residing in a multipatient room next to a patient with MDR *A. baumannii*.

We used risk set sampling (by matching for time at risk) but did not allow case-patients to be eligible to be controls before becoming cases. Since no clustering in time and place occurred, and controls were chosen from 35,000 admitted patients, this method of sampling should not have yielded biased results.

Despite the large number of cases that we identified, we were unable to understand the mode of spread and the reason for emergence of these organisms in our institution. This fact may be because only some of the isolates were available for typing or because of the complex mode of spread in our hospital. Further study will be required to more fully understand the intricate phenomenon of MDR *A. baumannii* spread.

Dr. Abbo is in clinical development at Biosense Webster (Israel). This work was part of his M.Sc. dissertation in internal medicine. His primary research interests include the clinical epidemiology of infectious diseases and cardiology.

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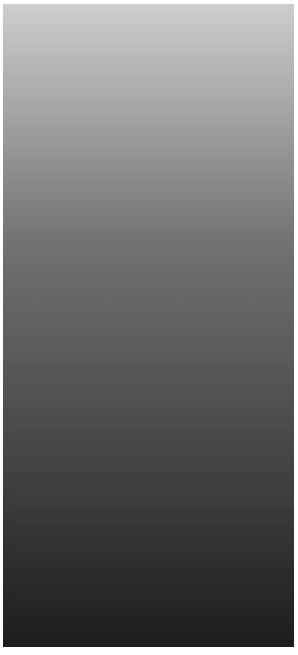

Hybrid *Vibrio vulnificus*

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The recent emergence of the human-pathogenic *Vibrio vulnificus* in Israel was investigated by using multilocus genotype data and modern molecular evolutionary analysis tools. We show that this pathogen is a hybrid organism that evolved by the hybridization of the genomes from 2 distinct and independent populations. These findings provide clear evidence of how hybridization between 2 existing and nonpathogenic forms has apparently led to the emergence of an epidemic infectious disease caused by this pathogenic variant. This novel observation shows yet another way in which epidemic organisms arise.

Vibrio vulnificus, a ubiquitous inhabitant of marine and estuarine environments, is considered one of the most dangerous waterborne pathogens. The case-fatality rate for *V. vulnificus* septicemia may reach 50% (1). Human infection is generally acquired through eating contaminated raw or undercooked seafood or through contamination of wounds by seawater or marine animals (2). Infected persons with preexisting liver disease, hemochromatosis, or compromised immune systems are at particularly high risk for fatal septicemia (3–8).

Human infections are sporadic and almost entirely caused by strains of biotype 1, while biotype 2 strains have been reported to cause disease mainly among eels and rarely infect humans (9). During the summer of 1996, a major outbreak of systemic *V. vulnificus* infections started among Israeli fish market workers and fish consumers (10,11). Molecular studies showed that the disease outbreak was caused by a previously undescribed biotype that exhibited a distinct phenotypic and molecular pattern, designated biotype 3 (11).

The origins of this emergent infectious disease have not been fully understood, although it was originally thought to arise mostly from human behavior and work practices (10). On the basis of these assumptions, new fish-handling procedures were introduced (11,12). However, disease continued, although at a lower incidence. Therefore, studies were undertaken to determine whether this novel outbreak of disease was caused by a specific lineage or clone. The emergence of this new biotype could not be resolved by conventional microbiologic and molecular typing approaches. We investigated this outbreak by combining a multilocus sequence typing approach (13) with molecular evolutionary analyses.

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Materials and Methods

Bacterial Isolates

To study the emergence of this new biotype, we examined a collection of 159 *V. vulnificus* isolates that represented all 3 biotypes from human disease and environmental sources that originated in Israel (n = 64), the United States (n = 54), Denmark (n = 7), Germany (n = 6), Spain (n = 5), Sweden (n = 5), Japan (n = 8), South Korea (n = 2), Singapore (n = 2), Thailand (n = 1), Indonesia (n = 1), and Taiwan (n = 1). In addition, 3 well-characterized reference strains that represented the 3 biotypes were included, the ATCC 27562 strain (biotype 1, isolated from human blood in the USA), the E-39 strain (biotype 2, isolated from diseased eel in Spain), and ATCC BAA-86 (biotype 3, isolated from human blood in Israel). Biotype 1 strains (n = 82) consisted of 39 isolates from human disease and 43 environmental isolates; biotype 2 strains (n = 15) consisted of 13 isolates from diseased eels, 1 from an infected person, and 1 from diseased shrimp. Biotype 3 strains were isolated from samples from persons with invasive disease in Israel (n = 61) and from fish-pond water (n = 3). Isolates were grown on blood agar plates and incubated overnight at 35°C in aerobic conditions. The lists of the isolates used in this study and their sources can be accessed at http://pubmlst.org/vvulnificus.

DNA Extraction

The DNeasy kit (QIAGEN GmbH, Hilden, Germany) was used to extract DNA with the gram-negative bacterial protocol as recommended by the manufacturer. Briefly, several colonies from a bacterial culture were picked off into phosphate-buffered saline solution and centrifuged at

7,500 rpm (5,000 x *g*) for 10 min. The cell pellet was resuspended in 180 µL of tissue lysis buffer, then 20 µL of proteinase K (10 mg/mL) was added, and the sample was incubated at 55°C until the tissue was completely lysed. Then 200 µL of lysis buffer was added and incubated at 70°C for 10 min. The DNA in the clear viscous lysates was precipitated with ethanol 95% (vol/vol) and added to DNeasy mini columns. Ethanol 70% (vol/vol)–based buffers (AW1 and AW2) were added sequentially to the columns and centrifuged at 8,000 rpm (6,000 x *g*). The supernatants were discarded, and the DNA was resuspended in AE buffer and used for amplification.

Multilocus Sequence Typing (MLST)

This bacterium has 2 chromosomes. Fourteen housekeeping genes (7 from each chromosome) that encoded enzymes responsible for intermediary metabolism were identified by searching the genome database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html) of *V. vulnificus* strain CMCP6, with gene sequences from other bacteria. Genetic loci were chosen for further investigation on the basis of the following criteria: chromosomal location, suitability for primer design, and sequence diversity in pilot studies. Ten loci were chosen for the MLST scheme, 5 from each chromosome. The following were chosen from the large chromosome: *glp*, the encoding glucose-6-phosphate isomerase; *gyrB*, the encoding DNA gyrase-subunit B; *mdh*, the encoding malate-lactate dehydrogenase; *metG*, the encoding methionyl-tRNA synthetase; and *purM*, the encoding phosphoribosylaminoimidazole synthetase. The following were chosen from the small chromosome: *dtdS*, the encoding threonine dehydrogenase; *lysA*, the encoding diaminopimelate decarboxylase; *pntA*, the encoding transhydrogenase alpha subunit; *pyrC*, the encoding dihydroorotase; and *tnaA*, the encoding tryptophanase. Their chromosomal location suggested that it was unlikely for any of the loci to be coinherited in the same recombination event, as the minimum distance between loci was 300 kb (Table).

Amplification and Nucleotide Sequence Determination

Polymerase chain reaction (PCR) products were amplified with oligonucleotide primer pairs designed from the *V. vulnificus* genome sequence. These primers provided reliable amplification from a diverse range of samples (available from http://pubmlst.org/vvulnificus). Each 50-µL amplification reaction mixture was made up of 10 ng of *V. vulnificus* chromosomal DNA, 100 pmol of each PCR primer (MWG Biotech, Ebersberg, Germany), 10 x PCR buffer with 1.5 mmol/L $MgCl_2$ (QIAGEN GmbH), 0.5 U of Taq DNA polymerase (QIAGEN GmbH), and 1.6 mmol/L deoxynucleoside triphosphates (ABgene, Epsom, UK). The reaction conditions were denaturation at 94°C for 1 min, primer annealing at 50°C for 45 s and extension at 72°C for 1 min for 30 cycles. The amplification products were purified by precipitation with 20% polyethylene glycol and 2.5 mol/L NaCl (14), and their nucleotide sequences were determined at least once on each DNA strand by using internal nested primers (available from http://pubmlst.org/vvulnificus) and ABI PRISM BigDye Terminators v 3.0 Reaction Mix (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Unincorporated dye terminators were removed by precipitation of the termination products with sodium acetate (3 mol/L, pH 5.2) and 95% ethanol, and the reaction products were separated and detected with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled from the resultant chromatograms with the STADEN suite of computer programs and edited to resolve any ambiguities (15). For each locus, every different sequence was assigned a distinct allele number in order of identification; these sequences were internal fragments of the gene, which contained an exact number of codons. Each isolate was therefore designated by a 10-integer number (the allelic profile), which corresponds to the allele numbers at the 10 loci in the following order: *glp*, *gyrB*, *mdh*, *metG*, *purM*, *dtdS*, *lysA*, *pntA*, *pyrC*, and *tnaA*. Isolates with the same allelic profile are assigned to the same sequence type (ST), which were

Table. Characteristics of loci included in the *Vibrio vulnificus* MLST scheme*

Locus	Size of sequenced fragment (bp)	No. of alleles identified	No. of polymorphic sites (%)	Position in *V. vulnificus* genome† (bp)
glp	480	38	46 (9.6)	Chromsome I (1379280)
gyrB	459	31	34 (7.4)	Chromsome I (999145)
mdh	489	29	30 (6.1)	Chromsome I (649619)
metG	429	31	37 (8.6)	Chromsome I (3091694)
purM	444	28	39 (8.8)	Chromsome I (1895474)
dtdS	417	46	56 (13.4)	Chromsome II (1621665)
lysA	465	41	78 (16.8)	Chromsome II (1110400)
pntA	396	32	35 (8.8)	Chromsome II (332656)
pyrC	423	35	50 (11.8)	Chromsome II (1752259)
tnaA	324	32	42 (12.9)	Chromsome II (926270)

*From the *V. vulnificus* genome (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html).
†MLST, Multilocus Sequence Typing.

numbered in the order of their identification (ST-1, ST-2, and so on). The data have been deposited (http://pubmlst.org/vvulnificus).

Inferring the Population Structure and Ancestral Sources

The program STRUCTURE was used to define the population structure and identify the ancestral sources of the 10 gene fragments from all the strains. STRUCTURE is a recently developed program that implements a Bayesian model approach for inferring population structure and ancestral sources from multilocus genotype data (16). Of the 4,326 nucleotides sequenced for each isolate from the 10 genes, 447 nt were polymorphic. For the purposes of the analysis, these nucleotides were used by STRUCTURE as individual loci. STRUCTURE can infer the population structure by using a variety of models, including the linkage model (17), which incorporates linkage disequilibrium due to correlations in ancestry between loci that reflects admixture between populations. This approach has recently been used to elucidate the structure and evolution of populations of the human pathogen *Helicobacter pylori* (18), and we have used the same method here. For the purposes of the analysis, the nucleotide sequence of the 10 housekeeping gene fragments of 2 clinical strains of *V. vulnificus*, CMCP6 and YJ016, whose complete genome sequence has been recently completed (http://www.ncbi.nlm.nih.gov/genomes/ MICROBES/Complete.html), were added to all the data sets.

Results

Genotypes Identified

The 159 isolates were resolved into 70 STs, 56 of which were present only once in the entire collection. Eighty-two isolates (51.6%) were represented by 1 of 4 STs: ST-8 was the most common and occurred 62 times (39%); ST-6 occurred in 11 isolates (6.9%); ST-32 occurred in 5 isolates (3.1%); ST-16 occurred in 4 isolates (2.5%). The remaining 21 isolates resolved into 10 sequence types. Strains of biotype 1 (n = 82) resolved into 66 STs. Biotype 2 (15 isolates) resolved into 4 STs; ST-6, ST-9, ST-10, and ST-48. ST-6 was the most common, occurring 11 times and consisting of all the indole-negative isolates. All biotype 3 strains (n = 62) were genetically identical and belonged to ST-8.

Population Structure and Ancestral Sources

The observed sequence variation between the 2 chromosomes was comparable. Initial analysis of sequence data from the 10 gene fragments showed that extensive recombination had occurred within all the genetic loci under study (data not shown). Constructing phylogenetic trees in the presence of recombination is problematic because different parts of the sequence may have different phylogenetic histories. Therefore, we analyzed the data with the program STRUCTURE. First, we tested the assumption that the 3 *V. vulnificus* biotypes represent 3 distinct predetermined populations of this pathogen (K = 3). The results of multiple analyses with STRUCTURE were incompatible with this assumption; in all cases, only 2 populations were identified, populations A and B (Figure 1 and data not shown). Further, while biotype 1 was present in both populations, biotype 2 was present only in population A (Figure 1A). Biotype 3 occupied an intermediate position between the 2 populations (Figure 1A). Figure 1B shows that an overrepresentation of human disease isolates occurred in population B and an overrepresentation of environmental isolates occurred in population A. And Figure 1C shows that both populations were globally distributed.

To identify the evolutionary processes underlying the emergence of the genotype responsible for the Israeli outbreak, we repeated the STRUCTURE analysis assuming only 2 populations (K = 2) (based on the findings from the first STRUCTURE analysis). This analysis identified the

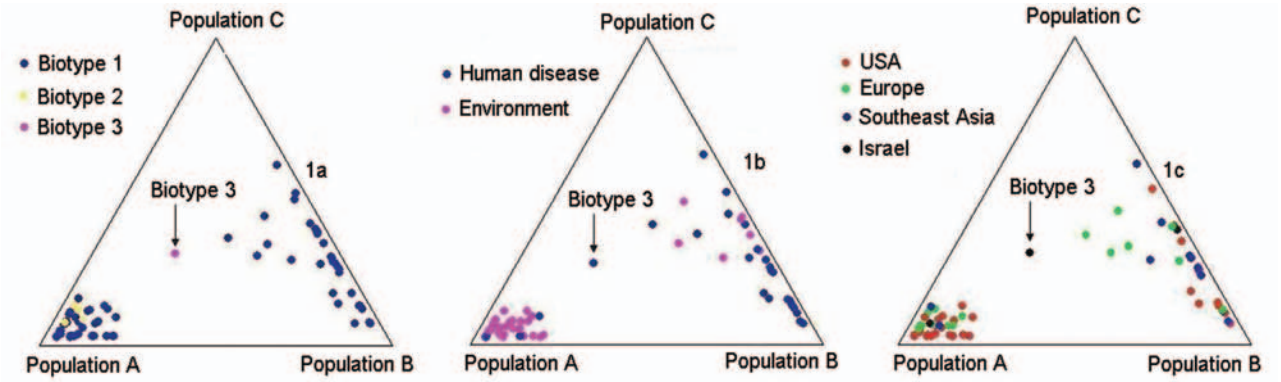

Figure 1. Triangle plots of STRUCTURE results. Only 2 populations were identified (A and B). A shows the distribution of the biotypes within the 2 populations. B shows the distribution of the strains according to their source (human or environmental). C shows the strains' distribution according to their geographic origin. These results were produced by the linkage model of STRUCTURE with K = 3.

ancestral sources of the individual strains (Figure 2). Each strain is represented by a thin vertical line partitioned into 2 (K = 2) most likely predetermined populations or genetic ancestries. Each line shows the proportion of polymorphic sites inherited from each of the 2 populations (shown in green and red). It shows that most biotype 1 and 2 strains have predominant contribution from 1 of the 2 genetic ancestries. However, strains of biotype 3 have almost equal contributions from both genetic ancestries. This analysis was further detailed to identify the ancestral sources of each of the polymorphic sites in each of the 10 gene fragments (Figure 3).These analyses confirmed that, notwithstanding the subdivision of *V. vulnificus* populations into 2 populations, recombination had occurred between these populations and that the Israeli outbreak genotype is a hybrid, with some genes originating from 1 population and some from another, while some genes have representation from both.

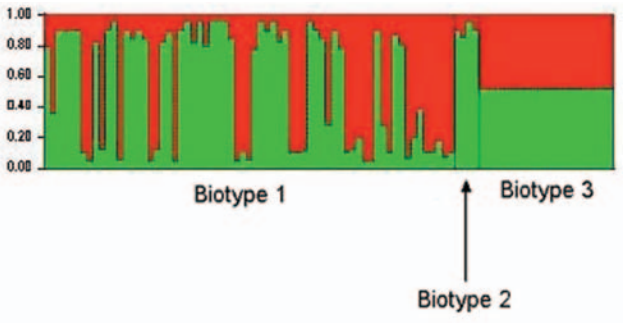

Figure 2. Results of a Bayesian cluster analysis by STRUCTURE. Each of the strains included in the analysis is represented by a thin vertical line, partitioned into 2 colored segments that represent the proportion of polymorphic sites inherited from each of the 2 genetic ancestries. For the representation of results, strains were grouped according to biotype. The analysis was carried out by using the linkage model with K = 2.

Discussion

We have shown that a hybrid virulent organism that acquired genes from 2 distinct and independent populations has caused the disease outbreak in Israel. To achieve this analysis, we studied large, carefully assembled, collections of *V. vulnificus* isolates. The human strains were collected from infected patients in Israel, the United States, Europe, and Southeast Asia. The environmental strains were collected from environmental sources in the United States, the Pacific Ocean, the Baltic Sea, inland fish farms in Israel, and eel farms in Europe.

The division of *V. vulnificus* populations into 2 major groups is consistent with results of multilocus enzyme electrophoresis studies (19). However, those studies placed the Israeli electrophoretic type within 1 of the 2 groups, in contrast to our findings, which placed the Israeli genotype in an intermediate position between the 2 populations (Figure 1A and Figure 3).

Hybridization within bacterial populations, i.e., the process whereby a hybrid results from the hybridization of the genomes of 2 or more populations of a species or an organism, has been the focus of much attention by scientists in the last decade (20–25); these events, which may be intra- or interspecies, could alter the genetic distances and the phylogenetic relationships within bacterial populations. The magnitude by which these events occur is crucially dependent on ecologic factors; different populations of a species must be present within the same niche for genetic exchange to have an impact on genetic variation (26). Multiple sampling of fish-farm water and fish documented the abundance of biotype 1 strains (11 and data not shown). These biotype 1 strains, representing both populations of *V. vulnificus*, were never implicated in disease among fish-farm fish, according to the Central Fish Health Laboratory, Kibbutz Nir David, Israel (www.moag.gov.il/english), or among humans in Israel (11). These observations are consistent with finding that these populations are not pathogenic to either humans or fish. The finding that this hybrid variant (biotype 3) was the only implicated organism in all disease cases from 1995 to 2003 is indicative of its pathogenicity. Furthermore, the finding that all 62 biotype 3 strains were genetically identical could suggest that this hybrid clone may have evolved by a relatively recent genome hybridization event.

Hybrid variants have been recently described among populations of *Staphylococcus aureus* (27) and *Chlamydia trachomatis* (28). However, our findings show the first bacterial variant that is clearly more pathogenic than the existing forms of the organism, i.e., the Israeli hybrid clone, is more pathogenic than the existing biotype 1 strains within the Israeli aquaculture system. These findings are consistent with observations among influenza viruses (29). This phenomenon has also recently been described also among populations of mosquitoes (30), in which hybridization between existing forms of a relatively nonpathogenic organism has apparently led to the emergence of a novel pathogenic variant that poses a particular threat to human health.

The Israeli genotype spread extensively after its emergence in 1995, and by 2003, most of the fish farms in Israel were the sources of *V. vulnificus* cases. This finding is consistent with the idea that this pathogen is circulating freely within the underground brackish water reservoirs that supply these fish farms. Despite the widespread use of inland fish farming around the world, no similar outbreaks have been reported. During the 1970s and 1980s, the introduction into Israel of stocks of *Tilapia* spp. from Africa, the Far East, and South America (31–33) (for experimental and commercial purposes) may have contributed to the

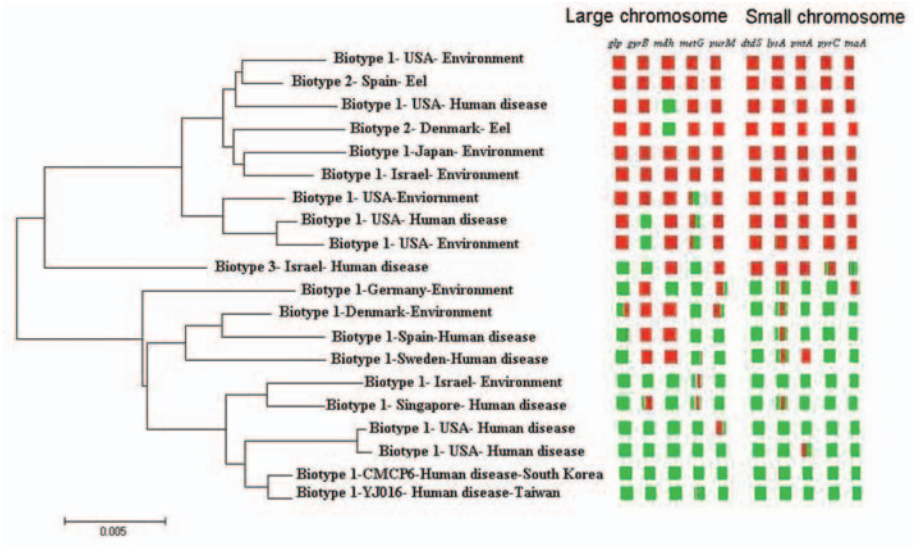

Figure 3. A neighbor-joining tree of representative isolates from the 2 populations is plotted with the inferred ancestral sources of individual polymorphic sites in each of the 10 genes. The names of the genes and to which chromosome they belong are indicated. These results are based on using the linkage model of STRUCTURE with K = 2.

evolution of this hybrid clone. In view of the widespread fish-trading industry, this hybrid clone may eventually emerge through exports of Israeli tilapia stocks, in remote geographic locations. In conclusion, these observations demonstrate the power of molecular and population genetic approaches in investigating the emergence of a novel pathogen and defining its nature. Our results show another way by which epidemic infectious diseases arise.

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Hypersensitivity to Ticks and Lyme Disease Risk

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Although residents of Lyme disease–endemic regions describe frequent exposure to ticks, Lyme disease develops in relatively few. To determine whether people who experience cutaneous hypersensitivity against tick bite have fewer episodes of Lyme disease than those who do not, we examined several factors that might restrict the incidence of Lyme disease among residents of Block Island, Rhode Island. Of 1,498 study participants, 27% (95% confidence interval [CI] 23%–31%) reported ≥1 tick bites, and 17% (95% CI 13%–21%) reported itch associated with tick bite in the previous year. *Borrelia burgdorferi* infected 23% (95% CI 20%–26%) of 135 nymphal *Ixodes scapularis* (*I. dammini*) ticks. The likelihood of Lyme disease infection decreased with ≥3 reports of tick-associated itch (odds ratio 0.14, 95% CI 0.94–0.03, p = 0.01). Prior exposure to vector ticks protects residents of disease-endemic sites from Lyme disease.

Although many residents of Lyme disease–endemic regions describe frequent exposure to ticks, relatively few become infected by the causative spirochetal agent, *Borrelia burgdorferi* (1–4). This disparity reflects both a relative paucity of spirochetal infection in vector ticks and the limited number of people actually bitten by ticks (5–6). Other variables that might restrict Lyme disease incidence include prompt removal of attached ticks before the pathogen is transmitted and acquired immunity to the salivary proteins of these ticks, the spirochetal pathogen, or both (7–11). Repeated exposure to tick bites has been associated with developing cutaneous hypersensitivity, which results in persistent itch and local swelling at the site of tick attachment (12–13). Itching provides an early sign of tick bite and may facilitate removal of the attached tick before the pathogen can be transmitted. Additional inflammatory reaction to tick salivary proteins also may help prevent transmission (10–11). The epidemiologic relevance of host immunity to tick bite for preventing Lyme disease remains unknown.

Acquired immunity to vector ticks may limit the incidence of Lyme disease by protecting persons who have been previously exposed to bites of vector ticks. Accordingly, we determined whether cutaneous hypersensitivity against tick antigens increases with the frequency of tick exposure and whether such reactivity protects against Lyme disease. In particular, we determined whether residents of Block Island, Rhode Island, who experienced itching associated with attached ticks have fewer episodes of Lyme disease than those who report no episodes of itching associated with tick attachment.

Methods

Study Site and Sampling Procedures

Block Island is located 15 km from the New England mainland, and Lyme disease is highly endemic there. Beginning in 1991, we invited all residents to participate in a serosurvey twice yearly (during October and April). We sought to identify all cases of infection due to *B. burgdorferi* among serosurvey participants with the help of the staff of the Block Island Medical Center, the sole medical facility on the island, and by a dedicated research nurse (3). Borrelial infections were identified among members of the study cohort who visited the medical center for an acute tickborne illness or who seroconverted against borrelial antigen during the study period. Written informed

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consent was obtained from all adult study participants and from the parents or legal guardians of minors, in accordance with human experimentation guidelines approved by the institutional review boards at Connecticut Children's Medical Center and the Harvard School of Public Health.

Data Collection

At the serosurvey visit, participants submitted a blood sample and responded to a questionnaire about tick bites, use of protective measures, exposure factors, and symptoms of tickborne illness during the previous year (Figure 1). Specifically, they were asked if they had experienced a tick bite in the previous year and whether their tick bites produced itch. A medical history, physical examination, and specific Lyme disease laboratory tests were performed on all symptomatic participants at the time of suspected Lyme disease illness and 4–6 weeks later. A medical history was repeated at least every 3 months until the participants became asymptomatic. *Ixodes scapularis* ticks (*I. dammini*) were collected in May through October from 1991 through 2000 by flagging at diverse sites on the island. Ticks were analyzed for *B. burgdorferi* by polymerase chain reaction (PCR).

PCR Assay for Spirochete DNA

Whole blood samples were analyzed and processed by personnel blinded to the clinical status of the donor, as previously described (14). DNA extraction was performed on blood from Lyme disease patients and on *Ixodes* ticks with a commercially available kit (IsoQuick Nucleic Acid Extraction Kit, ORCA Research, Bothell, WA) (14,15). A 294-bp portion of the *B. burgdorferi* OspA gene was targeted for amplification by using a previously described PCR protocol (14,15).

Assays for Antispirochetal Antibody

Serologic evidence of exposure to the Lyme disease spirochete was detected by enzyme-linked immunosorbent assay (16). A reactive serum was defined as one with a positive reaction at a dilution ≥1:320. All borderline or reactive sera were further characterized by immunoblot (16,17). Specimens were considered positive if the immunoglobulin (Ig) G immunoblot contained ≥5 of the 10 most common *B. burgdorferi*–specific bands (17).

Case Definition

To include both symptomatic and asymptomatic cases of Lyme disease, diagnosis of newly acquired *B. burgdorferi* infection during the course of the study required one of the following: 1) a physician diagnosis of erythema migrans consisting of an expanding, ringlike erythematous rash at least 5 cm in diameter; 2) influenzalike symptoms consistent with Lyme disease and laboratory evidence of recent infection; 3) seroconversion from an initial nonreactive serum to a subsequent reactive serum that contained anti-*B. burgdorferi* antibody. The influenzalike symptoms of Lyme disease include fever, chills, sweats, fatigue, headache, or myalgia. Laboratory evidence of recent infection included either amplification of *B. burgdorferi* DNA in blood by PCR, seroconversion, or a 4-fold rise in anti-*B. burgdorferi* antibody in paired acute-phase and convalescent-phase sera.

Predicted and Observed Lyme Disease Rates

A simple model of Lyme disease transmission would calculate the rate of Lyme disease infection as the product of 2 main factors: the proportion of persons who report being bitten by deer ticks and the proportion of these ticks infected by *B. burgdorferi*. We calculated the yearly incidence of Lyme disease by determining the number of serosurvey participants who met the Lyme disease case definition each year in relation to the number of participants enrolled each year. By comparing the projected incidence using the Lyme disease transmission model to actual incidence of Lyme disease, we could assess the overall importance of factors missing from the basic model, such as the effects of inflammatory reactions against tick salivary proteins and acquired immunity to the spirochetal pathogen.


1. How many years have you spent on Block Island? _____
During which months? All___
If not all, check all that apply: Jan_Feb_Mar_Apr_May_June_July_Aug_Sept_Oct_Nov_Dec_

2. How many hours a day do you spend out of doors near vegetation?
Less than 1 ___ Several ___ 5 or more ___

3. Do you keep a pet on Block Island?
Dog __ Cat __ Horse __ Other__

4. How frequently do you see deer around your BI residence?
Daily __ Weekly__ Less frequently__

5. Have you been bitten by a tick this year?
Yes__ No__
If yes, was it a deer tick__ wood/dog tick__
tiny__ large __
If yes, was it on Block Island? Elsewhere?

6. Do your tick bites itch? Yes_ No ___

7. When outdoors, what personal protection measures against ticks do you employ?
None__ Repellant__ Long pants/socks__ Avoid brush__ Tick check__

8. Do you try to control ticks around your residence?
Yes__ No __ Chemical spray__ Damminix__ Brush control__ Other _____

Figure 1. Standardized serosurvey questionnaire used to gather tick exposure history.

Statistical Analysis

All statistical calculations were performed with JMP 5.1 (SAS Institute, Cary, NC). To estimate the study sample frequency of tick bite and tick-associated itch, we averaged the individual reports of tick bite and tick-associated itch among study participants each year. To determine yearly Lyme disease incidence, we compared the number of new cases of Lyme disease each year to the total number of study participants who had enrolled in the study up to that time. To create 10-year individual measures, reports of tick bite and itch were summed across all visits for each participant. The results were analyzed with descriptive statistics (mean, proportion, and confidence intervals with 5% error). Bivariate logistic regression was used to estimate probability of itch for increasing tick bites. The relative contribution of risk factors to the acquisition of Lyme disease was evaluated from multiple logistic regression models to calculate odds ratios with associated confidence limits. The predicted probability of acquiring Lyme disease was estimated for significant risk factors.

Results

A total of 1,669 residents of Block Island, most of the island population, enrolled in our study from 1991 to 2000. We excluded those participants who did not report spending at least 1 month on the island during May through October, which resulted in a sample of 1,490. The mean age of the sample was 43 years (95% CI 42.2–44.1) and approximately half (51%) were female.

We determined how frequently the 1,490 persons in our sample recalled a deer tick bite. Each year an average of 27% (95% CI 23%–31%) of the study participants reported ≥1 tick bite during the prior year (range 20%–37%). An average of 17% (95% CI 13%–21%) of study participants reported itch associated with tick bite (Figure 2).

We then determined the prevalence of *B. burgdorferi* infection in nymphal deer ticks that contained *B. burgdorferi* DNA and that were swept from vegetation on Block Island throughout the course of this study. Of 135 such ticks tested, 23% (95% CI 20%–26%) contained amplifiable *B. burgdorferi* DNA. We therefore predicted that the maximum yearly incidence of Lyme disease would be 6.2% (95% CI 4.6%–8.1%) (27% with tick bite x 23% of ticks infected).

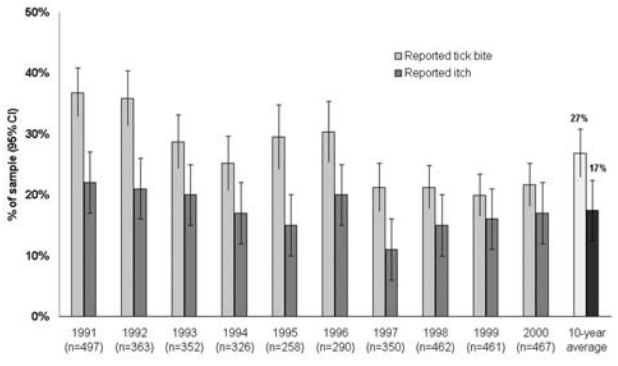

Figure 2. Reported tick bite and itch by serosurvey year on Block Island, Rhode Island, 1991–2000.

We next calculated yearly incidence rate of new Lyme disease cases among study participants from 1991 to 2000. The average incidence was 1.74% (95% CI 1.1%–2.4%) (Table). Thus, the predicted incidence of Lyme disease using the basic model (95% CI 4.6%–8.1%) was 3%–6% greater than the observed incidence. This difference would not be expected due to chance.

We next determined whether increasing exposure to ticks increases the probability of tick-associated itch. This analysis was limited to participants whose sera displayed no evidence of tickborne illness before study entry, who had >1 serosurvey visit, and who were >2 years of age. Among these 610 participants, 52% reported at least 1 tick bite (mean 2.2, 95% CI 2.0–2.4), and 32% reported itch with any tick bite. The probability of itch doubled as the number of reported tick bites increased from 1 to 2 (21% to 46%, respectively) and doubled again from 2 to 4 reported bites (46% to 97%, respectively; linear trend $p < 0.001$).

Finally, we tested the hypothesis that tick-associated itching is associated with decreased risk of Lyme disease. The acquisition of Lyme disease increased from 15% to 25% to 31% among participants who reported no itch, 1 episode of tick-associated itch, and 2 reports of itch, respectively. In contrast, the frequency of Lyme disease decreased to 13% in participants who reported 3 episodes of tick-associated itch and 10% in those with ≥4 such reports. We used a multiple logistic regression model to estimate the likelihood of acquiring Lyme disease for participants reporting none to 1, 2, and ≥3 reports of itch, controlling for number of study visits and reports of tick bite. Consistent with the bivariate analysis, the risk of acquiring Lyme disease was higher for 1 report of itch (OR 2.7, 95% CI 0.4–2.3), and decreased among those who reported itch at ≥3 study visits (range 3–12, OR 0.18, 95% CI 0.05–0.5) (Figure 3). Confining the analysis to only those who had Lyme disease illness by omitting participants whose evidence of Lyme disease was by seroconversion alone did not alter the inverse relationship between itch and developing Lyme disease. Persons who consistently report itching in association with tick bites are less likely to experience an episode of Lyme disease than do those who fail to react against tick bite.

Discussion

These observations suggest that residents of disease-endemic sites who experience persistent tick-associated itch are less likely to develop Lyme disease than are those

Table. Yearly incidence of Lyme disease among Block Island study cohort, 1991–2000*

Year	No. participants	No. (%) with first episode of Lyme disease
1991	731	25 (3.4)
1992	824	27 (3.3)
1993	933	17 (1.8)
1994	1,038	23 (2.2)
1995	1,114	15 (1.4)
1996	1,169	15 (1.3)
1997	1,246	5 (0.4)
1998	1,325	18 (1.4)
1999	1,432	14 (1.0)
2000	1,490	19 (1.3)

*10-year average = 1.74 (95% confidence interval 1.1–2.4).

who do not experience this reaction. Itch was reported at the site of tick bite, and the frequency of itch increases as the number of reported tick bites increase, which strongly suggests that tick-associated itch is linked to an acquired cutaneous hypersensitivity response. Such a relationship has definitively been established in the case of *I. ricinis*, the European analog of the North American deer tick. After having been bitten by these ticks repeatedly, persons experience both immediate and delayed cutaneous hypersensitivity reactions. They express tick-specific IgE antibodies, as well as dermal and perivascular infiltrates of CD8+ T lymphocytes and Langerhans' cells (13). Additional studies of other tick species also suggest that tick-associated itch is mediated by tick-specific antibody or cellular infiltration (12,18,19). People in our study who reported a single episode of tick-associated itch were more likely to acquire Lyme disease than those who did not report itch, probably because tick-associated itch is a marker for tick exposure. In contrast, persons who had repeated tick-associated itch were protected from developing Lyme disease. We are confident, therefore, that the people in our study who described repeated episodes of tick-associated itching were experiencing cutaneous hypersensitivity and that this immune reaction protected them from acquiring Lyme disease.

The antitick immune response that protects people from acquiring Lyme disease might act through any of several effector mechanisms. A heightened awareness by a person of an attached tick would result in the removal of the potentially infecting tick before pathogen transmission could occur. Because vector ticks must remain attached for at least 2 days before spirochetes are transmitted, prompt tick removal should prevent spirochetal infection (8,20). Vector ticks generally are removed by persons within such a time period (21–23). Alternatively, immunity against tick salivary antigens might interfere with pathogen transmission independent of early host recognition and removal of ticks. Indeed, preexposure of mice and guinea pigs to uninfected ticks prevents Lyme disease after challenge by spirochete-infected ticks using infestation conditions that prevent removal of feeding ticks by host grooming (24,25). Ticks feeding on such immune hosts detach sooner and retain less host material than do ticks feeding on nonimmune hosts. Although natural hosts, such as *Peromyscus leucopus*, do not develop robust antitick immunity, inbred strains of mice and guinea pigs become immune to the salivary antigens secreted by feeding ticks (10,26,27). This immunity results in reduced volume of engorgement, abnormal blood meal composition, prolonged feeding, and frequently death of the tick (10,27). Antitick immunity may specifically neutralize some components of tick saliva that ensure successful feeding and facilitate pathogen transmission, such as vasodilators, anticoagulants, and immunosuppressors (10,11,28). Prevention of *B. burgdorferi* transmission in guinea pigs is associated with antivector antibody (25). Persons who experience frequent deer tick bites produce an array of specific antitick antibodies (29–31). Cell-mediated immune factors against tick-derived antigen might similarly play a role in developing cutaneous hypersensitivity and protect against infection (13,19). A strong delayed cutaneous hypersensitivity response to sandfly bites in persons has been correlated with reduced transmission of leishmanial parasites (32,33). An array of antitick immune reactions may prevent Lyme disease and other tickborne diseases.

Our analysis incorporates several potentially confounding assumptions. Although our estimate of the prevalence of spirochetal infection in vector ticks is based on a small sample of ticks, this rate is consistent with that derived in other parts of southern New England at approximately the same time (5,6). Although our study participants live on Block Island where deer ticks predominate, some of our participants may have been bitten by other kinds of ticks or been bitten by ticks off the island. While approximately a quarter of our participants reported a tick bite, previous

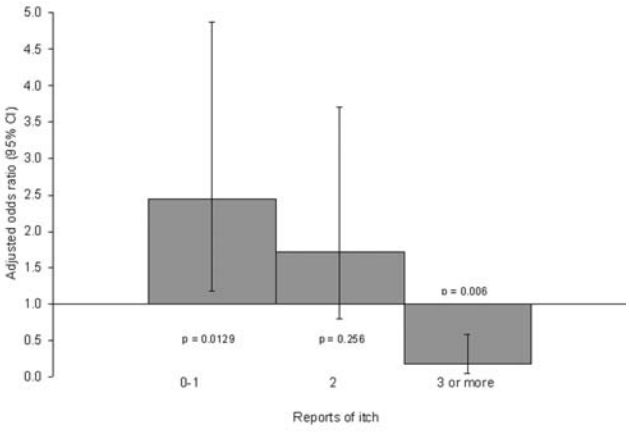

Figure 3. Risk of acquiring Lyme disease according to reports of tick bite–associated itch.

studies have shown that persons are often unaware that they have been bitten. From a third to three fourths of patients with Lyme disease cannot recall having been bitten by a tick (6,34–36). Assuming that a similar percentage of our study participants were unaware of a previous tick bite, the tick bite prevalence on Block Island would have varied from half to all of our participants. A tick bite prevalence of 71% was reported at another site where Lyme disease is highly endemic (37). Any underestimate of the frequency of tick attachment would increase the likelihood that other factors, including antitick immunity, help limit the incidence of Lyme disease. Finally, our predicted Lyme disease incidence assumes that people are bitten only once in any year. An increase in yearly tick bite frequency among persons would increase the likelihood that the tick bite is from an infected tick, thus similarly increasing the difference between predicted and observed incidence rates. We believe that none of these assumptions is notably confounding.

Several factors help prevent Lyme disease in persons who live in disease-endemic regions. These factors include the paucity of ticks that are infected with the Lyme spirochete, the limited number of persons who are bitten by ticks, acquired immunity against the spirochetal pathogen, and the immune reaction to ticks that develops in the course of tick attachment. Persons who express an immune reaction against the vector tick appear to acquire Lyme disease less frequently than do those who experience no such immune reaction. The protective effect of the immune response to tick salivary protein against the agent of Lyme disease in persons suggests that a tick protein-based vaccine might be developed that would protect against infection by the agent of Lyme disease and possibly other tickborne infections.

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Demand for Prophylaxis after Bioterrorism-related Anthrax Cases, 2001

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Media reports suggested increased public demand for anthrax prophylaxis after the intentional anthrax cases in 2001, but the magnitude of anthrax-related prescribing in unaffected regions was not assessed. We surveyed a random sample of 400 primary care clinicians in Minnesota and Wisconsin to assess requests for and provision of anthrax-related antimicrobial agents. The survey was returned by 239 (60%) of clinicians, including 210 in outpatient practice. Fifty-eight (28%) of those in outpatient practice received requests for anthrax-related antimicrobial agents, and 9 (4%) dispensed them. Outpatient fluoroquinolone use in both states was also analyzed with regression models to compare predicted and actual use in October and November 2001. Fluoroquinolone use as a proportion of total antimicrobial use was not elevated, and anthrax concerns accounted for an estimated 0.3% of all fluoroquinolone prescriptions. Most physicians in Minnesota and Wisconsin managed anthrax-related requests without dispensing antimicrobial agents.

Until recently, human anthrax infections in the United States have been rare and generally limited to agricultural workers with exposure to infected animals or animal products. The first bioterrorism-related cases of human anthrax occurred in late 2001, when spore-laden envelopes were mailed to news media facilities and government officials. Twenty-two cases of anthrax were identified from October 4 to November 20, including 5 fatalities (1). Cases occurred in New York, New Jersey, Pennsylvania, Virginia, Maryland, Connecticut, and Florida. The patients included targeted persons, people who worked with targeted persons, postal workers, and people who were exposed along the mail route of spore-containing envelopes.

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At the affected sites, ≈10,000 people were advised to take antimicrobial prophylaxis for at least 60 days to prevent anthrax, and an estimated 32,000 people initiated antibiotic prophylaxis (1,2). Most persons received initial prophylaxis with a fluoroquinolone (ciprofloxacin), a class of drug that is also important for treatment of community-acquired pneumonia and other serious infections (3,4). In public announcements regarding anthrax, the Centers for Disease Control and Prevention (CDC) emphasized the overall low risk to the population but also endorsed actions to minimize personal risk. These measures included not opening suspicious mail, keeping mail away from the face when opened, and washing hands after handling mail (5). Although national publicity generated a high level of public concern throughout the country, no evidence of anthrax spore release was found in any other regions of the United States.

Media reports in late 2001 reflected a high level of anxiety in the general public regarding anthrax. An ABC News/Washington Post poll in mid-October 2001 found that 65% of respondents were worried about letters contaminated with the anthrax bacteria, and 54% were worried about an anthrax attack on themselves, their close friends, or relatives (6). At the same time, anecdotal evidence suggested that ciprofloxacin sales were increasing at some pharmacies (7,8). New Web sites marketed ciprofloxacin "prevention packs" to anxious consumers (9). However, the magnitude of public demand for anthrax-related antimicrobial agents and for physicians to dispense them has not been assessed in regions of the country where no cases or exposures occurred.

Inappropriate use of fluoroquinolones raises concern for several reasons. They can potentially cause adverse reactions, and excessive hoarding could contribute to drug shortages. Fluoroquinolones are critical drugs for managing a variety of serious infections, and in recent years the rate of resistance has increased among both gram-positive

and gram-negative organisms because of the increasing use of these agents (10–12). Recent fluoroquinolone use is an independent risk factor for fluoroquinolone-resistant pneumococcal infections (13).

The purpose of this investigation was to assess patient demand for anthrax prophylaxis and changes in fluoroquinolone use in late 2001 in a region of the country in which no cases or exposures occurred. Clinicians were surveyed regarding patient demand for anthrax prophylaxis and use of prophylactic antimicrobial agents, and a commercial database was analyzed to assess changes in overall fluoroquinolone use.

Methods

Clinician Survey

A 1-page survey and cover letter were mailed to 400 primary care clinicians in Minnesota and Wisconsin in October 2002. Within each state, a simple random sample of 200 clinicians was selected from among those with an active medical license in any of the following specialties: emergency medicine, family practice, general practice, general internal medicine, pediatrics, nurse practitioner, or physician assistant. The sampling frame was obtained from the licensing agencies in each state and included 5,800 clinicians in Minnesota and 6,510 clinicians in Wisconsin. Two reminder letters were sent to all survey recipients to encourage participation.

The anonymous survey contained 12 questions to obtain information on specialty, county of practice, outpatient practice activity during the last quarter of 2001, requests for anthrax prophylaxis from October through December of 2001, and distribution of antimicrobials for anthrax prophylaxis or stockpiling. Clinicians who indicated that they prescribed or distributed anthrax-related antimicrobial agents were asked to specify the number and types of people (i.e., patients, family members, acquaintances, self) who received the drugs, and the specific drugs that were used. The occurrence of suspected anthrax-related exposures was not assessed. Clinicians who indicated they were not in full- or part-time outpatient medical practice from October through December of 2001 were asked to return the survey without answering the other questions.

The survey procedures were reviewed and approved by the institutional review board at Marshfield Clinic.

Outpatient Use of Antimicrobial Agents

The primary measures of fluoroquinolone use were based on prescribing data (Xponent, IMS Health, Inc., Plymouth Meeting, PA) and volume retail distribution data (DDD, IMS Health, Inc.) for the states of Wisconsin and Minnesota. The prescribing and distribution data represent independent measures of antimicrobial use that were obtained to evaluate a statewide program to promote appropriate antimicrobial use. Prescribing data were available for the years 2000 through 2002. Volume distribution data were available for the years 1999 through 2002. For this analysis, oral antimicrobial agents were grouped into the following three categories: fluoroquinolones (ciprofloxacin, levofloxacin, lomefloxacin, moxifloxacin, ofloxacin), tetracyclines (doxycycline, tetracycline, minocycline), and other oral antimicrobial agents (amoxicillin, amoxicillin-clavulanate, ampicillin, cephalosporins, extended-spectrum macrolides, macrolides, penicillin, and trimethoprim-sulfamethoxazole). The first two categories represented drug classes that were most likely to be used for anthrax prophylaxis or stockpiling, since they were recommended as first-line agents by CDC (14). The third category provided a measure of seasonal and annual trends in outpatient use of antimicrobials, since the drugs in this category (with the possible exception of penicillin and amoxicillin) would not be appropriate for managing anthrax exposure. Penicillin or amoxicillin use was recommended for anthrax use only when the first-line agents were contraindicated, and it is therefore unlikely that a substantial number of these prescriptions were generated for anthrax-related concerns. Prescribing and volume distribution data were not available for individual drugs within each class. Only oral agents in solid (capsule or tablet) formulation were included in the analyses to avoid including prescriptions for young children.

Xponent prescribing data included all dispensed prescriptions in Minnesota or Wisconsin from licensed prescribers. The total number of prescriptions written in each state was available by month for the years 2000, 2001, and 2002. The prescribing databases were compiled by IMS Health with proprietary methods. The Xponent prescribing database was derived from transactional data provided by 59% of all retail pharmacies in Wisconsin and Minnesota, including 65% of chain pharmacies and 51% of independent retail pharmacies. Prescriptions from unsampled stores were estimated on the basis of prescription totals from matched nearby stores with weighting to adjust for differences in total retail sales volume, which was available for nearly all stores. Estimates were also weighted to account for the distance between sampled stores and matched unsampled stores, with closer stores contributing more to the estimated prescription volume. The proportion of all prescriptions in each state based on estimated data from unsampled stores was 33%–37%.

Retail distribution was measured based on the volume of antimicrobial agents distributed to retail outlets on a monthly basis for the years 1999 through 2002 (DDD data, IMS Health, Inc.). Retail distribution data (measured in kilograms) were ultimately derived from wholesalers and distributors serving pharmacies in both states. Volume was

based on distribution to retailers rather than actual sales to patients, and distributed antimicrobial agents could be returned to the wholesalers without being sold. In this situation, returned antimicrobial agents were subtracted from the total distributed in a given month to yield the net retail distribution for each drug class. As a result, the retail distribution data may overestimate or underestimate actual distribution to patients, particularly in short time periods. Inpatient pharmacies, prisons, veterinarians, nursing homes, dialysis clinics, and federal government sites were excluded from the volume distribution data. The DDD database captured 93% of actual volume of antimicrobial distribution in these states.

Statistical Analysis

To account for the state-level sampling in the clinician survey, analysis weights were generated for each survey responder. These weights were computed by multiplying the inverse of the sampling probability for each responder by the inverse of the response rate in the appropriate state. Weighted analyses for complex sample designs were performed by using the SAS 8.2 (SAS Institute Inc., Cary, NC) and SUDAAN 8.0 (RTI International, Research Triangle Park, NC) software. The impact of the weighting was minimal in our study; estimated percents for the weighted and unweighted analyses differed by ≤l percentage point. We therefore present unweighted point estimates, except for the estimate of anthrax-related antimicrobial drug prescribing. All statistical tests for the clinician survey were based on weighted data to reflect the complex sample design. Categorical variables were compared by using the chi-square test.

For the Xponent prescribing data, we fit models for the absolute number and proportion of prescriptions for fluoroquinolones and tetracyclines. Absolute numbers of prescriptions were modeled by using linear regression, while proportions of prescriptions were modeled by using negative binomial regression. All of the models contained categoric effects for year and month and indicator variables for October, November, and December of 2001. We generated predictions for the fourth quarter of 2001 by computing linear combinations of the appropriate parameters from the above models. The modeling procedures for the DDD data were the same as those for the Xponent data, except that we modeled total retail distribution volume by month for each drug category.

Results

Clinician Survey

Surveys were returned by 239 (60%) of 400 clinicians, including 123 in Wisconsin and 116 in Minnesota. Twenty-nine (12%) of the 239 respondents were excluded from subsequent analyses because they were not engaged in full- or part-time outpatient practice during the last 3 months of 2001. The respondent medical practices were located in 68 counties; 58 (28%) practices were located in the 11-county Minneapolis–St. Paul metropolitan area, and 24 (11%) were located in the 4-county Milwaukee-Waukesha metropolitan area. One hundred fifty-seven (75%) were physicians (MD or DO), and 52 (25%) were nurse practitioners or physician assistants. Physician specialties included family practice (42%), internal medicine (30%), pediatrics (18%), emergency medicine (4%), and other (6%).

Fifty-eight (28%) of the clinicians reported that someone had asked them to prescribe an antimicrobial drug to prevent anthrax or stockpile in case of future bioterrorist attacks. Physicians were significantly more likely than nonphysicians to receive requests for antimicrobial agents (Table). The occurrence of patient requests by state, practice specialty, or practice location (Minneapolis–St. Paul and Milwaukee–Waukesha metropolitan areas vs. other areas) did not differ significantly.

Nine (4%) of the clinicians provided antimicrobial agents to 11 persons for anthrax prevention or stockpiling. Seven clinicians provided such agents for a single person, and 2 clinicians provided them for 2 persons. All 9 clinicians were MDs or DO, and 8 (89%) practiced in Wisconsin. Among 58 clinicians who received such requests, 8 (14%) provided them; 1 additional clinician did not receive requests but provided these drugs for family members. Nine (82%) of the 11 courses of antimicrobial agents were given to patients seen in the clinician's practice, and 2 were given to family members. One clinician reported that prophylactic antimicrobial agents were given to a patient who had exposure to a building in Washington, D.C., where anthrax release was confirmed; the risk for

Table. Characteristics of Minnesota and Wisconsin clinicians who received requests for antimicrobial agents to prevent anthrax during the last quarter of 2001

	Received requests (%)	p value
Prescriber type		
Physician	52/157 (33)	<0.001
Nonphysician	6/52 (12)	
Practice location		
Metropolitan area	22/82 (27)	0.82
Nonmetropolitan area	36/128 (28)	
State		
Minnesota	29/105 (28)	1.0
Wisconsin	29/105 (28)	
Physician specialty*		
Family practice	26/62 (41)	0.61
Internal medicine	14/42 (30)	
Pediatrics	8/28 (28)	
Emergency medicine	2/5 (33)	

*Nine physicians who listed 2 specialties and 2 who listed "other" as a specialty were excluded from the frequency distribution for physician specialty.

anthrax exposure was unknown for the other 10 persons. A fluoroquinolone (levofloxacin or ciprofloxacin) was used for 10 (91%) of the 11 courses; amoxicillin was given to 1 person. No other classes of antimicrobial agents were used.

Survey responses were used to estimate the total number of anthrax-related fluoroquinolone prescriptions written in Wisconsin and Minnesota during the last quarter of 2001. State medical licensing records indicate that ≈10,807 primary care clinicians were engaged in full- or part-time outpatient practice in Minnesota and Wisconsin at that time. If the survey responses are representative of this group, an estimated 420 clinicians (3.9%) in these states provided anthrax-related antimicrobial agents. The 95% confidence intervals (CI) for this proportion are 1.2% and 6.5%, corresponding to 135 and 706 clinicians, respectively. The total number of anthrax-related courses of antimicrobial agents prescribed during the last quarter of 2001 was estimated to be 523 (95% CI, 394–653) in the two states. If these prescriptions were mostly for fluoroquinolones, they would have represented ≈0.3% of all fluoroquinolone prescriptions written in Minnesota or Wisconsin from October through December of 2001.

Outpatient Use of Antimicrobial Agents

The annual number of fluoroquinolone prescriptions in Minnesota and Wisconsin increased 20% from 2000 to 2002, while the annual number of prescriptions declined during this period for tetracycline drugs and other antimicrobials. In October 2001, the number of fluoroquinolone prescriptions was 22% higher than in October 2000, and the number of fluoroquinolone prescriptions exceeded the 95% CI based on the predictive model (Figure 1A). The proportion of all antimicrobial prescriptions (excluding tetracyclines) represented by fluoroquinolones gradually increased from 2000 to 2002, and this proportion was higher in October 2001 (17.5%) than in October 2000 (15.5%). However, the observed proportion of fluoroquinolone prescriptions was lower than predicted in October and November of 2001, after adjusting for the secular trend (Figure 1B). Retail distribution of oral fluoroquinolones was significantly elevated in October and November of 2001 (Figure 2A). The fluoroquinolone proportion of total retail distribution of antimicrobial agents (excluding tetracyclines) also increased significantly in October 2001, but it was lower than predicted in November (Figure 2B).

The proportion of all antimicrobial prescriptions (excluding fluoroquinolones) that were for tetracyclines was near the upper limit of the 95% CI in October 2001, and it exceeded the upper limit in November 2001 (Figure 3). The proportion of antimicrobial drug retail volume represented by tetracyclines was not elevated during those months (data not shown).

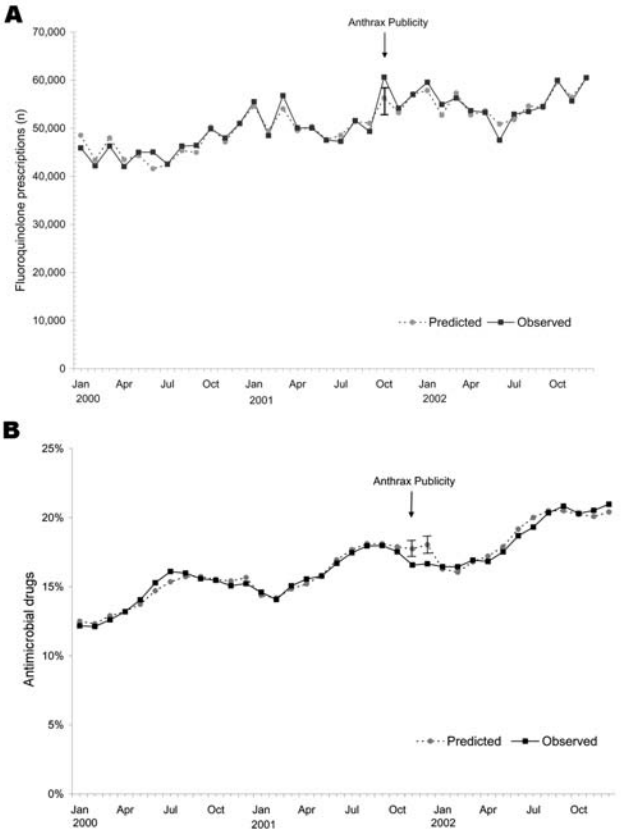

Figure 1. A) Actual and predicted fluoroquinolone prescriptions, January 2000 through December 2002. B) Actual and predicted fluoroquinolone prescriptions as a percentage of all outpatient antibiotic prescriptions, January 2000 through December 2002. Tetracycline and related antimicrobial agents were excluded from the denominator in each month. Vertical bars show 95% confidence intervals. All models included categorical effects for year and month and indicator variables for October, November, and December, 2001.

Discussion

The results of this study confirm that public demand for anthrax-related antimicrobial agents was substantial in Minnesota and Wisconsin, since one fourth of primary care clinicians received requests for these drugs. We found that physicians received these requests more often than nonphysicians. Despite requests from patients and family members, relatively few antimicrobial agents were dispensed for anthrax prophylaxis. Extrapolation of survey responses to all primary care clinicians in Minnesota and Wisconsin suggests that ≈500 anthrax-related antimicrobial drug courses were dispensed during the last quarter of 2001. Even if nearly all of these were fluoroquinolones, the volume of anthrax-related fluoroquinolone use represented only a small fraction of total use in Minnesota and Wisconsin during that period.

The prescribing and retail distribution data showed surges in fluoroquinolone use during October 2001, but

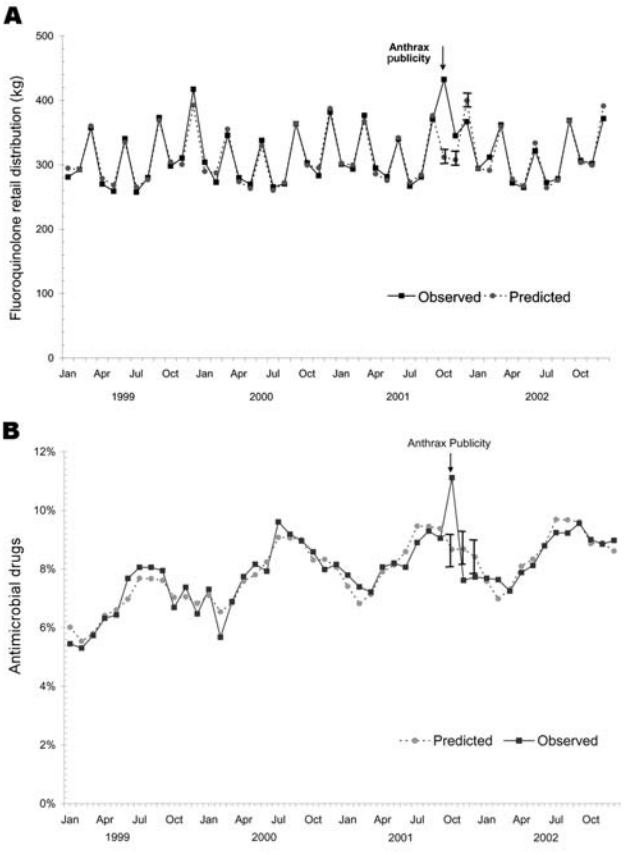

Figure 2. A) Retail distribution of fluoroquinolone antimicrobial agents, January 1999 through December 2002. Volume was measured in kilograms. B) Actual and predicted retail distribution of fluoroquinolone antimicrobial agents as a percentage of total antibiotic volume distribution (excluding tetracyclines), January 1999 through December 2002. Vertical bars show 95% confidence intervals.

total use of antimicrobials also increased for unknown reasons during that period. Fluoroquinolone prescriptions as a proportion of all antimicrobial prescriptions were not elevated, which was consistent with the findings from the clinician survey. Although tetracycline/doxycycline prescriptions as a proportion of all outpatient antimicrobial prescriptions (excluding fluoroquinolones) were elevated in October and November 2001, the clinician survey indicated that this increase was unrelated to anthrax prescribing. None of the survey respondents reported using tetracycline/doxycycline for this purpose, and ciprofloxacin was the anthrax drug that received most media attention in late 2001.

Two other studies have addressed national use of antimicrobial agents following the anthrax cases in 2001. In one study that used a national pharmacy claims database, the rate of ciprofloxacin use increased 9.8% in October 2001 relative to October 2000 (15). As expected, the greatest increase in use was observed in New York, the mid-Atlantic states, and Florida. Ciprofloxacin prescribing rates were not reported for Minnesota or Wisconsin. In this study, the denominator was defined as the number of covered persons who filled a prescription for any drug or eligible health product during that month. As a result, the observed rate differences may have been influenced by both changes in the numerator (number of ciprofloxacin prescriptions) and changes in the denominator (number of persons filling any prescription).

A similar study used IMS Health National Prescription Audit Plus7 data to compare national ciprofloxacin use from July to December of 2001 and 2000 (16). Comparison drugs included oral azithromycin and cefuroxime, which are commonly used in outpatient practice but not recommended for anthrax prophylaxis. Ciprofloxacin prescriptions increased by 42%; cefuroxime prescriptions declined by 3%. The results were not reported by region, and they included prescriptions in New York, Florida, and other affected regions. The authors did not assess monthly ciprofloxacin prescriptions as a percentage of all antimicrobial prescriptions. The results of our study suggest that short-term variations in single drug prescribing should be interpreted with caution when the specific diagnoses or prescribing indications are not known. We found that a short-term increase in fluoroquinolone use in Minnesota and Wisconsin was accompanied by an overall increase in antimicrobial drug use. Thus, factors unrelated to anthrax may have also contributed to the observed increase in fluoroquinolone use during October 2001, especially in unaffected regions of the United States.

Whether patterns of antimicrobial use in Minnesota and Wisconsin are generalizable to other unaffected regions of the United States is not known. For example, total ciprofloxacin prescriptions in October 2001 appeared to increase >25% in some unaffected states, including Nevada, California, and New Mexico (15). No information is available regarding the clinical indications for these prescriptions, and how much of this increase can

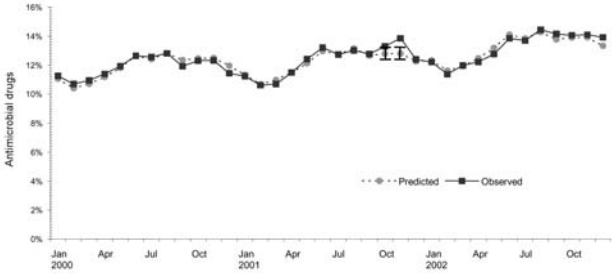

Figure 3. Actual and predicted tetracycline group prescriptions as a percentage of all outpatient antimicrobial prescriptions (excluding fluoroquinolones), January 2000 through December 2002. Vertical bars show 95% confidence intervals.

be attributed to anthrax-related prescribing is unclear. Other factors may also contribute to regional differences in prescribing, since physicians in the northeastern and southern United States are more likely to prescribe broad-spectrum antimicrobials than those in the midwestern or western regions (17).

The survey results in Minnesota and Wisconsin may have underestimated actual anthrax-related prescribing, since clinicians who dispensed antimicrobial agents may have been reluctant to return the survey. However, the cover letter and survey questions were neutral regarding the appropriateness of antimicrobial drug use, and the survey was anonymous. Poor recall is another potential source of error, since the survey was conducted approximately 1 year after the first cases of intentional anthrax occurred. Because we were asking about unusual events that were outside the scope of normal clinical practice, we assumed that clinicians would still recall any anthrax-related prescribing. Finally, the survey results did not allow us to determine if patients consumed these agents for anthrax prophylaxis, or if they were stockpiled for future use.

The human anthrax cases in 2001 and the related events illustrate how quickly demand for a critical drug can escalate as a result of heightened public anxiety and media attention. Most physicians in Minnesota and Wisconsin managed public and patient expectations without dispensing antimicrobial agents. However, social factors clearly influence prescribing decisions (18), and effective public and physician communication will be essential to promote rational behavior if similar or more extreme situations arise in the future. A communications strategy should be developed in advance that includes identifying key experts at the state and national level for news media communications and devising a plan for coordination and consistency of messages from different agencies.

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Dengue and Dengue Hemorrhagic Fever, Brazil, 1981–2002

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In the last 5 years, Brazil has accounted for ≈70% of reported dengue fever cases in the Americas. We analyzed trends of dengue and dengue hemorrhagic fever (DHF) from the early 1980s to 2002 by using surveillance data from the Brazilian Ministry of Health. Two distinct epidemiologic patterns for dengue were observed: localized epidemics (1986–1993), and endemic and epidemic virus circulation countrywide (1994–2002). Currently, serotypes 1, 2, and 3 cocirculate in 22 of 27 states. Dengue and DHF affected mainly adults; however, an increase in occurrence of DHF among children has been recently detected in northern Brazil, which suggests a shift in the occurrence of severe disease to younger age groups. In 2002, hospitalizations increased, which points out the change in disease severity compared to that seen in the 1990s. We describe the epidemiology of dengue in Brazil, characterizing the changing patterns of it and DHF during the last 20 years.

Brazil accounted for nearly 70% of the 3,141,850 reported cases of dengue fever in the American regions in the last 5 years (1). Dengue fever and a severe manifestation of the illness known as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are increasingly important health, environmental, and economic concerns in the Americas (2,3). Dengue/DHF is a febrile illness caused by a flavivirus with four known serotypes (dengue virus [DENV]1, 2, 3, 4), and infection is mainly transmitted by the mosquito *Aedes aegypti* (4). Currently, no vaccine is available for dengue fever, and disease control and prevention have mainly focused on vector control activities and surveillance that incorporates community participation (5,6). Despite vector control programs and heightened public awareness, outbreaks have occurred in several highly urbanized areas in Central and South America (7–9).

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The reemergence of dengue in Latin America occurred during the 1960s with epidemics in the Caribbean and Venezuela and in the 1970s in Colombia (3). Brazil remained free of *A. aegypti* until 1976 as a result of the successful eradication program to prevent urban yellow fever coordinated by the Pan American Health Organization (PAHO) in previous decades (10,11). The subsequent reinfestation of *A. aegypti* into urban areas of Brazil and the introduction of DENV in 1986 led to resurgence of dengue fever outbreaks and an increased risk for urbanization of yellow fever in the country (12).

The Brazilian system for dengue/DHF reporting relies on passive surveillance, with laboratory diagnosis for case ascertainment and identification of circulating serotypes. A parallel system for entomologic surveillance exists to monitor virus and vector dispersion. Analyses of data reported to the state and national level are used to evaluate the impact of disease, time trends, and geographic distribution, with the objective of supporting and improving public health interventions (13). In a previous report, we analyzed the dengue situation, focusing on the main policies regarding prevention and control strategies adopted in Brazil (8). Here, on the basis of national surveillance data, we analyze the trends of dengue/DHF from the early 1980s to 2002 and contrast the changes in the epidemiologic pattern of disease for regions in Brazil.

Methods

Brazil is the largest and most populated country in Latin America, covering >8 million km^2 with an estimated 2002 population of 174,632,932 inhabitants. High population density areas and cities (up to 12,901 inhabitants/km^2) are located mainly on the Atlantic Coast. Most of Brazil has a tropical climate; in the southern region, the climate is subtropical. The rainy season is observed in the first several months of the year, and the average temperature is >20°C (14).

Sources of Data

Dengue/DHF is a reportable disease in Brazil, and the ministry of health has implemented a surveillance system since the first epidemic in the early 1980s. We reviewed available data compiled from 1986 to 2003 by the official surveillance system. We also used the available computerized data from the Hospitalization Information System of the Unified Health System (SIH-SUS), which covers ≈70%–80% of overall hospitalizations in the country (15,16). This system permits recovery of information on hospital admissions according to the International Disease Classification system (ICD versions 9 and 10). For this analysis, we selected hospitalizations due to dengue fever and DHF stratified by state from 1990 to 2003.

Case Definition and Surveillance Forms

During the study period, case definitions used for suspected, probable, and confirmed dengue/DHF adhered to PAHO guidelines (17). Information on persons with suspected dengue/DHF is routinely reported by using standardized forms completed by clinicians or health staff and subsequently sent to local health surveillance officials for data checking. This dengue/DHF case investigation form includes information on basic demographic data, dates of symptom onset and sample collection, case classification (dengue fever, DHF, DSS, or discarded case), and outcome. Individual data are locally entered into the electronic information system and subsequently transmitted to state and national levels.

Laboratory Confirmation and Entomologic Surveillance

Laboratory confirmation was based on the following: 1) serologic tests (immunoglobulin M antibody-capture enzyme-linked immunosorbent assay) performed on serum samples collected ≥6 days after the onset of symptoms (18); or 2) virus isolation in C6/36 mosquito cell culture from blood samples collected <6 days after symptom onset (19). Immunohistochemical studies with formalin-fixed tissues were performed on samples from selected patients who died (20).

Initially, 3 public health laboratories were responsible for DENV laboratory confirmation: Evandro Chagas Institute (located in Belém, Pará State), which is the Ministry of Health reference laboratory; Oswaldo Cruz Foundation (Rio de Janeiro); and Adolfo Lutz Institute (São Paulo). During the 1990s, the Public Health Laboratory Network was set up for the dengue-endemic states to respond to increasing requests for laboratory confirmation. By 2001, a total of 27 public laboratories at state level were in charge of serologic tests and quality control. Information on circulating serotypes was obtained from viral isolation by the 3 national reference laboratories during the 1980s and subsequently from 13 public health laboratories at the state level. Laboratory results are routinely linked to individual data. Classification of DHF required laboratory confirmation throughout the study period. Approximately 30% of dengue fever cases are also laboratory-confirmed. During epidemics, dengue cases were mostly classified by clinical and epidemiologic criteria because of limits in laboratory capacity.

The number of municipalities documented with *A. aegypti* infestations and information on vector dispersion were abstracted from data compiled by the national vector information system for yellow fever and dengue.

Data Analysis

A descriptive analysis of dengue incidence was performed by region (using residence of reported cases) from 1986 to 2002. Incidence and hospitalization rates by age group were calculated by using census population data as denominators. The ratio of incident versus hospitalized cases was also calculated. Exploratory data analysis of the age group of reported patients focused on data from 1998 to 2002, since standardized data in electronic format for these variables were available for this period.

Results

The epidemiologic pattern of dengue fever in Brazil during the last 20 years can be divided into 2 distinct periods: 1) epidemic waves in localized areas (1986–1993) and 2) epidemic and endemic virus circulation countrywide (1994–2002) (Figure 1 and Table 1). The main dengue-related events occurring in these 2 periods are summarized in Table 1. In 1981, the first laboratory-confirmed cases of dengue (DENV1 and DENV4) occurred in an isolated area

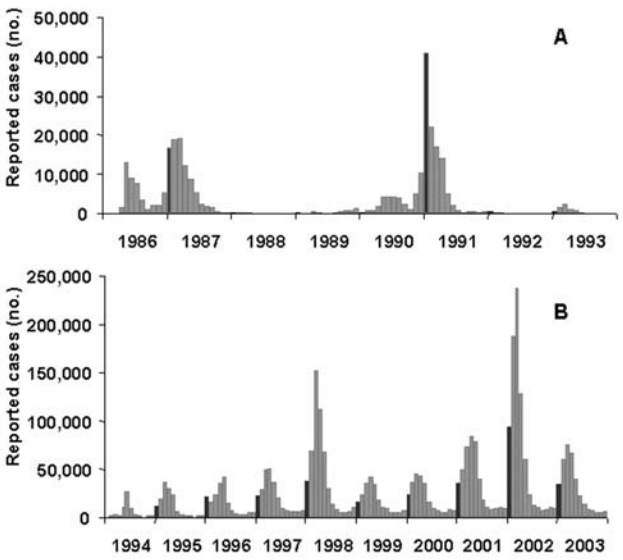

Figure 1. Number of dengue fever cases reported per month, Brazil. A) 1986–1993, B) 1994–2003. Dark bars represent January.

Table 1. Chronology of major dengue-related events in Brazil, 1981–2002

Period/y	Event
1981–1993: Epidemics waves in localized areas	
1981	Restricted outbreak (DENV1* and DENV4†) in Northwest Brazil (Roraima State)
1986	Introduction of DENV1 (Rio de Janeiro State)
1990	Introduction of DENV2‡ (Rio de Janeiro State) and first confirmed cases of DHF
1994–2002: Epidemic and endemic virus circulation countrywide	
1994–1999	Dispersion of *Aedes aegypti* nationwide
1998	Widespread outbreaks in 16 states (>534,000 reported cases)
2000	Introduction of DENV3§ (Rio de Janeiro State)
2002	Large outbreaks in 19 states (>794,000 reported cases)
	Deaths due to dengue hemorrhagic fever exceed deaths from malaria

*Dengue virus serotype 1.
†Dengue virus serotype 4.
‡Dengue virus serotype 2.
§Dengue virus serotype 3.

in the northwest Amazon region (Roraima State). After a 5-year interval without confirmed dengue fever, an epidemic due to DENV1 occurred in Rio de Janeiro State and was followed by several epidemics in highly populated cities in the southeast and northeast regions of Brazil. The number of dengue fever cases peaked at ≈100,000 in 1987 and 1991, probably because different serotypes (DENV1 and DENV2, respectively; Figure 2) were introduced. From 1986 to 1993, a total of 76.6% of the 294,419 reported dengue cases occurred in the rainy season from December to May, showing a marked seasonal pattern (Figure 1B). A cyclical pattern of 2-year intervals between large outbreaks was observed, which suggested low viral activity in the dry season (June to November).

In the second period (1994–2002), a total of 2,826,948 dengue cases were reported, indicating an upward trend in the incidence from 37 to 454 per 100,000 inhabitants. Although large outbreaks were observed in the rainy season, 482,163 cases were reported in the nonrainy season, which demonstrated increased dengue virus activity during the entire year. The bulk of incident cases were generated from metropolitan areas, although several outbreaks occurred in smaller urban settings in 25 out of 27 states. Two unprecedented epidemics occurred in 1998 and 2002, with 528,388 and 794,219 dengue fever cases reported, respectively. During this second period, vector surveillance to monitor *A. aegypti* infestation was extended to most of the country, covering 69.7% (n = 3,878) of the municipalities in 1996 and 89.6% (n = 4,985) in 2002. According to this vector information system, the number of infested municipalities ranged from 1,726 (44.5%) in 1996 to 2,905 (58.3%) in 2002. To date, Santa Catarina and Rio Grande do Sul States, located in southernmost Brazil, remain free of autochthonous dengue transmission; they report only imported cases.

The overall age distribution among reported cases during the last 5 years is presented in Figure 3. Approximately 50% of all reported dengue cases occurred in adults 20–40 years of age. During this period, dengue incidence was consistently higher in adults, reaching up to 432.7/100,000 population in the 30- to 49-year-old age group in 2002 (Table 2). The male:female ratio was constant at ≈1.1:1 during this 5-year period. Sex and age group distribution were very similar when the data for the 5 regions of the country were stratified (data not shown).

The first DHF cases were confirmed in 1990, after DENV2 was introduced into Brazil. During the decade that followed, 893 confirmed DHF cases with 44 deaths (rate of 4.9%) occurred; 75% of these deaths occurred in Rio de Janeiro State. During 2001–2002, a striking increase in the number of DHF cases was detected, with incidence rates of 2.9/100,000 population (n = 682) in 2001 and 12.9/100,000 population (n = 2,714) in 2002 (Figure 2). DHF cases increased 45-fold from 2000 to 2002, compared to a

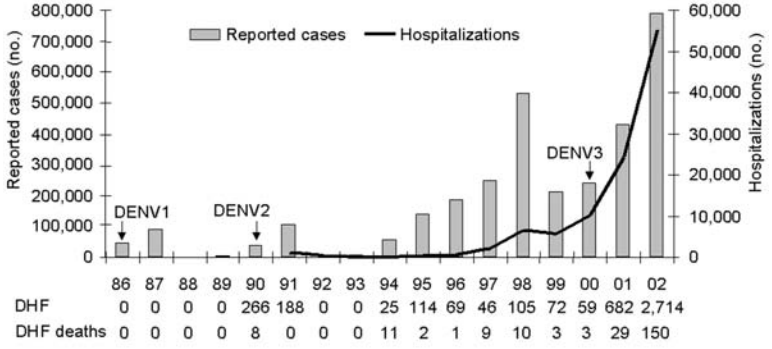

Figure 2. Number of reported cases and hospitalizations due to dengue and dengue hemorrhagic fever (DHF), Brazil, 1986–2002.

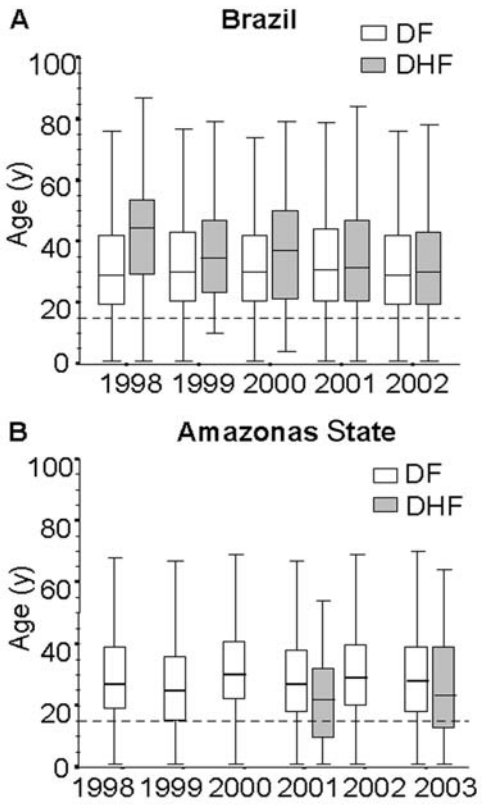

Figure 3. Age distribution of dengue fever (DF) and dengue hemorrhagic fever (DHF) cases for Brazil and Amazonas State, 1998–2003. Boxes encompass 25th and 75th percentiles. Black lines within boxes, medians. Outliers not shown. Dashed line, 15 years old.

3.3-fold increase in dengue fever cases during the same period. In the epidemic year of 2002, the overall ratio of dengue fever to DHF cases was 292.6; in Rio de Janeiro State, the most affected area, this ratio was 134.8. From 1998 to 2002, the case-fatality rate was 5.4% (195/3,632). During this period, the sex distribution of DHF was similar, and cases occurred mainly in adults (median age 33 years) (Figure 3A). Recently, a different pattern in the age distribution of DHF was observed in Amazonas State, where unlike the national level, a higher proportion of DHF cases occurred among children. In Amazonas State, 30.9% (17/55) and 28.8% (15/52) of DHF cases occurred among children <15 years of age in 2001 and 2003, respectively (Figure 3B).

Countrywide, an upward trend in hospitalized dengue cases has been apparent since 1994, peaking with >54,000 hospitalizations in 2002 (Figures 2 and 4). Hospitalization rates were consistently higher among adults from 1998 to 2002. Comparing the 2 largest epidemic years (1998 and 2002), hospitalization rates in all age groups increased ≈8-fold (Table 2). The ratio of reported to hospitalized cases decreased from 29.4 in 1998 to 10.6 in 2002, which represents 1 hospitalization for every 10 reported dengue cases. Despite this increased proportion of hospitalized cases, >90% of the total reported cases were considered mild or moderate cases by the health system.

In Brazil, the chronology of the appearance of DENV serotypes occurred in the following sequence: DENV1 in 1986, DENV2 in 1990, and DENV3 in 2000 (Figure 2). The simultaneous circulation of these 3 serotypes was first detected in multiple states during the last 2 years. Molecular epidemiology of DENV in Brazil has identified the Caribbean, Jamaican, and Sri Lankan genotypes for the DENV1, DENV2, and DENV3 viruses, respectively (21).

Discussion

Analysis of 20 years of dengue/DHF surveillance data showed 2 distinct periods: an initial phase from 1986 to 1993, characterized by localized, sporadic epidemic waves in urban centers, and a nationwide endemic and epidemic pattern from 1994 to 2002. In this latter period, several epidemics of dengue progressed towards hyperendemicity in multiple urban centers. Since 1999, dramatic increases in both the incidence and hospitalizations due to dengue fever and DHF have occurred, indicating a likely increase in disease severity in recent years. Changes in surveillance coverage might explain part of this increase, but this explanation seems unlikely since no major changes were made in surveillance definitions. The current intense virus transmission pattern can be explained by the number of municipalities infested with *A. aegypti* mosquitoes, the mobility of the population, and the introduction and cocirculation of 3 different virus serotypes (DENV1, 2, and 3).

The epidemiology of dengue in Brazil presents some unusual features, characterized by dengue/DHF affecting

Table 2. Dengue incidence and hospitalization (per 100,000 population) by age group, Brazil, 1998–2002

	1998		1999		2000		2001		2002	
Age (y)	Incidence	Hosp*	Incidence	Hosp	Incidence	Hosp	Incidence	Hosp	Incidence	Hosp
0–4	39.6	1.1	27.2	0.5	29.6	1.1	68.8	2.9	121.9	10.3
5–14	68.2	2.1	45.4	1.6	51.8	3.0	131.7	7.0	213.7	19.6
15–29	145.6	4.5	102.7	4.1	111.3	7.5	263.5	16.6	410.3	36.6
30–49	156.3	4.7	120.3	4.4	128.0	7.2	305.7	16.7	432.7	36.1
≥50	120.0	6.5	92.0	5.7	88.3	8.4	237.3	20.7	323.6	43.7
Total	117.2	4.0	85.7	3.5	92.3	6.0	225.3	14.1	335.3	31.6

*Hospitalization rates.

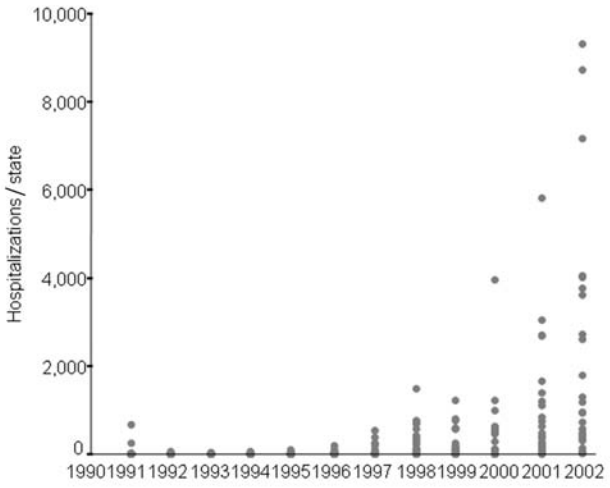

Figure 4. Number of dengue fever–related hospitalizations per state by year, Brazil, 1990–2002. Each dot represents the number of hospitalizations due to dengue/dengue hemorrhagic fever for 1 state by year. Source: Hospitalization Information System-Unified Health System (SIH-SUS).

mainly adults, with a predominance of milder infection in persons treated at outpatient clinics. In contrast, severe forms of the disease among children requiring hospitalization have been described in DHF epidemics in South and Central American countries (3,22,23). This endemic pattern has also been reported from Southeast Asia in recent decades (24,25). Cuban children with secondary infection by DENV2 showed a higher frequency of DHF and DHF-related deaths during the epidemic of 1981, which had been preceded by a DENV1 outbreak 4 years earlier (26). Apparently, similar events in Brazil, which also experienced the sequential introduction of DENV1 (during 1986) followed by DENV2 4 years later, led to a different outcome: most reported dengue/DHF occurred in adults. These findings may be explained by the distinct DENV2 strains circulating in Cuba (New Guinea) and Brazil (Jamaica) (21,27,28). Another possibility is the underdiagnosis of DHF in children in Brazil, although it seems unlikely that severe clinical manifestations would not likely be misdiagnosed or fail to draw the attention of public health authorities.

During the 20-year period examined, dengue/DHF in Brazil most commonly affected the adult population, even with the observed increase of dengue/DHF cases. Despite these findings, current data underscore a different pattern in the Amazon region, where an increased proportion of severe cases occurs among children. This finding represents a warning to pediatric practitioners and health officials. In the future, if the current intense DENV circulation persists, Brazil could resemble Southeast Asia, with DHF occurring mainly in younger age groups.

In 2002, the absolute number of deaths due to DHF (N = 150) exceeded malaria deaths for the first time in Brazil, demonstrating that malaria is not the only major endemic vector-borne disease in this tropical region. During that year, the largest epidemic yet recorded, including 250,000 dengue cases in the metropolitan area of Rio de Janeiro, caused major public health and political concern (7,8). Hemorrhagic fever and dengue-related deaths also clustered in this city. These findings probably reflect the recent introduction and predominance of DENV3, which suggests a possible role of this serotype in disease severity and the potential for additional DHF epidemics in the future (29). Another possible explanation for the increase in DHF is the association between secondary infection with disease severity (10); however, routine surveillance data only indicate patients' immune status. In addition, some highly urbanized areas have substantial proportions of the population living in crowded, impoverished areas with poor sanitation (30). These complex urban settings are present in many regions in Brazil, resulting in a major challenge for vector control activities (8).

Several seroepidemiologic studies in Brazil have shown that up to 70% of urban populations had previous dengue infection, outnumbering reported cases. These results suggest that seroprevalence increases with age and that subclinical outcomes are a common feature of DENV exposure (31,32). Access to health services is considered nearly universal in Brazil, particularly in urban settings; therefore, dengue/DHF reports should be representative of the disease in the population. Incidence and hospitalization rates by age group showed similar patterns when surveillance system and hospitalization database were analyzed. The consistent patterns of age and sex distribution for all regions during epidemic and nonepidemic periods also suggest reliability of these surveillance data.

The high case-fatality rate for DHF observed in the last 5 years may be due to difficulties in classifying case severity according to the standard World Health Organization/PAHO dengue case definition (33). The Brazilian Ministry of Health has been strongly committed to improving surveillance and patient care in a major effort to reduce dengue-related deaths (34). The aim of this policy is to rapidly identify patients at risk of developing DHF and to initiate prompt, adequate treatment (e.g., intravenous fluid infusion) to prevent DSS. As result of this early and effective replacement of plasma loss, hemoconcentration (one of the classification criteria for DHF) has become a less frequently observed event during the course of the disease, and therefore, a proportion of these cases may not fulfill DHF criteria. Different applications or interpretations of this case definition by countries may limit the ability to make valid comparisons, particularly in Latin America compared to Southeast Asia (23,33).

The current epidemiologic trend underscores the importance of dengue/DHF and the need for long-term improvements in disease control and surveillance in Brazil. We have described the changing patterns and epidemiologic profile of dengue/DHF during the last 20 years in one of the most severely affected countries in the Americas, highlighting the recent increase in disease severity.

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Genetic Background of *Escherichia coli* and Extended-spectrum β-Lactamase Type

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To assess the implication of the genetic background of *Escherichia coli* strains in the emergence of extended-spectrum β-lactamases (ESBL), 55 TEM-, 52 CTX-M-, and 22 SHV-type ESBL-producing clinical isolates involved in various extraintestinal infections or colonization were studied in terms of phylogenetic group, virulence factor (VF) content (*pap*, *sfa/foc*, *hly*, and *aer* genes), and fluoroquinolone resistance. A factorial analysis of correspondence showed that SHV type, and to a lesser extent TEM type, were preferentially observed in B2 phylogenetic group strains that exhibited numerous VFs but were fluoroquinolone-susceptible, whereas the newly emerged CTX-M type was associated with the D phylogenetic group strains that lacked VF but were fluoroquinolone-resistant. Thus, the emergence of ESBL-producing *E. coli* seems to be the result of complex interactions between the type of ESBL, genetic background of the strain, and selective pressures in ecologic niches.

Extended-spectrum β-lactamases (ESBL) that mediate resistance to oxyimino-cephalosporins, such as cefotaxime, aztreonam, and ceftazidime, are now observed worldwide in all species of *Enterobacteriaceae* (1). Traditionally, ESBLs are derived by point mutation from the common TEM and SHV-1 β-lactamases. However, recently, new families of ESBLs have been described (2). The CTX-M-type ESBLs have become particularly widespread and are mainly found in strains of *Salmonella* and *Escherichia coli* (3,4). These enzymes probably evolved from chromosomal β-lactamases of *Kluyvera* spp. by gene transposition from mobile elements and mutation (5,6). ESBLs are usually described as acquired β-lactamases that are encoded mainly by genes located on plasmids. Some ESBL-encoding genes are located within transposons or integrons, which facilitates transfer between organisms. ESBL-producing organisms are responsible for nosocomial infections, and many hospitals have experienced outbreaks (1,2,7). The lower digestive tract of colonized patients has been recognized as the major source of ESBL-producing organism (2,8). These organisms pose a therapeutic challenge, since they are frequently resistant to other kinds of antimicrobial drugs, including aminoglycosides, quinolones, and cotrimoxazole (2).

E. coli in humans is a commensal inhabitant of the gastrointestinal tract. It can also cause various intestinal and extraintestinal diseases (9). Strains causing infections harbor numerous virulence factors encoded on plasmids, bacteriophages, or the bacterial chromosome within pathogenicity islands (9). Several studies have shown that pathogenic *E. coli* strains may be derived from commensal strains by acquiring chromosomal or extrachromosomal virulence operons (10,11). Phylogenetic analyses have shown that *E. coli* strains fall into 4 main phylogenetic groups (A, B1, B2, and D) (12,13). Although virulence determinants are considered to be mobile, a link between strain phylogeny and virulence has been reported. Virulent extraintestinal strains belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal strains belong to groups A and B1. Strains of phylogenetic groups B2 and D often carry virulence determinants that are lacking in group A and B1 strains (10,14–17). In addition, a trade-off between resistance and virulence has been observed. Prevalence of antimicrobial resistance was shown to be greater in non-B2 phylogenetic group strains (18). In urinary tract infections, fluoroquinolone-resistant *E. coli* represented predominantly low-virulence phylogenetic groups A and B1 (19). These resistant strains were

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also associated with a decrease in the presence or the expression of some virulence factors and a decreased invasive capacity (20,21).

The intrinsic virulence potential of ESBL-producing *E. coli* is unknown. They may represent traditional virulence clones of extraintestinal pathogenic *E. coli* (ExPEC) or low-virulence opportunists whose ability to cause disease is largely limited to compromised hosts, in which antimicrobial resistance might provide relevant selective advantage. To assess the relationships between the genetic background of the strains and the presence of an ESBL, we analyzed a collection of ESBL-producing *E. coli* clinical isolates involved in various extraintestinal infections or in colonization in terms of phylogenetic grouping, virulence determinant content, and fluoroquinolone resistance.

Material and Methods

Bacterial Strains

We collected 157 *E. coli* isolates from clinical samples on the basis of their positive double-disk synergy test from 1997 to 2002 in different areas in France: Paris area (4 hospitals), Brest, and Amiens. From these isolates 129 strains were analyzed on the basis of 3 criteria: 1) the strains produced an ESBL, 2) the strains were epidemiologically unrelated, and 3) the strains were unambiguously classified as responsible for infection or colonization. ESBLs were characterized by isoelectric focusing with ceftriaxone and penicillin as substrates (7), specific polymerase chain reaction (PCR) amplification, and direct sequencing of PCR products. The oligonucleotide primer sets specific for the β-lactamase gene (*bla*) amplification and sequencing were taken from the literature (bla_{TEM} and bla_{SHV}) (22) or designed in this study (bla_{CTX-M}) (Table 1). As the family of CTX-M ESBLs belongs to 4 clusters on the basis of their protein sequences, the CTX-M-1 cluster (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15), the CTX-M-2 cluster (CTX-M-2, CTX-M-4 to CTX-M-7, Toho-1), the CTX-M-9 cluster (CTX-M-9, CTX-M-14, CTX-M-16, CTX-M-18, CTX-M-19, Toho-2), and the CTX-M-8 cluster, specific primers for each cluster of the CTX-M family were designed. PCR products of bla_{TEM} were subjected to direct sequencing to identify TEM-ESBLs, only when isolates produced a single β-lactamase indicated by isoelectric focusing. For isolates carrying a second β-lactamase of pI 5.4 or 5.6 shown by penicillin only (putative TEM-1 or TEM-2 β-lactamase), sequences were obtained after plasmid transfer into *E. coli* K-12 J53-2 rif^r (23). PCR product sequences were then compared to reported ESBL sequences and assigned to specific types or clusters. To identify any epidemiologic relationship between the strains, they were compared by using enterobacterial repetitive intergenic consensus (ERIC)-PCR with ERIC1 and ERIC2 as primers (24,25). When strains had identical electrophoretic profiles with both ERIC1 and ERIC2 primers, they were considered identical, and only 1 isolate per electrophoretic profile type was selected for further analysis. Among the collection of 129 strains selected for the study, 86 strains were involved in infections (urinary tract infection [UTI]: 64, bacteriemia: 7, pus production from miscellaneous infections: 15), and 43 strains were isolated from colonization (rectal samples: 39, gastric aspirate: 1, abdominal drainage: 1, vaginal sample: 1, tracheal aspirate: 1) (Table 2). The collection included 55 strains that produced a TEM-type ESBL, 22 strains produced a SHV-type ESBL, and 52 strains produced a CTX-M type ESBL (Table 2).

Susceptibility Testing, Phylogenetic Grouping, and Virulence Factors

Susceptibility to ciprofloxacin was tested by the disk diffusion technique according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (www.sfm.asso.fr) with MIC criteria of ≤1 mg/L (diameter ≥22 mm) used to define susceptibility. Phylogenetic grouping of the *E. coli* isolates was determined by a PCR-based method developed by Clermont et al. (26) that uses a combination of 3 DNA markers (*chuA*, *yjaA*, and an anonymous DNA fragment, TspE4.C2).

Table 1. Sequence of primers used to detect *bla* genes*

PCR target	Primer name	Primer sequence	Reference or accession no.
bla_{TEM}	A	ATGAGTATTCAATTCCG	(22)
	B	CTGACAGTTACCAATGCTTA	
bla_{SVH}	P4	GGTTATGCGTTATATTCGCC	(22)
	P5	TTAGCGTTGCCAGTGCTC	
bla_{CTX-M} (CTX-M-1 cluster)	MenA	AAGACTGGGTGTGGCATTGA	X92506
	MenB	AGGCTGGGTGAAGTAAGTGA	
bla_{CTX-M} (CTX-M-2 cluster)	M2A	CTGGAAGCCCTGGAGAAAAG	X92507
	M2B	TACCTCGCTCCATTTATTGC	
bla_{CTX-M} (CTX-M-9 cluster)	ToA	GCTTTATGCGCAGACGAGTG	AF174129
	ToB	GCCAGATCACCGCAATATCA	
bla_{CTX-M} (CTX-M-8 cluster)	A8	GCCTGTATTTCGCTGTTG	AF189721
	B8	TGTCATTCGTCGTACCATAA	

*PCR, polymerase chain reaction.

Table 2. Distribution of ESBL types according to strain origin*

ESBL type (no. strains)	No. strains isolated from		
	UTI	Other infections	Colonization
TEM (55)			
TEM-24	11	9	11
TEM-52	3	0	7
TEM-21	5	1	2
TEM-3	1	0	3
TEM-10	0	0	1
TEM-20	1	0	0
SHV (22)			
SHV2	3	1	2
SHV4	1	1	1
SHV5	2	0	1
SHV12	5	4	1
CTX-M (52)			
CTX-M-1 cluster	20	2	7
CTX-M-2 cluster	3	0	1
CTX-M-9 cluster	9	4	6

*ESBL, extended-spectrum β-lactamase; UTI, urinary tract infection.

Strains were assigned to phylogenetic groups on the basis of presence or absence of the 3 DNA fragments: *chuA*–, TspE4.C2–, group A; *chuA*–, *yjaA*–, Tspe4.C2+, group B1; *chuA*+, *yjaA*+, group B2; *chuA*+, *yjaA*–, group D. Because 2 possible profiles can be obtained for the groups A, B2, and D, each was subdivided as follows: *chuA*–, *yjaA*–, Tspe4.C2–, group A subgroup A_0; *chuA*–, *yjaA*+, Tspe4.C2–, group A subgroup A_1; *chuA*+, *yjaA*+, Tspe4.C2–, group B2 subgroup $B2_2$; *chuA*+, *yjaA*+, Tspe4.C2+, group B2 subgroup $B2_3$; *chuA*+, *yjaA*–, Tspe4.C2–, group D subgroup D_1; *chuA*+, *yjaA*–, Tspe4.C2+, group D subgroup D_2. Virulence genes (*pap*, *sfa*/*foc*, *hly*, *aer*) were detected from DNA by PCR as described previously (15,27). These genes code for 2 adhesins (pyelonephritis-associated pili system and S fimbrial adhesin), 1 toxin (α-hemolysin), and 1 iron captation system. These genes are good representatives of the intrinsic extraintestinal virulence of the strains (28).

Statistical Analysis

Data were summarized in 2 two-way tables, and each table had 129 rows, one for each *E. coli* strain. The first table had 16 columns corresponding to the variables, origin of the strains, phylogenetic group or subgroup, type of ESBL, and virulence factors. The second table had 12 columns corresponding to the variables, phylogenetic groups, type of ESBL, and resistance to ciprofloxacin. For each column, each strain was coded as a binary code: present = 2, absent = 1. A factorial analysis of correspondence (FAC) (29) was conducted from this table with SPAD.N software (Cisia, Saint Mandé, France). To confirm the significance of the correlation observed with FAC, χ^2 tests were carried out.

Results

Characterization of ESBL Strains

Among the 129 *E. coli* strains analyzed, phylogenetic group B2, which is the source of most ExPEC clones, was represented by 36.4 % of the strains (8.5% were subgroup $B2_2$ and 27.9% were subgroup $B2_3$). Phylogenetic group D, which is also a source of ExPEC but to a lesser extent, was represented by 25.5% of the strains (17% were subgroup D_1 and 8.5% were subgroup D_2). Of the remaining strains, phylogenetic groups A and B1 were represented by 27.9% (9.3% were subgroup A_0 and 18.6% were subgroup A_1) and 10 % of the strains, respectively. The virulence determinants most represented in the collection were *aer* and *pap*, with 53 (41%) and 38 (29.5%) strains carrying these genes, respectively. Less prevalent were *sfa*/*foc* and *hly* determinants, with only 18 (14%) and 19 (15%) positive strains, respectively. Fluoroquinolone resistance was present in 34.8% of the strains.

ESBL-producing strains were found in all *E. coli* phylogenetic groups. Of the strains, 60% and 24% harbored at least 1 or 2 extraintestinal virulence determinants, respectively. Coresistance to fluoroquinolones was frequent.

Multidimensional Analysis

To assess relationships between phylogenetic groups, VFs, type of ESBL produced, and origin of the strains (infection or colonization), a FAC was constructed with the 129 *E. coli* strains as individuals and the 16 characteristics as qualitative variables. Projections of the variables on the plane F1/F2 (Figure A), which accounted for 34.5% of the total variance, showed a correlation between the type of ESBL produced and several phylogenetic group/subgroups of *E. coli*. Thus, SHV type and subgroup $B2_3$ are projected on the positive values of F1 and negative values of F2, whereas TEM type and subgroup $B2_2$ are projected on the positive values of F1 and F2. CTX-M type and subgroup D_2 are projected on the negative values of F1 and F2. Correlation between SHV type and subgroup $B2_3$ was confirmed by χ^2 tests ($p < 0.001$) and the CTX-M type and the subgroup D_2 ($p < 0.001$) (Table 3).

As previously reported, *sfa*/*foc* and *hly* VFs were exclusively found in strains of the subgroups $B2_2$ and $B2_3$ (10,16). Pairwise comparisons between individual subgroups showed that subgroups $B2_2$ and $B2_3$ each had mean VF scores (1.45 and 1.8, respectively) significantly higher than either phylogenetic groups and subgroups A_0, A_1, B1, D_1, or D_2 ($p < 0.02$ for all comparisons), but they were not significantly different from one another. Likewise phylogenetic groups and subgroups A_0, A_1, B1, D_1, and D_2 were not significantly different from one another with respect to mean VF scores (mean scores 0.5, 0.66, 0.54, 0.5, and 0.63, respectively). When the type of ESBL produced was

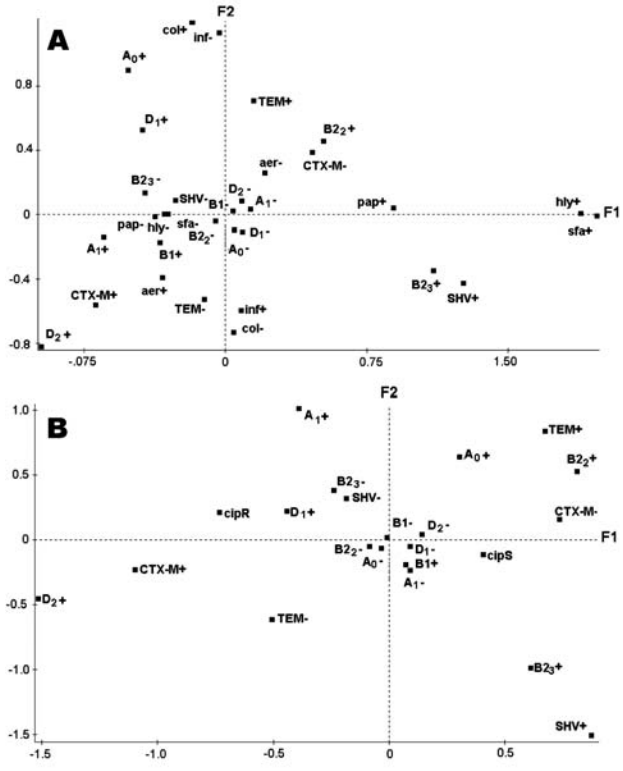

Figure. Graphic representation of the results of the factorial analysis of correspondence carried out with whole data from the 129 *Escherichia coli* strains. A) Projections of the variables on the F1/F2 plane: phylogenetic group and subgroups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1, and D_2), type of extended-spectrum β-lactamase (ESBL) (TEM, SHV, CTX-M), virulence factors (*pap*, *sfa*, *hly*, *aer*), and the source infection (inf) or colonization (col). B) Projections of the variables on the F1/F2 plane: phylogenetic group and subgroups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1, and D_2), type of ESBL (TEM, SHV, CTX-M), and ciprofloxacin resistance (cipR) or the ciprofloxacin susceptibility (cipS).

considered, the frequency of VFs was higher in SHV-producing strains (mean score = 1.8) than in TEM-producing strains (mean score = 0.96). The lowest frequency was found in the CTX-M-producing strains (mean score = 0.6). FAC stressed these 2 observations, as it showed that the *pap*, *sfa*/*foc*, and *hly* VFs were projected on the positive values of the first axis with the subgroup $B2_3$ and the SHV type. The correlation between SHV type and the presence of the 3 VFs was also confirmed by χ^2 tests (*pap*, $p < 0.01$; *sfa*/*foc*, $p < 0.001$; *hly*, $p < 0.001$). Aerobactin was found in all the phylogenetic groups and subgroups, and no correlation was observed with the FAC (Table 4).

Projection of the colonization and infection variables on the plane showed that they were clearly distinguished by the first factor and that there was a correlation with some phylogenetic groups (Figure A). The colonization characteristic was projected on the positive values of F1 with phylogenetic subgroups A_0 and D_1. The association was close to significance (A_0, $p = 0.05$; D_1, $p = 0.06$): strains of subgroups A_0 and D_1 were isolated more frequently from colonization (relative risk [RR] of 3.15 and 2.34, respectively) (Table 3). If we consider the clones usually to be the major source of ExPEC, strains of the subgroup $B2_2$ were equally distributed among the strains responsible for infection or colonization (8.1% versus 9.3%), but strains of subgroup $B2_3$ were more numerous among the strains responsible for infection than for colonization (32.5% versus 18.6%); the correlation was close to significance ($p = 0.09$, RR = 2.11) (Table 3). TEM type was also projected on the positive values of F2 with the colonization characteristic, and the χ^2 test confirmed the correlation ($p = 0.03$).

The mean VF score of the strains responsible for infection was significantly higher ($p = 0.03$) than the mean VF score of the strains responsible for colonization (1.1 and 0.76, respectively). However, when each VF was considered, only the frequency of aerobactin was significantly higher among the strains responsible for infection ($p = 0.03$) than the strains responsible for colonization.

To assess the relationships between phylogenetic groups and subgroups, ESBL type, and resistance to fluoroquinolones, a second FAC was performed, taking into account only these variables (Figure B). Projection of the variables on the plane F1/F2, which accounted for 34% of the total variance, showed a correlation between resistance to ciprofloxacin and type of ESBL produced. Thus, the ciprofloxacin-resistant characteristic was projected on the negative values of the first factor with CTX-M-type, and the ciprofloxacin-susceptible characteristic was projected on the positive values of the first factor with TEM and SHV types. Significant differences were observed between the rate of resistance to fluoroquinolones among the CTX-M- (51.9%) and among the SHV- and TEM-producing strains (13.6% and 27.7%, respectively): CTX-M type / SHV type, $p = 0.002$ and CTX-M type / TEM type, $p = 0.009$. FAC stressed also the correlation between the subgroup D_1 and the resistance to ciprofloxacin, which were projected together on the negative values of the first factor and on the positive values of the second factor. The correlation was confirmed by the χ^2 test ($p = 0.03$). Strains of phylogenetic subgroup D_1 had the highest resistance rate (54%), and strains of subgroups $B2_2$, $B2_3$, and A_0 had the lowest resistance rates (18%, 25%, and 25%, respectively). Group/subgroups B1, D_2, and A_1 had ciprofloxacin resistance rates of 30.7%, 36%, and 45%, respectively. No significant difference was seen in the frequencies of ciprofloxacin resistance among strains from infection or colonization (38.3% versus 27.9%). The mean VF score of the ciprofloxacin-susceptible strains was significantly higher ($p < 0.001$) than the one of the ciprofloxacin-

Table 3. Distribution of extended-spectrum β-lactamase types among *Escherichia coli* strains isolated from infection or colonization, according to phylogenetic group

Phylogenetic group	No. strains in the group (%)	No. (%) of isolates producing		
		TEM	SHV	CTX-M
Infection				
A_0	5 (5.8)	2 (6.4)	1 (5.8)	2 (5.2)
A_1	18 (20.9)	11 (35)	0	7 (18.4)
B1	9 (10.4)	2 (6.4)	3 (17.6)	4 (10.5)
$B2_2$	7 (8.1)	5 (16)	1 (5.8)	1 (2.6)
$B2_3$	28 (32.5)	8 (25.8)	10 (58.8)	10 (26.3)
D_1	11 (12.7)	3 (9.6)	2 (11.7)	6 (15.7)
D_2	8 (9.3)	0	0	8 (21)
All groups	86 (100)	31 (100)	17 (100)	38 (100)
Colonization				
A_0	7 (16.2)	5 (20.8)	0	2 (14.2)
A_1	6 (13.9)	2 (8.3)	0	4 (28)
B1	4 (9.3)	3 (12.5)	0	1 (7.1)
$B2_2$	4 (9.3)	2 (8.3)	1 (20)	1 (7.1)
$B2_3$	8 (18.6)	5 (20.8)	3 (60)	0
D_1	11(25.5)	6 (25)	1 (20)	4 (28.4)
D_2	3 (6.9)	1 (4.1)	0	2 (14.1)
All groups	43 (100)	24 (100)	5 (100)	14 (100)
All strains				
A_0	12 (9.3)	7 (12.7)	1 (4.5)	4 (7.6)
A_1	24 (18.6)	13 (23.6)	0	11 (21.1)
B1	13 (10)	5 (9)	3 (13.6)	5 (9.6)
$B2_2$	11 (8.5)	7 (12.7)	2 (9)	2 (3.8)
$B2_3$	36 (27.9)	13 (23.6)	13 (59)	10 (19.2)
D_1	22 (17)	9 (16.3)	3 (13.6)	10 (19.2)
D_2	11 (8.5)	1 (1.8)	0	10 (19.2)
All groups	129 (100)	55 (100)	22 (100)	52 (100)

resistant strains (1.2 and 0.6, respectively) (Table 4). We found *hly* and *sfa*/*foc* exclusively in ciprofloxacin-susceptible strains, and the frequency of *pap* was significantly higher among ciprofloxacin-susceptible strains ($p = 0.04$) than among ciprofloxacin-resistant strains. No difference was observed in the frequency of aerobactin between the 2 groups (Table 4). Although the frequency of CTX-M type was higher among UTI strains than among non-UTI strains (Table 2), FAC analysis and χ^2 tests did not show any significant association between UTI strains, phylogenetic group or subgroup, individual VFs, and ciprofloxacin resistance (data not shown), which could explain some of the previously observed correlations.

Therefore, strains harboring ESBL of SHV and TEM types belonged preferentially to the B2 phylogenetic group. They possessed extraintestinal VFs, but ESBL TEM-type strains were more likely to be isolated from cases of colonization; they were also susceptible to fluoroquinolones. On the other hand, strains harboring ESBL of CTX-M type were associated with D_2 phylogenetic subgroup, had few VFs, but were resistant to fluoroquinolones.

Discussion

This study was designed to assess the role of the genetic background of strains of *E. coli* in the emergence of ESBL. Strains were sampled from hospitals in several distant areas, which allowed us to build up a collection of strains producing variants of the most prevalent ESBL types. Thus 3 groups of ESBL-types were collected, TEM-, SHV-, and CTX-M-type, having enough strains in each group to be compared. Spread of clones of ESBL-producing organisms can occur from cross-contamination among patients (2,7,23). Therefore, to avoid redundant strains, we used ERIC-PCR as a typing method, and strains with similar profiles were eliminated.

Several studies suggested that extraintestinal pathogenic *E. coli* strains are mostly derived from the B2 phylogenetic group and to a lesser extend from the D group (15,16,30–34). It had been estimated in collections dating from before the emergence of ESBL, or in collections not selected for ESBL production, that group B2 strains account for approximately two thirds of all extraintestinal *E. coli* infections, including UTI, bacteremia, meningitis, and other miscellaneous infections. When all ESBL-producing *E. coli* strains were considered, whatever their types were, group B2 represented only 39.4% of the strains

Table 4. Frequency of virulence factors among ciprofloxacin-susceptible and ciprofloxacin-resistant *Escherichia coli* strains involved in infection or colonization, according to extended-spectrum β-lactamase (ESBL) type

ESBL type (no. strains)	No. (%) strains carrying				Virulence factor mean score
	pap	*sfa/foc*	*hly*	*aer*	
Ciprofloxacin resistance					
TEM (15)	4 (20)	0	0	8 (53)	0.8
SHV (3)	0	0	0	1 (33)	0.33
CTX-M (27)	2 (7)	0	0	12 (44)	0.51
All types (45)	6 (13)	0	0	21 (46)	0.6
Ciprofloxacin sensitivity					
TEM (40)	14 (35)	9 (22)	9 22)	9 (22)	1
SHV (19)	13 (68)	8 (42)	9(47)	10(52)	2.1
CTX-M (25)	5 (20)	1 (4)	1 (4)	13 (52)	0.8
All types (84)	32 (55)	18 (21)	19 (22)	32 (38)	1.2
All strains					
TEM (55)	18 (33)	9 (16)	9 (16)	17 (31)	0.96
SHV (22)	13 (59)	8 (36)	9 (4)	11 (50)	1.8
CTX-M (52)	7 (13)	1 (2)	1 (2)	25 (48)	0.6
All types (129)	38 (29.5)	18 (14)	19 (15)	53 (41)	1

responsible for infection in our study. Thus, production of ESBL among *E. coli* clinical strains isolated from infection was associated with shifts in phylogenetic distribution toward non-B2 phylogenetic groups, in particular groups D and A. The distribution of group B2 among strains isolated from infection or from colonization was not very different even if it was pointed out that subgroup $B2_3$ strains had a tendency to be isolated more frequently in clinical infections. Johnson et al., in 1991 (18), observed that *E. coli* strains belonging to phylogenetic groups other than group B2 have a greater prevalence of antimicrobial resistance, such as to ampicillin, tetracycline, chloramphenicol, streptomycin, and sulfonamide; express significantly fewer virulence factors; and invade more commonly compromised hosts. ESBL-producing organisms, which are resistant to β-lactams, except carbapenems and cephamycins, and are often resistant to other antimicrobial drugs, are responsible for nosocomial infections, mostly in immunocompromised patients. ESBL-producing organisms also frequently colonize the lower digestive tract, and therefore are a major source for ESBL propagation (8). This finding may explain why two thirds of the strains in our study were not traditional virulence clones of ExPEC but clones whose ability to cause infection is limited to compromised hosts, in whom antibiotic resistance might provide selective advantage.

ESBLs are acquired β-lactamases that are encoded mainly by genes located on plasmids (2). As such, they are a recent evolutionary development. Even if the genetic element that carries resistance is a mobile element, the multidimentional analysis showed a preferential association between the genetic background and the type of ESBL produced by the strains. Thus, an association was seen between SHV type and subgroup $B2_3$, between TEM type and subgroup $B2_2$, and CTX-M type and subgroup D_2. Even more, the *pap*, *sfa*/*foc*, and *hly* VFs were associated with the genotype SHV type/subgroup $B2_3$, defining a potentially high-virulence group of ESBL-producing *E. coli* strains. In contrast, the genotype CTX-M type/subgroup D_2, characterized by a low VF score, defined a potentially low-virulence group of ESBL-producing *E. coli* strains. The type of ESBL produced by *E. coli* could be a predictive factor for intrinsic virulence potential.

Organisms that produce ESBL are frequently resistant to other antimicrobial agents, such as aminoglycosides, tetracycline, and trimethoprim-sulfamethoxazole, as many of these additional resistance genes are encoded on the ESBL-associated plasmid. Fluoroquinolone resistance, which is also frequently associated with ESBL production, is usually chromosomally encoded, unlike the other coresistances. However, plasmid-mediated quinolone resistance has been discovered recently (35). Prevalence of fluoroquinolone resistance among ESBL-producing strains varies according to geographic regions (36), from 13.7% in Canada to 65.5% in the western Pacific. In our study, 34.8% of strains were resistant, which is close to the prevalence (34.2%) reported in Europe (36). Correlation with phylogenetic background and VF profiles showed highly fluoroquinolone-resistant strains of subgroup D_1, with the lowest VF score and association with colonization. In contrast, strains of phylogenetic group B2, which had the highest VF score, were among the strains with the lowest fluoroquinolone-resistance rates. These data agree with the work of Johnson et al. (19,37) and show a clear trade-off between resistance to fluoroquinolones and virulence. In addition, our study highlights an association between these fluoroquinolone-resistant strains and CTX-M-producing strains, which are devoid of VFs. However, the search for the gene responsible for plasmid-mediated quinolone resistance, *qnr*, by PCR was negative in our collection of

ESBL-producing strains (O. Zamfir, E. Denamur, C. Branger, unpub. data). Thus, the observed association is not due to a genetic link between resistance to expanded-spectrum β-lactams and quinolones on a mobile element, as was recently reported (38).

During the last 2 decades, most of the ESBL found in *E. coli* and, in general, in gram-negative bacilli, has been of TEM or SHV lineage. Recently TEM and SHV types have been replaced by CTX-M-type ESBL, whose emergence and proliferation are particularly noteworthy (39). The current spread may be explained in part by the ability of some insertion sequence elements to mobilize and promote the expression of β-lactamase (40). However, the high rate of fluoroquinolone resistance and the low virulence of the strains carrying CTX-M ESBL could provide them selective advantage to spread, especially under strong environmental antimicrobial pressure with fluoroquinolones.

In summary, mobile elements encoding ESBL are not randomly distributed among the genetic diversity of the *E. coli* species. The arrival, expression, and maintenance of such elements seem to be the result of complex interactions between the type of ESBL, the phylogenetic background, the intrinsic virulence of the strains, and the presence of associated fluoroquinolone resistance. Such complexity reflects very likely the diversity of ecologic niches with different selective pressures.

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Mycobacterium haemophilum and Lymphadenitis in Children

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Infections associated with *Mycobacterium haemophilum* are underdiagnosed because specific culture methods required for its recovery are not applied routinely. Using polymerase chain reaction (PCR) technology on fine needle aspirates and biopsied specimens from 89 children with cervicofacial lymphadenitis, we assessed the importance of *M. haemophilum*. Application of a *Mycobacterium* genus–specific real-time PCR in combination with amplicon sequencing and a *M. haemophilum*–specific PCR resulted in the recognition of *M. haemophilum* as the causative agent in 16 (18%) children with cervicofacial lymphadenitis. *M. avium* was the most frequently found species (56%), and *M. haemophilum* was the second most commonly recognized pathogen. Real-time PCR results were superior to culture because only 9 (56%) of the 16 diagnosed *M. haemophilum* infections were positive by culture.

Cervicofacial lymphadenitis is the most frequently encountered manifestation of nontuberculous mycobacterial (NTM) disease in children. In previous studies, *Mycobacterium avium* has been identified as the cause in >80% of the patients (1). Other mycobacterial species isolated from patients with lymphadenitis are *M. tuberculosis*, *M. malmoense*, *M. kansasii*, *M. scrofulaceum*, *M. intracellulare*, and *M. xenopi*. *M. haemophilum* has been described as the causative agent of lymphadenitis as well (2–7).

In an ongoing multicenter study in the Netherlands, the optimal treatment for NTM lymphadenitis is investigated. Diagnosis of mycobacterial infection is performed by using mycobacterial differential skin tests and fine needle aspiration biopsy. Biopsied specimens are subjected to acid-fast staining, mycobacterial culturing, and *Mycobacterium* genus–specific real-time PCR. Of 89 patients included in the study so far, mycobacterial species were identified in 55 cases, of which *M. avium* had been found in 50 patients (8).

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In addition, a mycobacterial infection without further identification was detected in 16 patients. An atypical mycobacterial infection was diagnosed in these patients because either acid-fast staining results were positive or the *Mycobacterium* genus–specific real-time polymerase chain reaction (PCR) was positive. Cultures or species-specific real-time PCR for *M. avium* and *M. tuberculosis* remained negative. Previously, an attempt to characterize these mycobacteria by sequence analysis of the genus-specific PCR fragment was successful in only 2 cases and showed *M. haemophilum* (8). In the current study, we further analyzed these uncharacterized mycobacteria.

M. haemophilum requires special growth conditions (9), and most of the diagnostic laboratories do not use these culture conditions. Furthermore, no molecular test is available to detect *M. haemophilum* directly in clinical materials. Therefore, *M. haemophilum* infection could be seriously underdiagnosed (4,10–12). In this study, we developed a species-specific real-time PCR to detect *M. haemophilum* directly in patient materials. This assay can show the actual prevalence of *M. haemophilum* in patients with mycobacterial lymphadenitis, but it could also be applied in other diseases and help elucidate the incidence and distribution of this species.

Materials and Methods

Bacterial Strains

Five *M. haemophilum* reference strains (all clinical isolates) were available for 16S and internal transcribed spacer (ITS) sequencing. Three strains were provided by the National Institute for Public Health and the Environment and 2 were provided by the Institute for Tropical Medicine (Antwerp, Belgium). The 25 mycobacterial strains used for specificity testing included *M. tuberculosis* complex, *M. kansasii*, *M. xenopi*, *M. avium*, *M. intracellulare*, *M. gordonae*, *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. scrofulaceum*, and *M. malmoense*. A complete list of all

strains (species and subspecies) has been published in Bruijnesteijn et al. (8). The strains were cultured in liquid Dubos medium at 35°C. The *M. haemophilum* strains were cultured at 30°C on solid Löwenstein-Jensen (LJ) medium with added iron citrate or in liquid Mycobacteria Growth Index Tube (MGIT) medium with X-factor-strip added (Becton-Dickinson, Alphen a/d Rijn, the Netherlands).

Patients and Samples

Clinical materials were obtained from patients included in the CHIMED-study. In CHIMED (a multicenter nationwide study on the optimal treatment for children with lymphadenitis), treatment is randomized between surgical and medical treatment. Pediatric patients were included on the basis of clinical appearance of atypical mycobacterial lymphadenitis and a positive skin test (13,14). Fine needle aspirates were taken from affected lymph nodes. In patients who underwent surgical treatment, the removed lymph nodes were also submitted for investigation. A control group to assess the specificity of the assay was assembled from 50 patients with lymphadenitis caused by *Bartonella henselae*.

Mycobacterial Diagnostics

Clinical materials were decontaminated with a NalcNaOH decontamination protocol (15). Auramine staining was performed on the decontaminated materials for detection of acid-fast rods. Standard mycobacterial culturing was performed at 35°C in liquid MGIT medium and on solid LJ medium. *M. haemophilum*–specific culturing was performed at 30°C on LJ medium with added iron citrate and in MGIT medium with X-factor-strip added. Mycobacterial species were identified by using the Inno-Lipa and more recently using the Inno-Lipa V2 assay (InnoGenetics, Gent, Belgium) (16). When no growth was detected after 12 weeks of incubation, the culture results were listed as negative. Samples were also investigated for the presence of other bacterial pathogens by conventional bacterial cultures and by PCR for *B. henselae* (17).

Nucleic Acid Isolation

All clinical materials were processed as described in Bruijnesteijn et al. (8). DNA was extracted from bacterial strains and clinical materials according to the method of Boom et al. (18) with an overnight incubation with proteinase K.

Primers and Probes

Genus-specific primers for sequencing the total ITS region of mycobacteria were described by Frothingham et al. (19). Primers described previously for a genus-specific real-time PCR (8) were also used for sequencing a part of the ITS region directly from clinical materials. Using these primer sets combined, we applied a seminested PCR approach to increase the amount of amplicon. The part of the ITS region used in this real-time PCR shows considerable variation between mycobacterial species (20) (Figure). The primers used in the real-time PCR are genus-specific, and for the design of the *M. haemophilum*–specific minor groove binding (MGB) probe, the intraspecies and interspecies variation in the amplified ITS region was investigated. Alignments were made of the sequences of the *M. haemophilum* strains and of different mycobacterial species. The *Mycobacterium* genus–specific probe is described in Bruijnesteijn et al. (8).

The *M. haemophilum*–specific probe sequence was checked by using the primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/) (21) and oligo-analyzer 3.0 (http://biotools.idtdna.com/analyzer/) (IDT Biotools, Coralville, IA), to ensure minimal self-complementary binding and to prevent the presence of secondary structures. By using the unique features of the MGB group (22), a short and highly specific probe could be designed. The probe was designed on the antisense strand to ensure an A/T rich MGB-site. An NCBI BLAST search was performed to check the specificity of

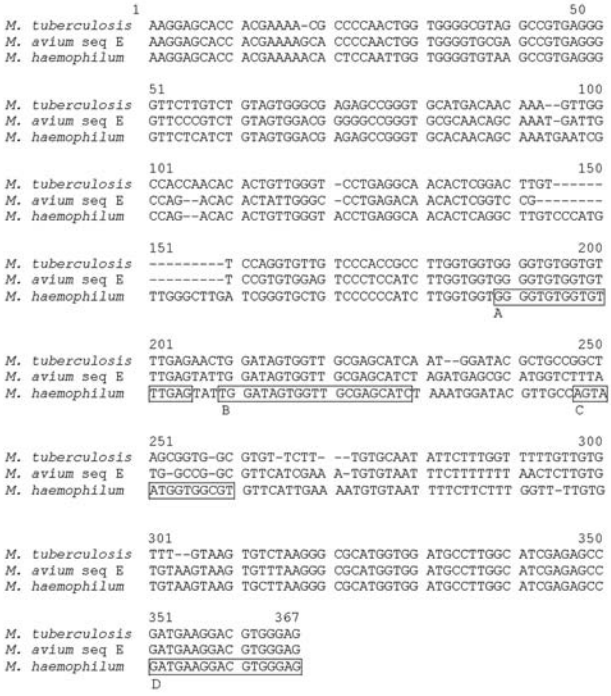

Figure. Alignment of internal transcribed spacers (ITS) and partial 23S sequences with primers and probes used for real-time polymerase chain (PCR) reaction. (nucleotides [nt] 1 to 301 make up the total ITS region; nt 302 to 367 are coding for partial 23S gene). The *Mycobacterium haemophilum* sequence was derived from 3 different patients, but no variation was found. A, forward primer for real-time PCR; B, *Mycobacterium* genus–specific probe; C, *M. haemophilum*–specific probe; D, reverse primer for real time–polymerase chain reaction.

the probe. The primers were prepared by Biolegio (Biolegio, Malden, the Netherlands), and the MGB probe was generated by ABI (Applied Biosystems Inc, Nieuwekerk a/d IJssel, The Netherlands). The broad range primers P1 and P4 were used for 16S sequencing. Primers and probes are listed in Table 1, and their positioning in the genome is illustrated in the Figure.

Sensitivity Testing

A plasmid with the ITS sequence of *M. haemophilum* was prepared by cloning the PCR product in a vector and was subsequently quantified (IQ corporation, Groningen, the Netherlands). Dilution series of this plasmid were tested in duplicate in the genus-specific and the *M. haemophilum*–specific real-time PCR.

Sequence Analysis

After amplification, PCR products were subjected to a cycle sequencing reaction with the Big Dye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems). Samples underwent electrophoresis and sequences were detected and analyzed on ABI model 310 DNA sequencer (Applied Biosystems).

Real-time PCR

Real-time PCR was performed in 50 μL of reaction mixture consisting of 25 μL of 2x IQ supermix (Bio-Rad, Veenendaal, the Netherlands), 20 pmol of each primer, 12.5 pmol of the genus-specific probe or 10 pmol of the *M. haemophilum*–specific probe, and 10 μL template. The PCR thermal profile consisted of an initial incubation of 3 min at 95°C for activation of the enzyme, followed by 50 cycles of 30 s at 95°C, 40 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with an iCycler IQ real-time detection system (Bio-Rad). The reaction mix and PCR profile were similar for both the genus-specific probe and the *M. haemophilum* probe.

Each DNA extract was tested by real-time PCR for the detection of the genus *Mycobacterium* and species *M. haemophilum*. As positive control for the genus-specific real-time PCR and extraction protocol, a dilution of *M. bovis* was used.

Results

Identification of *M. haemophilum* in Patient Material

In 16 (18%) of 89 patients from the CHIMED study, a mycobacterial infection was suspected, but initially no species identification could be established. After a positive signal was generated by the genus-specific real-time PCR and negative results from the cultures, the amplicons generated in real-time PCR were sequenced to determine the species. Sequencing of the ITS fragment formed in the real-time PCR was difficult, owing to the small amount of amplicon, but eventually the sequences of 4 patient samples were successfully derived. On 4 more samples, a seminested PCR was performed to increase the amount of specific amplicon. This enhancement of PCR resulted in the successful amplification and sequencing of all fragments. No variation was encountered between the ITS sequences of all 8 strains analyzed here. Because no *M. haemophilum* ITS sequences were available in the public databases, 3 complete ITS sequences from *M. haemophilum* strains isolated from different CHIMED patients were determined and submitted to the NCBI database (accession no. AY579398, AY579399, and AY579400). After specific culturing, the identity of the strains was confirmed by comparing partial 16S-gene sequences to the sequences in the NCBI (http://www.ncbi.nlm.nih.gov/) and the RIDOM database (http://www.ridom.com/). A variable part of the 16S gene of 330 base pairs was analyzed, and a 100% agreement was obtained with 16S sequences of 7 available *M. haemophilum* strains, including ATCC 29548. The *M. haemophilum* strains had at least 4 mismatches in the analyzed 16S PCR fragment in comparison to other mycobacterial species; therefore, all these strains were *M. haemophilum*. The identity was also confirmed because of a minimum of 4 mismatches in the 16S fragment between the *M. haemophilum* sequence and other mycobacterial species.

Table 1. Sequences of oligonucleotides used in this study*

Primer	Probe sequence (5′–3′)	Target sequence
ITS forward primer real-time PCR	GGGGTGTGGTGTTTGAG	Partial ITS
ITS reverse primer real-time PCR	CTCCCACGTCCTTCATC	Partial ITS
Forward primer Ec16S.1390p†	TTGTACACACCGCCCGTCA	Total ITS
Reverse primer Mb23S.44n†	TCTCGATGCCAAGGCATCCACC	Total ITS
16S forward primer P1‡	TAACACATGCAAGTCGAACG	16S
16S reverse primer P4‡	TCGTTGCGGGACTTAACCCAAC	16S
Mycobacterium genus–specific TaqMan probe	Fam-GGATAGTGGTTGCGAGCATC-Tamra	ITS
Mycobacterium haemophilum–specific MGB-probe	VIC–ACGCCACCATTACT-MGB	ITS

*ITS, internal transcribed spacer.
†Primers published in (19).
‡Primers published in (23).

Application of Real-time PCR to the Recognition of *M. haemophilum*

The real-time PCR was designed to the ITS region. The same conserved primers were used as described previously. The obtained ITS sequences were used to select an *M. haemophilum*–specific probe.

The detection limit of the *M. haemophilum*–specific real-time PCR was assessed at 1 copy per reaction by using a dilution series of the plasmid standard. The mycobacterial genus–specific PCR was tested simultaneously with the *M. haemophilum*–specific PCR and resulted in the same analytical sensitivity. As determined previously, the sensitivity of the primer set in clinical materials was estimated to be 1,100 CFU in pus (8). Specificity testing of the *M. haemophilum*–specific real-time PCR with 25 other species and subspecies showed no aspecific reactions. All 50 *Bartonella*-positive samples from the control group remained negative in the real-time PCR assay as well.

Of 16 patients with evidence for *M. haemophilum* infection, 9 (56%) were positive on auramine staining, and 9 (56%) were positive in *M. haemophilum*–specific cultures. In addition, in 1 patient (6%), the pathogen was able to grow on and in normal mycobacterial cultures. Thirteen patients (81%) had positive specimens in mycobacterial genus–specific real-time PCR, 11 of which were also positive in the *M. haemophilum*–specific real-time PCR (Table 2). In contrast, 2 genus-specific *M. haemophilum*–negative specimens were positive in the *M. haemophilum*–specific real-time PCR. Thus, the 2 PCRs combined yielded 15 positive (94%) patients. These 4 samples with inconsistent results all had high threshold cycle values, indicating that the amount of bacterial DNA present was close to the detection limit of the assays. This finding was confirmed by retesting the samples 5 times in both PCRs, which yielded 2 or 3 positive reactions in the genus-specific PCR and in the *M. haemophilum*-specific PCR. No correlation was found between the threshold cycle values in the real-time PCR assay and the culture or auramine-staining results. All 9 patients with positive specimens by auramine staining also had positive results in the real-time PCR assay. Three patients' conditions were diagnosed by real-time PCR only. Only 1 patient had a positive culture while results of the real-time PCR or auramine staining remained negative. Real-time PCR on the isolate cultured from this patient resulted in a positive signal.

The *M. haemophilum*–specific culturing method was less sensitive than the real-time PCR assay. The materials from the first 6 patients were cultured specifically for *M. haemophilum* after negative results were obtained from conventional culturing methods. The stored decontaminated materials were thawed and incubated at 30°C on enriched media. From these 6 patients, 2 samples (33%) yielded positive cultures. The materials from the 10 other patients were cultured directly and yielded positive results from 7 (70%) patients. *M. haemophilum*–specific real-time PCR was performed additionally on all positive cultures to confirm the specific growth of *M. haemophilum*.

From the 16 patients positive for *M. haemophilum*, 22 samples were collected: 9 tissue biopsy specimens and 13 fine needle aspirates. Of these samples, 19 (86%) were

Table 2. Results of diagnostics tests of 16 *Mycobacterium haemophilum*–positive patients

M. haemophilum–positive patient	Acid-fast staining	Culture 30°C*	Genus-specific real-time PCR	*M. haemophilum*–specific real-time PCR
1	+	−	+	+
2	−	+	−	−
3	+	−	+	+
4	−	−	+	+
5	−	−	+	+
6	+	+	+	+
7	+	−	+	+
8	+	+†	+	+
9	+	+	+‡	−‡
10	+	+	−‡	+‡
11	+	−	+‡	−‡
12	−	+	−‡	+‡
13	−	+	+	+
14	−	−	+	+
15	−	+	+	+
16	+	+	+	+
Total positive patients	9	9	13	13

*Löwenstein-Jensen (LJ) medium with added iron citrate or liquid MGIT medium with X-factor-strip added. Cultures at 30°C were performed after storage for patients 1 to 6.
†Patient material was also culture positive at 35°C.
‡Because of discrepant polymerase chain reaction (PCR) results with high threshold cycle values, the PCR was performed 5 times on these samples, which resulted in at least 2 specific positive signals for both PCRs on every sample. Therefore, the amount of mycobacterial DNA is estimated at the detection limit of the assay. The first obtained PCR result is described in the table.

positive in the real-time PCR assay, while 11 (50%) samples yielded positive results in auramine staining and 9 (36%) were positive in culture. No discrepancies were encountered in the real-time PCR assay when all samples instead of patients were considered. Application of the real-time PCR assay increased the diagnostic yield by 23%.

Discussion

M. haemophilum was found to be the causative agent of lymphadenitis in 16 (18%) of the children included in this study. Despite the use of specific enriched culture mediums, only 9 (56%) of the 16 *M. haemophilum* infections were culture-positive. In contrast, the real-time PCR assay was positive in 15 (94%) patients.

M. haemophilum infection is not diagnosed frequently and is therefore not considered a common cause of lymphadenitis. However, most studies on children with mycobacterial lymphadenitis have not used optimized cultures for *M. haemophilum*, and infection with this species is therefore likely underdiagnosed. Nevertheless, differences in geographic distribution may also contribute to the variable prevalence of *M. haemophilum*. For instance, no *M. haemophilum* was found in children with atypical mycobacterial lymphadenitis in a study in Ohio (24), whereas in a study in Israel, *M. haemophilum* was found in 12 of 29 patients (5). Both studies used appropriate culture conditions for *M. haemophilum*. Another reason for an underestimation of the occurrence of *M. haemophilum* infections is the misleading positive skin test. *M. haemophilum* can induce similar reactions in the Mantoux test as *M. tuberculosis* and could be misdiagnosed when no positive cultures are obtained (4,5).

The natural source of *M. haemophilum* infection is unknown. Its geographic distribution appears to be related to the occurrence of large bodies of water (6). A few natural reservoirs have been suggested (25–27), but studies focusing on the environmental reservoirs of NTM tend to culture without optimized conditions for *M. haemophilum*, which may be the reason the organism is rarely found. The temperature for culture is often too high (28,29), cultures do not contain hemin or iron citrate, or the incubation time is too short (30). Only 1 study detected *M. haemophilum* in water distribution systems, although the culture method was not optimal (26). Therefore, *M. haemophilum* may be widely distributed and present in several natural reservoirs; water is the most likely one (12).

M. haemophilum is slow growing, iron dependent, and has an optimal growth temperature from 30°C to 32°C. It is unable to grow on routine media such as LJ Middlebrook 7H9 and 7H10, or BACTEC broth. Media used to recover *M. haemophilum* on primary isolation include commercially available solid media or broth enriched with ferric ammonium citrate or hemin (31). Chocolate agar and lysed blood agar are mentioned as inexpensive and suitable alternatives (32,33). Little is known about the sensitivities of direct culturing of clinical materials for the recovery of *M. haemophilum*, and not all media have equal capacity for stimulating the growth (34).

Because application of culture conditions specific for *M. haemophilum* are not likely to become standard in clinical microbiologic laboratories, including this specific diagnosis might be useful for molecular methods. A species-specific real-time PCR was developed to identify *M. haemophilum* directly in patient materials. Because *M. haemophilum* was not expected to be an important pathogen, no specific culturing was applied initially. After the recognition of *M. haemophilum* by molecular methods, the culture methods were optimized, which resulted in 70% positive cultures. Additionally, all stored decontaminated materials from culture-negative specimens were recultured under *M. haemophilum*–specific conditions. Most likely because of these additional freezing and thawing steps, cultures were less sensitive for these materials and resulted in 33% positive cultures.

Identification of *M. haemophilum* in patient materials was performed by 16S sequencing (of cultures) and ITS sequencing. Two versions of a commercial reverse line hybridization assay (the Inno-Lipa assay and the V2 Inno-Lipa assay) were also used for the recognition of *M. haemophilum*, but these tests can only be applied on cultured isolates. The V2 Inno-Lipa can identify *M. haemophilum* by a specific probe, which was absent in the previous version of the Inno-Lipa assay. The reactions were uniformly positive only for *M. haemophilum* in the V2 Inno-Lipa.

The design of the real-time PCR MGB probe was based on the ITS sequences that were obtained from the patient materials and reference strains. An MGB probe enables specific detection of the target by using a shorter sequence than that of a TaqMan probe or a molecular beacon.

Sequencing of the ITS amplicons from the genus-specific real-time PCR on patient samples was difficult because of the small amounts of target sequence. To enhance the specific amplification, a seminested PCR was applied. Of the 8 clinical samples from which sequences were obtained, 4 samples also yielded positive cultures once the culture protocol was optimized. The ITS and 16S sequences derived from the cultured isolates confirmed the authenticity of the identified pathogen.

In this study, both fine needle aspiration and excisional biopsy were not applied as treatment options but as diagnostic procedures. Complete surgical excision of the affected lymph nodes is considered as the treatment of choice for atypical mycobacterial lymphadenitis (1,35,36). However, surgical excision leaves scarring and carries the

risk of damaging branches of the peripheral facial nerves (37,38). Antimicrobial therapy as a conservative treatment is currently the topic of our study. Incision and drainage increase the risk for sinus tract formation or recurrence of infection (33,35). This risk also applies to fine needle aspiration, but the usage of fine needle aspirate for PCR will provide a rapid diagnosis and thereby allow treatment to begin earlier and thus lower the risk for complications.

In conclusion, for detecting and identifying *M. haemophilum*, real-time PCR is a sensitive and specific assay suitable for direct application on clinical materials. In this study, by using the real-time PCR, *M. haemophilum* was shown to be an important pathogen involved in lymphadenitis. Because of special growth requirements, the clinical spectrum of diseases associated with *M. haemophilum* is largely unknown. Real-time PCR may be particularly useful for testing clinical samples such as sputum, cerebrospinal fluid, and synovial fluid for *M. haemophilum* to determine the role of *M. haemophilum* in more detail.

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HEPA/Vaccine Plan for Indoor Anthrax Remediation

Lawrence M. Wein,* Yifan Liu,* and Terrance J. Leighton†

We developed a mathematical model to compare 2 indoor remediation strategies in the aftermath of an outdoor release of 1.5 kg of anthrax spores in lower Manhattan. The 2 strategies are the fumigation approach used after the 2001 postal anthrax attack and a HEPA/vaccine plan, which relies on HEPA vacuuming, HEPA air cleaners, and vaccination of reoccupants. The HEPA/vaccine approach leads to few anthrax cases among reoccupants if applied to all but the most heavily contaminated buildings, and recovery is much faster than under the decades-long fumigation plan. Only modest environmental sampling is needed. A surge capacity of 10,000 to 20,000 Hazmat workers is required to perform remediation within 6 to 12 months and to avoid permanent mass relocation. Because of the possibility of a campaign of terrorist attacks, serious consideration should be given to allowing or encouraging voluntary self-service cleaning of lightly contaminated rooms by age-appropriate, vaccinated, partially protected (through masks or hoods) reoccupants or owners.

In addition to killing 5 of its 11 victims, the 2001 anthrax attack on the U.S. Postal Service and federal facilities also contaminated a number of buildings. The U.S. government spent several hundred million dollars recovering buildings with large-area contamination by using chlorine dioxide fumigation. The last of these federal facilities, the Hamilton, New Jersey Mail Sorting Facility, is not expected to reopen until early 2005, >3 years after the attack (1). A large-scale aerosol attack in a major metropolitan area could deny access to a portion of a city for years, with substantial economic and social consequences. While outdoor remediation would be challenging, the absence of sporicidal UV irradiation makes indoor remediation a particularly daunting task. Nonetheless, no federal agency has taken ownership of the wide-area remediation problem (2). A proactive plan to recover affected buildings quickly, safely, inexpensively, credibly, and with minimal collateral damage needs to be developed before such an event (2). To advance the analysis of these recovery options, we propose and evaluate a very simple HEPA/vaccine plan, where HEPA air cleaners continuously clean the indoor air and Hazmat workers use HEPA vacuums to clean the floors, walls, ceilings, and room contents on a twice-a-day basis; HEPA filters are 99.97% effective for 0.3-μm particles (3), which are 5–10 times smaller than a typical anthrax spore. In addition, residents are vaccinated before reoccupying the buildings. This strategy hypothesizes a nonzero standard for spore contamination and modest pre- and postremediation environmental sampling (in contrast, >5,000 negative environmental samples were taken after the fumigation of the Brentwood mail-processing facility [4]).The plan employs no sporicides, such as sodium hypochlorite (household bleach) or hydrogen peroxide, which can cause collateral damage to many hard surfaces, and does not discard carpets or furniture, which would generate profound solid waste problems. Using a hypothetical release in lower Manhattan, we compare the HEPA/vaccine and chlorine dioxide fumigation remedial options, in terms of anthrax cases among reoccupants, cost, and recovery time. No attempt is made to estimate the number of cases of cutaneous and gastrointestinal anthrax, which are less apt to be fatal. Although we focus on anthrax remediation, our framework may also be useful for indigenous agents of public health concern (e.g., tuberculosis, *Streptococcus*).

Materials and Methods

A mathematical model (see online mathematical model for details on model formulation and parameter estimation; www.cdc.gov/ncidod/eid/vol11no1/04-0635_mod.htm) was used to evaluate the HEPA/vaccine (Figure 1) and fumigation modalities. In the model, 1.5 kg of anthrax spores is released outdoors in lower Manhattan from a height of 2 m. We considered 92 different scenarios in total, depending upon the release location and the wind direction. A building inventory of lower Manhattan (5) and an atmospheric dispersion model (6) were used to calculate the concentration of spores in each building in the exposed region. We assumed that postattack environmental sampling and plume analysis allow at least some of the "exposed region" to be correctly diagnosed within 1 week

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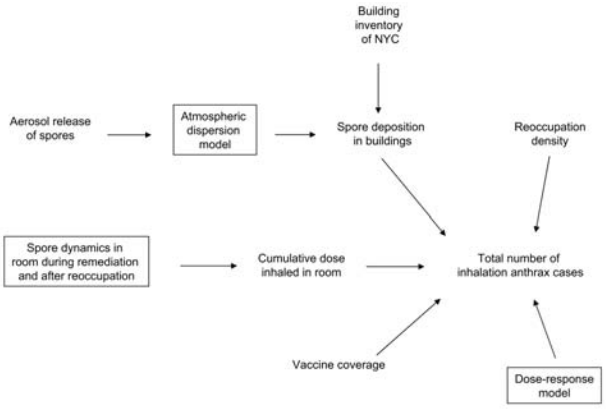

Figure 1. Graphic overview of the mathematical model. Mathematical submodels are in boxes. NYC, New York City.

after the attack, at which time remediation begins. We also assumed that by day 7, outdoor contamination would have subsided to the point where it did not affect indoor spore concentrations.

Since chlorine dioxide fumigation eliminated all detectable spores from the Hart Senate Office Building and several mail-sorting facilities, we assumed that it successfully eliminates all spores in the buildings of our model. In the 2001 attack, chlorine dioxide was used to decontaminate the 700-km^2 Brentwood postal facility, which took 1 year at a cost of $130 million (4); further discussion of this cost estimate appears in the online mathematical model. Because the technology was new, we assumed that 50% of the cost was a 1-time investment in technology development. We further assumed a 90% learning curve in both cost and time (at this time, only a small number of companies possess chlorine dioxide expertise); i.e., each time the area of anthrax decontamination doubles, the marginal cost and time are reduced by 10%.

To assess the HEPA/vaccine plan, we developed a differential equation model (Figure 2) of the spore dynamics within a generic 12x12x8-ft room in a building in the exposed region. The model measures the evolution of spore concentration in the air, on the room surfaces, and in the HVAC (heating, ventilation, and air-conditioning) system. A small fraction of spores adhere to the HVAC ducts as they enter the building, and then become slowly disengaged and enter the room. Rather than build multizone models of each building (7), we assumed that each room received air from a duct that is 50-m long, contains 360° of curvature, and has an air velocity of 1,000 ft/min. We implicitly assumed that all rooms within a building are remediated simultaneously, so as to minimize the effect of inter-room contamination within a building. Airborne spores in the room deposit on the room surfaces at a certain rate, and spores on the room surfaces, particularly the floor, reaerosolize at a rate that depends on the amount of activity in the room; more reaerosolization occurs during surface cleaning and reoccupation. The deposition rates and reaerosolization rates were derived by using data from the Hart Senate Office Building (8). HEPA air cleaners (achieving 10 air changes per h, possibly with the aid of dilution ventilation from the HVAC system) are used continually during the remedial period, which involves successive rounds of testing and vacuuming until n_s postcleaning samples suggest that the floor spore concentration in the room is below the target level $\bar{c}_f$; this approach is reminiscent of that taken during the asbestos remediation after the World Trade Center collapse (9). Rather than use a spatial model to capture spatial heterogeneity of spores within a room, we simply assume that the floor samples are log normally distributed, where 95% of within-room samples at a fixed point in time are within 1 order-of-magnitude (i.e., within $1/\sqrt{10}$ and $\sqrt{10}$ of the median), which is consistent with the sample variability in the Hart Senate Office Building (8). That is, in the initial testing of samples, we estimate the number of 2-h vacuumings of the room's surfaces and contents that are required to achieve the target concentration $\bar{c}_f$. After these vacuumings, a new set of n_s samples are taken. If the estimated concentration from these new samples is below $\bar{c}_f$ then remediation ceases; otherwise, another round of vacuuming and testing is performed. Consecutive vacuumings are 48 h apart, and testing (if needed) occurs midway between these 2 vacuumings, both to allow reaerosolized spores to resettle before testing and to permit the testing results to be received before the next scheduled vacuuming. We varied the 2 decision variables n_s and $\bar{c}_f$ to explore the tradeoffs among our performance measures.

After the floor concentration is believed to have dropped below $\bar{c}_f$ each generic room is reoccupied by 1 person for 12 h per day. After reoccupation, a portable HEPA air cleaner (at 3 air exchanges per h [10]) is used for 12 h every day, and 10 min of floor vacuuming occurs weekly at half the estimated efficiency of the remedial

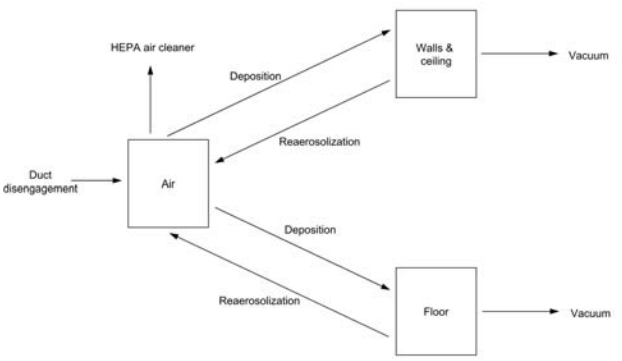

Figure 2. Graphic depiction of the compartments in the differential equation model and the spore movement among compartments.

vacuuming. We assumed that 85% of reoccupants are successfully vaccinated and will not become infected, regardless of the spore concentration in the room. The remaining 15% represent infants, the elderly, the immunocompromised, and persons for whom vaccination is contraindicated, who are assumed to have a dose-response curve that correspond to the lowest 30% of the probit dose-response model (11) with a 50% infectious dose (ID_{50}) of 8,000 spores (12) and a probit slope of 0.7 (13); e.g., the ID_{15} from the probit model in (11) (i.e., 253 spores) would infect half of the unvaccinated population. Here, ID_{50} denotes the dose that infects half of the population; because inhalational anthrax is nearly always lethal (in the absence of treatment), the ID_{50} coincides with the 50% lethal dose (LD_{50}). The differential equation model is used to measure the cumulative number of spores inhaled by each reoccupant in a 10-year period. Combining these cumulative doses, the dose-response model, the atmospheric dispersion model, and the population density of reoccupants allows us to compute the total number of inhalation anthrax cases.

The cost of the HEPA/vaccine plan includes $75/h for each Hazmat worker, who spends 4 h per 10-h shift vacuuming and the remaining 6 h resting, rehydrating, and handling protective gear; a $250 portable HEPA air cleaner for each 12x12x8-ft room; $25 for each environmental sample, which includes the costs for sampling, shipping, and laboratory testing; and $20 to vaccinate each person. If residents are vaccinated regardless of the remediation/reoccupation policy, the vaccination cost should be omitted from the comparison. The remediation time for the HEPA/vaccine plan was computed by assuming that 1,000 Hazmat workers (using level C protection) are available to perform remediation 10 h per day, which is ≈3 times larger than the labor force used at the Brentwood and Hart buildings, and that 200 samplers can each perform 24 samples in 4 h plus have 6 h for donning and removing protective gear, rest, and rehydration. The bottleneck for the total remediation time can be either sampling or vacuuming, depending upon the values of the concentration threshold ($\bar{c}_f$) and the number of samples per round (n_s).

Results

We averaged the 92 scenarios to obtain a base case. Figure 3A shows the depositional distribution averaged for the 92 scenarios, i.e., the number of square meters of indoor floor area that are contaminated at various levels. The particular forms of dips and peaks in Figures 3A and 3B are due to the irregular spatial distribution of tall buildings relative to the release location that caused the most indoor contamination. The total contaminated area in this average scenario is 5.73 x 10^7 m^2, which is >4 million 12x12x8-ft rooms. For this base-case scenario, the fumigation plan costs $2.7 billion and takes 42 years. Figures 4A–C express the expected number of cases, cost, and time of the HEPA/vaccine plan for the base-case scenario in terms of the floor concentration threshold ($\bar{c}_f$) and the number of floor samples per round (n_s). Because of the random sample measurements, 50 simulations were performed to estimate each of the points in Figure 4, and the 95% half-confidence intervals are < 0.05 times the sample mean in all cases. Figure 4A shows that the mean number of anthrax cases is nearly independent of the number of samples per round, and drops from ≈3,000 cases when the floor concentration threshold is 100 spores/m^2 to 28 cases when the floor concentration threshold is 0.1 spores/m^2. To put these numbers in perspective, we also found that 15,760 cases would occur if no cleaning was performed (i.e., $\bar{c}_f = \infty$). The total cost in Figure 4B varies from $1.7 billion to $6 billion and depends more on the spore concentration threshold than the number of samples per round. The mean remediation time ranges from 2.9 years to 39.3 years; since there are approximately 4 million rooms and vacuuming can be done at the total rate of 2,000 rooms/day, it would take 5.5 years to clean each room once. Vacuuming dictates the total remediation time in Figure 4C when n_s= 1 and $\bar{c}_f$= 0.1 or 1, and sampling is the bottleneck for the other values of tested. Because using n_s > 1 increases the cost and time without decreasing anthrax cases, we focus in Figure 4D on the cost versus time tradeoff by fixing n_s =1.

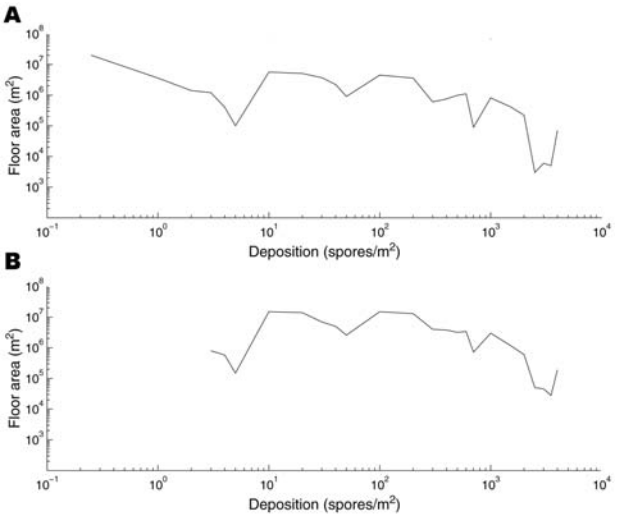

Figure 3. The amount of indoor floor area in lower Manhattan (vertical axes) that is contaminated at various anthrax concentration levels (horizontal axes) as a result of an outdoor release of 1.5 kg of anthrax spores. Plot A, an average of 92 scenarios (9 release locations in Manhattan times 8 wind directions, plus 20 release locations on the outskirts of Manhattan). Plot B, provides similar information for the scenario that generated the largest total area of contamination.

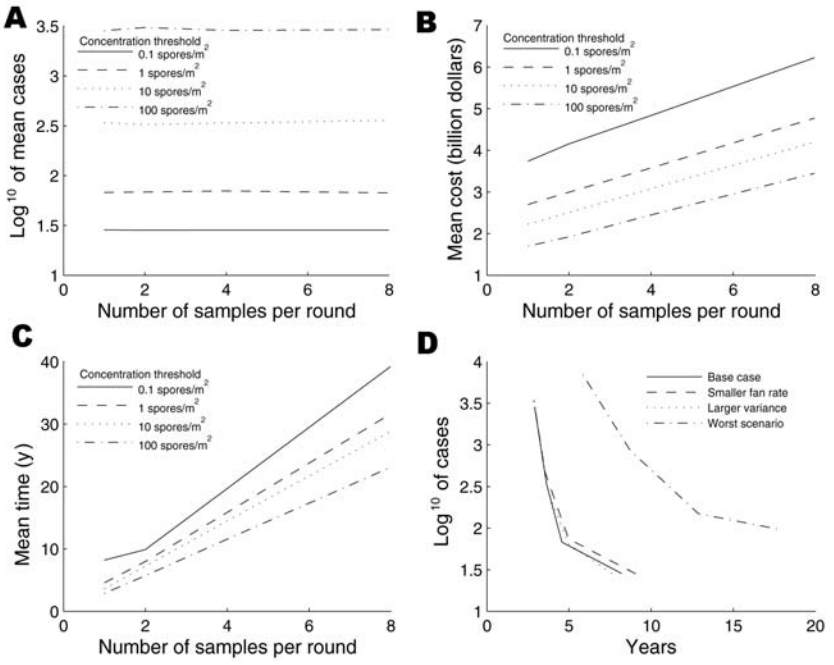

Figure 4. Performance of the HEPA/vaccine plan under the base-case scenario. The horizontal axes in plots A-C are the number of floor samples per round (n_s). Each of plots A-C have 4 curves, 1 for each value of the floor concentration threshold ($\bar{c}_f$). Cleaning stops after the estimated floor concentration from n_s samples per room is below the threshold $\bar{c}_f$. The vertical axes in plots A–C are A) the mean number of inhalation anthrax cases, B) the mean cost, and C) the mean recovery time. In plots A-C, the concentration threshold ($\bar{c}_f$) has a much bigger impact than the number of samples per room (n_s) on these 3 performance measures. Plot D shows the tradeoff of anthrax cases versus recovery time in the base case. The number of samples per room is assumed to be n_s= 1 in this plot, which is derived from plots A–C. Plot D also contains tradeoff curves for 3 sensitivity analyses: a lower air-cleaning rate, increased sampling variability of spore concentration, and the most severe of the 92 cases depicted in Figure 3B. This plot shows that the cases versus time tradeoff curve is very insensitive to changes in the air-cleaning rate and sampling variability.

Figure 5 depicts the mean cases and mean remediation time according to the amount of original spore deposition in the rooms. Figure 5A suggests a hybrid strategy that fumigates heavily contaminated rooms (>100 spores/m^2) and uses the HEPA/vaccine approach for lightly contaminated rooms (<100 spores/m^2). This hybrid approach results (on average) in only 2 anthrax cases, and the mean remediation time for the lightly contaminated rooms is 5.9 years. It takes 8.4 years to fumigate the highly contaminated rooms. Hence, the total remediation time ranges from 8.4 to 14.3 years, depending upon whether different workers are involved in the 2 decontamination modalities. For the 3 other threshold levels pictured in Figures 5B–5D, many of the anthrax cases occur right at the cutoff point, which is due to the tail behavior of the spore depositional distribution in Figure 3A. The hybrid strategy is not as helpful with these higher threshold levels; e.g., using a threshold of 1 spore/m^2 to decide between fumigation and vacuuming in Figure 5A, the plan would vacuum for 2 years and fumigate for 28 years.

Sensitivity Analyses

A number of aspects of the model contain considerable uncertainty: the cost and time of the fumigation plan, the indoor spatial deposition after an attack, the reaerosolization and deposition rates inside a room, spore dynamics in a duct, air-cleaning efficacy, vacuum efficacy, Hazmat logistics, the spatial heterogeneity in sampling, vaccine coverage, and the low end of the dose-response curve. Before discussing each of these 10 variables in turn, we note that our general approach to these uncertainties is to be conservative with respect to assessing the HEPA/vaccine option; i.e., we err on the side of overstating the mean number of anthrax cases that would result under this approach or understating the cost and time of the fumigation plan.

Although fumigation was successful during the cleanup after the 2001 postal attack, the fumigation of a skyscraper is a challenge that has yet to be tackled. Given the 42 years it would take to fumigate the exposed area, an alternative technology could be developed.

The estimated indoor spatial deposition contains orders-of-magnitude of uncertainty, depending upon the size of the release, the spore characteristics (e.g., dry versus wet, size, purity, viability, surface electrostatic properties), the weather conditions, building and canopy terrain in lower Manhattan, building HVAC infrastructure, and whether or not windows and vents were open. The goal of the atmospheric modeling is neither to accurately predict the probability distribution of indoor spatial concentrations for a possible future attack (such an attempt would be greatly limited by the irreducible uncertainty in the release size) nor to provide postattack situational awareness (which would require a much more detailed spatial model), but rather to generate a comparative set of plausible scenarios to evaluate remediation strategies before an attack. Hence, we focused on the average of 92 plausible scenarios. To give some sense of the upper range, we present in Figure 4D the results from the most severe of the 92 scenarios; the deposition distribution from this scenario appears in Figure 3B. This scenario contaminates

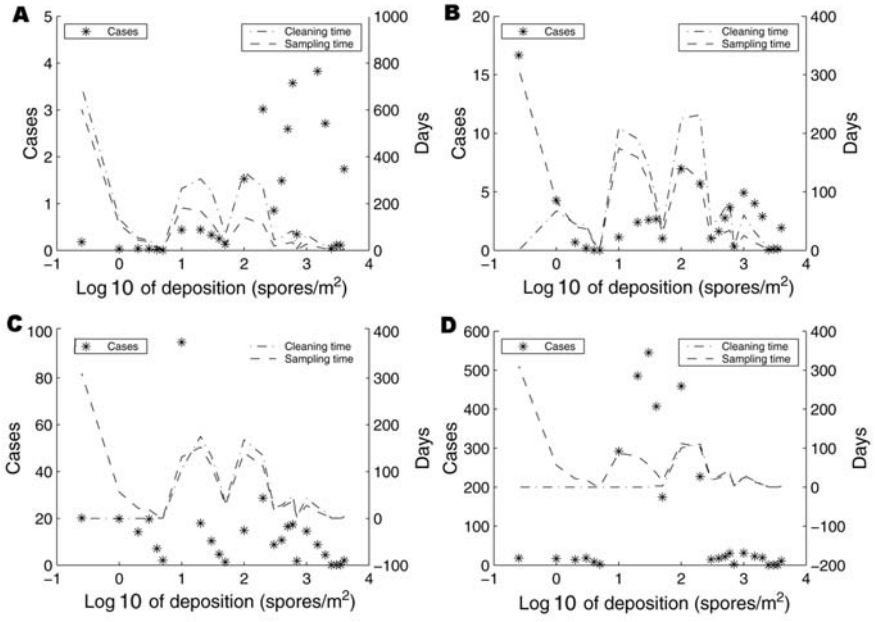

Figure 5. The horizontal axes in these 4 plots give the original room deposition level before remediation begins. These plots show how the total number of anthrax cases (the stars and the left vertical axes) are distributed across room deposition levels, e.g., in plot A, most of the anthrax cases occur in rooms with original deposition levels >100 spores/m^2. Similarly, the 2 curves and the right vertical axis of each plot show how much time is spent cleaning and sampling in rooms of various deposition levels. These 4 plots are identical except that the spore concentration threshold in spores/m^2 ($\bar{c}_f$), which dictates when remediation is stopped, is A) $\bar{c}_f$ = 0.1; B) $\bar{c}_f$ = 1; C) $\bar{c}_f$ = 10; D) $\bar{c}_f$ = 100. These plots motivate the hybrid policy, which fumigates heavily contaminated rooms and uses the HEPA/vaccine approach in lightly contaminated rooms.

≈7 million rooms and requires 17.7 years to reduce the number of cases to 98.

Because air and surfaces are concomitantly remediated, the number of anthrax cases is rather insensitive to the reaerolization and deposition rates in the room.

The large uncertainty with respect to duct modeling led us to adopt a worst-case approach and use the spore disengagement rate that maximizes the number of anthrax cases. Many new buildings and some retrofitted older buildings have HEPA filters built into the HVAC system (14), which would largely eliminate the risk for spore disengagement.

We have focused on portable air cleaners, whereas dilution ventilation, in which 15%–25% of the total airflow rate consists of outside airflow (15), may also play a key role in remediation. Figure 4D also presents results when we reduce the air-cleaning rate during remediation from 10/h to 3/h. The latter quantity, which can be achieved with an off-the-shelf air cleaner and an open window (10), generates only a minor change in the cases versus time tradeoff curve.

To the extent that reaerosolized spores resettled before or during postvacuum testing in the referenced study (16), we may have underestimated the vacuum efficacy. We conservatively assumed that all floors are carpeted and that sporicides such as sodium hypochlorite, hydrogen peroxide, or foams (17,18), which are much more effective than vacuuming for hard surfaces, are not deployed.

Our assumption that each Hazmat worker has 4 productive hours of work per day underestimates the rate that could be achieved over a several-week time frame but is prudent over a longer period of time and would help avoid worker fatigue and burnout.

Because the amount of spatial heterogeneity of spores in a room is difficult to assess, we considered the case where 95% of samples within a room fall within 2 orders of magnitude rather than 1. Figure 4D shows that the effect of this increased sampling variability is negligible and that the optimal amount of sampling did not change relative to the base case.

As noted in section 3.8 of the online mathematical model, our 85% vaccine coverage of reoccupants may be a considerable underestimate. No age groups are being left behind in the plans for the next-generation anthrax vaccine, and persons with weak immune systems may achieve partial protection.

We considered a cumulative dose during a 10-year period, whereas infection may be a result of a challenge over a shorter time horizon; our overestimate of cases is very modest because of the exponential decreases in spore concentration during the reoccupation period, and changing the horizon from 10 years to 6 months led to a negligible (<1%) reduction in cases. Our dose-response model assumed that the 15% unvaccinated population comes from the most vulnerable 30% of a widely used probit model, which itself has been criticized for greatly overestimating the number of cases at the lower end of the curve (19). If we used 95% vaccine coverage with the remaining 5% sampled from the lower 50% of the probit model, then the number of anthrax cases with $\bar{c}_f$ = 10 spores/m^2 and n_s= 1 sample per round would be reduced from 341 to 72. Even within the class of probit models, others have used a probit slope twice as steep, which results in many fewer cases (20). If we use a probit slope of 1.4 rather than 0.7, then the mean number of cases with $\bar{c}_f$ = 10 spores/m^2

and n_s=1 sample per round decreases from 341 to 3 x 10^{-5}, which highlights the value of further research into the low end of the dose-response relationship. However, in the online mathematical model we note that the slope of 0.7 is more consistent with data from the 2001 anthrax attack. Dahlgren et al. (21) estimated that goat-hair mill workers routinely inhaled about 500 (≤5 μm) anthrax spores per shift without accompanying illness or death, raising the possibility (although no subsequent work on this topic has been published) that chronic low-level exposure might induce adaptive or innate immunity. In any case, adaptive or innate immunity is unlikely to occur in the 15% of people in our model who are not successfully vaccinated. One assumption that is not conservative is that people reoccupy these rooms for 12 h per day. A small fraction of people may work at home, stay at home most of the day, or work and live in different buildings within the exposed region. We are underestimating the inhaled doses for these people by a factor of 2. Nonetheless, taken together, the numerical results reported here may overstate the actual number of anthrax cases by at least 1 order of magnitude, and perhaps many.

Discussion

The base-case release, which is an average of 92 different scenarios under various weather conditions and locations in lower Manhattan, contaminates the equivalent of 4 million 12x12x8-ft rooms. Our analysis suggests that an outdoor release would generate a more diffuse depositional distribution of spores than an indoor attack: we estimate that ≈10,000 spores/m^2 were deposited in parts of the Hart Senate Office Building (section 3.2 of the online mathematical model), which is considerably higher than the concentrations in Figure 3. As an alternative to a multidecade fumigation effort, the HEPA/vaccine plan appears capable of substantially reducing the number of anthrax cases but would require ≈8 years with the current estimated Hazmat labor pool. Both plans would require several billion dollars in direct costs. The HEPA/vaccine plan eventually experiences diminishing returns: from a base of 341 expected cases after 3.6 years of remediation, another year is required to reduce the mean number of cases to 67, but then an additional 3.6 years and $1 billion are needed to reduce the mean number of cases to 28. A hybrid HEPA/vaccine/fumigation plan, in which lightly contaminated buildings receive the HEPA/vaccine approach and heavily contaminated buildings are fumigated, could eliminate almost all of the anthrax cases. The required remediation time would be 8.4–14.3 years, depending upon whether the same Hazmat personnel carried out both operations.

A key finding of our study is that only a moderate amount of sampling appears to be required. In theory, additional sampling reduces type I and type II errors, thereby avoiding anthrax cases in rooms that were inadvertently thought to be sufficiently safe, and reducing unnecessary remediation of rooms that were mistakenly perceived as overly contaminated. However, the number of anthrax cases was essentially independent of the number of room samples per round, as long as at least 1 sample was taken. Indeed, with current vacuuming and sampling capacity, the only impact from taking >1 sample per 12x12x8-ft room is prolonged remediation and increased cost. However, in the absence of exhaustive environmental testing, on-site coordinators need to validate that work is performed according to the required standards (i.e., vacuuming is actually being done for the specified number of minutes/m^2).

Our results have several implications. First and foremost, field tests with simulants are required to accurately assess the real-world spore reduction that can be achieved—and the number of vacuumings required—by this HEPA/vaccine approach. If field tests confirm the model predictions, then the concentration threshold $\bar{c}_f$, the number of samples per round n_s, and the level of concentration that requires fumigation versus vacuuming should be determined with greater precision. These threshold values should be chosen so that the reoccupant risk level (in terms of quality-adjusted life years) is consistent with those for other hazards (e.g., asbestos, radiation).

Large-area urban remediation strategies must confront a number of difficult issues, the most important of which is surge Hazmat capacity. We have assumed that remediation and vaccination are initiated simultaneously 1 week after the attack. The initial vaccination of reoccupants would require ≈1 week; protective immunity is believed to develop at 35 days after initial vaccination (22). Hence, residents will be able to reoccupy buildings by 42 days after remediation is initiated. Presumably, most reoccupants would receive prophylactic antimicrobial agents because they would have been in these building during or soon after their exposure. Consequently, some of these residents may be interested in moving back in even earlier. Considering that 8.2 years is required to carry out the HEPA/vaccine plan in the base-case scenario, this reoccupancy delay may be viewed by the major stakeholders as unacceptable. Our analysis assumes the availability of 1,000 Hazmat personnel, compared to the 300 Hazmat workers (after attrition) used to perform the Brentwood cleanup and the roughly 3,000 licensed asbestos workers in New York State. To reduce the recovery delay from 8.2 years to 5 months requires a 20-fold increase in Hazmat labor, i.e., 20,000 personnel. To reduce the delay another 4-fold so as to allow reoccupation within 42 days is probably not realistic for this large-area scenario. Nonetheless, U.S. government coordination with the Hazmat, fumigation, and building protection industries—not just locally, but nationwide and perhaps including the U.S. military and

key allies—would be necessary to guarantee available capacity and resources. In addition, scheduling theory (23) implies that aggregate waiting time for reoccupants can be minimized by remediating the least-contaminated buildings first (i.e., use the shortest expected processing time priority rule).

There are other aspects to optimizing surge remediation and recovery capacity. Just as the worried well caused a surge in ciproflaxin sales in 2001, many people outside of the exposed region will attempt to buy HEPA air cleaners and vacuums. Hence, demand will come not only from the exposed area but also from surrounding regions. In the same way that the U.S. government is working with pharmaceutical companies to provide surge capacity of medical countermeasures (including anthrax vaccine) in the event of a biologic attack, it needs to develop cooperative agreements with building protection service companies so that equipment shortages do not block the critical path to recovering the exposed area.

Another key aspect of a detailed plan is exception management: the HEPA/vaccine plan will not work for 100% of the buildings in the exposed area. More aggressive remediation of critical assets (hospitals; nursing homes; daycare centers; emergency response facilities; electrical, water and sanitation facilities; transportation facilities) will be desirable. Some nonresidential buildings (such as the buildings contaminated in the 2001 attack) have extremely high ceilings, and achieving a high air-exchange rate in these spaces may be not be feasible with portable air cleaners. Another confounding issue is visitors to the impacted region. In the aftermath of a catastrophic anthrax attack, the public would expect nationwide voluntary mass vaccination. Visitors to the exposed areas should be offered an anthrax vaccine, and guidelines for unvaccinated visitors should be developed. Also, because the spore concentration continues to decrease exponentially during reoccupation (but not during semiquiescent periods), more vulnerable residents might delay their reoccupation until several months after the other residents. A significant logistical issue is the disposal of contaminated carpets, furniture, and other household goods. Some reoccupants will insist on discarding these items, even after they have been heavily cleaned. Reoccupant education and outreach measures, including perhaps temporal or financial disincentives for disposal, need to be taken to avoid overwhelming solid waste disposal capacity. Emergency plans (e.g., medical incinerator capacity) should be developed for the HEPA vacuum bags and other items that need to be discarded during remediation. Another difficult issue is postevent building maintenance, particularly of HVAC systems, which must minimize spore reaerosolization during maintenance and disposal of old ducts. Safe procedures to rid ducts of asbestos (asbestos fibers are roughly the same size as anthrax spores, but the U.S. Environmental Protection Agency limit for asbestos is 900 fibers/m^3 [9], which is larger than the postremediation spore concentrations considered here) and other materials have been developed (24); the important point is that HVAC cleaning should not block the critical path to reoccupation but rather should be performed asynchronously in a low-intensity manner over many years.

In summary, this study suggests that a HEPA/vaccine approach is viable for most buildings after a large-scale anthrax attack. This outcome is dependent on a qualitative increase in surge Hazmat remediation capacity to reduce the recovery delay to a level that would not invite permanent mass relocation. Detailed mass remediation plans need to be developed now; as noted by Danzig (2), without such a plan we are inviting economic and social disruption. Ultimately, the extent of restoration and sampling will be dictated by the reoccupants and building owners, and hence risk communication will be of the utmost importance. Inconvenience and cost may force relaxation of standards, and some thought should be given to whether voluntary "self-service" cleaning of minimally contaminated rooms by age-appropriate, vaccinated, partially protected (e.g., with N95 masks) reoccupants or owners would be allowed or encouraged. Indeed, in the face of a campaign of terrorist attacks (2), this self-service approach, with more effective masks or hoods, may be the only feasible response. Finally, a safe, effective, single-dose vaccine would have a profound impact on mitigating the undesirable consequences of this scenario.

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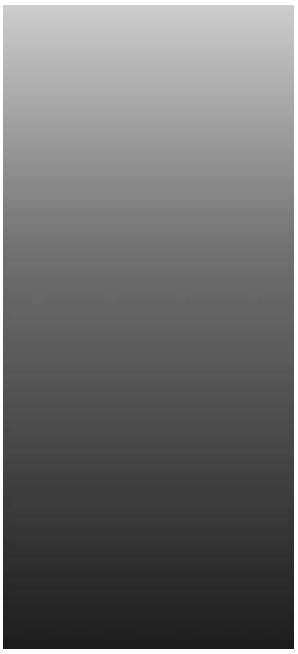

Invasive Group A Streptococcal Infections, Clinical Manifestations and Their Predictors, Montreal, 1995–2001

Maria-Graciela Hollm-Delgado,*† Robert Allard, *†‡ and Pierre A. Pilon*‡

We identified 306 invasive group A streptococcal infections (IGASI) by passive population-based surveillance in Montreal, Canada, from 1995 to 2001. The average yearly reported incidence was 2.4 per 100,000 persons, with a 14% death rate. Among clinical manifestations, incidence of pneumonia increased from 0.06 per 100,000 in 1995 to 0.50 per 100,000 in 2000. Over a span of 7 years, the odds of developing pneumonia increased (odds ratio [OR] = 1.21, 95% confidence interval [CI] 1.0–1.5), while they decreased for soft-tissue infections (OR = 0.86, 95% CI 0.7–1.0). Serotypes M1 and M3 accounted for 30% of IGASI. However, neither serotype was significantly associated with specific clinical manifestations, which suggests that manifestation development among IGASI might be attributable to host or environmental factors rather than the pathogen. In our study, these factors included age, gender, underlying medical conditions, and living environment, yet none explained temporal changes in risk for pneumonia and soft-tissue infections.

Since the mid-1980s, concern has grown that invasive group A streptococcal infections (IGASI) have been increasing in incidence and severity (1–3). In particular, the emergence of streptococcal toxic shock syndrome (STSS) during the 1980s is frequently cited as an example of increasing severity (4).

Person-to-person transmission of *Streptococcus pyogenes* (the causative agent for IGASI) primarily occurs through respiratory droplets, although it may also spread through body secretions from an infected patient (5,6). Additionally, M serotypes of *S. pyogenes* that cause severe disease in a patient are more likely to cause severe disease in subsequent patients (6). These serotypes include 3 (M1, M3, and M18) that are strongly associated with pathogenicity (7). Nonetheless, some evidence indicates that persons with IGASI from the same strain of *S. pyogenes* may have different clinical manifestations of this disease (8,9). Other risk factors for IGASI include patient's age and underlying medical conditions (e.g., varicella). However, what factors may be associated with different clinical manifestations of IGASI is unclear (10–22).

Some studies have examined the role of age, varicella, and chronic conditions such as diabetes mellitus and alcoholism as predictors for necrotizing fasciitis, soft-tissue infections, and STSS (21–24), yet little is known regarding other IGASI determinants. In this study, we describe the status of both IGASI and their clinical manifestations on the island of Montreal. We also identify predictors for clinical manifestations and death due to IGASI, which could explain temporal fluctuations in the incidence and severity of this disease.

Methods

Surveillance of IGASI

Data used in our study were collected during passive surveillance of IGASI among all residents of the island of Montreal (population = 1.8 million: 21,529 births per year from 1996 to 1999 [25]). Cases that occurred and were reported from January 1, 1995 (the year IGASI became a notifiable disease in the province of Quebec), through February 28, 2002, were included in our study.

Once the public health department had been notified of a potential case, usually by a hospital laboratory, a 6-part questionnaire was completed by using information from the physician or infection control nurse of the health

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center where the case-patient was identified or treated. Questions included the patient's demographic information, general medical information, laboratory results, diagnostic criteria, and medical history before the IGASI. With this information, all IGASI were classified into 1 of 3 groups: confirmed cases (*S. pyogenes* isolated from a normally sterile site), clinical cases (*S. pyogenes* isolated from a nonsterile site and toxic shock not attributable to any other cause), or noncases. Data on confirmed and clinical cases were entered into the regional notifiable infections computer database. This database was used for our study.

Laboratory Assessment of IGASI Isolates

Initial laboratory confirmation of *S. pyogenes* was made by using standard methods (26). Isolates were then collected and sent to the Canadian National Center for Streptococcus in Edmonton for further testing of the opacity factor, as well as M, T, and R surface proteins. The methods have been described in detail elsewhere (27). Briefly, antiopacity factor (AOF) typing was performed on any positive opacity factor sample. Although AOF testing does not possess the same type specificity as M typing, it is frequently used because of difficulties in producing antisera for certain M serotypes. Its use has been validated for most strains identified in industrialized countries. However, since 2000, the national center has supplemented AOF testing with *emm* gene sequencing for some nontypeable M serotype samples. Data on these results were not available for this study.

Classification of Outcomes

For our study, we looked at 5 dichotomous outcomes: STSS, soft-tissue infections, bacteremia, pneumonia, and death attributable to IGASI. All were invasive and defined in accordance with the classification of group A streptococcal infections (28).

STSS was defined according to the 1993 Working Group on Severe Streptococcal Infections consensus definition for a probable or confirmed case (28). Soft-tissue outcomes included fasciitis, myositis, cellulitis, or erysipelas. Bacteremia was characterized by a positive hemoculture, without any source of infection. Pneumonia attributable to IGASI was based on a clinical diagnosis made by the treating physician and could include STSS with respiratory distress.

Classification of Independent Variables

Age, calendar month, and year in which the IGASI case occurred were included in our study as continuous variables. Gender (male or female); underlying medical conditions (drug use, alcohol abuse, varicella, prior trauma or wound, cancer, and immunosuppression); type of living environment (hospital, daycare or preschool, school, work, other, and not available); as well as M, T, and R surface protein serotypes (presence or absence of a specific serotype) were all included as dichotomous variables. For those serotypes with identical strength of association with a given outcome, a single new dichotomous variable was created to represent the presence of one or the other (e.g., presence of either serotype M12 or M28 versus absence of both serotypes). Finally, since predominant site of infection (bacteremia, fasciitis, cellulitis or erysipelas, myositis, peritonitis, respiratory manifestations, septic arthritis, and other) was partially used in distinguishing between bacteremia, pneumonia, and soft-tissue infections, this variable was only considered a covariate of interest in models with STSS and death as their outcomes.

Statistical Analysis

The incidence (per 100,000), death rate attributable to IGASI, and proportion of IGASI cases due to a specific clinical manifestation were estimated by using data collected from 1995 through 2001. Incidence and proportion estimates were not calculated for 2002, given that only 2 months of data were available. Projected annual population estimates for Montreal were used when calculating the reported annual incidence of IGASI (25). Incidence and proportion of IGASI cases stratified by gender, calendar year, and age group were then calculated. Finally, temporal trends were assessed by using the chi-square test for trend.

For the inferential component of our study, we conducted unconditional logistic regression with SAS version 8.0 (SAS Institute, Cary, NC, USA). This test was initially performed by including in the model variables with a univariate likelihood ratio p value ≤ 0.20. Among these factors, those with the highest multivariate Wald chi-square p value were then individually dropped, until the lowest Akaike Information Criterion value was attained. The McGill University Faculty of Medicine Institutional Review Board approved the study.

Results

From 1995 through 2001, a total of 306 cases of IGASI were reported on the island of Montreal. The incidence of IGASI rose from 1.05 per 100,000 (19 cases) in 1995 to 1.71 (31 cases) in 1996 and 3.32 (60 cases) in 1997. After 1997, the incidence appeared to stabilize: 2.77 (50 cases) in 1998, 2.50 (45 cases) in 1999, 3.21 (58 cases) in 2000, and 2.37 (43 cases) in 2001. The average annual incidence of IGASI was 2.4 per 100,000. Most IGASI cases occurred in persons ≥ 40 years of age (172 [56%] of 306 cases) (Figure 1). The median age of patients was 46 years (range 1.5 months to 92 years).

Of the 306 reported IGASI cases, 112 (37%) were soft-tissue infections, 84 (28%) bacteremia, 32 (10%) pneumonia, and 29 (10%) STSS. Among patients with soft-tissue

Figure 1. Annual incidence and death rate of invasive group A streptococcal infections, by age, in Montreal, Canada, 1995-2001.

infections, 6 (5%) of 112 cases had myositis, 31 (28%) had cellulitis, and 76 (68%) had necrotizing fasciitis; 1 patient had both cellulitis and necrotizing fasciitis. We did not identify any significant trend over time with regard to the proportion of different clinical manifestations. As for specific clinical manifestations of IGASI, we estimated that bacteremia occurred, on average, in 0.66 per 100,000 persons each year, STSS in 0.23 per 100,000, soft-tissue infections in 0.89 per 100,000, and pneumonia in 0.25 per 100,000.

The predominant M serotypes included M1 (22%), M3 (12%), M28 (9%), M12 (8%), M4 (6%), and M6 (4%). Remaining serotypes accounted for <3% of isolates. Twenty percent of samples were nontypeable.

Pneumonia

The incidence of pneumonia appeared to significantly increase over time ($\chi^2 = 5.65$, $p = 0.018$), with an annual incidence of 0.06 per 100,000 in 1995 and 1996, 0.28 in 1997 and 1998, 0.39 in 1999, 0.50 in 2000, and 0.22 in 2001. This finding was confirmed by the odds of having pneumonia significantly increasing with each successive calendar year (adjusted odds ratio [aOR] = 1.21, 95% confidence interval [CI] 1.0–1.5). The proportion of women and girls with pneumonia (Figure 2) also significantly increased ($\chi^2 = 5.03$, $p = 0.025$), with women more likely to have pneumonia as compared to men (aOR 2.20, 95% CI 1.0–4.9). Gender was not associated with year in which the case occurred.

STSS

We did not detect a significant secular trend in the occurrence of STSS ($\chi^2 = 0.54$, $p = 0.46$). Persons who abused alcohol (aOR 7.66, 95% CI 1.9–30.3), were infected with serotype M9 (aOR 39.98, 95% CI 1.9–836), or who had fasciitis (aOR 10.21, 95% CI 4.1–25.7) were at a significantly greater risk of having STSS.

Soft-tissue Infections

No significant secular trend was apparent in the incidence of soft-tissue infections ($\chi^2 = 0.48$, $p = 0.49$). However, the odds of developing this manifestation as opposed to another significantly decreased with each successive calendar year (aOR 0.86, 95% CI 0.7–1.0). Drug use was weakly associated with soft-tissue infections (unadjusted OR 1.86, 95% CI 0.8–4.4). Given that trauma was a significant univariate risk marker for soft-tissue infections (OR 2.78, 95% CI 1.6–4.8), this association might have been attributable to injection drug use resulting in a trauma or wound. However, in our study, no correlation was seen between drug use and trauma ($r = 0.002$, $p = 0.97$). Furthermore, the association between drug use and soft-tissue infections became significant after adjusting for trauma or wound (OR 2.83, 95% CI 1.0–8.0).

Varicella and serotypes M6, M12, or M22 were significant predictors for developing soft-tissue infections with aORs of 5.69 (95% CI 1.4–23.1), 4.3 (95% CI 1.1–16.7), 9.1 (95% CI 1.3–64.5), and 27.9 (95% CI 2.7–289), respectively. None of these factors were correlated with calendar year.

Bacteremia

The incidence of bacteremia did not appear to change over time ($\chi^2 = 0.56$, $p = 0.45$). Only protective factors against bacteremia were identified: attending a school (aOR 0.15, 95% CI 0.0–0.7) and trauma or wound (aOR 0.4, 95% CI 0.2–0.9).

Death Due to IGASI

The death ratio from IGASI was 15% (42 deaths among 306 cases). The highest proportion of known deaths was among patients with pneumonia (38%, 12 deaths among 32 pneumonia cases), followed by STSS (35%, 10 among

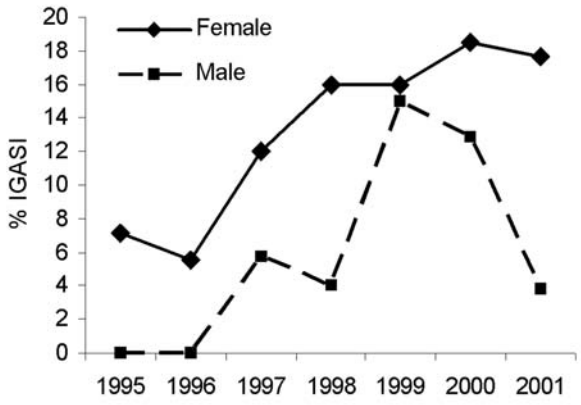

Figure 2. Pneumonia as a proportion of invasive group A streptococcal infections (IGASI) by gender, Montreal, Canada, 1995–2001.

Table. Adjusted odds ratio (OR) for factors associated with death attributable to invasive group A streptococcal infections, Montreal, Canada, 1995–2002

Variable	OR (95% CI)*
Age (y)	1.04 (1.0–1.1)†
Underlying medical conditions	
No cancer	Referent
Cancer	4.14 (1.6–10.5)
Primary site of infection	
Not cellulitis	Referent
Cellulitis	0.38 (0.1–1.0)
Not pneumonia	Referent
Pneumonia	3.62 (1.4–9.0)
Living environment	
Not working or living in hospital	Referent
Working or living in hospital	3.71 (1.0–13.6)
M serotypes	
Not M2	Referent
M2	10.69 (0.5–220)

*CI, confidence interval.
†Increase in risk per increase in year of age.

29), bacteremia (17%, 14 among 84), and soft-tissue infections (10%, 11 among 112). Within soft-tissue infections, necrotizing fasciitis had the highest risk for death among all age groups (16%, 5 deaths among 31 cases) followed by cellulitis and erysipelas (8%, 6 among 76). For myositis, among the 6 cases identified during a 7-year period, no deaths were recorded. No secular trends for death ratios were seen for any of the clinical manifestations of IGASI. Among those who died of IGASI, the most common serotypes were M1 (34%) and T1 (30%); however, neither was significantly associated with death (unadjusted OR for M1: 1.86, 95% CI 0.9–4.0; T1: 1.91, 95% CI 0.9–4.1). Predictors for death, after adjustment, are presented in the Table.

Discussion

When the results of our study are examined, several methodologic considerations must be taken into account. First, given that the administration of questionnaires for this study was not standardized, nondifferential misclassification could explain why certain factors in this study were not identified as potential markers for clinical manifestation outcomes.

An additional limitation of our study was the low statistical power. For some measures of association, the probability of detecting a true association was estimated to be as low as 3%. As a result, while this study can identify potential predictors, it cannot exclude them.

Additionally, given that this study was to a certain extent hypothesis-generating, some of the predictors found in this study (particularly those with weak associations) may have occurred by chance. Considering that an α level of 0.05 was used when testing $\approx$200 associations, at least 10 significant factors would be expected to be identified by chance. In our study, we identified 25 factors to be significantly associated with specific IGASI manifestations.

IGASI and STSS may be increasing in both incidence and severity (4). In particular, increasing trends in the IGASI incidence in the United States have been recorded in several hospital-based studies (29). Furthermore, past European studies noted a general increase in the incidence, although little evidence shows a trend occurring in the United States (14,30–32). While we documented a trend in the annual incidence of IGASI in Montreal during the first 3 years of our study, the incidence stabilized from 1997 onwards, which suggests that an initial rise in incidence might be attributable to underreporting immediately after IGASI became a notifiable disease. During the 7 years of our study, mortality did not appear to significantly change. Additionally, we could not identify any significant trends in the incidence and mortality of STSS. We did, however, ascertain that pneumonia attributable to IGASI significantly increased during 6 of the 7 years of our study. This finding was particularly evident among women. Our findings agree with those of a study in Ontario, which identified an increasing trend for pneumonia attributable to GAS from 1992 to 1999 (33).

To the best of our knowledge, no research has been published on transmission rates for the different clinical manifestations of IGASI. However, the primary mode of person-to-person transmission of *S. pyogenes* is through respiratory droplets (5,6). Additionally, *S. pyogenes* that causes severe disease in one patient is more likely to cause severe disease in subsequent patients (6). Considering these previous study findings, one could hypothesize that secondary contacts of patients with respiratory manifestations might be more likely to acquire an infection leading to severe disease, compared to contacts of patients with other IGASI manifestations.

Even though IGASI is a reportable disease, our results for pneumonia may be an underestimate of the true values. Given that <1% of community-acquired pneumonia is attributed to *S. pyogenes* (34), pneumonia caused by this bacterium may have been ascribed to other causes and hence not reported. Our findings are further complicated by difficulties in defining pneumonia (33). No standard clinical definition distinguishes IGAS pneumonia from respiratory distress caused by STSS. Although both clinical manifestations might differ with regard to pathophysiology, given that prophylaxis is required for secondary contacts of either manifestation in Quebec, difficulties in distinguishing between these manifestations will probably not affect the public health implications of our findings.

With regard to the generalizability of our results, when comparing our findings with previously published studies, we did not detect any geographic differences in the incidence of IGASI (17). Our data showed that the yearly inci-

dence of IGASI in Montreal (1.0–3.3 per 100,000) was similar to the incidence of IGASI in British Columbia (20), Ontario (16), Israel (35), Sweden (19,22), and the United States (14,18). Furthermore, death rates from IGASI in Montreal were comparable to death rates calculated for British Colombia (20) and Sweden (19,22). Only Arizona appeared to have a higher death rate due to IGASI, at 20% (3). This difference might be attributable to the elevated prevalence of diabetes (a risk factor for IGASI) in the Arizona community studied (3).

Along with these descriptive findings, we identified several factors associated with clinical manifestations of IGAS and associated death. Having varicella before IGASI increased the risk of developing a soft-tissue infection 6 times and the risk of dying 5 times. Although we could not identify any literature linking varicella infection with soft-tissue infections, given that soft-tissue infections are the predominant clinical manifestation of IGASI, our findings support previous research that suggests that varicella might be an important risk factor for developing IGASI (16,36).

Soft-tissue infections were almost twice as likely to develop in persons using drugs. This association could be attributable to injection drug use; however, it remains even after controlling for trauma. One explanation for this unexpected finding could be nondifferential misclassification. A subanalysis of drug use showed that 23% of patients indicated a trauma or wound. However, we were unable to determine the reliability of reporting. While a patient could have affirmatively answered to drug use, a wound inflicted by intravenous drug use may not have been considered sufficiently severe to indicate a trauma or wound.

Our descriptive analysis appears to support previous research findings that those <1 year of age and those ≥60 years of age have the highest incidence of IGASI (3,21–23). However, previous studies also suggest that children might have a lower incidence of STSS and be at a decreased risk of dying of IGASI (21). This research includes a study that identified a nonsignificant 5-fold rise in risk for death per year increase in age. In contrast, our study showed a 2%–4% increase.

Our study finding that M1 and M3 accounted for >30% of all isolates tested for M surface proteins was consistent with previous studies that reported these 2 M serotypes as the most common for IGASI (3,15,16,23,24). This finding is also consistent with the choice of serotypes to include in streptococcal vaccines being evaluated at the moment. The hexavalent vaccine (37) is composed of serotypes 1, 3, 5, 6, 19, and 24; these types represent 38% of isolates in our study. The types in the 26-valent vaccine (38) represent 70% of our isolates. This number includes M1 with 22%; M3 with 12%; M28 with 9%; M12 with 8%; M6 with 4%; M22 and M11 each with 3%; M89 with 2%; and M75, M2, M77, M43, M5, M76, and M33 each with 1%.

Furthermore, our study confirmed univariate model findings from a study by O'Brien et al. that found M3 to be significantly associated with STSS (14). However, this association did not remain in our multivariate analysis. Our findings would thus appear to concur with those of a another (case-control) study that found while M1 and M3 may be significant risk factors for IGASI, once a person is infected, environmental and host factors might have a role in determining the type of invasive disease manifestations (8,9). This finding could explain why IGASI may develop in patients infected with the same strain of GAS but have different clinical manifestations of the disease (e.g., STSS versus pneumonia) (9). Future epidemiologic studies of risk factors for clinical manifestations of IGASI might be designed to look at risk factors separately in patients identified with M1 and M3 serotypes. By doing so, nondifferential misclassification might be minimized and risk factors with weaker associations might be more easily identified.

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Ms. Hollm-Delgado is a doctoral student in public health at the Université de Montréal. This paper originates from research she completed at the Direction de Santé Publique (Montreal, Canada) while earning her Masters degree in epidemiology from McGill University. Her current research interests include respiratory infections and antimicrobial resistance.

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Malassezia pachydermatis Carriage in Dog Owners

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Yeasts of the genus *Malassezia* serve as both commensal microorganisms and pathogens on the skin of humans and domestic animals. Although rare, cases of life-threatening fungemia in people have been attributed to *Malassezia pachydermatis*, for which dogs are a natural host. Zoonotic transfer has been documented from dogs to immunocompromised patients by healthcare workers who own dogs. We investigated the role of pet dogs as risk factors for mechanical carriage of *M. pachydermatis* on human hands. Dogs and their owners were sampled as pairs, by fungal culture and nested polymerase chain reaction (PCR). Although fungal culture was not a reliable means by which to detect carriage of the yeast on human hands, PCR identified *M. pachydermatis* on most (≈93%) human participants. Human carriage of ubiquitous opportunistic pathogens such as *M. pachydermatis* underscores the importance of good hand hygiene by healthcare professionals.

Yeasts of the genus *Malassezia*, part of the normal cutaneous microflora of mammals, can cause life-threatening fungemia and other nosocomial infections in immunocompromised humans, especially in preterm neonates (1–3). While disease in humans is most commonly caused by *Malassezia furfur*, a commensal of human skin (4), it has also resulted from *M. pachydermatis*, for which dogs are a natural host (5–8). In some cases, the sources of human infections have been traced to pet dogs owned by healthcare workers (9).

In normal dogs with healthy skin, *M. pachydermatis* colonizes the stratum corneum in very low numbers (10). In dogs with allergic skin disease, however, the numbers of *M. pachydermatis* may increase dramatically on the skin and within the ear canals (11–13). The potential for human exposure to the organism is therefore quite great. While no evidence has shown that dogs represent an overt health concern to immunocompromised humans, the increasing incidence of immune suppression in humans worldwide suggests that a survey of the zoonotic potential of this organism is relevant to modern hospital hygiene practices.

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We hypothesized that mechanical transfer of *M. pachydermatis* from the inflamed skin of dogs with *M. pachydermatis* infection to the healthy skin of humans occurs commonly. We also hypothesized that atopic dermatitis of dogs, which is a widely documented risk factor for *M. pachydermatis* infection, would be a risk factor for human carriage. The purpose of this study was to evaluate the prevalence of *M. pachydermatis* in dogs and their owners as determined by microbiologic culture and polymerase chain reaction (PCR). The ultimate goal was to assess whether pet owners could be reservoirs for mechanical transfer of the organism.

Materials and Methods

Study Population

Approvals for privately owned animal use and sampling of human participants were obtained from the University of Pennsylvania's Institutional Animal Care and Use Committee and the biomedical institutional review board, respectively, and informed consent was obtained from participants.

Dogs referred to the Dermatology and Allergy Service of the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania (VHUP) for evaluation of allergic skin or ear canal disease were screened for secondary *M. pachydermatis* overgrowth (i.e., infection, commonly referred to as malassezia dermatitis or malassezia otitis) by using the tape strip and ear swab methods described below. Dogs with positive cytologic results and their human companions were recruited for the disease group.

A control group of healthy dogs and their human companions were recruited from the faculty, staff, and students at the VHUP. Samples were taken from dogs with normal skin and ear canals (defined as no episodes of skin disease in the preceding calendar year and no evidence of inflammation at the time of sampling) and their human companions by using the same techniques as for the disease group.

Cytology

The cytologic collection technique used for skin was the tape strip method (14). A piece of clear cellophane tape (5 cm x 2 cm) was applied to the surface of the skin 2 times in succession, removed, stained with a modified Wright's stain (Diff Quik, Dade Behring, Deerfield, IL), and applied to a glass slide for microscopic analysis. From dogs with atopic dermatitis, inflamed skin, which was typically alopecic from self-trauma due to pruritus, was sampled. One or more of the following regions were sampled from each dog: axilla, groin, chin, ventral neck fold, paronychium, and interdigital spaces (dorsal or plantar) according to clinical signs. From dogs with ear canal infections, cotton-tipped swabs of ear canal exudates were collected and streaked onto glass slides, which were then heat fixed and stained.

All slides were examined at 1,000x (high power under oil immersion) magnification. This technique allows for microscopic visualization of any microorganisms that reside on the surface of the skin or within the ear canal cerumen. When a minimum number of yeast cells per oil immersion field (oif) was exceeded (>1 yeast/oif on the skin, >5 yeast/oif in ear canal exudates) (10,15,16), excessive colonization by the organism (i.e., infection) was diagnosed, and these dogs were assigned to the disease group.

Microbiologic Analysis

For affected dogs, a tape strip was used to sample a positive skin site (an area adjacent to a site positive for yeast), and sterile cotton-tipped swabs were used to sample ear exudates. In healthy control dogs, only the chin and mucocutaneous junction of the lower lip were sampled, since this area is commonly colonized by *M. pachydermatis* (10). In the human companions, a single tape strip was used to sample the palms of both hands. To participate, each participant must have abstained from handwashing for at least 1 hour before sampling and must have handled the dog within that period. Veterinary personnel participating in the healthy control group were sampled at least 48 hours after last contact with a veterinary hospital patient.

Tape strips from each pair of participants were placed over drops of sterile olive oil, adjacent to one another, on the surface of a Sabouraud's dextrose agar plate. The agar was fortified with a drop of olive oil (source of medium- to long-chain fatty acids) to enhance growth of *Malassezia* spp., which are lipophilic (10). In cases in which canine ear exudate was sampled rather than skin, the swab was rolled across the surface of the agar incorporating a drop of sterile olive oil.

Plates were incubated at 32°C for up to 7 days. Any fungal colonies isolated were harvested from the tape strips with sterile cotton-tipped swabs and identified cytologically to be yeast by morphologic characteristics. Samples without yeast colonies were discarded. Yeast colonies were then stored at –80°C for future identification of species by polymerase chain reaction (PCR).

PCR

Samples for PCR were obtained from all dogs and their human companions. For dogs with malassezia dermatitis or otitis, a sterile cotton-tipped swab moistened with sterile saline was used to rub an affected area. For healthy control dogs, the chin and mucocutaneous junction of the lower lip was sampled. For human hands, a sterile gauze pad moistened with sterile saline was used to vigorously rub the hands (palms, fingers, and interdigital webbing) (17). Samples were stored in sterile saline at –80°C until used for PCR analysis.

DNA was extracted by using a MasterPure Yeast DNA Purification Kit (Epicentre Technologies, Madison, WI) with the following modifications. The cotton tipped swabs were stored in 1 mL sterile saline. The swabs were brought to room temperature and vortexed briefly. A 200-µL aliquot of saline was then removed from the cryotube and transferred to a sterile 1.5-mL centrifuge tube for DNA extraction. Gauze pads were stored in 10 mL of sterile saline. The pads were also brought to room temperature and were then agitated manually. A 1-mL aliquot was aseptically removed from the bag and transferred to a sterile 1.5-mL centrifuge tube. The tubes were centrifuged for 2 min at 13,000 rpm to pellet all cells, and DNA was extracted as described by the manufacturer.

Species characterization of malassezia DNA was performed by using a nested PCR assay developed by Sugita and colleagues (18). Briefly, organisms are identified with species-specific primers derived from the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene. After amplification of the ITS region, a small aliquot of the reactant is used in a second PCR to identify the *Malassezia* species. The protocol devised by Sugita and colleagues can identify 7 of the currently recognized *Malassezia* species, many of which have been isolated from canine skin. The sensitivity of the assay has been determined by Sugita and colleagues as 1 fg of DNA. As we were specifically interested only in *M. pachydermatis* for the purposes of this study, DNA samples were amplified with *M. pachydermatis*-specific primers. DNA from *M. pachydermatis* ATCC strain 14522 was prepared by American Type Culture Collection and used as a positive control in all reactions.

Statistical Analysis

To determine differences between culture and PCR in detecting *M. pachydermatis* on humans and dogs, the McNemar test was used. Where applicable, odds ratios

(OR) and 95% confidence intervals (CI) were calculated. To determine if the owners of dogs with malassezia dermatitis or otitis were more likely to harbor the yeast than owners of normal dogs, the Fisher exact test was used. Additionally, to assess concordance of culture and PCR results between owner and dog pairs, for both affected and normal groups, the McNemar test was performed. All analyses were performed by using statistical software (StatXact, Version 6, Cytel Software Corp., Cambridge, MA). A $p < 0.05$ was considered statistically significant.

Results

Fifty healthy dogs and 75 atopic dogs with malassezia dermatitis or otitis and their respective human companions made up the control and affected groups, respectively. Of the control group, 5 (10%) of 50 canine samples were positive for *M. pachydermatis* growth on lipid-enriched Sabouraud's dextrose agar, and 3 (6%) of 50 human samples were positive (Figure 1). No differences in rates of isolation were seen ($p = 0.6$).

Of the affected group, 61 (81.3%) of 75 canine samples were positive for *M. pachydermatis*, while 4 samples were overgrown with saprophytic molds before yeast colonies had grown, and 10 were negative. Of the human samples from this group, 29 (38.7%) of 75 were positive, while 5 were overgrown with saprophytic molds, and 41 were negative (Figure 1). For the 70 canine-human pairs with complete culture results, the dogs were more likely to have a positive result than their owners ($p < 0.0001$). Of the 61 dogs with positive cultures, only 49% had a concordantly positive owner (data for individual pairs not shown). However, all positive owners had dogs that were also positive. When comparing detection of *M. pachydermatis* between owners of normal dogs and owners of affected dogs by culture, the latter were 11.1 times more likely to be positive (95% CI 3.0–59.9, $p < 0.0001$, Figure 2).

PCR

Of the control group, 43 (86%) of 50 canine samples and 46 (92%) of 50 human samples were positive for *M. pachydermatis* (Figure 1). All participants (canine or human) with positive culture results were also positive by PCR; however, 38 dogs and 45 humans with negative cultures were positive by PCR. No difference was seen in the rate of detection ($p = 0.3$) between owner and dog.

Of the affected group, 73 (97.3%) of 75 canine samples and 70 (93.3%) of 75 human samples were positive by PCR for *M. pachydermatis* (Figure 1). Sixty-eight pairs had concordant positive results, and no negative pairs were found. No differences in rates of detection between the dogs and their owners were seen ($p = 0.45$). When comparing detection of *M. pachydermatis* between owners of affected dogs and owners of normal dogs by PCR, no differences ($p = 1.0$, 93% vs. 92%, respectively) were seen between groups (Figure 2).

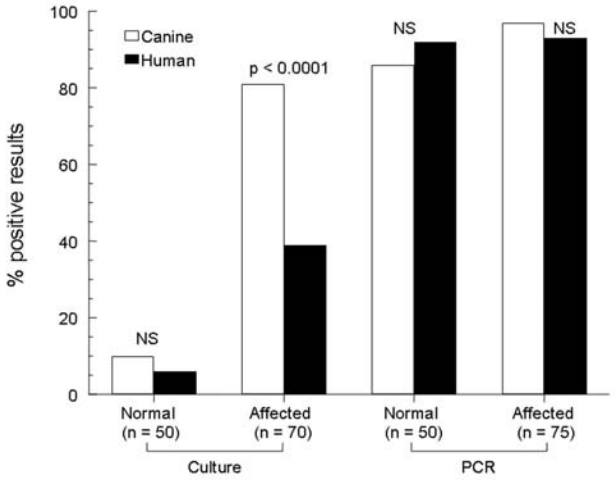

Figure 1. Rates of detection of *Malassezia pachydermatis* on canine and human skin by 2 laboratory techniques. A normal group of dogs and a group known to harbor *M. pachydermatis* infection, paired with their respective owners, are represented. NS, nonsignificant; PCR, polymerase chain reaction.

Microbiology versus PCR

When comparing PCR to culture, regardless of participant species or disease group, PCR was more likely to detect *M. pachydermatis* than culture. For dogs, PCR was 24 times more likely to be positive compared to culture (OR = 24, 95% CI 5.9–98.7), whereas for humans, PCR was 80 times more likely to be positive (OR = 80, 95% CI 11.1–574.9).

Discussion

Yeast organisms of the genus *Malassezia* are lipophilic fungi that occur as commensal inhabitants of the skin of mammals and birds in very low numbers (19). Ten distinct species are now recognized (20–22), and *M. pachydermatis*, *M. furfur*, *M. globosa*, and *M. sympodialis* are the best characterized with regard to clinical disease correlations (11–13,23,24). *M. pachydermatis* is part of the normal cutaneous microflora of dogs and many other mammals (19), while *M. furfur*, *M. globosa*, *M. sympodialis*, and *M. restricta* reside naturally on the skin of human beings (18,23,25). Lipophilic organisms exhibit the unique capability of using lipid as a source of carbon. All species except *M. pachydermatis* are entirely lipid dependent. While *M. pachydermatis* does not exhibit an absolute requirement for lipid, its growth is still enhanced by the addition of lipid substrates to culture media (20).

In normal dogs with healthy skin, *M. pachydermatis* can routinely be isolated by fungal culture, but proving the presence of the organism by skin surface cytology can be

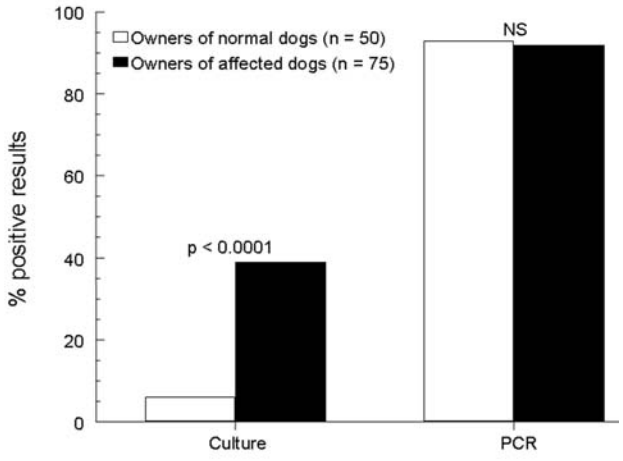

Figure 2. Comparison of rates of detection of *Malassezia pachydermatis* on the hands of dog owners by 2 laboratory techniques. NS, nonsignificant; PCR, polymerase chain reaction.

difficult (10). In dogs with allergic or seborrheic skin diseases, the homeostasis of the local cutaneous microenvironment is disrupted by inflammation and increased levels of moisture or sebum (26,27). Under these conditions, the number of *M. pachydermatis* organisms on the skin and in the ear canals may increase dramatically, making it possible to readily identify the organism with rapid cytologic screening (11–13). Atopic dermatitis may affect up to 10% of the canine population and is the most common reason that dogs are brought to our dermatology clinic for examination. It is also the most common predisposing factor for *M. pachydermatis* infections of the skin and ear canals. The potential for exposure of human beings to the organism is therefore great.

In human beings, especially preterm neonates and immunocompromised adults, *M. furfur* has been shown to cause a systemic bloodborne infection of patients receiving lipid-rich, parenteral nutritional infusions by catheter (4). Of zoonotic concern, *M. pachydermatis* has been documented to cause fungemia in similar patient populations (7,9); however, since this species is not lipid-restricted in its growth, lipid infusion is not a prerequisite for infection (9). Chang and colleagues suggested that the source of an outbreak in an intensive care nursery was pet dogs owned by nursing staff who worked in the neonatal intensive care unit (NICU). A single strain of *M. pachydermatis*, as determined by pulsed-field gel electrophoresis, was isolated from infants, the hands of a nurse, and from 3 dogs owned by other healthcare workers in the NICU. This observation suggested that *M. pachydermatis* could represent an emerging zoonotic pathogen.

A limited number of studies have investigated the prevalence of *M. pachydermatis* carriage by human beings. One report identified carriage of very low numbers of the organism on the scalp and palms of 24 (12%) of 200 normal volunteers from whom samples were collected by a washing technique for fungal culture, with subsequent speciation of yeast by biochemical methods (28). Although an association with pet ownership was speculated, such information was not collected from the participants. More recent reports have provided much lower estimates of human carriage. One report suggested that *M. pachydermatis* is present on the skin of <1% of normal persons but may be found in ≈2% of dermatitis patients (patients sampled by a swab technique for fungal culture) (23), while a second study failed to isolate the organism from either healthy human volunteers or dermatitis patients when application of transparent dressings for subsequent PCR detection was used as the sampling technique (22). To date, no single study has directly and systematically addressed the relationship between *M. pachydermatis* carriage on human skin and dog ownership. In the epidemiologic investigation of the NICU outbreak mentioned previously, a total of 53 pets (dogs, cats, and horses) were surveyed, and 12 (31%) of the 39 dogs were positive for *M. pachydermatis*, 3 of which matched the outbreak strain. However, only 1 of 9 nurses, who was not a pet owner, was positive for *M. pachydermatis* (9).

The cytologic and microbiologic results from dogs in this study mirror the literature regarding *M. pachydermatis* carriage on the skin of normal and atopic dogs (10,15,29). Ten dogs identified with malassezia infection by cytology were negative on culture. While this finding seems counterintuitive, it is not unusual in our clinical experience. The organism may have failed to grow because of suboptimal culture conditions or nonviable yeast cells. All positive cultures were confirmed to be *M. pachydermatis* by PCR, which confirms our ability to identify the species properly by cytology.

We were significantly less likely to isolate the organism from the skin of normal dogs than from atopic dogs in our study, but this bias was deliberate, since samples for culture were taken from sites that were known to be positive from rapid cytologic screening. However, when PCR was used, no significant difference was seen in detection rates, which reflects the commensal status of the organism on canine skin.

If the culture technique alone had been used, the significantly higher rate of yeast isolation from the hands of the companions of the disease group versus the control group (38.7% vs. 6%) would have supported our hypothesis that active malassezia infection of canine skin or ear canals is a risk factor for human carriage. However, when PCR was used as the detection technique, no significant difference was seen between detection rates on the hands of the 2 human groups (93.3% vs. 94%), which caused us to reject this hypothesis.

As part of the normal microflora of canine skin, *M. pachydermatis* is expected to be detectable by a technique as sensitive as PCR, even from sample sites that do not yield colony growth on culture. With our handwashing and canine skin and ear canal swabbing techniques, a larger surface area was sampled, especially on human hands, and the PCR was presumably able to detect low cell numbers within a large sample aliquot. The culture technique we used for human hands appears to be inadequate for screening purposes; however, we do not know the number or density of viable yeast cells on human hands that may be required to nosocomially spread infection in a clinical setting.

Since reports of *M. pachydermatis*–associated septicemia in humans are relatively scarce, our conclusion is that mechanical carriage of the organism is of low risk to public health. Dogs are commonly used for their therapeutic benefits in clinical settings, such as cancer therapy support groups for children and the elderly, and in psychiatric care facilities. The benefits of canine interaction have been documented (30). Advice to pet owners is available at http://www.cdc.gov/healthypets.

In intensive care units, where nosocomial infections are especially problematic, good handwashing practices among healthcare workers are imperative. Unfortunately, little is known about handwashing agents and techniques (e.g., contact time) that will effectively eliminate carriage of malassezia yeast from human hands, and disparate evidence is presented in the literature. In 1 report, improved handwashing practices seemed to eliminate an endemic problem with *M. pachydermatis* infections in a NICU (9), while in another, elimination of *M. furfur* from the surfaces of equipment was not achieved with routine hygienic measures (7).

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Anti–SARS-CoV Immunoglobulin G in Healthcare Workers, Guangzhou, China

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To determine the prevalence of inapparent infection with severe acute respiratory syndrome (SARS) among healthcare workers, we performed a serosurvey to test for immunoglobulin (Ig) G antibodies to the SARS coronavirus (SARS-CoV) among 1,147 healthcare workers in 3 hospitals that admitted SARS patients in mid-May 2003. Among them were 90 healthcare workers with SARS. As a reference group, 709 healthcare workers who worked in 2 hospitals that never admitted any SARS patients were similarly tested. The seroprevalence rate was 88.9% (80/90) for healthcare workers with SARS and 1.4% (15/1,057) for healthcare workers who were apparently healthy. The seroprevalence in the reference group was 0.4% (3/709). These findings suggest that inapparent infection is uncommon. Low level of immunity among unaffected healthcare workers reinforces the need for adequate personal protection and other infection control measures in hospitals to prevent future epidemics.

On January 2, 2003, a patient was admitted to the Traditional Medicine Hospital, Guangdong Province, with fever, cough, decreased leukocyte count, and abnormal chest radiographs. Shortly after the patient was admitted, 7 healthcare workers who cared for him became ill with similar symptoms. This index patient was retrospectively confirmed to be the first patient with severe acute respiratory syndrome (SARS) in Guangzhou (1). Subsequently, outbreaks of SARS occurred in several hospitals (2), and the disease rapidly spread to the Guangzhou community (1). In mid-February, the incidence of SARS reached a peak and gradually declined thereafter. When the last case was reported on May 9, 2003, a total of 1,284 probable SARS cases had been reported in Guangzhou (3).

In most cases, the disease was spread through close contact with an infected person (4). A high incidence of SARS was observed among healthcare workers, especially during the early stages of the SARS epidemic (5–8); healthcare workers were likely exposured to the SARS coronavirus (SARS-CoV) at the workplace. Also, SARS-CoV can survive for many hours on environmental surfaces (5); therefore, infection through contact with contaminated fomites is a distinct possibility, despite the absence of supportive epidemiologic evidence. A series of strict preventive measures, including specially designed wards to accommodate SARS patients and the use of gloves, eye protection, face masks, foot covers, and protective gowns, were adopted to control the spread of SARS to healthcare workers. Whether inapparent infections existed with this new epidemic was unclear. In this study, we explored the seroprevalence of antibodies to SARS-CoV in healthcare workers who had cared for SARS patients but did not have SARS and those working in hospitals with no SARS patients. We also determined the seroprevalence of antibodies to SARS-CoV in convalescent healthcare workers in whom SARS was diagnosed.

Methods

Study Populations

In mid-May 2003, ≈4 months after the initial SARS outbreak in Guangzhou, a cross-sectional survey was carried out on healthcare workers who worked with SARS patients in the First, Second, and Third Affiliated Hospitals of the Sun Yat-Sen University, Guangzhou. Outbreaks of SARS

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had occurred among healthcare workers in the Second and Third Affiliated Hospitals but not the First Affiliated Hospital. Of the 1,394 healthcare workers who cared for SARS patients in these 3 hospitals, 1,147 (82.3%) were surveyed. Those surveyed included healthcare workers from all departments that cared for SARS patients. All healthcare workers on duty were surveyed; only those who were off-duty during the survey were excluded.

For comparison, 709 healthcare workers were sampled from 2 hospitals with no SARS patients: the Affiliated Cancer Hospital, Guangzhou, where no SARS patients were admitted, and the Fifth Affiliated Hospital, Zhuhai, where no SARS cases were reported in the community. A total of 1,856 healthcare workers were surveyed.

Serum Collection and Interview

For each healthcare worker, 10 mL of peripheral venous blood was collected; the serum was separated and stored at –70°C. A standardized interview with a structured questionnaire was used to obtain information on sociodemographic characteristics and the history of SARS according to a case definition of SARS by the Ministry of Health, China (9).

A probable case-patient was defined as one who had close contact with a patient, was a member of an infected cluster, or infected other persons; had symptoms and signs of febrile respiratory symptoms, and had changes on chest radiograph. A patient was also considered to have a probable case if he or she visited or lived in an area where SARS was reported with secondary transmission within 2 weeks of illness onset, had symptoms and signs of febrile respiratory illness, had normal or decreased leukocyte count, and had changes on chest radiograph.

A suspected case-patient was defined as one who had close contact with a patient; was a member of an infected cluster, or infected other persons; and had symptoms and signs of febrile respiratory illness; and had normal or decreased leukocyte count. A patient was also considered to have a suspected case if he or she visited or lived in an area where SARS was reported with secondary transmission within 2 weeks of illness onset, had symptoms and signs of febrile respiratory illness, and had changes on chest radiograph. If a patient had no epidemiologic link to SARS but he or she had symptoms and signs of febrile respiratory illness, normal or decreased leukocyte count, and changes on chest radiograph, he or she was still considered to have a suspected case.

A person who had visited or lived in cities where SARS cases were reported with secondary transmission was placed under medical observation if he or she had symptoms and signs of febrile respiratory illness and had a normal or decreased leukocyte count.

Detecting Serum IgG against SARS-CoV

Immunoglobulin (Ig) G against SARS-CoV were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Beijing BGI-GBI Biotech Co., Ltd.) (10). This ELISA has a sensitivity of 89.9% and a specificity of 99.0% (11).

Statistical Analysis

Means and standard deviations were used to describe continuous variables. Proportions and prevalence rates were used to describe categorical variables. Chi-square tests were performed to test the association between SARS-CoV IgG seropositivity and the sociodemographic characteristics of the healthcare workers.

Results

Sociodemographic Characteristics

The mean age of the healthcare workers investigated was 30.78 years (SD 9.1 years); 71.6% were women. Details of their sociodemographic characteristics are presented in Table 1. General information related to SARS in the 5 hospitals is shown in Table 2.

Prevalence of IgG against SARS-CoV among Healthcare Workers

Among healthcare workers working with SARS patients, the prevalence of IgG against SARS-CoV was 88.9% (80/90) for those who contracted SARS and 1.4% (15/1,057) for those who did not (Table 3). By contrast, the seroprevalence was 0.5% (2/371) for healthcare workers working in the non-SARS hospital in Guangzhou and 0.3% (1/338) for healthcare workers in the hospital in SARS-free Zhuhai. The overall seroprevalence in this reference group of healthcare workers was 0.4% (3/709).

We also compared the prevalence of anti-SARS IgG in healthcare workers for each sociodemographic characteristic. We analyzed the data on healthcare workers who worked with SARS patients in the 3 hospitals. These results are presented in Table 4.

The results showed that the seroprevalence of anti-SARS IgG in healthcare workers $\leq$26 years of age was significantly higher than in those >26 years of age ($p < 0.05$). Women had a higher seroprevalence than men ($p < 0.01$). Those with a senior school or technical secondary school education had a higher seroprevalence than those with tertiary education. Seroprevalence was highest among healthcare workers working in departments of respiratory diseases, followed by those in departments of infectious diseases, then in intensive care units; the prevalence was <10% in all remaining departments ($p < 0.001$). No laboratory personnel had IgG against SARS. When healthcare workers were compared to those with senior positions,

Table 1. Sociodemographic characteristics of 1,856 healthcare workers

Sociodemographic characteristic	No. participants (%)
Hospital*	
First Affiliated Hospital	389 (21.0)
Second Affiliated Hospital	361 (19.5)
Third Affiliated Hospital	397 (21.4)
Affiliated Cancer Hospital	371 (20.0)
Fifth Affiliated Hospital	338 (18.1)
Age (y)	
<26	676 (36.5)
26–30	404 (21.8)
31–35	294 (15.8)
36–40	221 (11.9)
>40	261(13.0)
Sex	
Male	528 (28.4)
Female	1,328 (71.6)
Educational level	
Senior school	136 (7.3)
Technical secondary school	718 (38.7)
Junior college	367 (19.8)
University	333 (17.9)
MD/PhD	302 (16.3)
Department	
SARS ward	413 (22.3)
Emergency department/fever clinic	196 (10.6)
Infectious disease department	125 (6.7)
Respiratory diseases department	101 (5.4)
ICU	61 (3.3)
X-ray	74 (4.0)
Laboratory	66 (3.6)
Others†	820 (44.2)
Job title‡	
Doctor	567 (30.7)
Nurse	892 (48.3)
Health attendant	101 (5.5)
Technician in laboratory	74 (4.0)
Others	213 (11.5)

*All 5 hospitals are teaching hospitals of the Sun Yat-Sen University.
†Departments of internal medicine, surgery, and logistic service.
‡Missing for 9 healthcare workers.

those at a more junior level had a higher risk for infection by SARS-CoV ($p < 0.01$).

Discussion

Anti-SARS IgG can be detected 1–2 weeks after the onset of symptoms. Almost all SARS patients in the convalescent stage had anti-SARS IgG in their serum samples (11–14). In our study, all healthcare workers with SARS were in the convalescent stage, and SARS-CoV infected most while they were caring for the same index patient who was subsequently identified as a superspreader (15). The finding of a 100% seroprevalence of SARS IgG among 17 SARS-infected healthcare workers in the Third Affiliated Hospital was identical to the results by Li et al. (12), who tested SARS IgG at different stages among the same group of SARS-infected healthcare workers by using the same ELISA. By contrast, 63 (86.3%) of 73 healthcare workers with SARS in the Second Affiliated Hospital were seropositive for SARS IgG. Some of these healthcare workers might have been misdiagnosed, as the clinical diagnosis of SARS was not specific (16). Even allowing for this possibility, the overall high seropositivity rate of 88.9% among SARS patients is similar to findings by Wang et al. in Beijing, who used the same test (11); Chow et al. in Singapore, who used a different EIA (17); and Chan et al. in Hong Kong, who used an immunofluorescence assay (18). All of these studies indicate that serum IgG antibodies to SARS-CoV at the convalescent stage of the illness can be useful in confirming the disease.

The low seroprevalence o SARS IgG (0.3%–2.8%) in healthy healthcare workers with different levels of exposure to SARS patients is similar to that reported by Wang et al. (11). However, a similar study by Chow et al. in Singapore did not find any serologic evidence of subclinical infection among a population with a high likelihood of exposure to the virus. Our ELISA was 99% specific (11). This specificity could have produced a few false-positive results, which accounts for a low seropositive rate of 0.4% (3/709) among healthcare workers in the reference group, who had no exposure to SARS in their hospitals. One healthcare worker in Zhuhai, where no SARS occurred, was seropositive, which could be a false-positive result. However, we could not exclude the possibility of inapparent infection among healthcare workers in the 4 hospitals in Guangzhou. Another possibility is cross-reaction with other human coronaviruses. A more specific test, such as the indirect immunofluorescence test, should clarify this uncertainty (18).

The low seroprevalence of SARS IgG, at 1.4% (15/1,057) among apparently healthy frontline healthcare workers in all 3 SARS hospitals, suggests that inapparent infection is relatively uncommon. We did not, however, ascertain whether the healthcare workers with a positive antibody test result were carriers of SARS-CoV. Overall, the low seropositivity among healthy healthcare workers suggests that the level of immunity to SARS in the general population in Guangzhou was too low to constitute an effective immune barrier against the spread of SARS. Should the disease recur there, every effort should be made to protect healthcare workers and the general public from being infected by SARS patients.

The First Affiliated Hospital only admitted SARS patients after outbreaks had occurred among healthcare workers in the Second and Third Affiliated Hospitals. After these outbreaks, a series of protective measures were adopted in all 3 hospitals. Sufficient preparation, such as personal protection and designated SARS wards, is important to avert hospital outbreaks. The low seroprevalence of

Table 2. Severe acute respiratory syndrome (SARS)-related information in the 5 affiliated hospitals

SARS-related information	First Affiliated Hospital	Second Affiliated Hospital	Third Affiliated Hospital	Affiliated Cancer Hospital	Fifth Affiliated Hospital
In SARS-epidemic area?	Yes	Yes	Yes	Yes	No
No. healthcare workers exposed to SARS*	548	421	425	0	0
No. healthcare workers surveyed	389	361	397	371	338
No. days when SARS patients were in the hospital	120	110	102	0	0
No. probable SARS patients cared for	122	150	31	0	0
No. suspected SARS patients cared for	102	50	30	3	0
No. SARS patients who required tracheal intubation	10	0	1	0	0
No. SARS patients who required tracheotomy	0	1	2	0	0
Cared for the index patient?†	No	Yes	Yes	0	0
No. healthcare workers who had SARS‡ in SARS wards	0	80	20	0	0
No. healthcare workers who had from SARS in non-SARS wards	3	10	2	0	0

*This refers to healthcare workers caring for SARS patients and laboratory personnel handling specimens from SARS patients.
†The index patient was identified as a superspreader who subsequently infected >100 persons (both healthcare workers and other patients and family members in the Second Hospital and Third Hospital).
‡According to the clinical and epidemiologic case definition.

SARS IgG among healthcare workers working in the First Affiliated Hospital indicated the effectiveness of these measures. This finding is consistent with the study by Chow et al. (17).

The seroprevalence rates were significantly different among the healthcare workers who cared for SARS patients when classified by their age, sex, educational level, hospital, department, and job title. These differences could be due to the probability of exposure to the SARS index case. On January 30, 2003, the index patient was admitted to the Department of Respiratory Diseases of the Second Affiliated Hospital. On February 1, he was transferred to the Third Affiliated Hospital because of worsening dyspnea. During his stay in these 2 hospitals, where protective measures were lacking, he directly and indirectly infected 90 healthcare workers and 22 healthcare workers in the Second and Third Affiliated Hospitals, respectively. This finding accounts for the much higher seroprevalence of SARS IgG among healthcare workers in the departments of respiratory diseases and infectious diseases. Healthcare workers in SARS wards and fever clinics were fully equipped with personal protective measures (caps, gowns, multilayered cotton face masks, eye shields, gloves, and foot covers), which might explain their much lower seroprevalence. None of our laboratory healthcare workers, who performed serologic tests but not live viral tests, were seropositive, which suggests that the probability of SARS infection by handling serum samples of SARS patients was low. Of all occupations, healthcare attendants had the highest seropositive rate, which might be related to their general lower level of education and a lack of training in infection control measures. Future efforts to improve SARS containment should also address this problem among nonprofessional staff.

This study has several limitations. Even though the response rate among healthcare workers who cared for SARS patients was high (82.3%), some selection bias is inevitable. Moreover, the study was limited to 5 university hospitals, so we caution against the extrapolation of our findings to healthcare workers in other hospitals and to the general population. We have not tested serum samples from our healthcare workers against other pathogens, e.g., *Mycoplasma pneumoniae* and influenza virus, which limits our ability to exclude the nonspecific and atypical pneumonia caused by these agents (16).

In conclusion, this study shows that a high proportion of healthcare workers who have contracted SARS have IgG against SARS-CoV in their serum samples after they have fully recovered. Inapparent infection with SARS is uncommon. The low seropositivity against SARS among

Table 3. Severe acute respiratory (SARS) immunoglobulin G prevalence among healthcare workers with and without SARS in 5 affiliated hospitals

Affiliated hospital	Healthcare workers with SARS		Healthcare workers without SARS	
	No. participants	No. positive (%)	No. participants	No. positive (%)
First	0	0	389	4 (1)
Second	73	63 (86.3)	288	8 (2.8)
Third	17	17 (100)	380	3 (0.8)
Cancer	0	0	371	2 (0.5)
Fifth	0	0	338	1 (0.3)
Total/overall percentage	90	80 (88.9)	1,766	18 (1)

Table 4. Severe acute respiratory syndrome (SARS) immunoglobulin G prevalence for different sociodemographic characteristics

Sociodemographic characteristics	No. participants	No. positive for IgG	Prevalence (%)
Age (y)*			
<26	355	44	12.4
26–30	310	17	5.5
31–35	211	14	6.6
36–40	118	9	7.6
>40	141	11	7.8
Sex†			
Male	306	15	4.7
Female	743	80	9.7
Educational level*			
Senior school	112	14	12.5
Technical secondary school	401	42	10.5
Junior college	210	11	5.2
University	197	17	8.6
MD/PhD	227	11	4.8
Department‡			
SARS ward	409	13	3.2
Emergency/fever diagnoses	188	4	2.1
Infection	125	19	15.2
Respiratory	100	36	36.0
ICU	55	7	12.7
X-ray	57	2	3.5
Laboratory	66	0	0.0
Others	147	14	9.5
Job title†			
Doctor	388	24	6.2
Nurse	510	52	10.2
Healthcare attendants	91	12	13.2
Technician in laboratory	66	0	0.0
Others§	92	7	7.6

*$p < 0.05$.
†$p < 0.01$.
‡$p < 0.001$.
§Department of internal medicine, surgery and logistic service.

healthcare workers who have not been exposed to SARS patients suggests a lack of immunity in this group and in the general population, where the number of SARS cases is comparatively small.

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Norovirus and Foodborne Disease, United States, 1991–2000

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Efforts to prevent foodborne illness target bacterial pathogens, yet noroviruses (NoV) are suspected to be the most common cause of gastroenteritis. New molecular assays allow for better estimation of the role of NoV in foodborne illness. We analyzed 8,271 foodborne outbreaks reported to the Centers for Disease Control and Prevention from 1991 to 2000 and additional data from 6 states. The proportion of NoV-confirmed outbreaks increased from 1% in 1991 to 12% in 2000. However, from 1998 to 2000, 76% of NoV outbreaks were reported by only 11 states. In 2000, an estimated 50% of foodborne outbreaks in 6 states were attributable to NoV. NoV outbreaks were larger than bacterial outbreaks (median persons affected: 25 versus 15), and 10% of affected persons sought medical care; 1% were hospitalized. More widespread use of molecular assays will permit better estimates of the role of NoV illness and help direct efforts to control foodborne illness.

Foodborne infections are estimated to cause 76 million illnesses, 300,000 hospitalizations, and 5,000 deaths annually in the United States (1). Several high-profile outbreaks in the last 15 years have focused attention on the role of bacteria in severe foodborne illness (2–4) and led to serious efforts to prevent bacterial contamination of food during all levels of processing and handling—the "farm-to-fork" model. However, in more than two thirds of outbreaks of foodborne illness, no pathogen is identified (5).

Noroviruses (NoV), previously known as "Norwalk-like viruses," have long been suspected to be a frequent cause of foodborne outbreaks (6–11). Until recently, diagnosis of NoV infection relied on methods that were insensitive (electron microscopy [12]), difficult to set up (serologic testing with human reagents [13]), and available only in research settings. In 1982, epidemiologic and clinical criteria were formulated to help attribute outbreaks to NoV in the absence of a simple diagnostic test (14). Despite these criteria, the absence of any routine diagnostic assay for NoV has discouraged investigations into outbreaks of suspected viral etiology and thus limited assessment of the true impact of gastroenteritis associated with these pathogens. In 2000, for example, a survey of public health professionals in Tennessee found that only 9% cited viruses as a major cause of foodborne illness (15). Not unexpectedly, therefore, of the 2,751 foodborne outbreaks reported to the Centers for Disease Control and Prevention (CDC) from 1993 to 1997, only 9 (0.3%) were confirmed as due to NoV (5).[1]

In the early 1990s, sensitive and simpler assays were developed to detect NoV by identifying viral RNA after reverse transcription-polymerase chain reaction (RT-PCR) (16). In 1993, RT-PCR was adopted at CDC for the routine detection of NoV (17), particularly in outbreaks in which specimens test negative for common bacteria. A number of state public health laboratories subsequently adopted similar assays or began sending specimens to CDC for NoV testing. When RT-PCR was used, a NoV was identified as the etiologic agent in 93% of outbreaks of nonbacterial gastroenteritis submitted for testing to CDC from 1997 to 2000 (18). However, this selection was of specimens from outbreaks of illness characteristic of viral infection, and they usually have already tested negative for bacteria. The

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[1]Efforts in 1998 to improve outbreak reporting resulted in more outbreaks being retrospectively attributed to this period. The current figures for 1993 to 1997 are 65 (2%) of 3,257 outbreaks attributable to NoV and 67% of unknown etiology.

selection introduces bias since it does not permit an assessment of the true relative frequency of foodborne outbreaks of NoV disease. Therefore, we analyzed data from all foodborne outbreaks (irrespective of cause) reported to CDC by state health departments from 1991 through 2000 to assess how recent application of RT-PCR techniques might have improved understanding of the relative impact and role of NoV in these outbreaks in the United States.

Methods

We used 3 related datasets: 1) all foodborne outbreaks reported to CDC from 1991 through 2000 (N = 8,271), 2) a subset of these outbreaks reported from 1998 though 2000 when surveillance was enhanced and states began to use NoV diagnostics (N = 4,072), and 3) all foodborne outbreaks reported in 2000 in 6 selected states from which supplementary data on diagnostic testing were gathered (N = 600).

Foodborne Outbreak Reports, 1991–2000

Outbreaks of foodborne disease (excluding those on cruise ships) are voluntarily reported by state health departments to CDC for inclusion in the National Foodborne Outbreak Reporting System. Whether an outbreak is classified as foodborne or not is at the discretion of the state epidemiologist. Minimum data required for registering an outbreak report include the number of persons ill and the date of onset of the first case. The determination of outbreak cause is based on CDC's pathogen-specific guidelines (19). In 1998, the surveillance system was enhanced by annual data verification with states and solicitation of any unreported outbreaks.

We reviewed records of 8,271 foodborne outbreaks reported to CDC from 1991 through 2000. We also noted the year in which state laboratories set up the RT-PCR assay for NoV, and by cross-referencing with CDC laboratory logs, we determined whether an outbreak had been confirmed as attributable to NoV at a laboratory in a state or at CDC.

Foodborne Outbreak Reports, 1998–2000

This subset of foodborne outbreaks was selected for further analysis because, in addition to enhanced surveillance in this period, state public health laboratories had begun to test routinely for NoV, and these reports therefore included most outbreaks of confirmed NoV disease. Available variables included the laboratory-confirmed cause; clinical data (symptoms, median incubation period, median duration of illness); food vehicle; whether a foodhandler was implicated; and the number of persons exposed, ill, requiring medical attention, or hospitalized.

From January 1998 through December 2000, a total of 4,072 outbreaks were reported to CDC. We excluded 30 outbreaks involving multiple states and 10 occurring in the U.S. territories and further analyzed the remaining 4,032 outbreak reports.

To assess the differences between states in outbreak reporting and laboratory testing, each state was classified into 1 of 5 groups on the basis of the number of NoV-confirmed outbreaks that a state reported in 1998 to 2000 (≥20, 10–19, 5–9, 1–4, or none reported). The proportion of reported outbreaks with a known cause and the proportion confirmed to be due to NoV were calculated for each group. The number of reported outbreaks per 100,000 population per state for these 3 years was also calculated by using U.S. Census data for 2000.

To characterize the severity of illness and the settings associated with NoV outbreaks, we selected the 305 NoV-confirmed outbreaks and analyzed those with complete information on medical care (n = 112) and setting (n = 278). We calculated the proportion of persons seeking care and the proportion hospitalized by using the number of case-patients interviewed as a denominator.

To compare the epidemiologic and clinical features of outbreaks attributed to bacteria and viruses, we selected, from the 4,032 outbreaks of gastroenteritis, a subset of 1,216 reports with complete information on the number ill, duration of illness, incubation period, and the proportion of interviewed patients who reported vomiting or fever. Of these outbreaks, 136 were attributed to NoV, 173 to bacteria, and 907 to an undetermined cause. We further compared outbreak reports with information on implicated food types (n = 608) and whether or not an ill foodhandler was thought involved by the outbreak investigators (n = 760).

Data on Specimen Screening from 6 States, 2000

Data on the pathogens screened in a single outbreak are not reported to CDC; therefore, to estimate the proportion of outbreaks that would be NoV-confirmed if collected specimens were tested routinely not only for bacteria but also for NoV, we gathered additional data on the testing of stools gathered from foodborne outbreaks in 2000 from 6 states (Georgia, Minnesota, Ohio, Florida, Maryland, New York). These states were selected because they collected stools from a large number of outbreaks and had laboratory capability to test specimens for NoV.

We applied the proportion of all outbreaks tested for NoV that were NoV-positive in each state (≥1 positive specimens) to the number of outbreaks of undetermined etiology for which specimens had been gathered, had tested negative for bacteria, but had not been tested for NoV. We then added this figure to the total actual number of NoV outbreaks to estimate the proportion of all outbreaks with specimens in that state that would be attributable to NoV had specimens from all outbreaks been tested fully.

Results

Foodborne Outbreak Reports, 1991–2000

The number of foodborne outbreaks reported to CDC per year from 1991 to 2000 ranged from 411 outbreaks in 1992 to 1,414 in 2000, and increased markedly in 1998, when the reporting system was changed (Figure 1A). Of 8,271 outbreaks, 5,637 (68%) were of undetermined etiology. The number of NoV-confirmed outbreaks increased markedly from 11 outbreaks in 1996 to 164 (12% of all reported outbreaks) in 2000 (Figure 1B). This rise was initially due to laboratory confirmation of NoV by CDC, but by 2000, 100 (61%) of 164 NoV outbreaks were confirmed in state laboratories. Underreporting, however, remained an obvious problem since only 17 (34%) of 50 state public health laboratories tested for NoV, while the remaining 33 states (66%) either sent specimens to CDC for diagnosis (n = 12), or did not report any NoV outbreaks (n = 21).

Foodborne Outbreak Reports, 1998–2000

Of 4,032 outbreaks reported in this period of enhanced surveillance, only 1,146 (28%) were of determined cause and 2,886 (72%) were of undetermined etiology (Table 1). NoV-confirmed outbreaks comprised 305 (8%) of all 4,032 outbreaks or 27% of the 1,146 outbreaks with a determined cause. These 305 NoV outbreaks accounted for 13,527 (18%) of all 74,481 sick persons in all 4,032 outbreaks or 39% of 34,539 sick persons in 1,146 outbreaks of known cause.

NoV Reporting

A great disparity was observed in the reporting of NoV outbreaks. Of the 50 U.S. states and the District of Columbia, 15 (29%) reported no NoV outbreaks (Table 1 and Figure 2). Of the total of 305 NoV outbreaks, 232 (76%) were reported by 11 states, which each investigated ≥10 NoV outbreaks and accounted for 613 (53%) of all 1,146 outbreaks of determined cause.

We hypothesized that the proportion of outbreaks of determined cause reported in each state would be lowest in those states not reporting any NoV-confirmed outbreaks, but this hypothesis was not supported by the data. In fact, paradoxically, the 15 states that reported no NoV outbreaks in the study period determined a cause in 53% of all outbreaks, compared to 20%–45% in the 35 states that reported at least 1 NoV outbreak. The 11 states that reported >10 NoV outbreaks also reported, on average, more outbreaks per 100,000 population (2.3) compared with the 35 states that reported 0–10 NoV outbreaks (0.8–0.9). The number of NoV outbreaks reported by states, however, was not simply a function of total outbreaks reported; the percentage of NoV outbreaks of those outbreaks of determined etiology also increased significantly, from 0% to 57% (chi square for trend; $p > 0.001$), which suggests better outbreak investigation and testing for NoV.

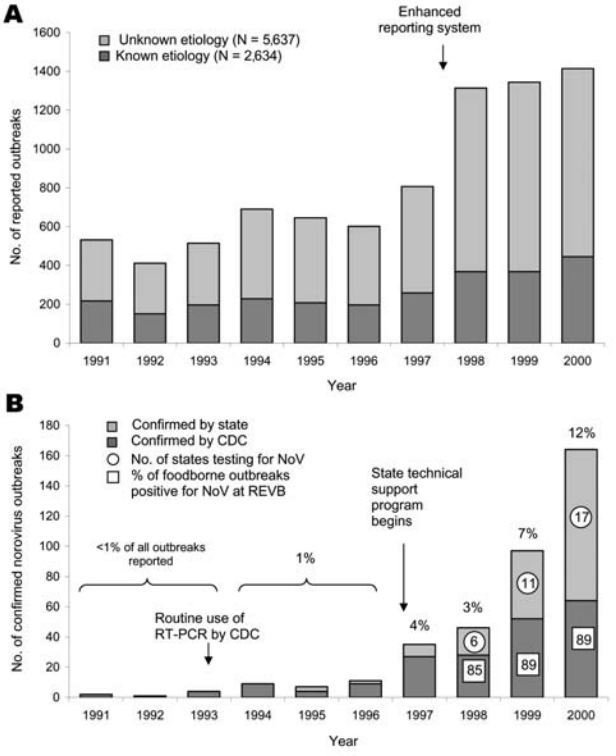

Figure 1. A) Foodborne outbreaks reported to the Centers of Disease Control and Prevention (CDC), United States, 1991–2000. B) Norovirus (NoV)-confirmed foodborne outbreaks reported to CDC, United States, 1991–-2000. REVB, Respiratory and Enteric Branch, CDC; RT-PCR, reverse transcription-polymerase chain reaction. Percentage value above bars represents proportion of all foodborne outbreaks reported to CDC that were laboratory-confirmed to be due to NoV by REVB and by some state public health laboratories.

Illness

Information on physician visits and hospitalization was complete in 112 (37%) of all 305 NoV outbreaks. Of 3,370 persons affected in these 112 outbreaks, 329 (10%) sought care from a physician, and 33 (1%) were hospitalized.

Setting

For 278 (91%) of the 305 NoV outbreaks where the site of food consumption or preparation was recorded, restaurants, caterers, or food outlets were associated with 108 (39%), private homes with 35 (13%), daycare facilities or schools with 27 (10%), workplace with 18 (6%), nursing homes or hospitals with 14 (5%), and other settings with 76 (27%).

Table 1. Foodborne outbreaks of gastroenteritis of known and unknown etiology by states grouped by number of reports of norovirus (NoV)-confirmed outbreaks, United States, 1998–2000

No. of NoV outbreaks reported by states	No. of states reporting	All reported outbreaks			NoV outbreaks reported	
		Total no. (R*)	Determined etiology (%)	Undetermined etiology (%)	No. (% of all outbreaks)	% of all outbreaks with determined etiology
>20	2	382 (2.3)	166 (43)	216 (57)	94 (25)	57
11–20	9	2,273 (2.3)	447 (20)	1,826 (80)	138 (6)	31
6–10	4	304 (0.8)	136 (45)	168 (55)	33 (11)	24
≤5	21	830 (0.9)	269 (32)	561 (68)	40 (5)	15
None	15†	243 (0.8)	128 (53)	115 (47)	0 (0)	0
Total	51	4,032 (1.4)	1,146 (28)	2,886 (72)	305 (8)	27

*R = outbreaks reported per 100,000 population, using U.S. Census data 2000.
†Includes District of Columbia.

Comparison of Bacterial and NoV Outbreaks

We compared selected epidemiologic and clinical features of NoV outbreaks (n = 136), bacterial outbreaks (n = 173), and outbreaks of unknown etiology (n = 907), where information was complete. Of the 173 bacterial outbreaks, 79 (46%) were attributed to *Salmonella* spp., 27 (16%) to *Clostridium* spp., 20 (12%) to *Staphylococcus aureus,* 19 (11%) to *Shigella* spp., 13 (8%) to *Escherichia coli*, 7 (4%) to *Bacillus cereus*, 6 (3%) to *Campylobacter* spp., and 2 (1%) to other bacterial pathogens.

NoV outbreaks were significantly larger than outbreaks of bacterial or unknown etiology (median number of cases per outbreak = 25 versus 15 and 7, respectively. Wilcoxon rank sum test: $p < 0.001$) (Table 2). Viral outbreaks had a shorter duration of illness compared to bacterial outbreaks but one similar to that of outbreaks of unknown etiology (median duration ≤48 hours = 82%, 40%, and 85% of outbreaks, respectively). Vomiting was more often a predominant symptom (reported by >50% of ill persons) in NoV outbreaks than in outbreaks of bacterial or unknown etiology ($p = 0.001$) and was reported in all 136 NoV outbreaks. Fever, however, was less often reported in outbreaks of NoV disease.

The median incubation period was significantly longer in outbreaks of NoV gastroenteritis: 85% of these outbreaks featured a median incubation period >24 hours compared with 39% in outbreaks of bacterial cause and 43% in outbreaks of unknown etiology. This finding may be explained by outbreaks caused by preformed toxins from certain bacteria (*S. aureus*, *Clostridium perfringens*, *B. cereus*), which tend to have shorter incubation periods.

NoV outbreaks were strongly associated with eating salads, sandwiches, and produce: these items were implicated in 56% of the 76 NoV outbreaks in which a food item was identified, compared with 19% of 124 bacterial outbreaks and 28% of 408 outbreaks of unknown etiology (chi-square test: $p < 0.05$) (Table 3). NoV outbreaks were significantly less often associated with meat dishes than bacterial outbreaks and outbreaks of unknown etiology (11% versus 44% and 34%, respectively: $p < 0.05$). A foodhandler was more likely to be implicated in a NoV outbreak (48% of 94 outbreaks with available data) than in either a bacterial outbreak (20% of 102 outbreaks) or an outbreak of unknown etiology (9% of 564 outbreaks) ($p < 0.001$).

Specimen Screening Data from 6 States, 2000

In the 6 states for which data on specimen testing were obtained, the percentage of outbreaks tested for NoV that were positive was 44%–100%, and the total percentage in all 6 states was 79% (Table 4). Even in these states, NoV testing was much less likely to be performed than was testing for bacteria. Of 220 outbreaks from which stool samples were collected, specimens from 85 (39%) were tested for NoV compared to 207 (94%) tested for bacteria. Specimens from 55 outbreaks (25%) tested negative for bacteria, but no further testing for viruses was performed. The overall percentage of all outbreaks with specimens that tested positive for NoV was 30%, but in 2 states that tested all specimens for NoV (Georgia and Minnesota), the average percentage was 43% (22/51) compared with 27% (45/169) in the 4 other states that did not test fully for NoV. Assuming that these 4 states had tested specimens from

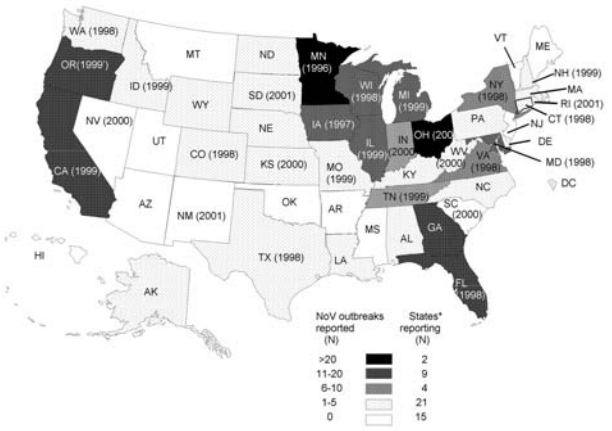

Figure 2. Norovirus-confirmed foodborne outbreaks by state, United States, 1998–2000 (N = 305). Years in parentheses indicate first year a state public health laboratory developed molecular assays for norovirus (as of December 2001). Includes District of Columbia.

Table 2. Selected epidemiologic and clinical features of foodborne outbreaks of gastroenteritis of noroviral, bacterial, and unknown cause, United States, 1998–2000*

Features†	Etiology of outbreak			p value‡
	Norovirus (N = 136) (%)	Bacterial (N = 173)(%)	Unknown (N = 907) (%)	
No. of persons ill				
≤10	22 (16)	65 (38)	544 (60)	< 0.001
>10	114 (84)	108 (62)	363 (40)	
Median no. of persons/outbreak (range)	25 persons (2–199)	15 persons (2–736)	7 persons (2–800)	0.001§
Median duration of illness (h)				
≤48	111 (82)	70 (40)	763 (85)	< 0.001¶
>48	25 (18)	103 (60)	134 (15)	
Median incubation period (h)				
≤24	21 (15)	105 (61)	517 (57)	< 0.001
25–48	82 (60)	13 (7)	266 (29)	
>48	33 (25)	55 (32)	124 (14)	
% of persons vomiting				
≤50	19 (14)	114 (68)	352 (39)	< 0.001
>50	117 (86)	59 (32)	555 (60)	
% of persons with fever				
≤50	90 (66)	100 (57)	752 (83)	< 0.001
>50	46 (34)	73 (42)	155 (17)	

*Data are no. (%), unless otherwise noted.
†No significant differences found in proportions of ill persons with diarrhea or abdominal cramping.
‡Chi-square test for unequal odds unless otherwise noted. p value refers to comparison of norovirus (NoV) outbreaks to both bacterial and unknown outbreaks separately unless otherwise noted.
§Wilcoxon rank sum test comparing median values.
¶Significant association only between NoV and bacterial outbreaks.

these outbreaks for NoV, 110 (50%) of the 220 outbreaks with specimens collected in all 6 states would have been confirmed as caused by NoV.

Discussion

The introduction of RT-PCR in the 1990s increased the percentage of all outbreaks attributable to NoV in the United States from <1% in 1991 to 12% in 2000. Nonetheless, noroviruses remain grossly underestimated as a cause of gastroenteritis outbreaks. From 1998 through 2000, most NoV outbreaks (76%) were reported from 11 states; 36 states, generally those with no PCR capability, reported either few or no outbreaks. Using data from 6 states, we estimated that if all specimens were tested for viruses, half of all foodborne outbreaks in the United States could be attributable to NoV. Even in these 6 states, bacteria were more likely to be tested for than viruses; specimens from 25% of outbreaks were negative for bacteria but not further tested. We also show that NoV outbreaks affect almost 50% more persons than in bacterial

Table 3. Role of different foods and foodhandlers in outbreaks of gastroenteritis of noroviral, bacterial, and unknown cause, United States, 1998–2000*

	Cause of outbreak			p value†
	Norovirus no. (%)	Bacteria no. (%)	Unknown no. (%)	
Total outbreaks with data on implicated food	76	124	408	
Implicated food				
Salad	20 (26)	20 (16)	73 (18)	NS
Sandwich	10 (13)	0	24 (6)	< 0.05‡
Produce/fruit	13 (17)	4 (3)	15 (4)	< 0.001
Meat dish	8 (11)	50 (40)	139 (34)	< 0.001
Fish dish	4 (5)	9 (7)	19 (5)	NS
Bakery product	5 (7)	2 (2)	15 (4)	NS
Oysters	2 (3)	2 (2)	12 (3)	NS
Other various§	14 (18)	37 (30)	111 (27)	ND
Total outbreaks with data on investigation of foodhandler	94	102	564	
Foodhandler implicated				
Yes	45 (48)	20 (20)	51 (9)	< 0.001
No	49 (52)	82 (80)	513 (91)	

* ND, not done; NS, not significant.
†Chi-square test: p value refers to comparison of norovirus (NoV) outbreaks with both bacteria and unknown outbreaks separately.
‡p < 0.001 when comparing NoV outbreaks with bacterial outbreaks.
§No difference noted for dairy products (3%–5%), cold meats (2%–4%), egg dishes (0%–1%), beverages (1%–4%), or ice (0%–1%).

Table 4. Laboratory testing of fecal specimens from foodborne outbreaks of gastroenteritis and projected number of norovirus (NoV)-confirmed outbreaks in 6 states, 2000

State	Total reported outbreaks	Total with specimens (% of total outbreaks)	No. positive/no. tested						No. with unknown etiology not tested for NoV*	Total NoV outbreaks (% outbreaks with specimens)	
			Tested only for bacteria	Tested only for NoV	Tested for both bacteria and NoV		Total for bacteria (%)	Total for NoV (%)			
					Bacteria	NoV				Actual	Projected†
MD	116	42 (36)	13/35	2/2	0/5	5/5	13/40 (33)	7/7 (100)	22	7 (17)	29 (69)
MN	41	32 (78)	10/10	0/1	2/21	15/21	12/31 (39)	15/22 (68)	0	15 (47)	15 (47)
GA	26	19 (73)	9/9	0/0	2/10	7/10	11/19 (58)	7/10 (70)	0	7 (37)	7 (37)
NY	60	35 (58)	19/28	1/2	0/5	4/5	19/33 (58)	5/7 (71)	9	5 (14)	11 (31)
FL	274	40 (15)	11/26	1/3	0/6	3/6	11/32 (47)	4/9 (44)	11‡	4 (10)	9 (23)
OH	83	52(63)	8/22	0/0	0/30	29/30	8/52 (15)	29/30(97)	13§	29 (56)	42 (81)
Total	600	220 (37)	70/130	4/8	4/77	63/77	74/207 (38)	67/85 (79)	55	67 (30)	110 (50)[b]

*Derived by subtracting nominator from denominator in column 4, i.e., the number of outbreaks tested only for bacteria and with negative test results.
†Calculated by multiplying value in column 10 by percentage in column 9 and adding to value in column 11.
‡Excludes 4 confirmed outbreaks of other causes (3 of *Cryptosporidium* and 1 of chemical cause).
§Excludes 1 confirmed outbreak of chemical cause.

outbreaks (median = 25 versus 15 persons affected). Although NoV outbreaks were generally of short duration, symptoms were sufficiently severe in 9.8% of patients to require medical care and in 1%, hospitalization.

In addition to a historic lack of diagnostic assays, a further reason for underrecognition of NoV is a lack of specimens and epidemiologic information gathered from outbreaks that exhibit clinical features characteristic of viral gastroenteritis. We expected states that do not test for NoV to report more outbreaks of unknown etiology, but this was not the case. In fact, states that reported no NoV outbreaks also reported the lowest percentage of outbreaks with an undetermined etiology (47%, Table 1). This bias in the etiologic distribution of reported outbreaks toward bacterial causes that can be easily determined is further suggested by the lower number of outbreak reports in states with ≤10 NoV outbreaks from 1998 though 2000 (0.8–0.9 outbreaks/100,000 persons) compared with those states that reported >10 NoV outbreaks (2.3 outbreaks/100,000 persons). Genuine differences in the incidence of NoV disease (e.g., rural/urban) or different patterns of reporting disease among communities in different states are also possible.

We found that >56% of foodborne NoV outbreaks were associated with eating salads, sandwiches, or fresh produce, which confirms that contamination of foods requiring handling but no subsequent heating is an important source of NoV infection (9,20–22). Despite their well-documented role in large multistate NoV outbreaks (23–25), oysters have not been frequently associated with NoV disease in the last 10 years in the United States. We excluded only 2 multistate NoV outbreaks from the analysis, 1 of which was linked to oysters. Restaurants or caterers were associated with 39% of NoV outbreaks, yet in >50% of NoV outbreaks, no foodhandler was implicated. This finding probably reflects a lack of positive evidence rather than the actual ruling out of a foodhandler's involvement. Although asymptomatic infections may play a role in transmission (26), and foodhandlers are likely to underreport illness, some outbreaks with no foodhandler implicated may be due to contamination of fresh produce at the source, as has been previously documented for NoV (21,27) and other foodborne viruses transmitted by the feco-oral route (28).

Our projected number of NoV outbreaks in each state may be overestimated because outbreaks that were tested for NoV were likely to have been more characteristic of NoV disease than those not tested. However, we only applied the proportion of outbreaks positive for NoV (79%) to outbreaks of unknown etiology that had already tested negative for bacteria. Moreover, between them, the 2 states that tested all nonbacterial outbreaks for NoV found 43% of outbreaks attributable to NoV, consistent with our estimate from all 6 states. Biases in surveillance data complicate straightforward extrapolation of our estimate of outbreaks with specimens from 6 states, to the group of reported outbreaks with no specimens collected in the same 6 states and in other states. Certain clinical characteristics of outbreaks of unknown etiology were similar to those of NoV outbreaks (e.g., percentage of patients vomiting); other epidemiologic characteristics were similar to those for bacterial outbreaks (e.g., implicated food). Etiologic make-up of outbreaks with no specimens collected is also likely to differ between states. Since specimens remain less likely to be collected from outbreaks of acute gastroenteritis of short duration, we think our estimate can be reasonably extrapolated to all outbreaks of unknown etiology.

Only a few small studies have looked at the relative impact of NoV as a cause of foodborne illness (Table 5), and none have fully tested for NoV with PCR. A small study of enhanced surveillance during 1 year in a Swedish municipality found 6% of all foodborne outbreaks, but 38% of 13 that were laboratory-confirmed, to be attributa-

Table 5. Estimates of the role of norovirus (NoV) in foodborne outbreaks of gastroenteritis*

Place (reference)	Years of data	No. of foodborne outbreaks	Method used to attribute to NoV	% of foodborne outbreaks attributable to NoV
United Kingdom (31)	1995–1996	341	Electron microscopy	6
Sweden (30)	1998–1999	85	RT-PCR	6
Sweden (29)	1994–1998	92	Electron microscopy	72
New Zealand†	2000–2002	383	RT-PCR	12
The Netherlands‡	2002	59	RT-PCR	27
United States (6)	1982–1989	1049	Epidemiologic criteria	33
United States (8)	1981–1998	295	RT-PCR and epidemiologic criteria	41
United States§	2000	600	RT-PCR and extrapolation	50

*RT-PCR; reverse transcription–polymerase chain reaction.
†N. Boxall, pers. comm.
‡Y. van Duynhoven, pers. comm.
§Current study.

ble to caliciviruses (30). Our estimate of 50% of foodborne outbreaks being attributable to NoV is higher than estimates that rely on epidemiologic criteria (33%–41%) (6,8), consistent with the low sensitivity of such criteria (CDC, unpub. data). Our estimate of percentage of outbreaks attributable to NoV is lower than Mead's figure of 66% of all foodborne illness of known etiology being caused by NoV (1). However, our finding that NoV outbreaks are >50% larger than bacterial outbreaks suggests that the total number of cases associated with our estimate of outbreaks is comparable to Mead's estimate. We may have overestimated the size of NoV outbreaks and the proportion of persons seeking care since these larger outbreaks of more serious illness may be more likely to be reported. However, our estimates are not inconsistent with a study in the United Kingdom that reported the median size of NoV outbreaks to be 21 persons and the hospitalization rate to be 0.3% (32). The very low infective dose of NoV (33) allows for extensive transmission by means of contaminated food and subsequent person-to-person spread. Data on other variables may also be biased. For instance, that 61% of bacterial outbreaks would have a median incubation of <24 hours is surprising, given that 69% of the analyzed bacterial outbreaks were attributed to *Salmonella* spp., *Shigella* spp., *Campylobacter spp*., and *E. coli*, which have longer incubation periods. Finally, since no standard criteria are required for an outbreak to be classified as foodborne and since NoV are more often spread from person-to-person than bacteria, the dataset from 6 states that we used may have resulted in an overestimate of the impact of foodborne NoV.

Efforts are required to increase the capacity of states to investigate outbreaks, irrespective of suspected cause, and include improved specimen collection and more widespread testing for viruses. Evaluation of epidemiologic criteria is needed to assess how best these can be used to guide testing strategies when laboratory resources are limited. Better appreciation of the exact causes of the large number of outbreaks of undetermined etiology will help better target measures to prevent foodborne disease. Furthermore, to be able to identify novel and intentionally introduced pathogens, the ability of state health departments to quickly investigate outbreaks and discount common causes is critical. "Real-time" collection systems of epidemiologic and sequence data from different outbreaks, such as developed in Europe (34) and the United States, can provide insights into the epidemiology of NoV (35) and will allow for rapid comparison of data to rapidly identify common risk factors (such as foods contaminated at source) and implement control measures. While these initiatives are developed, however, the high disease impact of outbreaks of NoV illness should prompt prioritization of development and implementation of prevention measures, such as foodhandler education, by food safety agendas.

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Human Parechovirus 3 and Neonatal Infections

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A third serotype of human parechovirus (HPeV) has been recently isolated from stool specimens of a young Japanese child with transient paralysis. We report 3 additional cases of neonatal sepsis caused by HPeV-3 in the fall of 2001 in Canadian infants 7–27 days old. All children were hospitalized with high fever, erythematous rash, and tachypnea for a median of 5 days. The viruses isolated from nasopharyngeal aspirates grew slowly on tertiary monkey kidney cells and were successfully passaged on Vero cells. The predicted amino acid identity of the VP0-VP3-VP1 region of the three viruses was 74.6%–74.8%, 73.4%–73.6%, and 97.0%–97.1% when compared to HPeV-1, -2, and –3 prototype strains, respectively. Although different, our isolates were closely related; amino acid identity was 99.6%–100% for the last 3 proteins.

Viruses belonging to the *Picornaviridae* family have been recently reclassified into 9 genera based on acid lability, serum neutralization, and sequence homologies (1). The *Parechovirus* genus is 1 of 5 picornavirus genera, along with enteroviruses, hepatoviruses, rhinoviruses, and kobuviruses, known to infect humans. Human parechoviruses (HPeV) 1 and 2, previously known as echoviruses 22 and 23, were first isolated in 1956 (2). These viruses, in particular, HPeV-1, have been associated with gastrointestinal and respiratory tract infections, as well as occasional cases of encephalitis and flaccid paralysis (3–7). A new serotype of HPeV (HPeV-3) was described in 2004; the strain was isolated from a stool specimen of a 1-year-old Japanese girl with transient paralysis (8). Preliminary HPeV-3 seroepidemiologic studies from Japan indicated a seroprevalence of 85% in children entering elementary school (8). However, clinical information related to this viral infection is limited, and the virus has not been reported outside Japan. We report 3 cases of neonatal infection caused by HPeV-3 in Canada.

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Case Reports

Case 1

A 27-day-old boy, born at term, was hospitalized on September 5, 2001, for fever of unknown origin. The fever appeared the day before without respiratory or gastrointestinal symptoms. His father had had sinusitis, and his 2-year-old brother had had an upper respiratory tract infection in the preceding few days.

On initial examination, the baby was alert, with a temperature of 39.4°C, a respiratory rate of 40 breaths per minute, heart rate of 143 beats per minute, and oxygen (O_2) saturation at 98% (in room air). Blood pressure was not recorded. Results of physical examination were otherwise normal, with no lung rales, normal tympanic membranes, and no rash. Leukocyte count was 4.5 x 10^9 cells/L (30% neutrophils, 0% band forms, 60% lymphocytes), hemoglobin level was 123 g/L, and platelet count was 166 x 10^9 cells/L. C-reactive protein was undetectable. Urinalysis results were normal. A lumbar puncture was performed, and analysis of the cerebrospinal fluid showed erythrocytes with no leukocytes, a glucose level of 2.8 mmol/L (normal 2.2–4.4 mmol/L), and a protein level of 0.44 g/L (normal 0.15–0.45 g/L). Chest radiograph results were normal. The patient was admitted with a diagnosis of probable viremia, and intravenous antimicrobial drugs (ampicillin and gentamicin) were administered until the results of blood cultures were available.

The following day, the infant experienced an episode of O_2 desaturation (90%) with increased respiratory rate (70/min) and temperature (39.7°C). A maculopapular rash was also noted on the trunk and extremities. Chest radiograph results remained normal. Because of the infant's tachypnea, a nasopharyngeal aspirate (NPA) was obtained for human respiratory syncytial virus (RSV) antigen testing (Test Pack, Abbott Laboratories, Abbott Park, IL) and direct immunofluorescence assays (Bartels, Carlsbad, CA) for adenoviruses and parainfluenza viruses 1–3. Results of all rapid antigenic tests were negative. NPA was inoculated into numerous cell lines, including Madin Darby kidney, LLC-MK2 (tertiary monkey kidney), Hep-2, human

foreskin fibroblast, Vero, Mink lung, human lung adenocarcinoma (A-549), human rhabdomyosarcoma, transformed human kidney (293), and human colon adenocarcinoma (HT-29) cells. The patient's condition gradually improved over the next 3 days, fever disappeared, and O_2 saturation returned to normal. Four days after admission, the patient was afebrile and eupneic. A pale macular rash was still present on the trunk. The leukocyte count was 12.5 x 10^9 cells/L (8% neutrophils, 1% band forms, and 75% lymphocytes), with hemoglobin at 138 g/L and a low platelet count of 101 x 10^9 cells/L. Blood and cerebrospinal fluid cultures remained negative, and the urine culture was interpreted as bacterial contamination. The patient was discharged after 5 days of hospitalization with a presumptive diagnosis of viremia and mild reactive thrombocytopenia. Intravenous antimicrobial drugs were switched to oral amoxicillin for 5 more days. The viral culture of NPA was positive for a nonhemadsorbing virus (CAN01-81235) that initially grew on LLC-MK2 cells after 18 days of incubation.

Case 2

A 20-day-old girl, born at term, was hospitalized on September 27, 2001, for high fever and irritability. She was seen 2 days earlier with an acute upper respiratory tract infection with rhinorrhea and conjunctivitis, for which she was receiving topical erythromycin. The day before her hospitalization, her mother also noted a diffuse macular eruption. The patient had been in contact with her 3-year-old brother who had an upper respiratory tract infection a few days before.

On initial physical examination, the patient was conscious but irritable. Her temperature was 39.0°C, blood pressure was normal for age, respiratory rate was 48 breaths per minute, and heart rate was 190 beats per minute. No O_2 desaturation and no rales at lung auscultation were noted. A pale erythematous rash was present on the trunk and the limbs. Otitis media was not observed. At admission, she had a leukocyte count of 5.6 x 10^9 cells/L with 36% neutrophils, 10% band forms, and 33% lymphocytes. Hemoglobin level was 104 g/L, and platelet count was 296 x 10^9 cells/L. The C-reactive protein level was normal at 4 mg/L. Urinalysis results were normal, and examination of the cerebrospinal fluid showed 2 x 10^6 leukocytes/L, a glucose level of 3.2 mmol/L, and a protein level of 0.53 g/L. Chest radiograph results were normal, and the child was hospitalized with a diagnosis of viremia or bacteremia. Intravenous ampicillin and gentamicin were administered after blood and urine samples were obtained for cultures.

The next day, the patient had an episode of O_2 desaturation (89% in room air), which led to her transfer to the intensive care unit for O_2 administration and monitoring. Her temperature was 39.4°C, with a respiratory rate of 60 breaths per minute and a heart rate of 210 beats per minute. The rash had disappeared by that time. Results of chest radiograph remained normal. NPA was obtained for rapid antigenic tests and viral culture. Antigenic test results were negative. The infant responded well to O_2 supplementation and was discharged from the intensive care unit after 24 hours. Fever disappeared 3 days after admission, and respiratory rate and O_2 saturation gradually normalized. On day 3, the leukocyte count was 13.5 x 10^9 cells/L, with 13% neutrophils, 1% band forms, and 79% lymphocytes. The hemoglobin level was 102 g/L, with a platelet count of 180 x 10^9 cells/L. Antimicrobial drugs were discontinued after 4 days, since blood, urine, and cerebrospinal fluid cultures remained negative. The infant was discharged on day 5 of hospitalization with a diagnosis of viral infection of the upper respiratory tract accompanied by conjunctivitis. Viral culture of the stools collected the day after admission was negative. Viral culture of the NPA specimen was positive for a nonhemadsorbing virus (CAN01-81554) after 14 days of incubation on LLC-MK2 cells.

Case 3

A 7-day-old girl, who was born at term without complications, was brought to the hospital on December 16, 2001, for a recent onset of fever, irritability, and loss of appetite. Medical history showed that her mother had an upper respiratory tract infection 3 days before with cough, fever, sore throat, and a faint rash on her arms. She had been treated with clarithromycin for sinusitis during the last month of her pregnancy. The patient had a 2.5-year-old brother who was in good health.

On initial medical evaluation, the infant was irritable and refused breastfeeding but was conscious. She had no history of respiratory or gastrointestinal infections. Her rectal temperature was 39.2°C, with a heart rate of 150 beats per minute, blood pressure of 82/39 mm Hg, and a respiratory rate of 62 breaths per minute. No bulging of the fontanel was observed, and the lungs were clear on auscultation. An erythematous rash involved the trunk and axillary area without petechiae. Initial laboratory tests showed a leukocyte count of 8.0 x 10^9 cells/L, with 71% neutrophils, 5% band forms, and 18% lymphocytes. Hemoglobin level was 162 g/L, and platelet count was 199 x 10^9 cells/L. C-reactive protein level was normal at 11 mg/L. Urinalysis results were normal, and cerebrospinal fluid contained 147 (144 erythrocytes and 3 leukocytes) x 10^6 cells/L, with a glucose level of 3.1 mmol/L and protein level of 0.74 g/L. A chest radiograph did not show lung infiltrates. The child was hospitalized with an initial diagnosis of bacteremia, and treatment with intravenous ampicillin and gentamicin was instituted after blood and urine samples were collected for culture.

The next day, the erythrodermialike rash became more diffuse and confluent. Rectal temperature was at 38.5 C, and the infant was tachypneic but with good O_2 saturation (97%) in room air. A physical examination showed bilateral acute otitis media, which was confirmed by an ear-nose-throat specialist. The peripheral leukocyte count increased to 11.8 x 10^9 cells/L, with 69% neutrophils and 20% lymphocytes, whereas the platelet count had dropped to 145 x 10^9 cells/L. Liver transaminases were normal, and the chest radiograph showed no infiltrates. Because of the infant's tachypnea, an NPA was obtained. All rapid antigenic test results were negative, and the NPA was cultured for virus. At this point, the regimen was changed to cefotaxime and gentamicin.

Over the next 4 days, the patient's condition progressively improved: fever decreased, breastfeeding improved, and respiratory rate normalized. In addition, the rash gradually faded, and areas of desquamation were noted. On her last day of hospitalization (5 days after admission), the infant was afebrile and eupneic. The leukocyte count was 13.5 x 10^9 cells/L, with 20% neutrophils and 66% lymphocytes, and the platelet count had dropped to 127 x 10^9 cells/L. Results of a streptozyme test were negative. All bacterial cultures of specimens collected at the time of admission (urine, blood, and cerebrospinal fluid) were negative. The patient was discharged from the hospital with diagnoses of bilateral acute otitis media, cutaneous eruption related to a viral infection, and reactive thrombocytopenia. On follow-up, 2 days after discharge, the infant was in good health. Viral cultures of urine and stools collected during the hospitalization were negative. However, viral culture from the NPA specimen was positive for a nonhemadsorbing virus (CAN01-82853) growing in LLC-MK2 cells after 16 days of incubation.

Laboratory Findings

All 3 viruses initially grew in LLC-MK2 cells after 14–18 days. On passage, however, the viruses grew rapidly (in ≈4–5 days) in Vero cells, with cytopathic effects similar to those of enteroviruses. The viruses could not be neutralized by pools of antisera against human enteroviruses or by specific antisera for HPeV-1 and -2 (formerly echovirus-22 and -23). Immunofluorescence assays using antibodies against common respiratory pathogens (influenza A and B, parainfluenza viruses 1–3, RSV, adenovirus) and reverse transcription–polymerase chain reaction (RT-PCR) assays for human metapneumovirus (9) were all negative.

More than 2 years after these patients were treated, and after the initial description of HPeV-3 (8), 2 RT-PCR assays for HPeV-3 were performed with infected Vero cell culture supernatants and were positive. The first set of primers amplified an 810-bp fragment between the 5′ untranslated region (5′ UTR) and the VP0 gene, whereas the second set amplified a 2,030-bp fragment between the VP0 and 2A genes of HPeVs as reported (8). Amplified products were sequenced with an automated DNA sequencer (ABI 377A; Perkin-Elmer Applied Biosystems, Foster City, CA), and the nucleotide and deduced amino acid sequences were aligned along with representative strains from other HPeVs by using ClustalW.

When compared to prototype strains representative of HPeV-1 (GenBank accession no. S45208), HPeV-2 (no. AF055846), and HPeV-3 (no. AB084913), the nucleotide identity of the 3 Canadian strains for part of the 5′ UTR region was 88.0%–88.9% (HPeV-1), 80.6%–82.9% (HPeV-2), and 93.5%–94.3% (HPeV-3) (Figure 1). Nucleotide identity was 97.2%–98.1% between the 3 Canadian isolates for the same region. The nucleotide identity of the Canadian strains for the VP0-VP3-VP1 region (2,313 nucleotides, 771 amino acids) was 70.9%–71% (HPeV-1), 69.5%–69.5% (HPeV-2), and 96.0%–96.2% (HPeV-3). Similarly, the predicted amino acid identity for this region was 74.6%–74.8%, 73.4%–73.6%, and 97.0%–97.1% when compared to HPeV-1, -2 and -3 prototype strains, respectively (online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol11no01/04/0606_app.htm). The Canadian strains were closely related, with nucleotide and amino acid identities of 99.2%–100% and 99.6%–100%, respectively, for these 3 capsid proteins. Contrasting with HPeV-1 and -2 strains, but similar to the HPeV-3 prototype, the RGD motif located near the

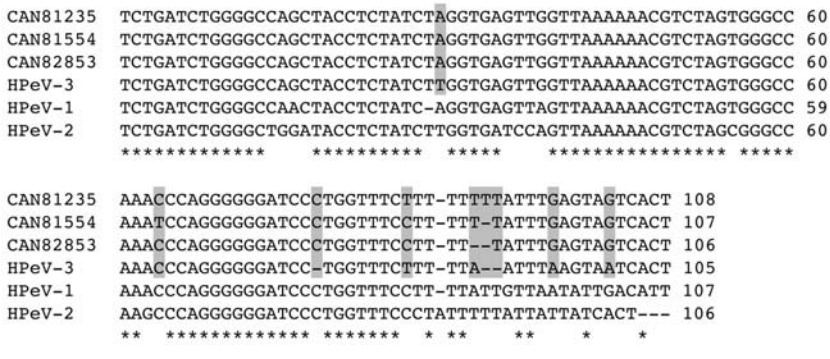

Figure 1. Comparison of the partial nucleotide sequences of human parechovirus (HPeV)-3 Canadian isolates no. 81235, 81554, and 82853 with reference sequences of HPeV-1, -2 and -3 (GenBank accession no. S45208, AF055846, and AB084913, respectively) for the 5' untranslated region (5' UTR; corresponding to nucleotides 595 to 699 of the HPeV-3 A308/99 strain, accession no. AB084913) (8,10). Asterisks denote identical nucleotides in all strains, whereas shaded nucleotides highlight differences between HPeV-3 isolates.

C-terminus of VP1 was absent in all Canadian isolates (Appendix Figure). A phylogenetic tree confirming the relationship between Canadian HPeV strains and other HPeV-3 isolates is shown in Figure 2.

Conclusion

We report the first 3 cases of HPeV-3 infection outside Japan and the first description of neonatal sepsis caused by this virus. The initial article on HPeV-3 infection by Ito et al. mentioned a case of transient paralysis with fever and diarrhea in a 1-year-old infant, as well as 3 other cases of gastroenteritis, exanthema, and respiratory illness, although no other clinical information was provided (8). In our study, viral infections occurred in the first month of life, and all patients had relatively similar clinical signs, including high fever, tachypnea, and erythematous rash. Acute otitis media and conjunctivitis were also noted in 1 patient each. All infants had a complete microbiologic work-up for possible bacteremia, including blood, urine and cerebrospinal fluid cultures, and all patients received at least 4 days of intravenous antimicrobial drugs for suspected bacterial sepsis. Neonatal infections caused by other HPeVs and many enteroviruses have been reported, including outbreaks of respiratory diseases on neonatal wards (11). Neonatal enteroviral infections can be severe, depending on the timing of maternal infection relative to time of delivery, and can progress to systemic disease, which may include hepatic necrosis, myocarditis, and necrotizing enterocolitis with or without neurologic involvement (12). Similar to some of our cases, a preceding maternal infection has been reported for 59% to 68% of neonates with enteroviral infections (12).

Most symptomatic HPeV-1 infections have been reported in young children, and most have been in children <1 year of age (3,5). For both HPeV-1 and the newly described HPeV-3, the proportion of seropositive children increases rapidly, reaching almost 100% by 5–6 years of age in Japan (8,13). HPeV-3 does not seem to be a recently emerging pathogen, as 87% of Japanese adults >40 years of age have preexisting antibodies (8). The reasons for the late description of this virus are unclear; as postulated for the newly described human metapneumovirus (14), a slow and restricted initial growth on a few cell lines (e.g., tertiary monkey kidney cells) might be one possibility, although additional epidemiologic and virologic studies are needed. In addition, as previously reported (8), HPeV-3 strains could not be neutralized with any antisera against human picornaviruses, including those directed against HPeV-1 and HPeV-2. The absence of the RGD motif near the C-terminus of the VP1 protein may be one of the epitopes involved in this event, since an antiserum specific for this region exhibited neutralizing activity against HPeV-1 (15). Also, the absence of the RGD motif in HPeV-3 is probably related to receptor usage since this region has been shown to interact with integrins (16,17). One of the HPeV-1 antigenic sites described by Joki-Korpela et al. (15), located near the N-terminal region of the VP0 protein (amino acid 79–90), was also conserved in our HPeV-3 strains as well as in reported HPeV-2 isolates (except for 1 residue), which suggests that it might represent a group antigen (Appendix Figure). All 3 Canadian strains isolated in the fall of 2001 were clearly related to the prototype HPeV-3 strain from Japan recovered in 1999 and reported in 2004 (97.0%–97.1% amino acid identity for the VP0-VP3-VP1 region), although point mutations could discriminate all our strains (Figure 1 and Appendix Figure).

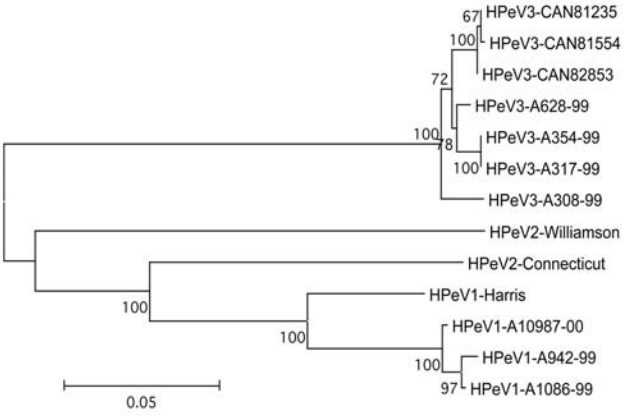

Figure 2. Phylogenetic tree showing the relationship between human parechovirus (HPeV)-3 Canadian isolates no. 81235, 81554, and 82853 and other HPeV-3 (A628-99, GenBank accession no. AB112484; A354-99, no. AB112483; A317-99, no. AB112482; A308-99, no. AB084913), HPeV-2 (Williamson, no. AJ005695; Connecticut, no. AF055846) and HPeV-1 (Harris, no. S45208; A10987-00, no. AB112487; A942-99, no. AB112486; A1086-99, no. AB112485) strains based on amino acid differences in capsid proteins (VP0-VP3-VP1 region). The tree was constructed by using the neighbor-joining method. Numbers represent the frequency of occurrence of nodes in 500 bootstrap replicas.

In summary, we provide additional confirmation for a third serotype of HPeV that could not be neutralized with antisera against other known members of the *Parechovirus* genus. Our case reports expand the clinical spectrum of previously-reported HPeV-3 infections and highlight the need for additional research work to characterize clinical and epidemiologic features of this newly described pathogen. Of particular interest are the proportion of asymptomatic viral infections in childhood and the occurrence of reinfections in the adult population.

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A Novel Paramyxovirus?

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In public databases, we identified sequences reported as human genes expressed in kidney mesangial cells. The similarity of these genes to paramyxovirus matrix, fusion, and phosphoprotein genes suggests that they are derived from a novel paramyxovirus. These genes are sufficiently unique to suggest the existence of a novel paramyxovirus genus.

The identification of novel viruses, particularly those with the potential to cause human disease, has important public health and scientific implications. Examples of emerged or recently identified viruses affecting humans include HIV (1), hepatitis C virus (2), West Nile virus (3), severe acute respiratory syndrome (SARS)-associated coronavirus (4), human coronavirus from the Netherlands (HCoV-NL) (5), and Ebola virus (6). Among the paramyxoviruses, Nipah virus, Hendra virus, and human metapneumovirus were recently described as causing disease in humans (7–9).

We now report as viral sequences particular nucleotide sequences previously considered to be human genes these sequences were named Angrem52 and Angrem104. These genes' expression in human kidney mesangial cells was reportedly upregulated by treatment with angiotensin II (10,11). However, these genes appear to encode viral proteins with striking homology to those of paramyxoviruses. A careful analysis of these sequences suggests that they actually belong to the *Paramyxoviridae* and represent a novel genus in this virus family. Given the identification of these putative "orphan paramyxovirus" (putative OPmV) sequences from human cells, the putative OPmV may cause human infections.

The Study

A BLAST search restricted to mammalian protein sequences was performed through the NCBI Web page (http://www.ncbi.nlm.nih.gov/BLAST) by using the Nipah virus matrix protein sequence as the query. One sequence, accession number AAK76747, with homology to the Nipah virus matrix protein, was identified. This protein, called Angrem52 (for angiotensin II-induced, renal mesangial cell gene 52), displays 53% amino acid identity and 73% amino acid similarity over 337 amino acids (aa) to the Nipah virus matrix protein (data not shown). The Angrem52 protein sequence is derived from a theoretical translation of an open reading frame (ORF) from nucleotides (nt) 16 to 1038 within the 3170-nt long Angrem52 nucleotide sequence (GenBank accession no. AY040225). This notable homology suggests that Angrem52 is actually a paramyxovirus M gene (Figure 1A).

Upon further analysis of sequences within the Angrem52 cDNA downstream of the putative matrix gene, several relatively short ORFs were found to encode peptides with homology to paramyxovirus fusion (F) proteins. Modification of the reported Angrem52 sequence in several positions yields what appears to be a full-length or near full-length paramymyxovirus fusion (F) protein gene, which would be separated from the M ORF by 355 nt (Figure 1A). Specifically, the F ORF within the original Angrem52 sequence begins at position 1393 but appears to terminate prematurely with a stop codon at 2118. To obtain what appears to be a "full-length" F ORF, several modifications were made to the reported sequence in order to to incorporate the additional "F-like" sequences. An A at position 2110 was deleted. A T at position 2155 was deleted. A single nucleotide, either C or T, was added between positions 2296 and 2297. A T was deleted at 2461. The theoretical translation of this modified ORF yields a protein of 546 aa, the same length as the Nipah virus F protein (data not shown). A pairwise alignment of the resulting protein with the F protein of Nipah virus shows 32% identity and 53% similarity over 509 aa (data not shown). Within this protein, a putative fusion peptide is readily identifiable based on homology to those of other paramyxoviruses (Figure 1B). Although the cleavage site adjacent to the fusion peptide is typically a basic amino acid, the reported Angrem52 cDNA sequence has an acidic glutamic acid at this position (Figure 1B). Both Nipah and Hendra viruses possess F proteins that are cleaved at the expected site (Figure 1B) but are apparently processed by novel but ubiquitous proteases. Cleavage of these sites can occur even when the residue immediately left of the cleavage site is mutated to a nonbasic residue (A. Maisner, R.E. Dutch, pers. comm.).

Other features common to paramyxovirus fusion proteins, type I transmembrane glycoproteins, are a signal sequence, a transmembrane domain, and 2-heptad repeats. The 2- heptad repeats play an essential role in membrane fusion and are able to form trimeric coiled coils (13). For the putative OPmV F, a potential signal sequence from residues 1 to 23 and a potential transmembrane domain is found between residues 497 and 516. Further, the putative OPmV F has heptad repeats in the positions expected for a paramyxovirus F protein (residues 108–190 and 428–481).

Paramyxovirus genes are separated by cis-acting elements in the genome. The signals that lie between genes include a gene-end signal, an intergenic sequence, and a gene-start signal. The Angrem52 sequence, which contains

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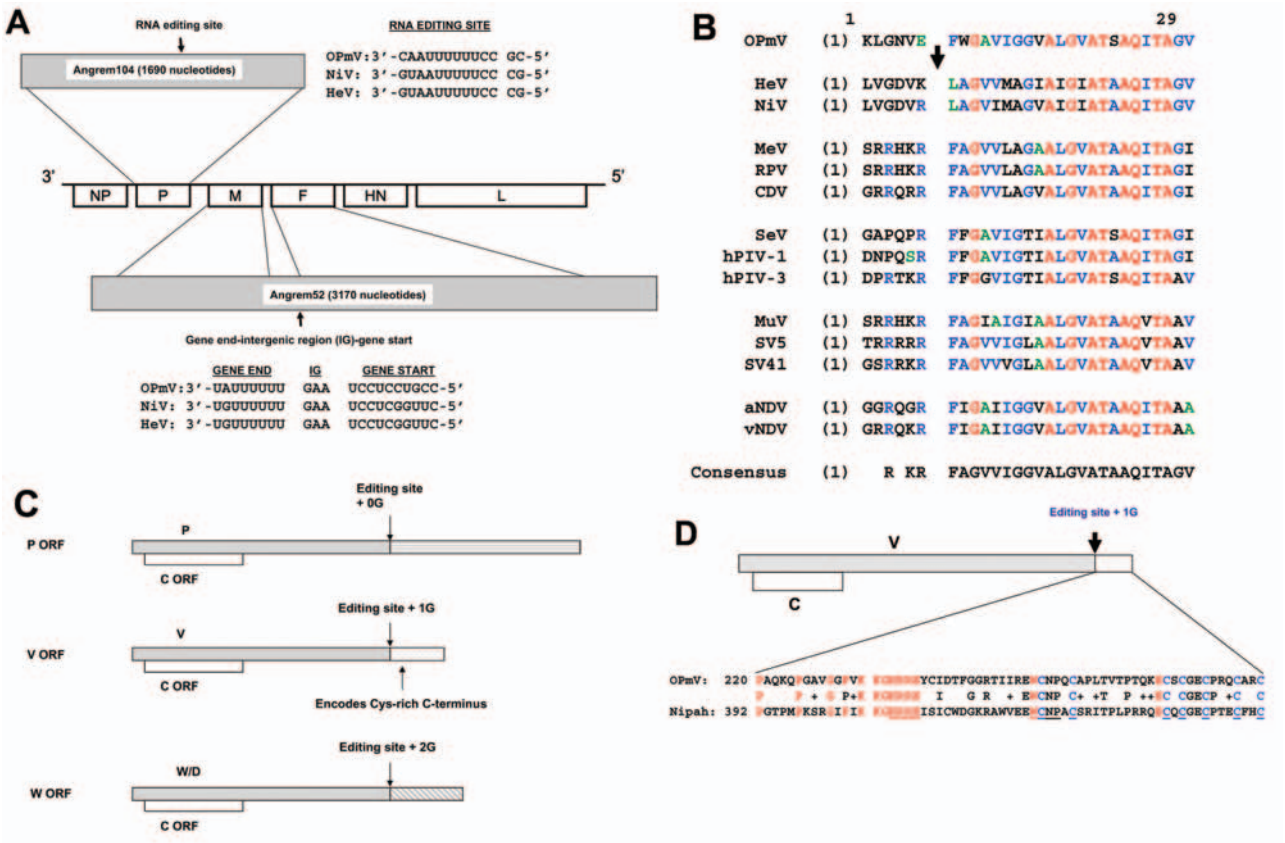

Figure 1. Angrem52 and Angrem104 appear to be paramyxovirus genes. A) Gene positions of a generic paramyxovirus and predicted genome position of Angrem104 (top), the phosphoprotein (P) gene, Angrem52 (bottom), the matrix protein (M) and fusion protein (F) genes. A potential editing site (nucleotides 783–795), which might allow production from the OPmV P gene of V and W/D proteins, is shown in genomic (negative) sense aligned with the proposed editing sites of Nipah virus (NC_002728) (1) and Hendra viruses (NC_001906). The full-length P open reading frame (ORF) was obtained by inserting an additional nucleotide in the reported Angrem104 sequence (see text). Angrem52 is predicted to be a "read-through" product of the M and F genes of a novel paramyxovirus. The full-length F ORF was obtained by making 5 changes to the reported Angrem52 sequence (see text). Putative gene-end, intergenic (IG), and gene-start transcription regulatory signals lying between OPmV M and F genes are shown aligned to the corresponding signals from Nipah and Hendra virus (shown in genomic sense [12]). B) The putative OPmV F protein contains a fusion peptide. The sequences surrounding the F protein cleavage sites, including most fusion peptides, of several paramyxoviruses, including putative OPmV, were aligned by using the AlignX program of the Vector NTI6 software package. The arrow indicates the cleavage site. Residues in red are absolutely conserved. Residues in blue are conserved in most sequences. C) Organization of the putative OPmV P gene, allowing translation of P, V, W, and C ORFs. D) Alignment of cysteine-rich carboxy-termini of the putative OPmV and Nipah virus V proteins. The conserved carboxy-terminal regions of the V proteins were aligned by using the AlignX program of the Vector NTI6 software package. Conserved residues are indicated in red, except for conserved cysteines, which are in blue. Underlined residues are conserved among all paramyxovirus V proteins.

the continuous sequence for the M and F genes, possesses a sequence with similarity to the regulatory sequences in other paramyxoviruses (Figure 1A).

Another reported angiotensin II-induced gene, Angrem104 (accession no. AF367870), appears to be a paramyxovirus phosphoprotein (P) gene. The reported Angrem104 sequence is 1690-nt long (10,11). The ATG that begins the P ORF is at position 90. Based on the reported sequence, an ORF is present from nt 90 to 1133, and the theoretical translation of this ORF yields a protein with homology to paramyxovirus P proteins but which is shorter than reported P proteins. However, the insertion of a single T residue between nucleotides at positions 1130 and 1131 results in a single reading frame that ends at position 1579 of the reported Angrem104 sequence and encodes a protein of 496 aa. Alignment of the modified protein sequence to the Nipah virus P protein shows 20.1% amino acid identity over the length of the putative OPmV protein.

Paramyxovirus P genes frequently encode multiple proteins (13). For example, C proteins are encoded by alternate ORFs near the 5′ end of P genes in a number of paramyxoviruses. In addition, through the process of "RNA editing," the site-specific insertion of nontemplate

encoded nucleotides by the viral polymerase, additional proteins, such as V and W proteins, can be produced (13). These latter proteins share amino-terminal sequences with the P proteins but differ after the editing site and thus have distinct carboxy-terminal ends (13). In the case of V proteins, the unique carboxy-terminus is characterized by a relatively conserved cysteine-rich domain. Analysis of the modified Angrem104 sequence identifies a C ORF (from positions 109 to 598) potentially encoding a 163-aa protein. In addition, a possible RNA editing signal similar to that found in the P gene of other paramyxoviruses is present (Figure 1A). One or 2 additional G residues added to the newly synthesized mRNA transcribed from this template sequence (i.e., the singly edited mRNA sequence would then be AAAAAAGGG) would give rise to mRNAs encoding a V or W protein (Figure 1C). The V ORF would begin at nt 90 of our modified Angrem104 sequence and end at position 964 of the modified Angrem104 sequence (this numbering does not count the additional G residue found in the edited mRNA). The carboxy-terminus of the predicted V protein is cysteine-rich, as expected for a paramyxovirus V protein (Figure 1D). The W ORF would also begin at nt 90 but would end at nt 1027 of the original Angrem104 sequence (again not including the 2 extra G nucleotides introduced by editing [Figure 1C]).

Based on the similarity of the predicted Angrem52 and Angrem104 sequences to paramyxovirus protein sequences, a reasonable conclusion is that Angrem52 and Angrem104 are actually paramyxovirus genes. Phylogenetic comparison of the putative OPmV P, M, and F proteins suggests that the putative OPmV belongs to a previously uncharacterized genus in the paramyxovirus family. Comparison of the M proteins may provide the most compelling argument for the uniqueness of the putative OPmV, given that an intact ORF was present in the Angrem52 nucleotide sequence and did not require additional manipulation before analysis. The putative OPmV M is found to be slightly more similar to the Henipah virus genus than to other paramyxoviruses but distinct from even Nipah and Hendra virus (Figure 2A). Likewise, the putative OPmV F protein shows the highest degree of sequence identity with the F protein of tupaia paramyxovirus (Figure 2B). Finally, analysis of the putative OPmV P gene places the putative OPmV protein on a separate branch with slightly greater similarity to the P proteins of Hendra and Nipah virus (Figure 2C). Final evidence for classifying the putative OPmV in a distinct phylogenetic group is the fact that the nucleotide sequences of its genes do not show substantial similarity to other paramyxoviruses (data not shown). Typically, notable nucleotide identity is seen between members of a paramyxovirus genus but is not seen when nucleotide sequences are compared across genera. For example, morbillivirus M genes share nucleotide identity with one another but not with the M genes of Henipah viruses (data not shown).

Conclusions

The Angrem52 and Angrem104 genes were identified by a reverse transcriptase–polymerase chain reaction (RT-PCR)–based method from primary human mesangial cells (10,11). Basal expression of each gene was detected, but each gene was identified as an angiotensin II–induced gene (10,11). The apparent presence of viral genes in a primary human cell culture system is intriguing. The presence of these genes could reflect the presence of a virus in any of several states. These include the presence of an actively replicating, fully competent virus; the presence in the cells of a persistent virus infection; or the presence of a replicating but defective virus (14). Although unlikely, these genes could also reflect the integration into the cellular genome of a viral genome as cDNA (15,16).

We have performed several experiments in an effort to determine whether these paramyxoviruslike sequences are universally present within the human genome or whether they represent a very common infection found in human mesangial cells. Searches of publicly available human and mouse sequence databases have not identified proteins or predicted proteins with homology to the putative OPmV sequences (data not shown). We obtained a human 12-tissue, multiple tissue, Northern blot from Clontech and probed this with recombinant probes corresponding to the putative OPmV M and P genes. No specific signal could be obtained under conditions in which a β-actin probe efficiently recognized its mRNA (data not shown). We also screened 4 lots of Clonetics primary human mesangial cells (Cambrex, East Rutherford, NJ) for the presence of the putative OPmV mRNAs and products when the cells were untreated and after treatment with a range of concentrations of human angiotensin II (Sigma Chemical Co., St. Louis, MO). RT-PCR analysis using a number of primer sets for each of the putative OPmV genes yielded consistently negative results, and antibodies raised against recombinant forms of the putative OPmV M and P proteins failed to detect the putative OPmV proteins (data not shown). Based on these data, it appears that the putative OPmV genes are not human genes and are not universally expressed in human mesangial cells.

The source of OPmV, the genes of which were identified as Angrem52 and Angrem104, remains unclear. Infection of the cells after their establishment in culture cannot be excluded, nor can contamination of the PCR reactions used to identify Angrem52 and Angrem104 be ruled out. However, the cells may also have been infected in vivo before the generation of the primary cell culture. In this respect, the putative OPmV might be similar to simian

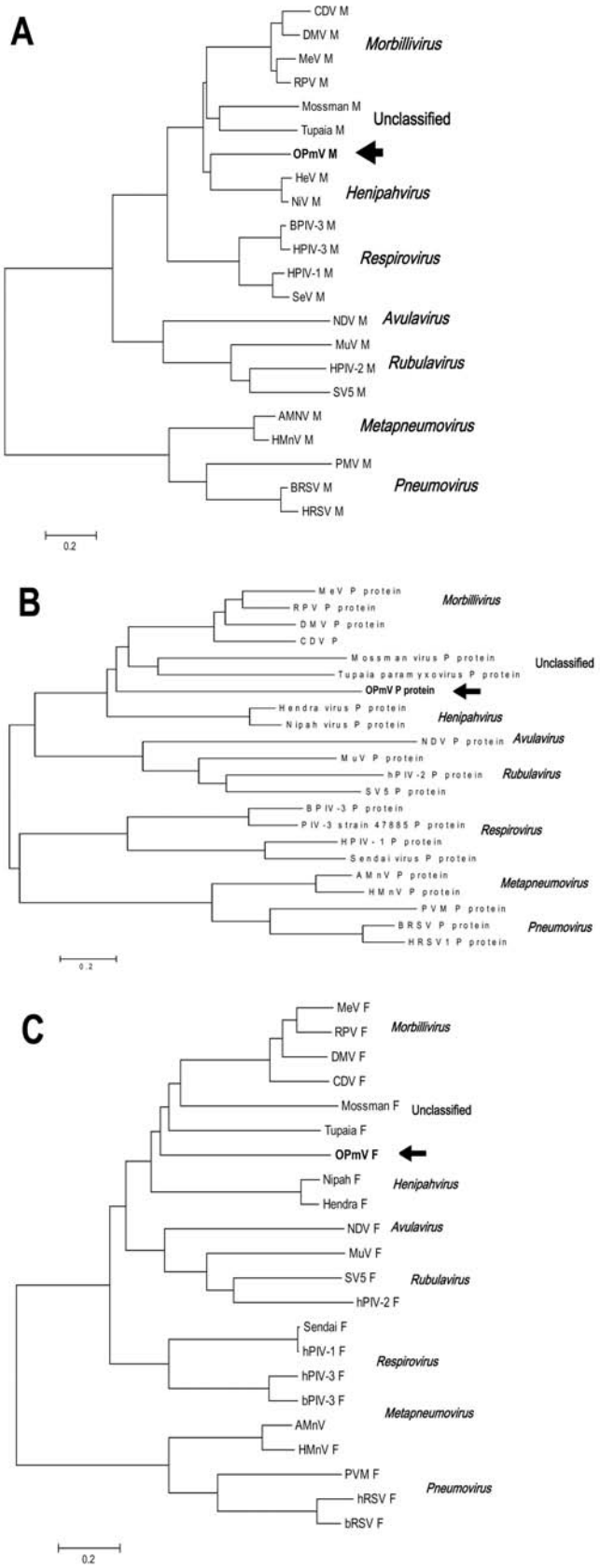

Figure 2. Phylogenetic comparison of OPmV proteins to other paramyxovirus proteins. A) Phylogenetic tree showing the relationship of the putative OPmV M protein to the M proteins of representative members of the various genera in the family Paramyxoviridae. B) Phylogenetic tree showing the relationship of the putative OPmV F protein to the F proteins of other representative paramyxoviruses. C) Phylogenetic tree showing the relationship of the putative OPmV P protein to the P proteins of other representative paramyxoviruses. Sequence alignments were made with the ClustalW method of the AlignX program of the Vector NTI6 software package. The trees were generated from these alignments by using neighbor-joining methods through the computer program MEGA version 2.1 (available from http://www.megasoftware.net/). The position of the putative OPmV sequences are indicated by arrows; distance bars, which represent 0.2 amino acid changes per position, are shown below the trees. The sequences from which the trees were constructed are as follows: Mossman, Mossman virus (NC_005339); Tupaia, Tupaia paramyxovirus (NC_002199); NiV, Nipah virus (NC_002728); HeV, Hendra virus (NC_001906); SeV, Sendai virus (AB065188); hPIV-1, human parainfluenza virus 1 (NC_003461); hPIV-3, human parainfluenza virus type 3 (NC_001796); bPIV-3, bovine parainfluenza virus type 3 (AF178655); hRSV, human respiratory syncytial virus (GI:133665); BRSV, bovine respiratory syncytial virus (NC_001989); PMV, pneumonia virus of mice (AY573814)AMnV, avian metapneumovirus (AY028582); HMnV, human metapneumovirus (NC_004148); SV5, simian paramyxovirus SV5 (D13868); hPIV-2, human parainfluenza virus type 2 (NC_003443); MuV, mumps virus (AY309060); NDV, Newcastle disease virus (NC_002617); MeV, measles virus (AF266288); RPV, rinderpest virus (AB021977, M21514, M34018); DMV, dolphin morbillivirus (NC_005283); CDV, canine distemper virus (NC_005283).

virus 5 (SV5), which can cause persistent infection in monkey kidneys and be detected in monkeys for long periods after initial infection (14). Given the possibility that the putative OPmV infects human cells, the potential association of the putative OPmV with human disease is worth exploring.

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Estimate of Illnesses from *Salmonella* Enteritidis in Eggs, United States, 2000

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Results from our model suggest that eating *Salmonella enterica* serovar Enteritidis–contaminated shell eggs caused 182,060 illnesses in the United States during 2000. Uncertainty about the estimate ranged from 81,535 (5th percentile) to 276,500 illnesses (95th percentile). Our model provides but 1 approach for estimating foodborne illness and quantifying estimate uncertainty.

Foodborne salmonellae are estimated to cause ≈1.3 million illnesses, 15,000 hospitalizations, and 500 deaths per year in the United States (1). *Salmonella enterica* serovar Enteritidis is a leading cause of foodborne salmonellosis. After its emergence in the northeastern United States during the late 1970s, the *S.* Enteritidis epidemic spread throughout the country. It was detected in the Atlantic region in 1984 and the Pacific region in 1993 (2,3). Culture-confirmed *S.* Enteritidis infections peaked at ≈4/100,000 population in 1995 and declined to ≈2/100,000 in 1999 (4). Eggs and egg-containing foods are the primary vehicles of *S.* Enteritidis infection, having been implicated in 298 (80%) of the 371 known-source *S.* Enteritidis outbreaks reported to the Centers for Disease Control and Prevention (CDC) from 1985 through 1999 (4). Nevertheless, the annual number of shell egg–associated *S.* Enteritidis illnesses in the United States is unknown. One estimate suggested that 200,000 to 1 million *S.* Enteritidis illnesses occurred in the United States in 1996 (2), but it was not specific for those attributed to shell egg consumption. Using data from the Foodborne Diseases Active Surveillance Network (FoodNet), we developed a model to estimate the number of shell egg–associated *S.* Enteritidis illnesses in the United States for 2000. The model was also designed to quantify estimate uncertainty.

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The Study

The estimated number of illnesses from shell egg–associated *S.* Enteritidis in the United States for the year 2000 (Ill_{SE}) was calculated as: $I11_{SE}$=F1×F2×F3×F4×F5 (equation 1), where

Ill_{SE} = number of *S.* Enteritidis illnesses from eating shell eggs in 2000.

F1 = number of culture-confirmed salmonellosis cases ascertained by FoodNet in 2000 = 4,330.

F2 = the proportion of culture-confirmed salmonellosis cases ascertained by FoodNet for which isolates were serotyped as *S.* Enteritidis. From the 4,330 culture-confirmed salmonellosis cases ascertained for 2000, 3,964 *Salmonella* isolates were serotyped, 585 of which were identified as *S.* Enteritidis (585/3,964 = 0.148).

F3 = the proportion of *S.* Enteritidis cases from eating shell eggs. In 2000, FoodNet ascertained 15 *S.* Enteritidis outbreaks in which food vehicles were identified: 12 were egg-associated (12/15 = 0.8). This proportion was used as a surrogate for the proportion of sporadic *S.* Enteritidis illnesses from eating shell eggs.

F4 = a multiplier to account for cases of salmonellosis that occurred in the FoodNet catchment area but were not confirmed by fecal culture, and subsequently, not ascertained by FoodNet. The value used for this multiplier was 38.6 (5).

F5 = a multiplier to extrapolate from the FoodNet catchment area to the U.S. population. For 2000, the population in the 8 FoodNet catchment sites was 30,500,000 persons, thus representing 10.8% of the U.S. population at that time (6). The multiplier was computed by taking the inverse of the proportion of the U.S. population represented by the catchment area (1/0.108 = 9.2).

Thus, based on equation 1 above, the Ill_{SE} point estimate was calculated as: 4,330 x (585/3,964) x (12/15) x 38.6 x (281,400,000/30,500,000) = 182,060.

Uncertainty for the estimate of Ill_{SE} was also determined. As illustrated in equation 1, multipliers F2, F3, F4, and F5 adjusted the number of culture-confirmed salmonella illnesses ascertained by FoodNet in 2000 (F1) to estimate the number of *S.* Enteritidis illnesses due to eating shell eggs. Uncertainty associated with each multiplier contributes to the overall uncertainty associated with the estimate of Ill_{SE}. The distributions described below were incorporated into a Monte Carlo simulation (@RISK, version 4.0, Palisade Corp., Newfield, NY) of 100,000 iterations to estimate the range of potential values for Ill_{SE} (Figure).

F2 assumed the proportion of *Salmonella* illnesses attributable to *S.* Enteritidis ascertained by FoodNet was equal to the proportion of *Salmonella* illnesses attributable to *S.* Enteritidis throughout the United States. The β distribution (585 + 1, 3,964 – 585 + 1) was used to describe uncertainty around the F2 point value.

F3 assumed that the proportion of *S.* Enteritidis outbreaks and sporadic infections attributable to eating shell eggs was equivalent. The β distribution, (12 + 1, 15 – 12 + 1), was used to model the uncertainty around the proportion of *S.* Enteritidis cases assumed to have resulted from shell egg consumption.

F4 assumed that the impact of diarrheal illness, and the behavior of persons with diarrhea and their healthcare providers, was the same in the FoodNet catchment area as in the U.S. population. It also assumed that the proportions of case-patients who 1) sought medical attention, 2) provided a specimen for fecal culture, and 3) were confirmed as salmonellosis patients contributed equally to case ascertainment, but that these proportions differed for patients who experienced bloody diarrhea compared to those who experienced nonbloody diarrhea. A triangular distribution with a minimum value of 9.8 and a maximum value of 67.7 around the point estimate of 38.6 was specified to quantify uncertainty associated with F4.

F5 assumed that the population of the FoodNet catchment area in 2000 was representative of the U.S. population. Because this assumption was qualitative, uncertainty associated with the multiplier could not be modeled.

Conclusions

We estimated that 182,060 illnesses due to egg-associated *S.* Enteritidis occurred during 2000 (Figure). Based on previous estimates that suggested that the ratio of illnesses to hospitalizations to deaths for nontyphoidal salmonellosis is roughly 2,426 to 28 to 1 (1), our estimate extrapolates to ≈2,000 hospitalizations and 70 deaths. In recognition of the fact that descriptions of the impact of illness from foodborne pathogens are inexact, our model was designed to characterize uncertainty about the estimate of illnesses resulting from eggborne *S.* Enteritidis. Ninety percent of the model iterations resulted in estimates of the number of shell egg–associated *S.* Enteritidis illnesses from 81,535 (5th percentile) to 276,500 (95th percentile) (Figure). Because the proportion of *S.* Enteritidis illnesses attributed to eggs was based on a relatively small number of outbreaks (15 outbreaks), the uncertainty about this multiplier was an important contributor to the overall uncertainty in our estimate. Angulo and Swerdlow (2) estimated that 200,000 to 1 million *S.* Enteritidis infections occurred in the United States in 1996. The lower range of our estimate in part reflects that it was computed for only those *S.* Enteritidis infections from eggs and that the relative number of *S.* Enteritidis infections reported by FoodNet was lower in 2000 than in 1996 (7).

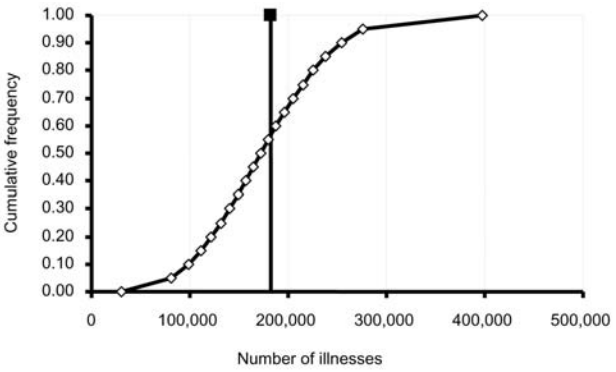

Figure. Estimated number of illnesses from *Salmonella enterica* serovar Enteritidis in shell eggs, United States, 2000. The point estimate of 182,060 illnesses is indicated by the filled box and solid vertical line. The open diamonds and attached line indicate the range of estimate uncertainty (5th percentile = 81,535 illnesses, 95th percentile = 276,500 illnesses).

Several assumptions were made in this study. First, all culture-confirmed salmonellosis cases in the FoodNet catchment area were assumed to have been ascertained through FoodNet, a reasonable assumption considering FoodNet is an active surveillance system. Second, the proportion of *Salmonella* isolates identified as *S.* Enteritidis in FoodNet sites was assumed to be comparable to that identified nationally. The proportion derived from FoodNet (≈15%) was similar to that reported for 2000 through the Public Health Laboratory Information System (PHLIS) (≈19%) (8). Third, the proportion of *S.* Enteritidis cases from shell eggs was assumed to be similar between the FoodNet catchment area and the nation. The value derived from FoodNet (80%) for 2000 was identical to that for 1985 through 1999, as reported through CDC's National *Salmonella* Surveillance System (4). Fourth, the multiplier for underascertainment was assumed to be correct. Granted, FoodNet data are limited to diagnosed illnesses, whereas most foodborne illnesses are neither diagnosed nor reported: nevertheless, the value of 38.6 used here was derived specifically for the estimation of salmonellosis cases from FoodNet data (5). Lastly, the population of the FoodNet catchment area in 2000 was assumed to be representative of the U.S. population, although FoodNet findings may not be generalizable to the nation.

Findings of this study suggest eggborne *S.* Enteritidis was an important public health problem in the United States during 2000. The findings also illustrate the potential for uncertainty in estimating the impact of foodborne illness. The model we described here provides but 1 approach for estimating foodborne illness and quantifying estimate uncertainty.

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Dr. Schroeder is a risk analyst with the U.S. Department of Agriculture. His general research interests include risk assessment and foodborne illness.

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Mosquitoborne Viruses, Czech Republic, 2002

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Specimens from residents (N = 497) of an area in the Czech Republic affected by the 2002 flood were examined serologically for mosquitoborne viruses. Antibodies were detected against Tahyna (16%), Sindbis (1%), and Batai (0.2%) viruses, but not West Nile virus. An examination of paired serum samples showed 1 Tahyna bunyavirus (California group) infection.

The 2002 flood in Bohemia struck the Czech Republic just a few years after the 1997 flood (in Moravia and Silesia). Apart from Prague, extensive rural areas along the Vltava and Labe Rivers were flooded in August 2002. In the Melník area, which offers favorable habitats for mosquitoes under normal conditions (1), mass mosquito breeding (largely *Ochlerotatus sticticus*, *Oc. cantans*, *Aedes vexans*, and *Ae. cinereus*) occurred after August 20. This increased mosquito population peaked September 3–9, with a biting frequency of 70 bites per person per minute. The mosquito population declined during the second half of September and disappeared by November.

The Study

To estimate the risk for infections with mosquitoborne viruses, we screened the human population of the flooded area (Figure 1) for antibodies against the viruses known to occur in central Europe (2): Tahyna (TAHV), *Orthobunyavirus* of the California group, *Bunyaviridae*; West Nile (WNV), *Flavivirus* of the Japanese encephalitis group, *Flaviviridae*; Sindbis (SINV), *Alphavirus*, *Togaviridae*; and Batai (BATV), *Orthobunyavirus* of the Bunyamwera group, *Bunyaviridae*.

We subdivided the flooded area into 4 risk zones according to quantities of mosquitoes. Zone A was a forested floodplain along the Labe River between Obríství-Kly and Lobkovice-Kozly (11 villages), with large quantities of mosquitoes. Zone B was an intermediate area between zones A and C (5 villages, 1 small town), with fewer breeding sites but possibility for mosquito migration from zone A. Zone C was the area along the Vltava and Labe Rivers between Kralupy and Horní Pocaply (25 villages and small towns), with no floodplain forests and few breeding sites for mosquitoes. Zone D was a control zone, with only sporadic occurrences of mosquitoes (mainly in Prague).

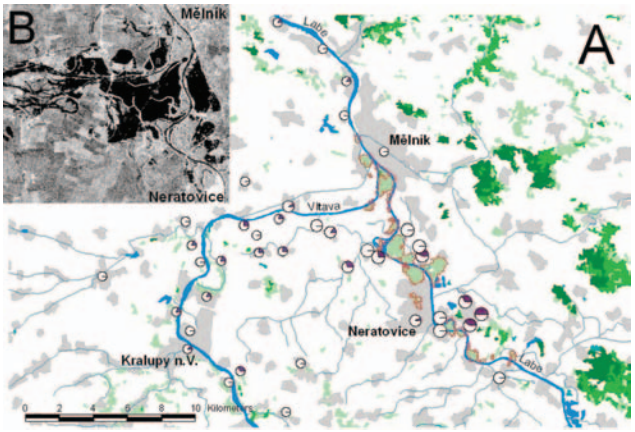

Figure 1. A), Potential foci of mosquitoborne viruses in the Melník area. Floodplain forests identified on the Landsat MSS satellite images (dotted red line), with hydrology and settlement in background (DMU-200, VTOPÚ Dobruška), and proportion of Tahyna virus seropositive residents at particular localities (large, medium, and small circles indicate the risk zones A, B, and C, respectively). B) [inset] radar satellite image of the conflux of the Labe and Vltava Rivers on August 17, 2002 (2 days after the flood culmination), showing extent of floodwater (dark areas). Inundated forests, with subsequent mass occurrences of *Ochlerotatus* and *Aedes* mosquitoes, are visible as lighter areas surrounding the Labe River upstream of the confluence; scattered lagoons (dark areas) in arable fields along both rivers far left and right turned into breeding sites of predominantly *Culex* mosquitoes.

Informed written consent and serum samples were obtained from 497 survey participants of various ages from September 6 to September 13, 2002 (3 weeks after the flood culmination and 2 weeks after the mosquito emergence). Paired serum samples were taken from 150 of the survey participants 7 months later, from April 9 to May 15, 2003 (34 in zone A, 43 in zone B, 73 in zone C).

Serologic examination was performed with the hemagglutination-inhibition (HIT) and plaque-reduction neutralization tests (PRNT) in microplates (3–5). The strains used for HIT were TAHV 92, WNV Eg101, BATV 184, and SINV Eg339; a commercial control antigen (Test-Line Ltd., Brno, Czech Republic) of Central European tick-borne encephalitis virus (CEEV) was used. All serum samples were acetone-extracted and tested with sucrose- and acetone-processed antigens by using 8 hemagglutinin units; titers >20 were considered positive. For PRNT, TAHV T16, WNV Eg101, CEEV Hypr, and BAT 184 viral strains were used. The test was conducted on Vero or SPEV (embryonic pig kidney: for CEEV) cells. All serum

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samples were heat inactivated and screened for antibodies at 1:8; those reducing the number of virus plaques by 90% were considered positive and titrated to estimate dilutions causing plaque–number reduction by 50% ($PRNT_{50}$) and 90% ($PRNT_{90}$). The serum samples reacting with WNV were examined for cross-reactivity with CEEV. PRNT with BATV was used only as a confirmatory test for the serum samples reacting with BATV in HIT.

Against TAHV, 82 (16.5%) of 497 study participants had neutralizing antibodies, and 74 (14.9%) were seropositive in HIT. In $PRNT_{50}$, the titers were 32–2048 (geometric mean titer [GMT] 260), in $PRNT_{90}$ 16–1024 (GMT 119), and in HIT 20–40 to 160 (GMT 40). Figure 2 illustrates the distribution of neutralizing antibody titers. No difference occurred in neutralizing antibody prevalence between sexes, 32 (15.8%) of 202 males and 50 (16.9%) of 295 females ($\chi^2 = 0.11$; $p = 0.744$). The prevalence rate increased significantly with age (Table 1: $\chi^2 = 39.809$; $p < 0.001$); TAHV antibodies were found infrequently in persons <19 years of age. Neutralizing antibody distribution, with respect to the residence location (Table 2, Figure 1), showed the highest seroprevalence in zone A (28%), lower seroprevalences in zones B and C, and 5% in the control zone D ($\chi^2 = 14.57$; $p = 0.002$). Significant differences were found between zone D and all other zones, and between zones A and C ($\chi^2 = 7.243$; $p = 0.007$), but not between zones A and B or B and C; HIT yielded similar results. The seroprevalence in relation to the proximity of study participants' locations to the nearest floodplain forest within zones A, B, and C demonstrated decreasing seroprevalence with increasing distance to the forest ($\chi^2 = 8.51$; $p = 0.003$) (Table 2).

Against WNV, no specific reactions were found. Although serum samples from 34 (6.8%) study participants reacted in HIT with the WNV at titers 40 to 80, all of them also reacted with CEEV at titers similar or higher (≤160). CEEV could have occurred in the area, and some study participants may have been vaccinated against tick-borne encephalitis. In $PRNT_{90}$, 6 study participants (1.2%) reacted with WNV but at low titers of 8 to 16; these serum samples also reacted in PRNT with CEEV; thus, the results were considered to be crossreactions as well. Additionally, 42 (8.5%) seroreactors against WNV appeared in the less stringent $PRNT_{50}$, but all titers were low (8–32) and cross-reacted with CEEV.

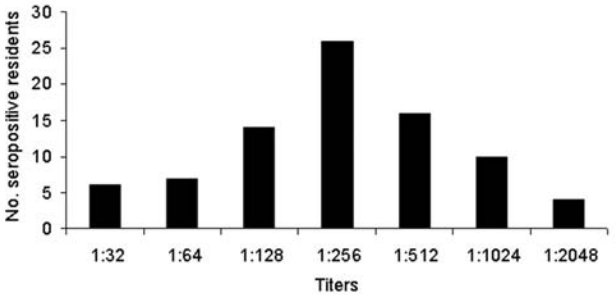

Figure 2. Distribution of 50% plaque-reduction neutralization titers of antibodies to Tahyna virus.

Against SINV, antibodies were tested with HIT only and detected in specimens from 7 (1.4%) study participants, with low titers of 20 to 40. Of the BATV, specimens from 7 study participants reacted in HIT at a low titer of 20. By confirmatory testing of these serum samples in PRNT, only 1 (0.2%) showed specific antibodies to BATV; the titer was 64 in $PRNT_{50}$ and 32 in $PRNT_{90}$.

Seroconversion (≥4-fold rise in titer) was found with TAHV only. After the flood the infection episode occurred in one 55-year-old woman from Obříství (zone A), as shown by the seroconversion in both HIT (<20/40) and $PRNT_{50}$ (<8/512). Three other study participants seroconverted in 1 test only: a 40-year-old man from Chlumín, zone B (HIT 20/80; PRNT 128/128); a 32-year-old man from Chlumín (HIT <20/20–40; PRNT 128/64); and an 80-year-old woman from Obříství (HIT 20/80; PRNT 64/32). These results are less convincing. Upon our request, local general practitioners did not corroborate consistent signs of a disease reported by these 4 study participants from October 2002 to April 2003. In general, clinical symptoms of TAHV infection are milder in adults than in children (7). Seroconversion against mosquitoborne viruses was not detected in any of the 73 study participants in zone C.

Table 1. Comparison of the prevalence of neutralizing antibodies to Tahyna virus by age groups after the floods in central Bohemia in 2002 and southern Moravia in 1997*†

Age (y)	CB 2002, n	% positive	SM 1997, n	% positive
0–9	18	5.6	39	0.0
10–19	53	0.0	49	8.2
20–29	74	5.4	128	19.5
30–39	69	17.4	79	63.3
40–49	62	11.3	80	62.5
50–59	86	19.8	90	81.1
60–69	78	32.1	59	79.7
≥70	57	28.1	95	88.4

*CB, central Bohemia; SM, southern Moravia; n, number of residents examined.
†Source (6).

Table 2. Prevalence of neutralizing antibodies to Tahyna virus after the 2002 flood, Central Bohemia*

	n†	% positive
Risk zone		
A	75	28.0
B	83	20.5
C	279	14.7
D	60	5.0
Distance to FPF (km)		
<1.0	78	28.2
1.0–2.9	75	21.3
3.0–5.9	70	17.1
≥6.0	214	13.6

*As related to the residence location: risk zones A to D; and distance to floodplain forest (FPF, within zones A, B, and C only).
†n, number of residents examined.

Conclusions

On the basis of this serosurvey, recent infections with WNV (in contrast to South Moravia after the 1997 flood [5,6]), SINV, and BATV have not been found in Central Bohemia after the flood. However, activity of another mosquitoborne virus, TAHV, has been found in a natural focus along the Labe River at Neratovice. This focus has so far gone unnoticed (8). Lower frequency of TAHV antibodies has been detected along the lower reaches of the Vltava River. The prevalence of antibodies to TAHV increased with risk-zone ranking (from zone D to the highest risk zone A) and with decreasing distance to floodplain forests, the primary breeding habitat of vector mosquitoes (9–11).

In disease-endemic areas, the proportion of residents with antibodies against California group viruses increase with age (6,12). A similar situation occurred in the Central Bohemian flooded area, where antibodies to TAHV were detected in a low proportion of residents <20 years of age. Nevertheless, TAHV seems to be active in the area. At least 1 seroconversion among 150 residents (attack rate ≈0.67%) against TAHV has been proven. With ≈100,000 inhabitants in the risk zones (1992 census), ≈670 (95% confidence interval 20–3,719) persons could have been infected after the flood.

Environmental factors, such as heavy rains followed by a flood, artificial inundation of floodplain forests, or rehabilitation of wetlands that support mosquito-vector populations, could give rise to preconditions for an increased incidence of mosquitoborne infectious diseases, even in temperate climates. Under such circumstances, the optimum strategy is an epidemiologic surveillance that includes monitoring, especially of infection rate of mosquito populations and incidence of mosquitoborne diseases in humans. The surveillance results could then be used in integrated mosquito control.

Acknowledgments

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Mycobacterium lentiflavum Infection in Immunocompetent Patient

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Mycobacterium lentiflavum is a recently described nontuberculous mycobacterium that has mainly clinical importance in young children with cervical lymphadenitis and in immunocompromised patients. We describe a case of chronic pulmonary infection in an immunocompetent patient. Our observation confirms clinical, diagnostic, and treatment difficulties in the management of *M. lentiflavum* infection.

Mycobacterium lentiflavum was described as a nontuberculous mycobacterium in 1966 (1,2). Most of the isolates represented fortuitous isolations, although recently its identification has posed concerns about its possible clinical importance. *M. lentiflavum* was mainly isolated from lymph nodes of young children, while isolations from other sites (lung specimens included) were described only in immunocompromised patients (3–9).

We describe, for the first time, a chronic pulmonary *M. lentiflavum* infection in an elderly immunocompetent woman. Our report confirms the emergence of this nontuberculous mycobacteria infection in immunocompetent patients and underlines the clinical, diagnostic, and therapeutic difficulties in its management.

The Study

In February 2000, dyspnea, productive cough, hemoptysis, weight loss, weakness, and slight fever developed in a 67-year-old woman with a previous diagnosis of pulmonary tuberculosis (47 years earlier). A chest radiograph showed post-tuberculous fibrodystrophy of the right upper lobe. Ziehl-Neelsen smear gave positive results, and a nontuberculous mycobacterium was isolated. Drug-susceptibility tests, performed on agar medium by proportion method, showed sensitivity to rifampin, ethambutol, and pyrazinamide and resistance to streptomycin and isoniazid. Treatment with isoniazid, pyrazinamide, ethambutol, and rifampin was begun for 3 months without any microbiologic changes. The persistence of acid-fast bacilli and nontuberculous *Mycobacterium*–positive cultures in the sputum were interpreted as a chronic nontuberculous mycobacterial colonization not associated with true pathogenic damage, and no further treatment was undertaken.

In March 2002, the patient was admitted to our hospital because of productive cough, weakness, dyspnea, hemoptysis, fever, and weight loss. Radiograph and computed tomography scan showed worsening chest abnormalities, with the appearance of a widespread reticulonodular alteration and an opacity in the left middle lobe (Figure A).

The sputum smear was still positive for acid-fast bacilli (polymerase chain reaction [PCR] specific for *M. tuberculosis* and *M. avium* DNA was negative), and routine cultures for mycobacteria yielded unidentified scotochromogenic mycobacterium. HIV test was performed to investigate a possible cause of immune impairment, but it was negative. Lymphocyte subsets by flow cytometry were studied and showed normal values. In addition, a killing test to evaluate macrophage activity was performed, and a

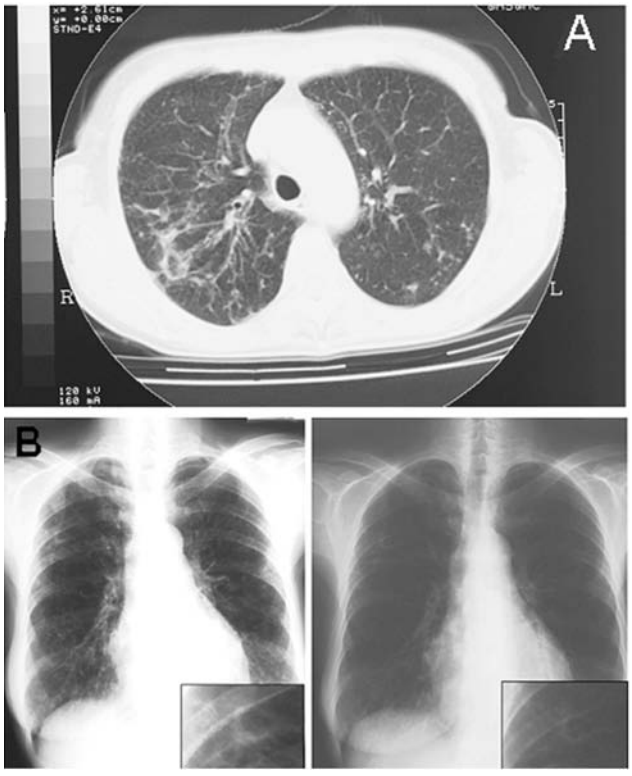

Figure. A) Pulmonary computed tomographic scan representation of *Mycobacterium lentiflavum* lesions. Radiologic image shows the appearance of a widespread reticulonodular alteration and an opacity in the left middle lobe. B) Chest radiograph results before and after 3 months of treatment shows a sustained improvement of the visible alterations to the left pulmonary middle lobe.

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diagnosis of chronic granulomatosis disease was excluded. Concomitantly, cultures from sputum and from broncholavage were performed for either standard bacteria or fungi (including *Pneumocystis carinii*). These cultures did not identify other pathogens. Serologic tests to detect *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella* species (including urinary antigens) were performed and produced negative results, thus confirming the pathogenic role of the *Mycobacterium lentiflavum*.

Susceptibility tests, using proportion methods, showed sensitivity to clarithromycin, ethambutol, isoniazid, streptomycin, rifabutin, cycloserine, and terizidon and resistance to rifampin, amikacin, kanamycin, pyrazinamide, and ofloxacin. According to the clinical history, microbiologic results, and susceptibility pattern (clarithromycin MIC 2 μg/mL), treatment with clarithromycin was initiated. The patient was released after 10 days with no fever; a slight, yet progressive, improvement of radiologic features; and a substantial recovery of the clinical conditions.

All conventional identification procedures, including cultural, biochemical, and enzymatic tests, failed to properly identify the species. Moreover, many questions remained unresolved, and further clarification was needed about the origin and effect of treatment on the clinical response. Because of the need to reach a definitive diagnosis, we sent the unidentified *Mycobacterium* culture to one of the Italian reference laboratories for mycobacteria.

Two months after hospitalization, the diagnosis of *M. lentiflavum* was obtained by analyzing cell wall mycolic acids by using high-performance liquid chromatographic test and by nucleic acid sequence analysis of PCR-amplified 16S ribosomal RNA gene fragments. After 3 months of clarithromycin treatment, the patient completely recovered, and chest radiograph showed sustained improvement (Figure B). Mycobacterial investigations, which had produced negative sputum samples after 1 month of treatment, once again gave positive results. After a new evaluation of drug susceptibility that showed no change in drug-resistance pattern, a further treatment with clarithromycin, ethambutol, rifabutin, and ciprofloxacin was undertaken, but it was prematurely ended because of poor patient compliance.

At present, after a 3-year follow up, the patient complains of intermittent hemoptysis, weakness, and dyspnea. Radiographic examinations still show the known widespread reticulonodular alterations, and sputum cultures are persistently positive for acid-fast bacilli.

Conclusions

M. lentiflavum is a recently described nontuberculous mycobacterium (1,2). Most isolates have represented fortuitous isolations that required critical evaluation about their clinical importance. Indeed, as summarized in the Table, *M. lentiflavum* identification has been shown to cause disease in only few cases. All of these cases were described in Europe. Most reports describe isolates from cervical lymphadenitis of very young children (3–9); other anatomic sites are less frequently implicated (1,7,8). The few *M. lentiflavum* pulmonary cases were described in immunocompromised patients only (5,8,9).

We describe, for the first time, a chronic pulmonary infection due to *M. lentiflavum* in an immunocompetent patient. Our observation provides further evidence that this species should be added to the growing list of nontuberculous mycobacteria, which can cause pulmonary disease in both immunocompromised and immunocompetent patients.

Traditional identification techniques are widely insufficient in providing a correct diagnosis, and more sophisticated diagnostic methods need to be improved. Susceptibility tests have reliability problems.

When the difficulties in reaching a diagnosis are considered, the identification standard techniques, based on the rate of growth, pigmentation, and biochemical tests, even if well established and relatively inexpensive, are unable to identify most nontuberculous mycobacteria. Laboratory methods that perform better, such as high-performance liquid chromatography and genetic investigations of the 16S rRNA gene fragments, through the use of nucleic acid probes, sequencing, and amplification, have to be added to diagnostic protocols for nontuberculous mycobacteria diagnosis. Moreover, new reference centers should be created and organized. Cooperation programs among research laboratories, which have provided a structured experience with complex methods, have to be implemented, and an effective collaboration should be created.

In addition to the diagnostic uncertainty, interpreting the sensitivity tests is also of concern. Information provided by tests performed on solid medium was discordant and created serious misunderstanding in the therapeutic choices. Thus, in the presence of nontuberculous mycobacteria, the susceptibility profile must be performed on liquid media, and tests on agar by proportion method should be avoided because of the risk of obtaining false-resistant results. Because of the difficulties in executing these methods and interpreting their results, we recommend that the tests be performed by experienced laboratories that can maintain feedback for both peripheral laboratories and clinicians.

In 1998, the Italian National Institute of Health launched, in accordance with the World Health Organization and International Union Against Tuberculosis and Lung Disease, a project to implement proficiency testing; results from the first (1998–1999) and second surveys (2000) showed substantial improvement in the accuracy of drug-susceptibility testing in the network.

Table. Summary of clinical features for 14 patients with *Mycobacterium lentiflavum* infection*

Patient no. (ref. no.)	Age	Sex	Concomitant disease	Intercurrent treatment	Side of infection	Susceptibility test	Antimycobacterial therapy	Clinical outcome
1 (3)	19 mo	M	No	No	Cervical lymph node	No	Surgical excision	Recovery (resolved)
2 (4)	42 mo	M	No	No	Cervical lymph node	No	Surgical excision	Recovery
3 (4)	33 mo	M	No	No	Cervical lymph node	No	Surgical excision	Recovery
4 (2)	6 y	F	No	No	Cervical lymph node	ND	Rif, clm/3 wk surgical excision	Recovery
5 (2)	4 y	F	ND	ND	Cervical lymph node	ND	Inh, rif/† surgical excision	Recovery
6 (2)	4 y	M	ND	ND	Cervical lymph node	ND	surgical excision	Recovery
7 (6)	3 y	M	No	No	Cervical lymph node	ND	Clm, eth/6mo	Persistent suppuration
8 (I7)	52 y	F	Antisynthetase syndrome	Corticosteroid	Synovial fluid of wrist	inh R, rif R, str R, eth R, pza R, cys S	inh, rif, eth, pza/† fus, levo, clm/1wk	Exitus
9 (8)	49 y	M	HIV infection	HAART	Blood, lung	clm S, rib S	clm, rib, eth/4mo	Recovery
10 (1)	85 y	F	Diabetes mellitus	ND	Thoracic vertebrae	No	inh, rif, pza/3mo Inh, rif/6mo	Improvement
11 (2)	58 y	M	Rheumatoid arthritis	Corticosteroid	Lung	ND	inh, rib, eth, pza/4mo	No improvement
12 (2)	61 y	F	COPD, ovarian carcinoma	Reiterated chemotherapy	Lung	ND	rif, inh, pza/† rib, eth, clm, cip/†	No improvement (unchanged)
13 (2)	45 y	M	HIV infection, NHL	HAART	Hepatic nodular lesion	ND	rib, clm, eth, cip/2 mo Rib, clm/4mo	Recovery
14 (Molteni)	70 y	F	COPD, lung fibrodystrophy	No	Lung	inh R, str R, rif R, amik R, km R, pza R, oflox R, clm S, eth S, cys S, ter S, rib S	cip, inh/1mo inh, pza, eth, rif/3mo clm/3mo clm, eth, rib, cip/2wk	No improvement

*M, male; F, female; ND, not done; COPD, chronic obstructive pulmonary disease; HAART, higly active antiretroviral therapy; amik, amikacin; clm, clarithromycin; cys, cycloserine; eth, ethambutol, fus, fusidic acid; inh, isoniazid; km, kanamycin; levo, levofloxacin; oflox, ofloxacin, pza, pyrazinamide; rib, rifabutin; rif, rifampin, str, streptomycin; ter, terizidon.
†Treatment duration not determined.

These data demonstrate that establishing and coordinating a focused monitoring program were effective in improving the quality of drug susceptibility results (10).

By describing a recurrent chronic pulmonary infection, our report provides further evidence of the possible role of *M. lentiflavum* as an emerging human pathogen. Our data also stress the diagnostic and therapeutic difficulties in the management of this nontuberculous *Mycobacterium* infection.

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G, N, and P Gene-based Analysis of Chandipura Viruses, India

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An encephalitis outbreak in 2003 in children from India was attributed to Chandipura virus. Sequence analyses of G, N, and P genes showed 95.6%–97.6% nucleotide identity with the 1965 isolate (G gene, 7–11 amino acid changes); N and P genes were highly conserved.

Chandipura virus (CHPV, family *Rhabdoviridae),* was implicated as the cause of a large outbreak of encephalitis in children, involving 329 cases with 183 deaths, from Andhra Pradesh State, India in 2003 (1). On the basis of serologic investigations conducted during the epidemic, CHPV infection led to different clinical manifestations, including subclinical cases, mild fever, and encephalitis; some patients died within 48 hours, while others recovered (1). CHPV was described for the first time in India in 1965, when it was isolated from the serum of a patient with febrile illness (2) during an outbreak of dengue and Chikungunya viruses. The virus was isolated again in 1980 from an encephalopathy patient during an outbreak in children (3). However, the magnitude of the 2003 outbreak was unique. The present study was conducted to understand the relationship of the 2003 isolates with the 1965 strain and to assess association of mutations in G, N, and P genes with different clinical manifestations.

The Study

During the outbreak investigations, 5 CHPV isolates were obtained in cell culture. Table 1 provides details about these isolates. The 1980 isolate was not available for further analysis. These isolates were subjected to reverse transcription–polymerase chain reaction (RT-PCR), according to the previously described method (1). The primers listed in Table 2 were designed on the basis of published sequences and used to amplify and sequence the G, P, and N genes (4,5). The PCR products were purified by using Wizard PCR preps DNA purification Kit (Promega, Madison,WI) and sequenced by using Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an automatic sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

Multiple alignment of nucleotide/amino acid sequences was carried out by using software ClustalX v.1.83. Phylogenetic analyses based on the G, N, and P genes (1593, 1269, 882 nucleotides [nt], respectively) were carried out employing maximum likelihood method in Phylo_win software (6). The reliability of different phylogenetic groupings was evaluated by using the bootstrap test, with 1,000 bootstrap replications, available in Phylo_win. CHPV sequences representing 3 encephalitis cases, including 1 fatal case (patient 2, Table) and 2 febrile cases, were compared.

G gene analysis led to the correction of the sequence reported for the 1965 isolate (accession no. J04350). As compared to the 1965 isolate, the only sequence available in the GenBank database, the following differences were noted for all the 2003 epidemic isolates: 1) an addition of 17 nt after position 1457 base; 2) additions at positions 804, 902, and 1558; and 3) deletions at positions 854 and 869. To confirm these mutations, we sequenced the 1965 isolate available with the repository of the institute and noted that the 1965 sequence did not exhibit the deletions or additions mentioned above. The corrected 1965-CHP-G gene sequence was deposited in GenBank (accession no. AY614717) and used for comparisons. When compared with the corrected sequence, the 2003 epidemic isolates did not exhibit the mutations mentioned above. Although Walker and Kongsuwan resequenced part of the G gene of the 1965 isolate (262 nt) and made necessary corrections (7), these were not deposited in GenBank.

The epidemic isolates exhibited 97% ± 0.3% nucleotide identity (PNI) with each other and 95.6%–96.1% PNI with the 1965 isolate. For CIN0360 and CIN0327 isolates, grown in 2 different cell lines, the PNIs were 100% and 99.9%, respectively. Comparison of partial G gene sequences from clinical samples (N = 3) with the corresponding cell-line isolates documented that, although sequences derived from different clinical samples exhibited unique mutations, except for l substitution in CIN0331M isolate (A1167C), no changes were noted (see online Figure 1; available from http://www.cdc.gov/ncidod/ EID/ vol11no01/04-0602-G1.htm).

Alignment of deduced amino acid sequences of the G protein (530 amino acids [aa]) from different isolates is depicted in online Figure 2 (available from http://www.cdc.gov/ncidod/EID/vol11no01/04-0602-G2.htm). A total of 7 aa substitutions were noted for the epidemic isolates: Leu19Ser, Tyr22Ser, Thr219Ala, Gly222Ala, Arg264Lys, His269Pro, and Thr279Ala. In addition, the brain-derived isolate exhibited 4 more

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Table 1. Details of the Chandipura viral isolates examined

Patient no	Place (state)	Isolate/date of origin	Cell line	Inoculum	Clinical category	Accession no.
1	KarimNagar (AP)*	CIN0327M July 2003	MDCK	Throat swab	Encephalitis	G gene: AY382603 N/P gene: AY614725
1	Karimnagar (AP)	CIN0327R July 2003	RD	Throat swab	Encephalitis	G gene: AY614718 N/P gene: AY614726
2	Karimnagar (AP)	CIN0360R July 2003	RD	Brain	Encephalitis	G gene: AY614719 N/P gene: AY614731
2	Karimnagar (AP)	CIN0360V July 2003	Vero	Brain	Encephalitis	G gene: AY614720 N/P gene: AY614730
3	Karimnagar (AP)	CIN0331M July 2003	MDCK	Throat swab	Encephalitis	G gene: AY614721 N/P gene: AY614729
4	Karimnagar (AP)	CIN0309R July 2003	RD	Throat swab	Fever	G gene: AY614723 N/P gene: AY614728
5	Karimnagar (AP)	CIN0318R July 2003	RD	Throat swab	Fever	G gene: AY614722 N/P gene: AY614727
6	Chandipura (Maharashtra)	CIN6514V† June1965	BS-C-1	Serum	Fever	G gene: AY614717 N/P gene: AY614724

*AP, Andhra Pradesh
†1965 isolate.

substitutions: Ile16Val, Asn30Ser, Ile218Val, and Arg502-Lys. This isolate did not replace Pro → Met at position 367 seen in other epidemic isolates. Two amino acid substitutions (Lys40Arg and Leu424Val) were seen in the isolates from encephalitis cases (CIN0327M and CIN0327R). One isolate from a febrile patient, CIN0309R, showed an additional substitution, Asp213Val.

N gene analysis showed that the 1965 isolate was 96.5%–97.6% identical at the nucleotide level with the epidemic isolates, whereas the epidemic isolates were 97.7% ± 0.3% identical with each other. The isolates grown in different cell lines exhibited 99.3%–99.5% PNI. A single amino acid substitution, Lys37Arg, was noted for all epidemic isolates (online Figure 3; available from http://www.cdc.gov/ncidod/EID/vol11no01/04-0602-G3.htm). In all isolates except CIN0331M, Asp substituted Glu at 364. Additional substitutions, Val413Ile (brain-derived isolate) and Ala163Thr (CIN0309R, from a febrile case), were present.

For the P gene, among epidemic isolates, the PNI was 97.4% ± 0.4%, whereas 95.8%–96.8% identity was observed with the 1965 isolate. The isolates grown in different cell lines were 99%–99.7% identical at the nucleotide level. Glu64Asp substitution was present in all

Table 2. Primers used for amplification and sequencing

Gene	Primers
G gene	
CHAND-G-F1	27-5′ ATGACTTCTTCAGTGACAATTAGT 3′-50
CHAND-G-F2	425-5′ GTCTTGTGGTTATGCTTCTGT 3′-445
CHAND-G-F3	853-5′ TGTGTCCGACCGGGATCAGAGGT 3′-875
CHAND-G-F4	1278-5′ GACAATGAACTACACGAGCT 3′-1297
CHAND-G-R1	1741-5′ TCATCCACCGGGTTGAGATCCAT 3′-1708
CHAND-G-R2	1342-5′ TGAGCATGAGGTAGCTGTGGAT 3′-1321
CHAND-G-R3	30-5′ TCCTCTGAATCTCTGAGGTC 3′-911
CHAND-G-R4	471-5′ TGATTACCAAGAACTCAGAGT 3′-451
N / P gene	
CHAND-N-F1	31-5′ TATAGTAGTACACGAACACT 3′-50
CHAND-N-F2	481-5′ TCTTTGGTCTTTATCGTG TGT 3′-501
CHAND-N F3	871-5′ TTGACCAAGCTGATTCCTACAT 3′-892
CHAND-N-F4	1279-5′ TAGGAGATATTCGAGTGAACT 3′-1299
CHAND-N-F5	1742-5′ TGAGTGCTCTCCAACTTCTGCAGT 3′-1765
CHAND-N-F6	2281-5′ CAGATTCTCTGTTGCTTACCACT 3′-2306
CHAND-N-R1	531-5′ TCTTCTTGTACTCGACCTGT 3′-512
CHAND-N-R2	942-5′ TTGAAGAGTAAGGAGACTTCGT 3′-921
CHAND-N-R3	1320-5′ TCCTGGCGTACTCTGCAACT 3′-1301
CHAND-N-R4	1830-5′ TGTGCTGATCTGCAACAGCCT 3′-1810
CHAND-N-R5	2331-5′ TTCTTCAGAGCTTGCATCTTGAT 3′-2309
CHAND-3′-F	11-5′ TATGTCTTATAAGAATGCTATT 3′-32

the epidemic isolates (online Figure 4; available from http://www.cdc.gov/ncidod/EID/vol11no01/04-0602-G4.htm). A unique single amino acid substitution was noted for 3 isolates: Gln103Arg in CIN0309R (febrile case), Ile180Val in brain-derived isolates, and Asn257Thr in CIN0327M (encephalitis case). In addition, Gly112Glu substitution was recorded in 4 isolates (CIN0327R, CIN0327M, CIN0309R, and CIN0331M); Ala214Val was present in all except CIN0327R, CIN0360R, and Ile270Val in 3 isolates (CIN0318R, CIN0309R, and CIN0331M).

The Figure presents the phylogenetic status of different epidemic isolates. Overall, different CHPV isolates were not very divergent from each other. For G and P gene–based analyses, the brain-derived isolate was closer to the 1965 isolate. No segregation of fever and encephalitis case-derived isolates was noted. Although the topology for the unrooted N gene–based tree was similar, the 1965 isolate remained on a separate branch.

Conclusions

This study showed that the 2003 epidemic isolates were closely related to the 1965 isolate. PNIs were 95.6%–96.1% for the G gene, which is responsible for virus entry into cells and induction of neutralizing antibodies; 96.5%–97.6% for the N gene, mainly associated with cytotoxic T-lymphocyte responses; and 95.8%–96.8% for the P gene, associated with RNA polymerase. Thus, the epidemic was not associated with extensive mutations in these genes. Adaptation to cell cultures did not result in changes in the partial G gene sequences, except for l nt change (A to C at position 1167) for 1 isolate.

The comparison of the deduced amino acid sequences of G protein of 1965 and 2003 isolates documented 7 differences for the epidemic isolates. None of these were in the transmembrane region sequence (482–502 aa) or in the intracytoplasmic region sequence (503–530, the carboxyl end of the protein). No change in the signal sequence was noted for CIN0309R, the only isolate sequenced completely in this region. Additional amino acid substitutions were recorded for the brain-derived isolate. These included Ile16Val, the signal sequence, and Arg502Lys, the transmembrane region sequence. Both N and P proteins were highly conserved, with only 1 aa substitution at positions 37 and 64, respectively. Importance of the amino acid substitutions in these proteins in the pathogenesis of CHPV infection remains to be determined. As modeled by Walker and Kongsuwan (7), major antigenic sites for Vesicular stomatitis virus (New Jersey) neutralization escape mutations correspond to the CHPV G domain exhibiting multiple amino acid changes in epitope VII (Thr219Ala and Gly222Ala) and epitope VI (Arg264Lys and His269Pro).

Phylogenetic analyses based on G and P genes (Figure) showed that the brain-derived isolate clustered with the

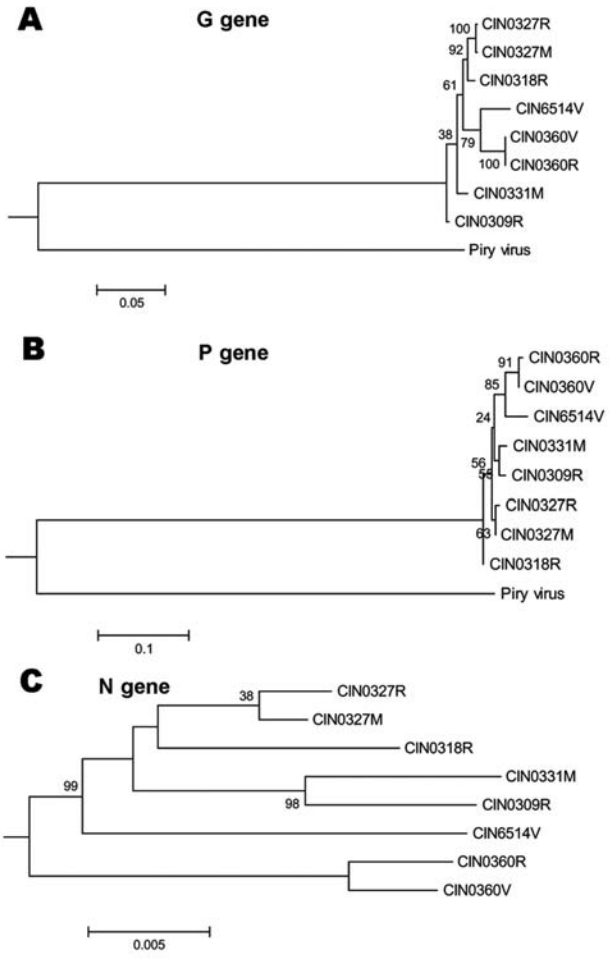

Figure. Phylogenetic analyses of complete G gene (A), P gene (B), and N gene (C) of Chandipura virus isolates. For details on isolates, see Table 2. Percent bootstrap support is indicated by the values at each node. For G and P gene-based analyses, Piry virus (GenBank accession no. D26175) was used as an outgroup. For N gene, an unrooted tree was constructed because the sequence for Piry virus was not available.

1965 isolate. No segregation of the isolates from encephalitis and febrile cases was noted, regardless of the type of the viral gene examined, a finding that suggests the importance of host factors in influencing the outcome of the infection.

In conclusion, the present study shows that Chandipura viruses isolated from human cases in India in 1965 and 2003 were not very divergent. Although several amino acid differences were recorded in G protein, the importance of these changes in the pathogenesis of CHPV infection needs to be determined. Generation of infectious cDNA clones for 1965 and 2003 isolates and assessment of individual genes in the pathogenesis of CHPV infection may help in understanding the relationship of structure to outcome for CHPV infections.

Dr. Arankalle, deputy director of the National Institute of Virology, has been working on hepatitis viruses for 23 years with special contributions to the understanding of hepatitis E. She is also a member of a team that investigates outbreaks of unknown etiology.

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Emergent Strain of Human Adenovirus Endemic in Iowa

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We evaluated 76 adenovirus type 7 (Ad7) isolates collected in Iowa from 1992 to 2002 and found that genome type Ad7d2 became increasingly prevalent. By 2002, it had supplanted all other Ad7 genome types. The association of Ad7d2 with severe illness and death calls for heightened public health concern.

Human adenoviruses are the cause of a wide spectrum acute and chronic diseases. The associations of adenovirus with keratoconjunctivitis, upper respiratory tract infections, pneumonia, gastroenteritis, cystitis, and encephalitis have long been recognized. Recently, molecular methods have shown adenoviruses to be associated with bronchopulmonary dysplasia (1), chronic obstructive pulmonary disease (2), and mycocarditis (3). Adenovirus infections cause severe illness and death in immunocompromised persons, particularly bone marrow transplant recipients (4–6).

In 2002, Erdman et al. (7) reported that 2 emergent genome types of adenovirus type 7 (Ad7) had recently been detected in North American populations. From restriction enzyme studies of 166 archived specimens, the available data suggested that Ad7d2 and Ad7h first appeared in North America in 1993 and 1998, respectively. Both genome types had been associated with epidemics, severe illness, and deaths in populations outside the United States. Since Ad7d2 has been associated with 3 military and 3 civilian epidemics and at least 19 deaths in the United States since 1993, the 2002 report voiced concern regarding a shift in the prevalence of U.S. adenovirus strains and the need to increase surveillance for adenoviral disease. We present a retrospective study of Ad7 isolates in Iowa.

The Study

By using a previously described DNA restriction analysis procedure (7), we studied 76 archived adenovirus isolates collected among influenzalike-illness surveillance sites across Iowa from 1992 to 2002.

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Among the 76 isolates, 40 (53%) were Ad7d2, and 6 (8%) were Ad7h (Figure). The first Ad7d2 specimen was isolated in March 1994 from a child living in south-central Iowa. The first Ad7h specimen was isolated in November 1993 from a child living in north-central Iowa. The latter specimen is the earliest Ad7h detected in North America.

Ad7d2 caused illness among patients in Iowa ranging in age from 3 months to 49 years. Of the patients, 75% were male (cause of overrepresentation is unknown). Although the clinical details are sparse, a number of patients were thought to have influenza or were diagnosed with respiratory distress syndrome. At least 4 children from an Ad7d2 October 2000 epidemic at a long-term care facility in Des Moines, Iowa, died. Ad7d2 isolates were obtained from 12 different sites in Iowa. Ad7h was detected in 4 Iowa counties.

Beginning in 1994, Ad7d2 became increasingly more prevalent across Iowa, displacing Ad7b, the predominant genome type circulating in the United States since the early 1970s (8). In 2002, data suggest that Ad7d2 supplanted all other Ad7 genome types (9 of 9 Ad7 isolates were A7d2) (Figure).

Conclusions

Ad7d2 and Ad7h have only recently been recognized. Ad7d2 was first detected in Israel in 1992; beginning in 1995, it was associated with epidemics of unusually severe respiratory disease with high fevers among children in Japan (9,10). Ad7h was first detected in South America in 1986; since then it has supplanted the previous most prevalent genome type, Ad7c, in Chile, Uruguay, Argentina, and possibly other countries (11). Ad7h has caused pediatric respiratory epidemics, and infected children had longer hospitalizations, had higher temperatures, and required more supplemental oxygen (12). In at least 1 study, up to 94% of adenovirus deaths were attributed to Ad7h (11).

Whether these strains are truly more virulent or whether they better evade the host's immune system is a matter for future study. What does seem to be clear is that a simple mutation (Ad7d2) (9) or recombination (Ad7h) (13) may

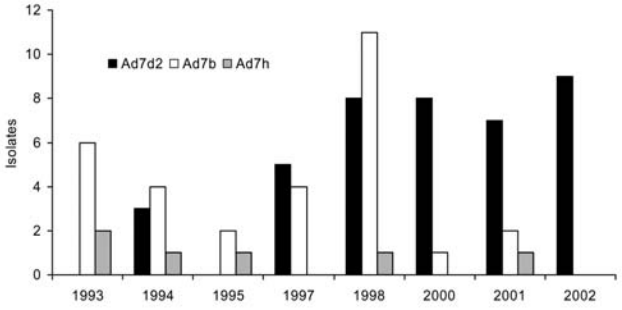

Figure. Number of adenovirus (Ad) isolates collected in Iowa during influenzalike illness surveillance by genome type and year.

generate new adenovirus strains that could result in more epidemics and higher death rates. These strains may then quickly migrate to new areas and cause more epidemics.

Current U.S. surveillance for adenovirus is passive and incomplete. The number of immunocompromised patients in the United States is increasing, and they, in addition to young children, may be at increased risk for severe disease from emergent adenovirus strains. Developing molecular typing strategies for emerging Ad strains seems prudent, as does improving local and national surveillance for adenovirus illness. Considering adenovirus to be a potential nosocomial pathogen seems wise, and researchers should seek to identify effective antiviral therapy for outbreak interventions. These actions will help public health officials better understand the changing epidemiology of adenovirus infections. Because of increased adenovirus morbidity (14,15), the U.S. Department of Defense recently contracted to again produce Ad4 and Ad7 vaccines for military trainees. If civilian populations were identified to be at high risk for serious Ad4 or Ad7 disease, they might also benefit from these vaccines.

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Vibrio parahaemolyticus Diarrhea, Chile, 1998 and 2004

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Analysis of clinical isolates of *Vibrio parahaemolyticus* from outbreaks in Chile in the cities of Puerto Montt in 2004 and Antofagasta in 1998 indicated that 23 of 24 isolates from Puerto Montt and 19 of 20 from Antofagasta belonged to the pandemic clonal complex that emerged in Southeast Asia in 1996.

Vibrio parahaemolyticus infections that caused most seafoodborne gastroenteritis were associated with multiple diverse serotypes until 1996. Since then, however, most cases have been caused by the O3:K6 serotype (1). Molecular studies with pulsed-field gel electrophoresis (PFGE) (2), arbitrarily primed polymerase chain reaction (AP-PCR) (3,4), and multilocus sequence typing (MLST) (5) have shown that these pandemic strains are clonally related. Most isolates of this pandemic complex exhibit a unique sequence within the *toxRS* operon (*toxRS /new*) (4) and possess a unique open reading frame, *orf8* (6), which corresponds to an associated filamentous phage. Other common properties of pandemic strains are the presence of the structural *tdh* gene and the absence of *trh* and urease gene (7). By using the criteria stated above, strains of the pandemic clone have been identified as dominant isolates from clinical cases of diarrhea reported in various Southeast Asian countries, including India, Japan, Thailand, Bangladesh, Taiwan, and Vietnam, as well as from some cases in the United States (1,2,4,8,9) but not in the Southern Hemisphere. Most epidemic isolates initially identified were classified within serovar O3:K6, however, more recently, isolates classified in serovars other than O3:K6 have been identified as also forming part of the pandemic clone (4,8,10,11).

From 1992 to 1997, the Institute of Public Health (ISP) reference laboratory in Chile received 30 isolates from regional hospital laboratories for identity confirmation. However, an outbreak that occurred primarily in the northern city of Antofagasta (23°39′S 70°24′W) from November 1997 to March 1998 caused this number to increase to >300 isolates (12). A second outbreak affecting approximately 1,500 persons (Unidad de Epidemiología, Servicio de Salud Llanquihue-Chiloé-Palena, Chile, pers. comm.) occurred from January to March 2004, mainly in Puerto Montt (41°29'S 72°24'W), a region of usually cold coastal water. Apart from its public health impact, this last outbreak had important economic and social repercussions because this region is one of the main shellfish-producing areas in Chile. The presence of *tdh*-positive *V. parahaemolyticus* was confirmed in random shellfish samples, and the extraction of seafood had to be suspended during the season of high demand.

The Study

To investigate the identity and relationship of the *V. parahaemolyticus* strains causing these two outbreaks to the pandemic strains, we determined their phenotypic and genotypic properties. *V. parahaemolyticus* isolates from Puerto Montt were obtained from rectal swabs from 24 patients 6 to 69 years of age with acute diarrhea. The 20 clinical isolates from Antofagasta were provided by the National Institute of Public Health of Chile. Eleven previously well-characterized strains of the pandemic complex, isolated in Southeast Asia, including strain RIMD2210633 (VpKX), whose genome has been sequenced (13), were included for comparison. VpKX was directly obtained from the culture collection. The other bacterial strains of the pandemic group were provided by Mitsuaki Nishibuchi, Center for Southeast Asian Studies, Kyoto University. They are KXV225, VP2, VP47, VP81, 97LVP2, JKY-VP6, AN-5034, AN-8373, OP-424, KXV737. Three other *V. parahaemolyticus* nonpandemic strains, ATCC17802T (VpD), RIMD 2210856 (VpAQ), 2210086 (VpI), and the *V. alginolyticus* strain ATCC17749 (Va) were directly obtained from the indicated culture collections. The identification of the isolates used in this study was confirmed by API-20E for enterobacteria (bioMérieux, Inc., Hazelwood, MO), according to the manufacturer's instructions. The O and K antigens of the *V. parahaemolyticus* strains were determined by slide agglutination with rabbit antisera obtained from Seiken (Denka Seiken. Co. Ltd. Tokyo, Japan), as described by the supplier.

For PCR, bacterial DNA was extracted from overnight cultures in Luria Bertani broth (supplemented with 1% NaCl) by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). DNA concentration was assessed by the intensity of the DNA band after agarose gel electrophoresis and staining with ethidium bromide. Known amounts of λ-DNA were used as a standard. PCR assays were performed by using approximately 10 ng per

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reaction tube, except for AP-PCR, in which 25 ng were employed. Amplifications of the different markers were performed as previously described: *tdh* and *trh* (7), *orf8* (11), *toxRS/new* (4), and AP-PCR (11). The AP-PCR patterns were recorded and analyzed with GelCompar II (Applied Maths, Sint-Martens-Latern, Belgium). Genetic distance was calculated on the basis of the number of shared bands between isolates, and similarity matrices were calculated by using the Dice coefficient (14). Clustering was achieved by using the unweighted pair group method with arithmetic mean (UPGMA). Kanagawa and urease tests were performed as described by the U.S. Food and Drug Administration/Bacterial Analysis Manual (15).

Twenty *V. parahaemolyticus* isolates obtained from the outbreak in Antofagasta, Chile, in 1998 and 24 from the outbreak of Puerto Montt, Chile, in 2004 were characterized. All of the isolates, excluding 1 from Antofagasta and 6 from Puerto Montt, tested positive for every property of the pandemic clonal complex under analysis (Table). Only 1 isolate from Antofagasta (ATC 230) and 1 from Puerto Montt (PMC 59) differed from the pandemic group in more than 1 property. Five isolates from Puerto Montt diverged from the main group in only 1 of the tested properties; 4 were *toxRS/new* negative, and 1 was serovar O4:K12. The isolates were also analyzed by their AP-PCR patterns. For this analysis, the patterns for 11 South East Asia isolates of the pandemic clone were initially obtained. All of these isolates could be clustered into only 2 groups when either primer P1 or P3 was employed. The patterns obtained with the isolates from Chile were then compared with those of 3 isolates from Southeast Asia, representing the 2 AP-PCR patterns observed within this last group. Three nonpandemic *V. parahaemolyticus* strains and 1 *V. alginolyticus* strain were also included. Every isolate from Chile, except ATC230, clustered with the pandemic isolates when using primer P1 (11) for PCR (Figure A). A

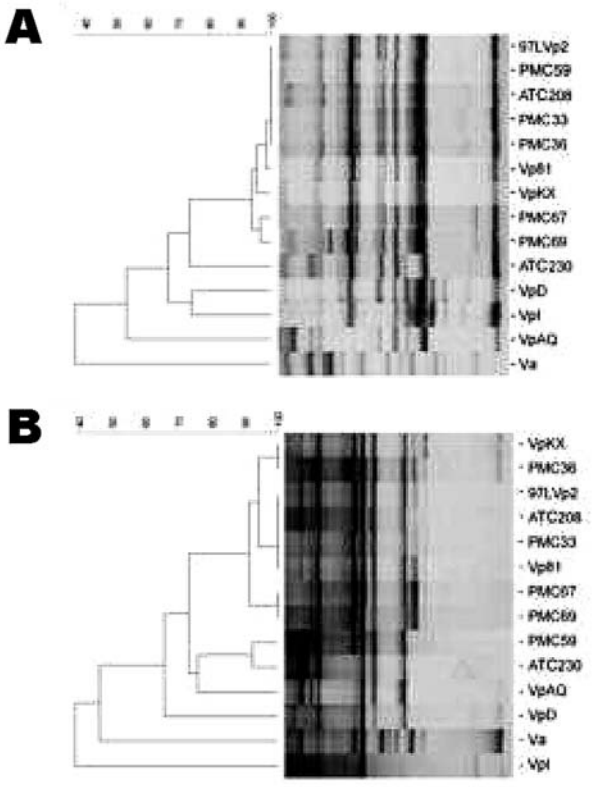

Figure. Representative arbitrarily primed polymerase chain reaction (AP-PCR) patterns for *Vibrio parahaemolyticus* DNA from strains of the outbreaks in Chile and Southeast Asia and dendrogram illustrating the clustering of the patterns by similarity. The percentage of similarity is shown above the dendrogram. A and B are AP-PCR with primers P1 and P3, respectively. The corresponding isolates are indicated on the right. PMC and ATC correspond to isolates from the outbreaks in Puerto Montt and Antofagasta, respectively. VpD, VpKX, VpAQ and VpI correspond to culture collection strains ATCC17802T, RIMD2210633, RIMD 2210856, and RIMD 2210086, respectively. 97LVp2 and Vp81 correspond to strains obtained from Mitsuaki Nishibuchi of the Center for Southeast Asian Studies, Kyoto University.

Table. Properties of *Vibrio parahaemolyticus* strains isolated from outbreaks in Antofagasta, 1998, and Puerto Montt, 2004, in Chile and of strains from culture collections

Strain	Serotype	*tdh*	*trh*	Urease	*orf8*	Ka	*toxRS/new*
Southeast Asia							
VpD	O1:K1			+	−	−	−
VpI	O4:K12	+	−	−	−	−	−
VpAQ	O3:K6	+	+	+	−	−	−
VpKX	O3:K6	+	−	−	+	+	+
Chile, Antofagasta, 1998							
ATC: 208, 210, 211, 213, 214, 216, 217, 218-227, 231, 232	O3:K6	+	−	−	+	+	+
ATC 230	O1.K56	+	+	+	−	+	−
Chile, Puerto Montt, 2004							
PMC:33, 34, 36, 41, 42, 47-49, 52, 53, 55, 57, 58, 60, 62, 65, 69, 72	O3:K6	+	−	−	+	+	+
PMC-46	O4:K12	+	−	−	+	+	+
PMC-61, 66, 67, 68	O3:K6	+	−	−	+	+	−
PMC-59	O4:K12	+	+	+	+	+	−

similar result was observed with primer P3 (Figure B), although in this case PMC59, the isolate that differed from those in the clonal complex in more than 1 property (Table) clustered with ATC230.

Conclusions

Our results show that the spread of the pandemic clonal complex reached the Southern Hemisphere as early as 1998, only 2 years after the strain abruptly appeared in Calcutta, India, in 1996 (3). The diversity observed among the isolates obtained in Puerto Montt did not appear among those from Antofagasta. Five isolates differed in only 1 property, 1 was from a different serovar, and 4 lacked the *toxRS/new* sequence. These findings raise the question of whether this diversity was originally present in the introduced pandemic strain or whether it was locally generated. The serovar O4:K12 observed in 1 of these isolates (PMC46) has been previously observed among isolates from southern Thailand (11). How and when the pandemic strain arrived to these remote regions in the Southern Hemisphere, and why it caused the outbreaks during those years, remains a matter for speculation. The outbreak in Puerto Montt was likely triggered by higher than normal temperatures during the summer months in this region, which is normally cool in all seasons and has an average daily superficial water temperature <16°C year round (Dirección Meteorológica de Chile, pers. comm.).

Acknowledgments

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Plasmodium vivax Malaria

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We report 11 cases of severe *Plasmodium vivax* malaria in Bikaner (western India). Patients exhibited cerebral malaria, renal failure, circulatory collapse, severe anemia, hemoglobinurea, abnormal bleeding, acute respiratory distress syndrome, and jaundice. Peripheral blood microscopy, parasite antigen–based assays, and parasite 18s rRNA gene–based polymerase chain reaction showed the presence of *P. vivax* and absence of *P. falciparum*.

Plasmodium vivax malaria is prevalent in many regions of the world. It accounts for more than half of all malaria cases in Asia and Latin America. Despite the high prevalence of disease caused by this parasite, research into its effects has lagged disproportionately (1).

Organ dysfunction seen in *P. falciparum* malaria is not seen in *P. vivax* infections. Thus, severe malaria is reported with *P. falciparum* but not with *P. vivax* infection. If a patient with *P. vivax* exhibits severe malaria, the infection is presumed to be mixed. When patients have a mixed infection, *P. vivax* may lessen the effect of *P. falciparum* and cause the disease to be less severe. Luxemburger et al. observed that severe malaria is 4.2 times less common in patients with mixed *P. falciparum* and *P. vivax* infections than in those with *P. falciparum* alone (2).

The Study

During the post-rainy season epidemic of malaria from August to December 2003, many persons along the India–Pakistan border had severe malaria caused by *P. vivax*. During the last few outbreaks, we made similar observations, but in 2003 the number of cases was comparatively higher. Clinically severe cases and complications of malaria are commonly due to *P. falciparum* and not to *P. vivax*. Beg et al. reported a patient from Pakistan with central nervous system (CNS) involvement with *P. vivax*, in which the diagnosis was confirmed by polymerase chain reaction (PCR) studies. Beg et al. reviewed the *P. vivax* cases with CNS involvement reported before 2002; however, most were diagnosed by examination of peripheral blood films (PBF) (3).

We searched available literature and could find only isolated reports of severe *P. vivax* malaria with cerebral malaria, thrombocytopenia, disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS), and renal involvement caused by *P. vivax*. In most cases, the diagnosis was made by PBF examination without molecular diagnostic confirmation, thus allowing for potential errors in species diagnosis (4–13). Although detection of *P. vivax* in PBF is the standard, its presence does not rule out undetected mixed infection. To rule out this possibility, all the patients received a thorough diagnostic evaluation, which included PBF examination, a rapid diagnostic test for malaria (OptiMAL test, DiaMed AG, Switzerland, which is based on detecting specific *Plasmodium* LDH antigen by using monoclonal antibody directed against isoforms of the enzyme), and PCR. Our findings are shown in Tables 1 and 2.

All patients were admitted to an intensive care ward dedicated to malaria control. Clinical, biochemical, and radiologic examinations were conducted to establish the diagnosis. Severe malaria was categorized and a treatment regimen of intravenous quinine was instituted according to World Health Organization guidelines (14). Formal approval of the hospital's ethical committee and consent of the patients were obtained for further studies.

The PCR studies were targeted against the 18S rRNA gene of the parasite and were based on conditions reported earlier (15) utilizing 1 genus-specific 5′ primer and 2 species-specific 3′ primers in the same reaction cocktail. Some of the primer sequences were modified for this study: 1) 5′ATCAGCTTTTGATGTTAGGGT ATT 3′–genus specific, 2) 5′ TAACAAGGACTTCCAAGC–*P. vivax* specific, and 3) 5′GCTCAAAGATACAAATATAAGC 3′–*P. falciparum* specific (Figure). Our PCR results in each sample ruled out the possibility of coinfection with *P. falciparum*. Each sample was subjected to a minimum of 4 rounds of PCR with varying template amounts to eliminate the possibility of overlooking *P. falciparum* coinfection. In this report, we have not included 2 samples that showed *P. vivax* infection in PBF examination but showed evidence of mixed infection in PCR examination. The result of PCR analysis of 1 sample is shown in lane 8 of the Figure.

Conclusions

The essential pathologic feature of severe malaria is sequestration of erythrocytes that contain mature forms of the parasite in the deep vascular beds of vital organs, thus producing cerebral malaria, renal failure, hepatic dysfunction, or ARDS. However, severe anemia and thrombocytopenia that causes bleeding diathesis is produced by hemolysis, reduced cell deformity of parasitized and nonparasitized erythrocytes, increased splenic clearance, reduction of platelet survival, decreased platelet production, and increased splenic uptake of platelets, and can be

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Table 1. Clinical characteristics of severe vivax malaria patients

Patient No.	Age (y)/sex	Clinical presentation*	Parasitemia (density) (*P. vivax*/mm^3)	Diagnostic tests for malaria			Other relevant information	Outcome
				PBF	RMDT OptiMAL test†	PCR		
1	30, F	ARDS	6,000	+	Positive	+	Skiagram chest suggestive of pulmonary edema	Died
2	17, M	Renal failure, bleeding diathesis	20,000	+	Positive	+	PT >60 (Control – 16) BT, CT, PT – N	Recovered
3	53, M	Jaundice‡	35,000	+	Positive	+	Epistaxis, hemoglobinurea BT, CT, PT – N	Recovered
4	20, F	Cerebral (GCS– 3) anemia, ARDS, PCF	15,000	+	Positive	+	BP <70 mmHg (systolic) CSF – N CT scan head – could not be done	Died within 5 of admission
5	45, M	Renal failure, jaundice‡	36,000	+	Positive	+		Recovered
6	22, F	Cerebral (GCS – 6) anemia	8,000	+	Positive	+	Puerpural period 3rd gravida CSF – N CT scan head – N	Recovered Baby died on 14th day at residence
7	18, F	Cerebral (GCS – 5) anemia	10,000	+	Positive	+	Primigravida CSF – Normal CT scan head – N	Recovered PMNS - Psychosis Premature delivery Baby survived
8	28, F	Renal failure, ARDS, PCF	44,000	+	Positive	+	Gross hematuria BP <70 mm Hg systolic	Recovered
9	25, F	Jaundice‡, haemoglobinurea	90,400	+	Positive	+	Secondgravida	Recovered Pregnancy continued
10	50, M	Jaundice‡	18,000	+	Positive	+		Recovered
11	18, F	Renal failure, anemia, pulmonary edema	34,000	+	Positive	+	Skiagram chest – pulmonary edema	Recovered Underwent hemodialysis

*All patients were fully conscious except patients 4, 6, and 7. ALT, alanine aminotransferase; ARDS, acute respiratory distress syndrome; AST, aspartate aminotransferase; BT, bleeding time; CT, clotting time; CT, computerized tomography scan of head (non-contrast); F, female; GCS, Glasgow coma scale; LDH, lactate dehydrogenase; M, male; N, normal; PBF, peripheral blood film; PCR, polymerase chain reaction; PMNS, post malarial neurologic syndrome; PT, prothrombin time; TLC, total leukocyte count; RMDT, rapid malaria diagnostic test.
†Positive for *Plasmodium vivax*, *malariae*, or *ovale* because of common LDH isoenzyme antigen and negative for *P. falciparum*.
‡Relevant investigations were done to rule out viral hepatitis and leptospirosis in all patients with jaundice.

produced by *P. vivax* and *P. falciparum* infection. Our clinical data from these patients strongly indicate that *P. vivax* can cause both sequestration-related and nonsequestration-related complications of severe malaria, including cerebral malaria, renal failure, circulatory collapse, severe anemia, hemoglobinurea, abnormal bleeding, ARDS, and jaundice, all of which are commonly associated with *P. falciparum* infections. None of the patients described in this study had evidence of *P. falciparum* infection at the level of antigen (parasite LDH) and 18S rRNA–based PCR test, apart from PBF examination.

This is the first detailed report of severe *P. vivax* malaria. We cannot comment on a pathogenic mechanism causing multiple organ dysfunction and the characteristics of host-parasite interrelationship responsible for it. A detailed prospective study is required to address these issues.

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Vishal Saxena received a fellowship from the Council of Scientific and Industrial Research.

Dr. Kochar is professor and head of the Department of Medicine at S.P. Medical College, Bikaner, India. He is currently engaged in clinical research on malaria.

Table 2. Hematologic and biochemical characteristics of severe vivax malaria patients*

Patient Number	Hb (gm/dL)	TLC (10^3/mm^3)	Platelet count (10^3/mm^3)	Blood sugar (mg/dL)	Blood urea (mg/dL)	Serum creatinine (mg/dL)	Serum bilirubin total/conjugated (mg/dL)	Serum ALT (IU/L)	Serum AST (IU/L)
1	7	10.0	92	94	24	1.0	1.0/0.4	40	30
2	8	5.8	100	90	95	3.5	0.9/0.3	30	27
3	9.5	5.8	50	105	54	1.0	10.3/6.4	360	278
4	5	6.6	120	60	70	1.7	2.6/1.2	36	32
5	10	9.0	87	120	90	3.0	3.1/2.2	39	33
6	6	8.4	80	80	25	0.8	0.9/0.3	25	36
7	6	9.0	96	60	24	1.0	1.0/0.4	40	30
8	8.4	6.0	150	76	60	3.0	1.6/0.6	90	80
9	9.0	8.4	75	138	72	0.6	16/10.9	510	546
10	7.2	8.1	110	80	48	0.8	4.0/3.0	187	136
11	6	10.0	121	90	82	4.5	1.0/0.3	36	32

*Hb, hemoglobin; TLC, total leukocyte count; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

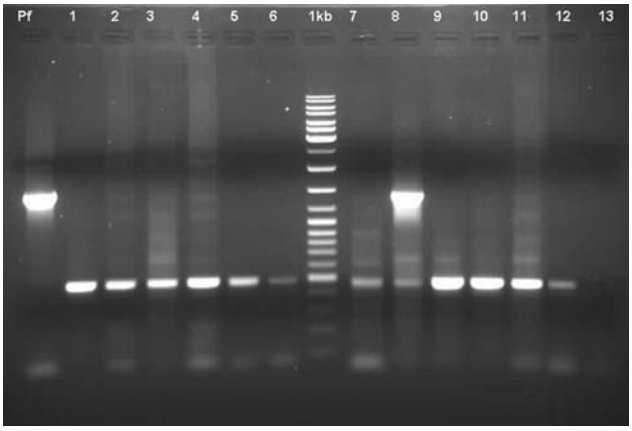

Figure. Polymerase chain reaction analysis of patient samples: lane Pf = positive control showing *Plasmodium falciparum* band at position 1,400 bp; lanes 1–7 and 9–12 = *P. vivax*–positive samples showing band at ≈500 bp; (lanes 1–7 correspond to cases 1–7, and lanes 9–12 correspond to patients 8–11 numbered in Tables 1 and 2.); lane 8 = sample showing bands at ≈1,400 bp and 500 bp, indicating mixed infection; lane 13 = negative control, normal human DNA; lane 1kb = 1kb DNA ladder mix (MBI Fermentas, SM#0331)

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SARS Clinical Features, United States, 2003

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We compared the clinical features of 8 U.S. case-patients with laboratory-confirmed severe acute respiratory syndrome (SARS) to 65 controls who tested negative for SARS coronavirus (SARS-CoV) infection. Shortness of breath, vomiting, diarrhea, progressive bilateral infiltrates on chest radiograph, and need for supplemental oxygen were significantly associated with confirmed SARS-CoV infection.

The clinical course and outcomes of cases of severe acute respiratory syndrome (SARS) in Asia and Canada have been well described (1–6). Most of these studies defined cases based on clinical and epidemiologic criteria with or without laboratory evidence of SARS-associated coronavirus (SARS-CoV) infection. In the event of a subsequent outbreak, distinguishing clinical features associated with SARS-CoV infection may help inform decisions regarding patient evaluation and infection control practices while laboratory results are pending. We describe the clinical characteristics of patients in the United States with laboratory-confirmed SARS and compare them to persons who tested negative for SARS-CoV but had similar illnesses

The Study

We defined a case-patient as a U.S. resident who met the clinical and epidemiologic criteria for suspected or probable SARS and had laboratory evidence of SARS-CoV infection (7). Laboratory evidence of SARS-CoV infection was defined as 1) isolation of SARS-CoV, 2) detection of SARS-CoV RNA by polymerase chain reaction (PCR), or 3) detection of antibodies against SARS-CoV by using enzyme-linked immunosorbant assay or indirect fluorescent-antibody assay (8,9).

After obtaining verbal consent, health officials used a standard questionnaire to interview by telephone patients with suspected or probable SARS and their healthcare providers. Data collected included clinical symptoms, past medical history, relevant exposures, physical examination, radiographic and laboratory findings, and clinical course and outcome.

Case-patients with laboratory-confirmed SARS were compared to a convenience sample of persons who met the clinical and epidemiologic criteria for suspected or probable SARS but subsequently tested negative for SARS-CoV infection. Controls had negative findings on all testing performed for SARS-CoV, including the absence of antibody against the virus in convalescent-phase serum samples obtained >21 days after onset of symptoms. Statistical analysis was performed with SAS software version 8.2 (SAS Institute, Cary, NC). Univariate odds ratios, 95% confidence intervals, and p values for association were calculated by using exact likelihood methods.

We identified 8 case-patients with laboratory-confirmed SARS-CoV infection in the United States. Dates of onset of symptoms were from February 22 to May 24, 2003. The median age of case-patients was 43 years (range 22–53 years); 4 were women. Two case-patients were pregnant (8 weeks' and 19 weeks' gestation) at the onset of their illness. No other major underlying medical conditions were noted.

Seven case-patients reported travel to an area with community transmission of SARS in the 10 days before illness onset, including Hong Kong (n = 4), Toronto (n = 2), and Singapore (n = 1). One case-patient returned to the United States 13 days before illness onset after traveling to Hong Kong with her spouse, who was also a laboratory-confirmed SARS patient. Three (38%) patients visited a healthcare facility during their travel in the 10 days before illness onset, and 4 patients stayed at a hotel associated with a well-defined SARS cluster (7).

Over the course of their illness, findings suggestive of a lower respiratory tract infection developed in all 8

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patients with laboratory-confirmed SARS; these findings included dyspnea (n = 8), rales (n = 5), and hypoxia (n = 5) (Table 1). Symptoms indicative of an upper respiratory tract infection, including rhinorrhea and sore throat, were reported less often. The most common symptoms at illness onset included fever (n = 8), chills (n = 6), and headache (n = 5). Four (50%) patients reported at least 1 respiratory symptom at illness onset. In the remaining 4 patients, respiratory symptoms began 3–7 days after illness onset. The median duration of symptoms before a patient sought medical evaluation was 6 days (range 3–14 days). When patients were first evaluated, the median recorded temperature was 38.6°C (range 37.0°C–40.0°C); the median recorded oxygen saturation on room air was 95% (range 87%–100%).

Gastrointestinal symptoms were also prominent. Six patients reported diarrhea, and 5 reported vomiting during the course of their illness. When present, diarrhea occurred a median of 3 days after onset (range 2–3 days) and was noted before (n = 4), or within 48 hours (n = 2) of receiving antimicrobial therapy. Vomiting began a median of 5 days after onset (range 3–9 days).

All 8 case-patients had radiographic evidence of pulmonary infiltrates during the course of their illness (Table 2). Bilateral pulmonary infiltrates developed in 7 patients during the course of illness with both interstitial and alveolar involvement. Of these, 6 demonstrated worsening chest radiographic findings in week 2 of illness.

The first abnormal chest radiograph was obtained a median of 7 days after onset of symptoms (range 1–14 days). Six patients had an abnormal chest radiograph when first evaluated, including 3 with bilateral infiltrates. Two patients had unremarkable initial chest radiographs on days 6 and 8 after onset, respectively, but were subsequently noted to have infiltrates on chest imaging obtained on days 8 and 11 of their illness.

During the course of their illness, all 8 case-patients received antibacterial therapy. Three patients also received oseltamivir; none was treated with ribavirin. One patient received corticosteroids. Seven patients were hospitalized for a median of 8 days (range 6–15 days). Two patients were admitted to the intensive care unit for 7 and 9 days, respectively; no deaths occurred (Table 2).

Antibodies against SARS-CoV developed in all 8 patients; 3 had positive PCR findings in clinical specimens (1 sputum and 2 stool specimens) (7). Variable levels of clinical laboratory testing were performed (Table 2).

The 8 patients with laboratory-confirmed SARS were compared to 65 SARS-CoV–negative controls (≥18 years old), of whom 14 (22%) had radiographic evidence of pneumonia. Forty-four (68%) controls tested negative for antibodies to SARS-CoV on serum obtained >28 days after symptom onset; the remaining 21 (32%) controls had a negative serologic finding for SARS-CoV 22–28 days after illness onset.

Table 1. Signs and symptoms of patients with laboratory-confirmed SARS-CoV infection, United States, 2003 (N = 8)*

Signs and symptoms	Any time during illness, n (%)	At illness onset, n (%)
Temperature ≥38.0°C	8 (100)	8 (100)
Room air oxygen saturation <94%	5 (63)	NA
Chills/rigors	7 (88)	6 (75)
Headache	6 (75)	5 (63)
Rhinorrhea	2 (25)	0 (0)
Sore throat	1 (13)	0 (0)
Cough	8 (100)	2 (25)
Sputum production	4 (50)	0 (0)
Dyspnea	8 (100)	1 (13)
Rales	5 (63)	NA
Vomiting	5 (63)	0 (0)
Diarrhea	6 (75)	0 (0)

*SARS-CoV, severe acute respiratory syndrome–associated coronavirus; NA, not available.

Patients were similar to controls with regard to age and sex. Fifty-eight (89%) controls reported travel to an area with community transmission of SARS in the 10 days before illness onset. However, patients were significantly more likely than controls to have visited a healthcare facility during their travel (3/8 vs. 4/65; p = 0.03) or to have stayed at the hotel associated with the SARS cluster (3/8 versus 1/65; p < 0.01).

Univariate analysis of clinical features showed that dyspnea, hypoxia, rales, vomiting, and diarrhea were more common among SARS-CoV–positive patients than SARS-CoV–negative controls (Table 3). Case-patients were also significantly more likely than controls to report fever as an initial symptom (8/8 vs. 29/65; p < 0.01) and to have an abnormal chest radiograph at the time of first evaluation

Table 2. Clinical, radiographic, and laboratory features of patients with laboratory-confirmed SARS-CoV infection, United States, 2003 (N = 8)*†

Finding	n (%)
Radiographic findings	
Abnormal chest radiograph	8 (100)
Bilateral infiltrates	7 (88)
Prolonged progression of infiltrates‡	6 (75)
Pleural effusions	3 (38)
Acute respiratory distress syndrome	1 (13)
Laboratory findings	
Hematocrit <36%	2 (25)
Leukocyte count <4,000 cells/mm^3	2 (25)
Absolute lymphocyte count <1,500 cells/mm^3	7 (88)
Platelets <150,000/mm^3	2 (25)
Clinical course and outcomes	
Hospitalized	7 (88)
Admitted to intensive care unit	2 (25)
Received supplemental oxygen	6 (75)
Required mechanical ventilation	1 (13)
Died	0 (0)

*Features present at any time during course of illness.
†SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
‡Radiographic worsening of infiltrates >7 days after onset of symptoms.

Table 3. Univariate analysis for distinguishing clinical features of SARS-CoV–positive cases from SARS-CoV–negative controls, United States, 2003*

Clinical feature	SARS-CoV–positive cases (N = 8), n (%)	SARS-CoV–negative controls (N = 65), n (%)	OR (95% CI)	p value
Dyspnea	8 (100)	32 (49)	10.9 (1.6, ∞)	0.01
Hypoxia	5 (63)	9 (14)	9.9 (1.6, 75.0)	0.01
Rales	5 (63)	15 (23)	5.4 (0.9, 38.9)	0.06
Sore throat	1 (13)	39 (60)	0.1 (0.0, 0.8)	0.03
Vomiting	5 (63)	3 (5)	30.5 (4.0, 315.4)	< 0.01
Diarrhea	6 (75)	18 (28)	7.6 (1.2, 83.6)	0.02
Radiographic evidence of infiltrates at first evaluation	6 (75)	12† (23)	10.0 (1.8, 56.2)	< 0.01
Lymphopenia	7 (88)	24‡ (53)	6.0 (0.7, 288.9)	> 0.10

*SARS-CoV, severe acute respiratory syndrome–associated coronavirus; OR, odds ratio; CI, confidence interval.
†Among 52 controls who had chest radiograph performed.
‡Among 45 controls who had complete blood counts performed.

(6/8 versus 12/52; $p < 0.01$). When the analysis was limited to patients with radiographic evidence of pneumonia, dyspnea and vomiting remained associated with SARS-CoV infection. In addition, SARS-CoV–positive cases were significantly more likely to have bilateral multifocal infiltrates (7/8 cases versus 4/14 controls; $p = 0.02$) and radiographic progression of pulmonary infiltrates into week 2 of illness (6/8 cases versus 0/14 controls; $p < 0.01$).

Conclusions

We compared the 8 U.S. patients with laboratory-confirmed SARS to SARS-CoV–negative controls who met the clinical and epidemiologic criteria for suspected or probable SARS. Our findings indicate that SARS-CoV infection is associated with significant lower respiratory tract disease. Patients with laboratory-confirmed SARS were more likely than controls to have dyspnea, hypoxia, and rales. Patients were also more likely than controls to have an abnormal chest radiograph at the time of first evaluation. These clinical findings are similar to those reported in case series from Asia and Canada, and contrast the clinical manifestations of SARS-CoV with most viral respiratory pathogens including other human coronaviruses (1–5,10). When compared to controls with radiographic evidence of pneumonia, patients with SARS were more likely to manifest dyspnea and progressive bilateral pulmonary infiltrates. This radiographic progression to multifocal infiltrates has been a prominent finding in several previous studies and may prove to be a hallmark feature of the later stages of this disease (1–3,6,11). Among U.S. case-patients, diarrhea and vomiting were also significantly associated with SARS-CoV infection. While gastrointestinal symptoms were a relatively uncommon feature in some previous reports (1,3), diarrhea was frequently reported in other case series, including a major community outbreak at a Hong Kong apartment block (2,4,5,12).

Although previous studies have described the clinical features of patients with laboratory-confirmed SARS, none compared the characteristics of these patients with SARS-CoV–negative controls. Our findings suggest that the combination of gastrointestinal symptoms, dyspnea, and bilateral pulmonary infiltrates may warrant a higher level of suspicion for SARS-CoV infection. By contrast, patients with findings of only upper respiratory tract infection may be unlikely to have SARS. Although moderate lymphopenia was prominent among U.S. case-patients, it was also a fairly common finding among controls who likely had other viral sources of infection. The small number of persons with laboratory-confirmed SARS in the United States limited our power to identify independent clinical predictors of SARS-CoV infection. Further data are needed to describe the full clinical spectrum of SARS-CoV infection and to clarify when specific clinical findings are most likely to occur during the course of illness (13,14).

Early recognition of possible SARS-CoV infection and rapid initiation of infection control precautions are currently the most important strategies for controlling SARS (15). Identifying persons who warrant further investigation for SARS-CoV infection may be difficult on the basis of clinical symptoms alone, especially early in the course of illness. Appropriate preparedness for SARS will thus require vigilant clinicians and public health officials to integrate timely epidemiologic information, astute clinical evaluation, and improved laboratory diagnostic tools.

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Melioidosis in Mauritius

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We report the first case of human melioidosis from Mauritius, where *Burkholderia pseudomallei* has never been isolated. The patient was immunocompromised, had never traveled abroad, and had a history of regular exposure to mud. She became ill at a time when rainfall was higher than the monthly average.

Melioidosis is an infectious disease of humans and animals caused by *Burkholderia pseudomallei* (1). Most documented cases occur in Southeast Asia and northern Australia (2). In recent years, melioidosis has increasingly been recognized in the Indian subcontinent (3). Only a few cases have been reported in South America (4) and Africa (5). In the islands of the southwest Indian Ocean, although no human cases have been reported, *B. pseudomallei* has been isolated from pigs in Madagascar as far back as 1936 (6) and from the soil in Madagascar and La Reunion (7). We report the first case of human melioidosis in the southwest Indian Ocean island of Mauritius, where *B. pseudomallei* has not been isolated previously.

The Case

A 40-year-old patient was admitted to the hospital on January 29, 2004, with fever, generalized weakness, diarrhea, and vomiting. Her temperature on admission was 39.2°C. Results of physical examination were unremarkable. Initial blood tests showed hemoglobin level of 8.5 g/dL, leukocyte count of 4.9 x 10^9/L, and platelet count of 110 x 10^9/L. Erythrocyte sedimentation rate was elevated at 88 mm/h. Her serum glucose level was 8.5 mmol/L, and urea and electrolyte values were normal. She was started on intravenous ciprofloxacin, but her fever persisted, and she became increasingly confused. Two days after admission, therapy was changed to cefotaxime and metronidazole. A blood culture was collected the next day. The following day, she had cellulitis of the right leg. She remained feverish and intermittently drowsy and confused. She died 9 days after being admitted. Her old hospital records became available shortly after her death, and we noted that she had been diagnosed with systemic lupus erythematosus (SLE) in 1994. When she last attended the outpatient department 3 months before her hospital admission, she was prescribed 50 mg azathioprine and 5 mg prednisolone daily. She was a housewife and lived in Cité La Cure, a poor suburb of the capital city Port-Louis. She had never traveled abroad. According to her mother, her home becomes very muddy after heavy rainfall, and her feet were often in mud while performing her household duties.

After 5 days of incubation, an oxidase-positive, gram-negative bacillus was isolated from blood cultures. It produced colonies that appeared dry and rugose on the plates after 48 h and was identified as *B. pseudomallei* by using API 20NE (BioMérieux, Marcy l'Etoile, France) with the profile 1156577. Antimicrobial susceptibility testing by disc diffusion showed the organism to be resistant to colistin, ampicillin, cephalexin, gentamicin, and ciprofloxacin and susceptible to co-amoxiclav, tetracycline, cefotaxime, ceftriaxone, ceftazidime, piperacillin, and meropenem. A large zone of inhibition was seen around the co-trimoxazole disc, within which a thin film of growth was observed.

Conclusions

This case represents the first time *B. pseudomallei* was isolated in Mauritius. The patient must have been infected in Mauritius because she never traveled abroad. We are not aware of any study looking for the organism in soil in this country. Veterinary cases do not appear to have been reported previously in Mauritius (V.B. Groodoyal, pers. comm.). Whether human cases of melioidosis have been missed in the past is not known, and cases may be missed currently. Recognizing the disease depends on awareness on the part of clinicians and on the ability of microbiology laboratories to identify the causative organism (1,8). Before 1998, oxidase-positive, gram-negative bacilli other than *P. aeruginosa* were not identified to species level in laboratories in Mauritius. Since then, at our laboratory, which receives specimens for bacteriologic investigations from all government healthcare institutions, such organisms are routinely identified by API 20NE when isolated in pure culture from blood, but only occasionally when isolated from nonsterile sites such as sputum and pus swabs. Thus, nonsepticemic cases of melioidosis in Mauritius could easily have been missed. Diagnosis also depends on appropriate specimens being sent to the laboratory. Some clinicians routinely request blood cultures from patients with high fever before starting antimicrobial drugs, although in practice, the specimen is often collected by nursing staff after the first dose has already been administered. Other clinicians only request blood cultures if fever persists after a few days of empiric antimicrobial therapy. In the case reported here, prior administration of cefotaxime may have delayed *B. pseudomallei* culture from blood until 5 days of incubation, when the median time to obtain a positive blood culture result is typically 48 hours (1).

An association between rainfall and melioidosis has long been recognized; most cases in Thailand (9) and

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northern Australia (10) occur during the wet season. The increased number of cases noted during the rainy season may be caused by the movement of *B. pseudomallei* from deeper layers toward the surface when dry topsoil is moistened by rainfall (2).

In Mauritius, the rainy season is December to March. In January 2004, 196 mm rainfall was recorded in Port-Louis, which is 37% higher than the 1971–2000 mean rainfall for the region during this month. January 2004 was the sixth wettest January of the past 30 years in Port-Louis. Similarly above-average rainfall was recorded throughout the island in 2004.

Recent reviews have suggested a predominant role for percutaneous *B. pseudomallei* infection in the pathogenesis of melioidosis (11). Studies carried out in regions where melioidosis is endemic have shown that exposure to wet soil and water are associated with increased risk for disease (9). The feet of our patient were regularly exposed to wet soil during rainy periods.

In melioidosis-endemic areas, although a large percentage of the population has been exposed to *B. pseudomallei*, as determined by seroprevalence studies, only a few develop melioidosis (12). Most cases occur in patients with underlying illnesses, such as diabetes mellitus, renal disease, and alcoholism (9,10) or in those who are immunosuppressed (1). Our patient had SLE and was on immunosuppressive drugs. Septicemic melioidosis has been reported in patients who have SLE (13).

This first case of melioidosis in Mauritius occurred in an immunosuppressed patient who had a history of prolonged and regular exposure to mud during a year when rainfall was higher than average. This combination of 3 risk factors does not occur regularly, and it is possible that few additional cases will be recorded in Mauritius in the future. However, clinicians and laboratory staff must remain aware of this disease, particularly because in a noncommunicable disease survey carried out in 1998, almost 20% of the Mauritian population >20 years of age were found to have type 2 diabetes mellitus (14), the most common predisposing condition for melioidosis (1). Determining the distribution of *B. pseudomallei* in soil in Mauritius by conducting environmental investigations will also be useful.

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Dr. Issack trained as a clinical microbiologist. He has been head of the bacteriology section at the Central Health Laboratory of the Ministry of Health in Mauritius since 1997. His main interests include the epidemiology of infectious diseases and antimicrobial drug resistance.

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Distribution and Characteristics of *Escherichia coli* Clonal Group A[1]

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Among 1,102 recent *Escherichia coli* clinical isolates, clonal group A was identified in 17 of 20 (U.S. and non-U.S.) geographic locales, mainly among U.S. isolates (9% vs. 3%; $p < 0.001$) and those resistant to trimethoprim-sulfamethoxazole (10% vs. 1.7%; $p < 0.001$). The extensive antimicrobial resistance and virulence profiles of clonal group A may underlie its recent widespread emergence.

The Study

The recently recognized *Escherichia coli* clonal group A (CGA) accounts for up to 50% of trimethoprim-sulfamethoxazole (TMP-SMZ)–resistant *E. coli* from U.S. women with acute uncomplicated cystitis and pyelonephritis (1–3). Available data show CGA to exhibit a stereotypical virulence factor (VF) profile and a conserved multidrug antimicrobial resistance phenotype, i.e., to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and trimethoprim (ACSSuTTp), which is conjugally transferable on a large plasmid (1,2). Together with CGA's unusual O antigens (O11, O17, O73, and O77), these findings suggested that CGA represents a newly emerged virulent clonal group (1,2). CGA's homogeneity across geographic locales, and the indistinguishable pulsed-field gel electrophoresis (PFGE) profiles of clustered CGA isolates within 1 community (1), suggested recent and possibly ongoing dissemination, a novel paradigm for extraintestinal pathogenic *E. coli* (ExPEC) (4).

To date, CGA has been studied only within the United States, predominantly among women with uncomplicated urinary tract infections (UTI). Its occurrence in some locales has been questioned (5), and its antimicrobial susceptibility profile has been assessed for only 12 drugs (1,2,6). Accordingly, we sought to more fully define the global distribution, host range, virulence characteristics, and resistance phenotypes of CGA.

Twenty clinical microbiology laboratories (10 U.S., 10 non-U.S.) each provided approximately 25 consecutive TMP-SMZ–resistant and TMP-SMZ–susceptible *E. coli* isolates, except for 2 laboratories that provided resistant isolates only (Table 1). Isolates were distributed by specimen type (urine/other) and source (inpatient/outpatient). Antimicrobial drug–resistant and –susceptible isolates were collected approximately concurrently during 2001 from each site, except London (1999) (7) and Columbus (1999 and 2002). When available, data regarding specimen type and host gender, age, and inpatient/outpatient status were provided.

Phylogenetic group (A, B1, B2, or D) was defined by triplex polymerase chain reaction (PCR) (8). Group D isolates were defined as CGA if by random amplified polymorphic DNA (RAPD) analysis they resembled CGA controls with primers 1254 and 1290, or with one of these plus ≥2 of primers 1247, 1281, or 1283 (2). PFGE analysis used *Xba*I (1).

CGA isolates and (2:1) geographically matched controls selected randomly based on the local TMP-SMZ resistance prevalence were tested for 35 ExPEC-associated virulence markers and 13 *papA* alleles by PCR (2). Such typing predicts experimental in vivo virulence (9). Isolates were defined as ExPEC if positive for ≥ 2 of *papA* and/or *papC*, *sfa/foc*, *afa/dra*, *iutA*, and *kpsM* (10). The aggregate virulence score was the number of different virulence markers detected in an isolate, adjusted for multiple detection of certain operons.

O typing was carried out by the *E. coli* Reference Center (University Park, PA). O antigens O1, O2, O4, O6,

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Table 1. Sources of *Escherichia coli* clinical isolates and local prevalence of *E. coli* clonal group A (CGA) by trimethoprim-sulfamethoxazole (TMP-SMZ) phenotype*

Location and type of institution	Patient population	Specimen type(s)	I/P or O/P†	Local TMP-SMZ resistance rate (%)	Total no. isolates	Prevalence of CGA, proportion (%)‡		p value
						TMP-SMZ–susceptible	TMP-SMZ–resistant	
Curitiba, Brazil University medical center	Mostly adults	Urine	Unk	33	60	1/30 (3)	2/30 (7)	
Montreal, Canada University medical center	Mostly adults	Urine, other	I/P, O/P	33	50	0/25 (0)	0/25 (0)	
London, England Teaching hospital	Mostly adults	Urine	Unk	29	46	n.a.	0/46 (0)	
Brest, France University medical center	Mostly adults	Urine, blood	I/P, O/P	30	59	0/29 (0)	0/30 (0)	
Toulouse, France Children's hospital	Children	Urine, other	I/P, O/P	24	50	0/25 (0)	5/25 (20)	0.05
Münich, Germany University medical center	Mostly adults	Unknown	Unk	24	55	0/27 (0)	2/28 (14)	
Afula, Israel Outpatient clinic	Women	Urine	O/P	30	100	1/51 (2)	3/49 (6)	
Barcelona, Spain Private medical center	Mostly adults	Urine, other	I/P, O/P	37	74	2/30 (7)	0/44 (0)	
Göteborg, Sweden University medical center	Mostly adults	Unknown	Unk	10	48	0/25 (0)	1/23 (4)	
Bangkok, Thailand Government hospital	Adults	Urine, other	I/P, O/P	67	48	0/24 (0)	1/24 (4)	
Baltimore, MD, USA Student health center	Mostly women	Urine	O/P	29	62	2/24 (8)	7/38 (18)	
Billings, MT, USA Private medical center	Mostly adults	Urine, other	I/P, O/P	11	50	0/25 (0)	3/25 (12)	
Birmingham, AL, USA University medical center	Mostly adults	Urine, other	I/P, O/P	18	48	1/23 (4)	1/25 (4)	
Chicago, IL, USA County medical center	Mostly adults	Urine	O/P	24	24	NA	7/24 (29)	
Columbus, OH, USA University medical center	Children	Urine	I/P, O/P	13	59	0/33 (0)	6/26 (23)	0.005
Iowa City, IA, USA (Multiple)	Unknown	Urine, other	Unk	18	58	0/27 (0)	4/31 (13)	
Houston, TX, USA Children's medical center	Children	Urine, other	I/P, O/P	36	60	1/32 (3)	7/28 (25)	0.02
Rochester, MN, USA Private medical center	Mostly adults	Urine, other	I/P, O/P	16	54	1/28 (4)	0/26 (0)	
Seattle, WA, USA Teaching hospital	Adults	Unknown	Unk	15	50	0/24 (0)	6/26 (23)	0.02
Tucson, AZ, USA Veterans' medical center	Men	Unknown	Unk	25	47	0/34 (0)	2/13 (15)	

*Isolates were collected in 2001 for all sites except London, England (1999), and Columbus, OH, USA (1999 for susceptible isolates and part of resistant isolates; 2002 for remaining resistant isolates).
†I/P, inpatient; O/P, outpatient; Unk, unknown.
‡Eighteen centers contributed both susceptible and resistant isolates (total, 47 to 100 isolates each; median, 55). Two centers contributed only resistant isolates (Chicago, IL, USA: 24 isolates; London, England: 46 isolates). Specimen type was known for 55 of the CGA isolates and was urine for 42, nonurine for 13 (10 blood, 1 tracheal aspirate, 1 fecal, 1 unspecified nonurine).

O7, O16, O18, O25, and O75 were considered UTI-associated (O-UTI) (10).

Susceptibility testing of the isolates to 30 drugs was carried out by broth microdilution (11) or disk diffusion (nitrofurantoin) (11), by using *E. coli* ATCC 25299 for reference and National Committee for Clinical Laboratory Standards (NCCLS)–specified interpretative criteria (12), except for kanamycin, spectinomycin, and streptomycin (resistant at MICs of ≥25 mg/L, ≥128 mg/L, and ≥8 mg/L, respectively). The aggregate resistance score was the number of different agents to which an isolate exhibited resistance.

Statistical analysis used a Fisher exact or a chi-square test for comparisons of proportions and the Mann-Whitney U test for comparisons of scores. Multiple variables were assessed as predictors of selected outcomes by multivariate logistic regression or multiple linear regression.

The 1,102 clinical *E. coli* isolates were divided evenly by U.S. and non-U.S. origin and TMP-SMZ resistance status (Table 2). Overall, phylogenetic group B2 predominated (44%), followed by group D (27%). Group D was significantly more prevalent among U.S. than non-U.S. isolates (34% vs. 21%: $p < 0.001$). TMP-SMZ–resistant isolates exhibited significant shifts from group B2 toward groups A, D, or both. The nearly 2-fold greater prevalence of group D

Table 2. Prevalence of phylogenetic groups and *Escherichia coli* clonal group A (CGA) according to locale and resistance phenotype

	Prevalence of indicated *E. coli* group by locale and TMP-SMZ* phenotype, no. (column %)											
	Total (N = 1,102)				United States (N = 512)				Non–United States (N = 590)			
E. coli group†	Overall	Susceptible (n = 516)	Resistant (n = 586)	p value‡	Overall	Susceptible (n = 250)	Resistant (n = 262)	p value‡	Overall	Susceptible (n = 266)	Resistant (n = 324)	p value‡
ECOR A	168 (15)	65 (13)	103 (18)	0.02	63 (12)	28 (11)	35 (13)	NS	105 (18)	37 (14)	68 (21)	0.03
ECOR B1	143 (13)	70 (14)	73 (12)	NS	59 (12)	27 (11)	32 (12)	NS	84 (14)	43 (16)	41 (13)	NS
ECOR B2	483 (44)§	277 (54)§	210 (35)	<0.001	213 (42)	134 (54)§	79 (30)§	<0.001	270 (46)	143 (54)§	127 (39)§	<0.001
ECOR D	298 (27)§	98 (19)§	200 (34)	<0.001	176 (34)	60 (24)§ ¶	116 (44)§#	<0.001	122 (21)	38 (14)§ ¶	84 (26)§#	0.001
CGA	68 (6)	9 (1.7)	59 (10)	<0.001	48 (9)	5 (2)	43 (16)	<0.001	20 (3.4)	4 (1.5)	16 (5)	0.02

*ECOR, *E. coli* reference
†TMP-SMZ, trimethoprim-sulfamethoxazole.
‡p values (by Fisher exact test) for comparison of susceptible and resistant isolates are shown when <0.05. NS, not significant.
§For prevalence of group B2 (versus group D) within same column; p ≤ 0.01, McNemar's test.
¶For prevalence of group D among U.S. (vs. non-U.S.) susceptible isolates; p = 0.005 (Fisher exact test).
#For prevalence of group D among U.S. (vs. non-U.S.) resistant isolates; p < 0.001 (Fisher exact test).

among TMP-SMZ-resistant isolates reversed, for U.S. isolates, the "B2 > D" pattern of susceptible isolates.

By RAPD analysis, 23% of group D isolates (6% overall) represented CGA (Table 2). CGA was strongly associated with TMP-SMZ resistance (10% of resistant isolates, 1.7% of susceptible: $p < 0.001$) and a U.S. origin (9% vs. 3%: $p < 0.001$). CGA accounted for 15% of U.S. TMP-SMZ–resistant isolates and 5% of those from abroad ($p < 0.001$), but for a similarly low proportion of U.S. (2%) and non-U.S. (1.5%) susceptible isolates. According to multivariate logistic regression analysis, after accounting for TMP-SMZ resistance and U.S. origin, none of the other source variables (specimen type and host gender, age, and inpatient/outpatient status) was significantly associated with CGA status or appreciably altered the association of TMP-SMZ resistance or U.S. origin with CGA status (not shown).

CGA occurred in all but 3 locales (1–10 isolates per locale), accounting for up to 29% of local resistant isolates (median, 9.5%) (Table 1). CGA was highly prevalent (20%–25%) among resistant isolates from the 3 pediatric centers (Columbus, Houston, and Toulouse) and the veterans' hospital (Tucson). Toulouse exhibited the highest non-U.S. CGA prevalence, whereas nearby Brest lacked CGA (Table 1). CGA accounted for 29% of the ostensibly non-clonal (5) TMP-SMZ–resistant isolates from Chicago. Consistent with this, *Xba*I PFGE profiles of 6 Chicago and 2 reference CGA isolates were highly similar (Figure 1).

CGA isolates differed significantly from controls, according to almost all characteristics analyzed; they were significantly enriched with the O11, O17, and O77 antigens, certain VFs (F16 *papA* allele, *papAHCEFG*, *papG* allele II, *iha*, *iutA*, *kpsM* II, *traT*, and *ompT*), and ExPEC status, but lacked other traits (Figure 2). Aggregate virulence scores were similar for CGA and controls (medians, 8.0) but were less diverse for CGA (range 5–9, vs. 1–13).

By multivariate logistic regression, both CGA status and phylogenetic group B2 were significant independent predictors of ExPEC status, with CGA exhibiting an odds ratio (OR) of 21.9 (95% confidence interval [CI], 6.1–78.2; $p < 0.001$), and group B2 an OR of 2.4 (CI, 1.7–3.4; $p < 0.001$). Additionally, by multiple linear regression, CGA status (standardized regression coefficient [β] = 0.57; $p < 0.001$) and group B2 status ($\beta = 0.596$; $p < 0.001$) predicted VF score better than did O-UTI ($\beta = 0.32$; $p = 0.002$).

Antimicrobial resistance phenotypes were compared between CGA isolates and a subset of the controls, which had been selected to provide an unbiased distribution of TMP-SMZ resistance (Figure 2). CGA exhibited a higher prevalence of resistance to 10 individual drugs, any drug, and ACSSuTTp, whereas controls exhibited a low but measurable prevalence of resistance to 7 drugs to which all CGA isolates were susceptible. Accordingly, resistance

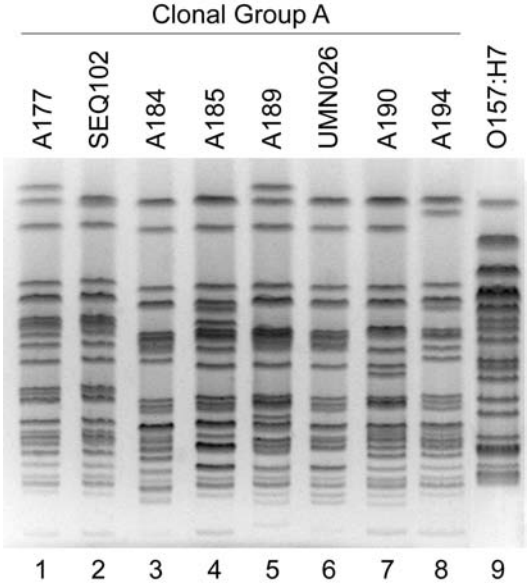

Figure 1. *Xba*I pulsed-field gel electrophoresis profiles of *Escherichia coli* clonal group A (CGA) isolates and *E. coli* O157:H7. Lane numbers are shown below the gel image. Six CGA isolates from Chicago, IL ("A" series identifiers; lanes 1, 3, 4, 5, 7, and 8) exhibit similar profiles to reference CGA isolates SEQ102 (from California; lane 2) and UMN26 (from Minnesota; lane 6) (1). *E. coli* O157:H7 isolate G5244 (lane 9) exhibits a distinctive profile.

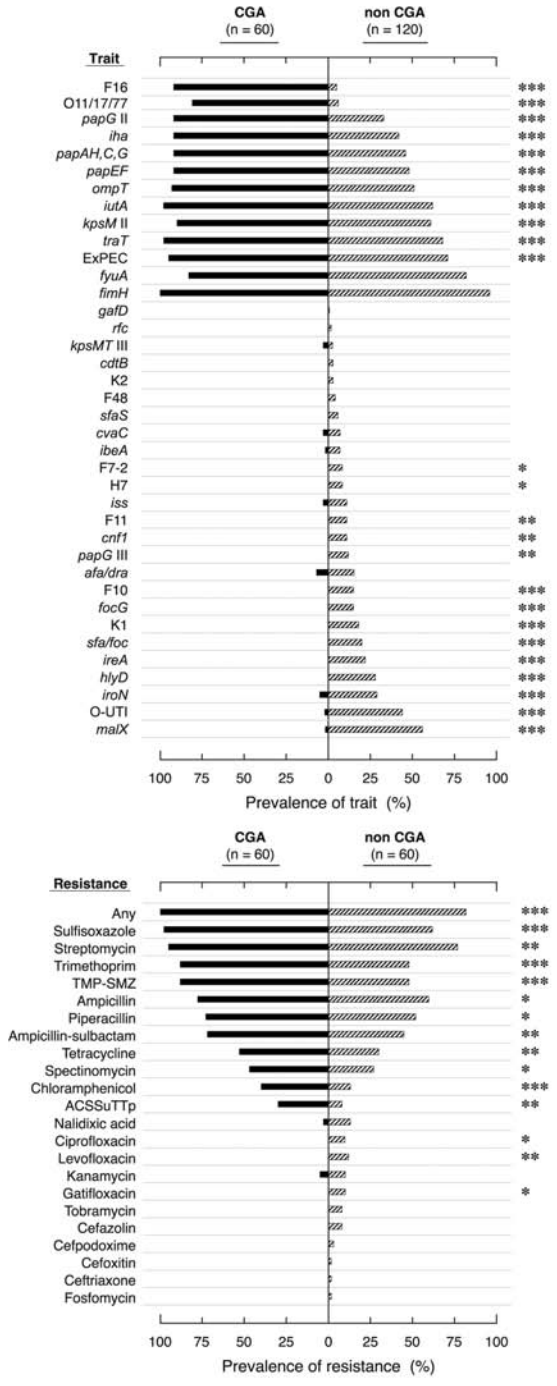

Figure 2. Virulence traits (top) and antimicrobial resistance phenotypes (bottom) of *Escherichia coli* clonal group A (CGA) isolates versus non-CGA controls. Percentage of isolates positive for each virulence trait (top) or resistance phenotype (bottom) is shown by black bars to left of midline (for CGA) or grey bars to right of midline (for controls). No isolates were positive for *bmaE* (M fimbriae), or resistant to amikacin, aztreonam, cefepime, ceftazidime, ertapenem, imipenem-cilistatin, nitrofurantoin, piperacillin-tazobactam, or ticarcillin-clavulanate. p value symbols at right of each chart, from Fisher exact or χ^2 test, indicate significant differences between CGA and non-CGA. *, p <0.05; **, p ≤0.01; ***, p ≤0.001.

scores were significantly higher for CGA than controls (median 8.0 [range 1–11] vs. 5.5 [range 0–15]; p = 0.001). Among TMP-SMZ–resistant isolates, TMP-SMZ MICs ranged from 32/608 mg/L to >520/9,880 mg/L; 61% of resistant isolates had MICs ≥520/9,880 mg/L. CGA accounted for 67% of this highly resistant population (p = 0.01 vs. controls).

The U.S. and non-U.S. CGA isolates exhibited only 2 statistically significant differences for VF profiles, O antigens, and resistance phenotypes, with *iroN* (p = 0.03) and absence of *ompT* (p = 0.008) associated with non-U.S. isolates.

Conclusions

We found that the recently described *E. coli* CGA is globally but heterogeneously distributed and more prevalent within the United States than abroad. Clonal group A affects diverse host populations (including inpatients, outpatients, adults, children, men, and women), infects urinary tract and nonurinary tract sites, is strongly associated with TMP-SMZ resistance (particularly high-level resistance), and exhibits a robust virulence profile suggesting enhanced extraintestinal virulence. This combination of resistance and virulence may account for CGA's recent emergence as a broadly disseminated "epidemic clone" (1–3,6,13,14).

The greater overall prevalence of CGA within the United States suggests a U.S. origin for CGA. However, it also could reflect more rapid clonal expansion within the United States due to enhanced dissemination or more favorable conditions for outgrowth, including possibly less competition from other potential occupants of the same niche(s). The variable prevalence of CGA among even closely situated locales, both within the United States and abroad, might reflect true geographic heterogeneity, versus locale-specific differences in patient populations, selection criteria, and collection intervals. Studies that compare well-defined, homogeneous, concurrent populations from different locales are needed.

The new evidence of CGA as a prominent TMP-SMZ–resistant pathogen among children and veterans extends the known host range of CGA, consistent with a recent report of CGA as a community-wide TMP-SMZ-resistant pathogen in Denver, Colorado (6). This finding illustrates CGA's pathogenic versatility.

That 29% of the TMP-SMZ–resistant isolates from Chicago, Illinois, proved to be CGA and resembled reference CGA isolates by PFGE, despite a previous PFGE-based assessment of nonclonality (5), illustrates the limitations of conventional PFGE analysis as a screen for CGA. We have developed a CGA-specific PCR assay, based on a single nucleotide polymorphism within *fumC*, to provide improved screening for CGA (15).

CGA's robust, highly homogeneous consensus VF profile (F16 *papA* allele, *papG* allele II, *iutA*, *kpsM* II, *traT*, and *ompT*) suggests considerable extraintestinal virulence potential, an inference supported by experimental data indicating that CGA is able to compete successfully with classic group B2-derived pathogens in a mouse UTI model (J.R. Johnson, unpub. data). Determination of which VFs of CGA contribute most to pathogenicity may identify future targets for preventive interventions.

In our study, antimicrobial resistance was more extensive and, with TMP-SMZ, more potent for CGA than controls. These factors may be contributing to CGA's global emergence. Conceivably, high-level TMP-SMZ resistance could allow CGA to out-compete even other TMP-SMZ–resistant strains. CGA's resistance involved primarily older agents, whereas fluoroquinolone and cephalosporin resistance was confined to controls. However, the emergence of fluoroquinolone resistance within the closely related O15:K52:H1 clonal group (2,13) suggests that this potential exists also for CGA.

The study's strengths include large population, broad geographic sampling, matching of CGA and control isolates, and extensive range of traits analyzed. Limitations include the heterogeneous inclusion criteria, gaps in global surveillance, possible type I errors from multiple comparisons, and limited sampling per locale.

In summary, we found CGA to be a globally disseminated, multidrug-resistant clonal group of pathogenic *E. coli* with a broad range of human hosts. Although more prevalent within the United States than abroad, CGA exhibits characteristic O antigens, resistance markers, and virulence traits wherever encountered. Further study is needed of the origins, virulence mechanisms, geographic distribution, clinical associations, and modes of dissemination of CGA.

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Invasive Group A Streptococcal Infection in High School Football Players, New York City, 2003

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After being notified that 2 high school football teammates from New York City were hospitalized with confirmed or suspected invasive group A streptococcal infections, we conducted an investigation of possible spread among other team members. This investigation highlights a need for guidelines on management of streptococcal and other infectious disease outbreaks in team sport settings.

Group A *Streptococcus* (*S. pyogenes*; GAS), a bacterium commonly found on the oropharynx and skin, causes infections ranging from relatively mild and localized to invasive and potentially life-threatening (1). In October 2003, the New York City Department of Health and Mental Hygiene was notified of 2 high school varsity football teammates hospitalized on the same day, one with culture-confirmed GAS septic thrombophlebitis and another with suspected GAS pyoderma.

Case Studies

A 17-year-old male high school student (patient 1) visited the emergency department of a local hospital in Bronx, New York, on October 20; he had bilateral groin pain that had begun 2 days previously, shortly after he played in a high school football game. He complained of pain with swallowing but denied fever or chills. His oropharynx was erythematous but without tonsillar exudate. Abdominal examination showed suprapubic tenderness and voluntary guarding. His thighs were tender bilaterally. A rapid streptococcal antigen throat swab test result was positive. Computed tomographic (CT) scan of the abdomen suggested acute appendicitis, and CT and duplex ultrasound confirmed a right external iliac vein thrombus. Exploratory laparotomy showed a normal appendix and no evidence of acute pathology. Blood cultures were positive for GAS. He was treated with antimicrobial agents and anticoagulation for septic external iliac thrombus.

Another 17-year-old male high school student (patient 2) from the same football team was hospitalized at the same hospital on October 20 for a fluctuant right leg mass. Two weeks previously, he had sought medical care for sore throat and erythematous skin overlying his right posterior calf, where he was hit by a helmet during a game. A rapid streptococcal antigen throat swab test result was positive, and he was treated with amoxicillin for 10 days. Two days before admission, he noticed increased swelling and a blister on his right calf. He was afebrile, and an oropharyngeal examination was normal. His right lower leg was swollen posteriorly from behind the knee to the ankle. Blood culture was negative. The right calf swelling was drained, and ≈1 L of purulent fluid was removed. Results of a rapid streptococcal antigen test of the aspirated fluid were positive, but Gram stain and cultures were negative.

On October 24, 2003, hours before the team's homecoming game, the New York City Department of Health and Mental Hygiene was notified about these hospitalized teammates. The health department also learned that other varsity teammates recently had sore throats and skin lesions. Given the close physical contact and sharing of equipment and water bottles among players, the health department launched an epidemiologic investigation that evening at the high school. Teammates reporting symptoms consistent with GAS infection were to be excluded from play. However, since none of the players reported having symptoms consistent with acute GAS disease, the homecoming game was allowed to proceed.

A screening questionnaire was designed to identify persons with current or recent throat and skin infections and risk exposures for GAS. Thirty-three varsity players and 5 coaches were interviewed. Throat cultures from the 33 varsity players and 5 coaches were screened for GAS by using standard methods (2). Isolates from positive culture specimens were characterized by using pulsed-field gel electrophoresis (PFGE) with *Sma* I and *Sfi* I enzymes (3). GAS isolates were tested for susceptibility to chloramphenicol, clindamycin, erythromycin, penicillin G, tetracycline, and vancomycin by using standard agar disc diffusion (Kirby-Bauer, Remel, Lenexa, KS).

Positive isolates were characterized at the World Health Organization Collaborating Center for Reference and Research on Streptococci at the University of Minnesota Medical School. Isolates were serologically grouped and

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further subtyped by opacity factor, T-agglutination pattern, and *emm* sequencing (2,4). To prevent additional cases of GAS, antimicrobial prophylaxis with either penicillin and rifampin or azithromycin was recommended for all varsity teammates and coaches.

The 33 varsity players and 5 coaches ranged in age from 15 to 55 years (median age 17 years). None reported symptoms consistent with acute GAS infection. Among the 38 throat cultures obtained, 3 (8%) were positive for β-hemolytic GAS (Table). The only reported exposure to ill persons was contact with the 2 patients.

One GAS isolate from an asymptomatic player (specimen C) was *emm*-type 6, and another (specimen B) was *emm*-type 75. The isolate from a third asymptomatic player (specimen D) was indistinguishable from that of the blood culture (specimen A) from patient 1 by PFGE analysis and *emm* sequencing (type 82).

During the investigation on October 24, the New York City Department of Health and Mental Hygiene learned that the junior varsity team shared equipment and water bottles with varsity players but did not have the same close, skin-to-skin contact with the patients as did varsity teammates. Therefore, the health department screened all junior varsity players on October 27 but provided treatment only for those whose throat cultures were GAS-positive. Among 51 junior varsity team members and 3 coaches screened, 4 (7%) had GAS-positive throat cultures (Table). Two of the 4 reported current or recent symptoms, including headache and stomachache. The other 2 denied having symptoms or contact with ill persons. The positive isolates from junior varsity players were unrelated by PFGE or *emm* typing to strains from other varsity or junior varsity players, including patient 1.

Conclusions

GAS infections are typically spread through contact with mucus from infected persons or with infected skin lesions (6). GAS infections in athletic settings could be transmitted by person-to-person contact, airborne or droplet spread, or exposure to shared-use items (7).

In 2002, guidelines for preventing invasive GAS among household contacts of case-patients were published (8). These guidelines address the management of household contacts but not of other types of close, physical contacts (e.g., athletic teams). General guidelines exist regarding preventing infectious diseases in athletic settings (7,9). However, no specific recommendations have been published regarding prevention strategies after invasive GAS cases have been identified among athletes in contact sports such as football, a setting where spread of GAS is possible because of repetitive and forceful skin-to-skin contact and resultant trauma.

Although the recommendations advise against routine screening and prophylaxis for household contacts, prophylaxis is recommended in certain situations where host factors are associated with increased risk for invasive disease (8). We hypothesized that teammates are at least as likely to share drinks from common sources and have close, skin-to-skin contact as are household contacts. Moreover, we believed that football teammates of our case-patients were potentially at greater risk for invasive GAS than typical household contacts because of their greater risk for skin trauma. Any resultant skin disruption could provide a portal of entry for a more virulent GAS strain from an infected teammate, potentially leading to invasive GAS disease even among otherwise healthy persons (10).

Because of the severity of the patients' illnesses and the theoretically increased risk for invasive GAS, we screened and provided antimicrobial prophylaxis to the varsity team during the initial investigation without awaiting final culture results. However, the 8% GAS positivity rate was consistent with published estimates of the overall background colonization rates among schoolchildren (11%–25%) in nonoutbreak settings (11–13), and lower than the second-

Table. Characterization of group A *Streptococcus* isolates from high-school varsity and junior varsity football players, New York City, 2003

Specimen origin	Site	Antimicrobial susceptibility*	PFGE†	M/OF†/*emm* type
Varsity player				
A‡	Blood	Susceptible to all antimicrobial agents tested		82
B	Throat	Resistant to erythromycin, susceptible to all others	Unrelated§	75
C	Throat	Susceptible to all antimicrobial agents tested	Unrelated	6
D	Throat	Intermediate to tetracycline, susceptible to all others	Indistinguishable	82
Junior varsity player				
E	Throat	Susceptible to all antimicrobial agents tested	Unrelated	89
F	Throat	Susceptible to all antimicrobial agents tested	Unrelated	44/61
G	Throat	Susceptible to all antimicrobial agents tested	Unrelated	28
H	Throat	Susceptible to all antimicrobial agents tested	Unrelated	118

*Antimicrobial agents tested: chloramphenicol, clindamycin, erythromycin, penicillin G, tetracycline, vancomycin.
†Entries represent putative genetic relatedness to the case-patient no. 1 strain A based on *Sma*I and *Sfi*I DNA restriction patterns by using categories as defined by Tenover et al. (5). The results obtained with *Sfi* I correlated completely with the results obtained with *Sma*I; PFGE, pulse-field gel electrophoresis; OF, opacity-factor.
‡Case-patient #1.
§Specimen B was nontypable with *Sma*I but was typable with *Sfi*I.

ary carrier rate among household contacts of persons with streptococcal illness (14). Only 1 varsity player carried the same GAS strain as his septicemic teammate. One of the unrelated strains isolated from an asymptomatic player was resistant to erythromycin. This finding emphasizes the possibility that in some situations macrolide antimicrobial agents may not be the most effective for prophylaxis. Given the absence of any additional cases of invasive GAS or increased GAS carriage rates among the varsity team, we screened the junior varsity team but treated only players with positive throat cultures.

Both hospitalized patients tested positive on rapid streptococcal antigen throat swab tests. Only patient 1 had culture-confirmed *emm*-82 invasive GAS; no positive culture was obtained from patient 2. When patient 2 was assessed for a fluctuant leg mass, he had already received antimicrobial agents for streptococcal pharyngitis. No throat culture was obtained at initial diagnosis, and subsequent leg fluid culture was sterile. Although not approved for testing specimens other than throat swabs, rapid antigen testing (Thermo BioStar Acceava Strep A Test, Thermo Electron Corporation, Louisville CO) of the leg fluid was positive for streptococcal antigen, suggesting that GAS was the etiologic agent. The inability of microbiologic techniques to yield a GAS isolate from patient 2 prevented definitive linkage of the 2 invasive cases. However, the epidemiologic link and temporal proximity of infections suggest that they probably were infected by the same GAS strain. Furthermore, the high sensitivity and specificity of rapid antigen detection in diagnosing GAS pyoderma in children have been demonstrated (15). Of note, *emm*-82 accounted for only 3.1% of sterile site isolates in the United States from 1995 to July 2001 (16). Thus, even relatively uncommon GAS strains can cause serious infections.

How many cases, if any, might have been prevented by our efforts cannot be determined. However, we received no additional reports of invasive GAS that were epidemiologically linked to this football team in the 10 months after the investigation.

More information is needed regarding the appropriate preventive measures for GAS outbreaks among contacts of invasive GAS patients in athletic settings. Healthcare professionals who care for athletes should be reminded of the potential for outbreaks of infectious diseases. Public health authorities should share their experiences so additional information can be gathered on which to establish consensus guidelines for prevention and control of future invasive GAS clusters or outbreaks occurring among contact sport participants.

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Mycobacterium tuberculosis Beijing Genotype, Northern Malawi

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In a 7-year population-based study in Malawi, we showed that Beijing genotype tuberculosis (TB) increased as a proportion of TB cases. All the Beijing genotype strains were fully drug sensitive. Contact histories, TB type, and case-fatality rates were similar for Beijing and non-Beijing genotype TB.

The Beijing genotype family of *Mycobacterium tuberculosis* may be spreading worldwide. The genotype may be particularly virulent and have a predilection for developing drug resistance; the multidrug-resistant W strain is a member of this family (1). Few studies have examined trends in the prevalence of Beijing genotype strains over time, and little is known about their distribution or characteristics in Africa (2), although drug-resistant Beijing genotype strains have been identified in Cape Town and Nairobi (3,4). We describe Beijing genotype tuberculosis (TB) in a population-based, 7-year study in Malawi.

The Study

As part of the Karonga Prevention Study, a longstanding epidemiologic study of TB and HIV in a rural area of northern Malawi (5,6), persons suspected of having TB in Karonga District are identified at the hospital and peripheral clinics. Sputum is taken for smear and culture. Cultures are also performed on lymph node aspirates and other samples as appropriate. Treatment follows Malawi National TB Control Programme guidelines, and drug resistance has remained low, with initial isoniazid resistance ≈6% during this study (7,8).

Positive cultures from all patients in whom TB has been diagnosed since late 1995 have been DNA fingerprinted (9). Fingerprinting used IS*6110* restriction fragment length polymorphism (RFLP) following standard guidelines (10), and RFLP patterns were compared by using computer-assisted (Gelcompar 4.1, Applied Maths, Kortrijk, Belgium) visual comparison. Beijing genotype strains were identified by comparing each RFLP pattern to 19 reference RFLP patterns (https://hypocrates.rivm.nl/bnwww/index.html) with 1% position tolerance. Taking strains with patterns with ≥80% similarity to any of the reference patterns as Beijing genotype, and spoligotyping strains showing 75%–80% similarity, this method has been shown to identify Beijing genotype strains with a sensitivity of 96% to 100% and specificity of 98% to 100%, using spoligotyping as the gold standard (11). In the present study, we spoligotyped all strains with RFLP patterns with >70% similarity to the reference patterns in order to maximize the sensitivity and specificity for identifying Beijing genotype strains.

From late 1995 to early 2003, a total of 1,194 specimens were fingerprinted from 1,044 persons (84% of patients in whom culture-positive TB was diagnosed in this period). After excluding likely laboratory errors (9) (25 isolates) and multiple isolates per person, we found 406 different RFLP patterns in samples from 1,029 patients. Overall, 757 patients (73.6%) were clustered, i.e., they had an isolate with an RFLP pattern that was identical to that of at least 1 other patient in the study.

Thirteen different RFLP patterns from isolates from 45 patients matched the Beijing genotype reference patterns by ≥75%. At least 1 isolate from each of the 13 strains was spoligotyped: 12 patterns were confirmed as Beijing genotype. The RFLP pattern from the strain with the non-Beijing genotype on spoligotyping, present in only 1 patient, matched the RFLP pattern of a reference strain by 81% but showed a <73% match with the other reference RFLP patterns and a <71% match with the other 12 Beijing

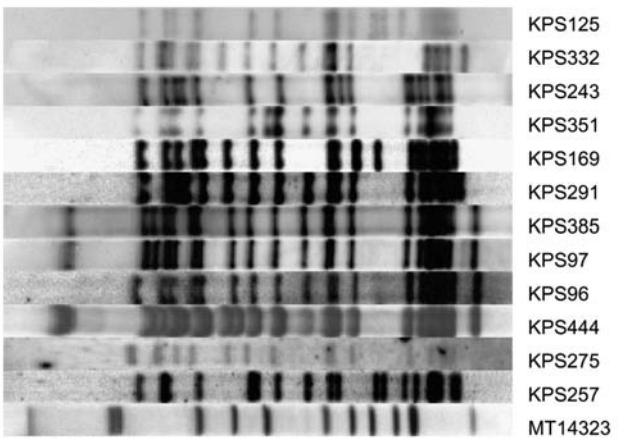

Figure 1. Restriction fragment length polymorphism patterns of 12 Beijing strains from Karonga District, Malawi. All strains were 79% related to at least 1 of the other Beijing strains found in the district. Strain MT14323 is a reference strain.

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Table. Comparison of persons with Beijing and non-Beijing genotype *Mycobacterium tuberculosis*

Characteristic	Beijing genotype, n (%)	Other genotypes, n (%)	p value
Sex			0.001
Male	10 (22.7)	472 (47.9)	
Female	34 (77.3)	513 (52.1)	
Age group			0.3
<15	2 (4.6)	16 (1.6)	
15–29	17 (38.6)	306 (31.1)	
30–44	16 (36.4)	423 (42.9)	
≥45	9 (20.5)	240 (24.4)	
Born in Malawi			0.08
Yes	38 (90.5)	747 (79.2)	
No	4 (9.5)	196 (20.8)	
Moved in last 5 y			0.8
No move	16 (40.0)	343 (41.2)	
Within district	10 (25.0)	188 (22.6)	
Outside district	9 (22.5)	226 (27.1)	
Outside country	5 (12.5)	76 (9.1)	
HIV status			0.4
Positive	15 (57.7)	396 (65.6)	
Negative	11 (42.3)	208 (34.4)	
Previous tuberculosis (TB)			0.9
Yes	3 (6.8)	74 (7.6)	
No	41 (93.2)	904 (92.4)	
BCG scar			0.7
Yes	23 (74.2)	486 (69.7)	
No	6 (19.4)	176 (25.3)	
Doubtful	2 (6.5)	35 (5.0)	
Contact with TB patient in district			0.6
Yes	22 (50.0)	453 (46.0)	
No	22 (50.0)	532 (54.0)	
Type of TB			0.4
Smear-positive pulmonary	33 (75.0)	711 (72.2)	
Smear-negative pulmonary	10 (22.7)	202 (20.5)	
Extrapulmonary	1 (2.3)	72 (7.3)	
Drug resistance			0.2
Sensitive	44 (100)	920 (93.9)	
Resistant isoniazid only	0	38 (3.9)	
Resistant isoniazid plus	0	22 (2.2)	
Died during treatment			0.7
Yes	13 (32.5)	224 (29.6)	
No	27 (67.5)	532 (70.4)	

genotype RFLP patterns from Malawi. In the whole dataset, only 3 other strains had RFLP patterns that matched the reference set by >70% (and <75%): spoligotyping confirmed that these strains were not Beijing genotype. The RFLP patterns of the 12 Beijing genotype strains from Karonga District are shown in Figure 1.

Overall 44 (4.3%) of 1,029 patients had Beijing genotype TB (Table). Beijing genotype strains were found in persons from all areas of the district, and they were no more common in those who had lived outside Malawi. Of the 8 people with Beijing genotype TB who had been born outside Malawi or lived outside Malawi in the last 5 years, 4 were from Tanzania and 4 from Zambia. Among TB patients, no association was seen between Beijing genotype and HIV status. Beijing genotype strains were more common in women than in men, and this difference persisted after adjusting for age and year of diagnosis (adjusted odds ratio [OR] 2.9, 95% confidence interval [CI] 1.4–6.0).

The proportion of TB due to Beijing genotype strains increased over time (Figure 2, OR 1.2, 95% CI 1.0–1.4 for each year of study, p = 0.03) and was slightly lower in older persons (p = 0.2, test for trend). Of the 12 different Beijing genotype RFLP patterns, 5 were shared by ≥2 patients (i.e., were clustered), and the remaining 7 were unique in the dataset. The proportion of patients who were clustered was slightly higher in those with Beijing genotype strains than among those with other strains (84% vs. 71%, p = 0.07, after excluding those with <5 bands on the RFLP pattern).

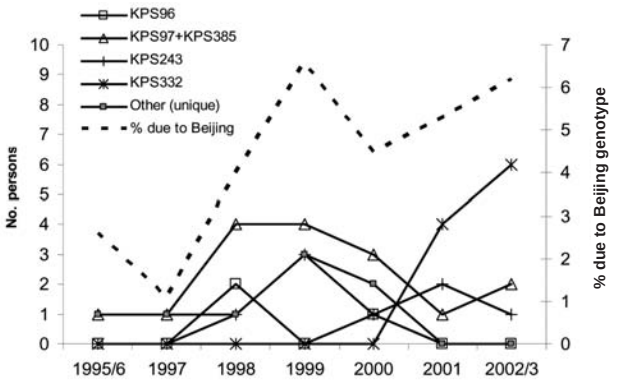

Figure 2. Beijing genotype tuberculosis (TB) in Karonga District, Malawi, over time. The solid lines show the number of persons with each Beijing genotype restriction fragment length polymorphism (RFLP) pattern, and the dotted line shows the proportion of culture-positive TB cases that are due to the Beijing genotype. Because strains KPS97 (14 patients) and KPS385 (2 patients) differed by only 1 band on RFLP, they are shown together.

Both the time trend and the association with age suggest an increase in Beijing genotype TB, and the association with clustering might suggest higher transmissibility. These results were influenced by the appearance of strain KPS332 from October 2001 onwards, affecting 10 patients. This strain was 83% similar to strain KPS96 and more distantly related to the other strains, so this strain was probably newly introduced into the district. However, an increase over time was also apparent in the data before 2001 (OR 1.3, 95% CI 0.98–1.8, for each year of study, p = 0.07), and the trend with age was stronger for this earlier period (p = 0.07).

Since 1997, patients have been asked about contacts they had had with persons in whom TB had been diagnosed. Further potential source contacts were identified from the project database by identifying relatives and those sharing a household. The diagnosis of TB in these named contacts was checked with the project database (9). Two patients with strain KPS332 were relatives.

Overall, approximately half of those with Beijing genotype TB and of those with other strains had identified contact with previous TB patients (Table). Among the 219 persons who were named as potential source contacts and had RFLP results, 10 (4.6%) had Beijing genotype strains, similar to the proportion in the whole population. Some contacts were named more than once. The proportion of case-patients who had identical RFLP patterns to these source contacts, confirming transmission, was similar for contacts with Beijing genotype strains (3 [20%] of 15 contact pairs) and those with other strains (61 [21%] of 289, p = 0.9). Since the proportion of contacts with the Beijing genotype is similar to the proportion with Beijing genotype in the whole population, and since the proportion of transmissions that were confirmed by RFLP was similar for those with Beijing genotype and those with other genotypes, this analysis provides no evidence of increased transmissibility of Beijing genotype strains. In addition, those with Beijing genotype were no more likely to be smear-positive than were those with other genotypes (Table).

Drug resistance was tested to isoniazid and rifampicin (rifampin). If resistance was found, sensitivity to other drugs was tested as well. None of the Beijing genotype strains had any drug resistance, compared to 6.1% of other strains (p = 0.1).

Outcome data were available for 92% of patients. After excluding 154 patients (3 with Beijing genotype) who were lost to follow-up or transferred, we found that the proportion who died during treatment was similar for those with Beijing genotype strains as for the other patients (Table). These results were little altered by adjusting for year of diagnosis, age, sex, or HIV status.

Conclusions

Beijing genotype strains of *M. tuberculosis* are present in northern Malawi. The variety of RFLP patterns suggests several different introductions of Beijing genotype strains into the district. The origin of these strains is unknown, but they may have come directly from Asia, where they are common (12): Chinese people have worked in the district, for example, as agricultural advisors, in the last 20 years. The proportion of TB cases caused by Beijing genotype strains increased over the period of the study. This increase was exacerbated by 10 cases with a single RFLP pattern occurring within the last 12 months, which suggests an outbreak, although epidemiologic links were only established for 2 cases. No association with drug resistance was seen, and no evidence of increased severity or transmissibility of TB was seen in those with Beijing genotype.

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Norovirus Transmission on Cruise Ship

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An outbreak of norovirus gastroenteritis affected passengers on two consecutive cruises of ship X and continued on 4 subsequent cruises despite a 1-week sanitization. We documented virus transmission by food and person-to-person contact, persistence of virus despite sanitization onboard, introduction of new strains, and seeding of an outbreak on land.

We describe an investigation of a norovirus gastroenteritis outbreak aboard a cruise ship affecting 6 consecutive cruises and the use of sequence analysis to determine modes of virus transmission. Noroviruses (NoV), are the most common cause of infectious acute gastroenteritis and are transmitted feco-orally through food and water, directly from person to person and by environmental contamination (1). These viruses are often responsible for protracted outbreaks in closed settings, such as cruise ships, nursing homes, and hospitals (2,3).

On November 20, 2002, cruise ship X recorded an elevated number of persons with acute gastroenteritis symptoms reporting to the ship's infirmary (84 [4%] of 2,318 passengers) during a 7-day vacation cruise from Florida to the Caribbean. According to federal regulations, when the incidence of acute gastroenteritis among passengers and crew exceeds 3%, an outbreak is defined and requires a formal investigation (4). The outbreak continued on the subsequent cruise (cruise 2), after which the vessel was removed from service for 1 week of aggressive sanitization. Despite cleaning, gastroenteritis also developed in 192 (8%) of 2,456 passengers and 23 (2.3%) of 999 crew on the following cruise (cruise 3). To determine the source of this continuing outbreak and to better understand the mechanisms of NoV transmission, we began an investigation on cruise 1 and collected stool specimens from persons with gastroenteritis on this cruise and the next 5 cruises.

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The Study

We surveyed all 2,318 passengers on cruise 1 to determine dates of illness onset, symptoms, cabin locations, activities, and food consumption. We also performed a sanitary inspection of the ship. We suspected that initial infection among passengers on cruise 1 originated from a common food or water source and then continued to spread from person to person. Therefore, we conducted a case-control study with all passengers in whom illness developed early in the cruise (days 3 and 4) after embarkation (defined as day 1) and also with passengers who became ill later (day 5). Controls were systematically selected among passengers who reported no symptoms of gastroenteritis throughout the entire cruise. We continued to monitor the number of cases of acute gastroenteritis on the subsequent 5 cruises and collected fecal specimens from ill persons on all 6 cruises. During our shipboard investigation, we also obtained stool specimens from ill persons in a long-term care facility affected by an outbreak of acute gastroenteritis, in which the index patient was a passenger who returned ill to the facility after disembarking from cruise 1. All stool specimens were tested for NoV by reverse transcription–polymerase chain reaction, as previously described (5). The positive amplicons were sequenced, and sequences were compared for genetic diversity.

The outbreak began abruptly on day 2 of cruise 1 and continued on cruise 2 with new passengers. Despite sanitization of the ship for 1 week after cruise 2, illness was also reported among passengers on cruise 3 (Figure 1). On the subsequent cruises (4–6), the number of ill persons reporting to the infirmary remained above background levels but below 3%. Of the 2,318 surveyed passengers on cruise 1, 1,276 (55%) returned questionnaires, of these, 212 case-passengers and 265 control-passengers were enrolled in our study. We identified that eating breakfast at restaurant A on

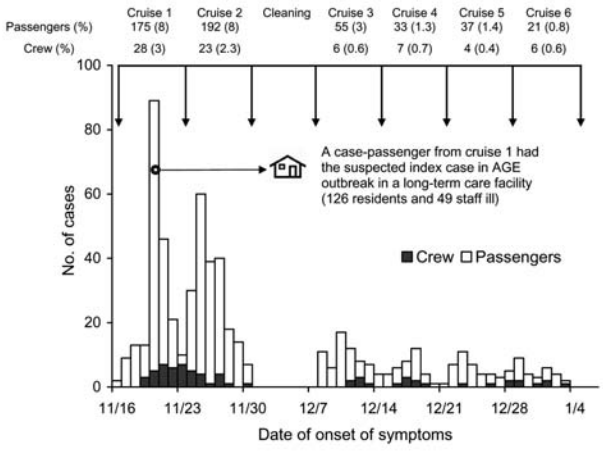

Figure 1. Number (%) of cases of acute gastroenteritis among 513 passengers and 74 crew by date of symptom onset reported to the infirmary on 6 consecutive cruises of ship X, November 2002–January 2003. Arrows indicate start and end of each cruise.

day 2 of the cruise was associated with illness among case-passengers with onset of symptoms on day 3 (odds ratio [OR] 4.04, $p < 0.01$) and that eating dinner at the same restaurant on day 2 was a risk factor for illness among case-passengers who became ill on day 4 (OR 2.8, $p < 0.005$). We also found that eating dinner at restaurant B aboard ship on day 3 was associated with illness among case-passengers with onset on day 5 (OR 2.3, $p < 0.05$). Restaurants A and B did not share a galley. Case-passengers with later onset of illness on day 5 were more likely than controls to have a cabin mate in whom gastroenteritis developed on days 3 or 4 (OR 2.01, $p = 0.01$), which suggests either infection by environmental contamination or by person-to-person spread.

Of 55 tested stool specimens from all 6 cruises, 25 (45%) were positive for NoV and belonged to 6 strains (Table). Norovirus was detected in at least 1 stool sample from all cruises, except cruise 4, where no stool samples were found positive, and in 2 samples of ill persons from the long-term care facility. The genetic sequences detected on cruises 1 and 2 were identical in regions B and C and belonged to a lineage of NoV within genogroup II (GII), cluster 4 (Figure 2), which has been provisionally described as the Farmington Hills strain (6). Five of the 8 NoV-positive specimens on cruise 3, which sailed after sanitization, contained 3 different sequences (X, Y, and Z). Sequence X was found in 1 sample and was identical to the sequence detected on cruises 1 and 2, which suggested that this strain may have persisted onboard despite cleaning. Sequence Y was found in 3 samples and differed from sequence X by 3 nucleotides (nt) in region C, which suggested that it was the predominant strain and probably newly introduced by passengers or crew at the start of cruise 3. Sequence Z was detected in 1 sample and belonged to the same lineage of NoV as the strain found on cruises 1 and 2 but to a different cluster (cluster 3), which suggested that it was also newly introduced onto cruise 3. Single stool samples from persons on cruises 5 and 6 contained a sequence that differed from the Farmington Hills strain by 3 nt and 1 nt, respectively, which suggested probable continuous reintroductions of closely related viruses aboard the ship. A sequence indistinguishable from that found on cruises 1 and 2 was also detected in stool samples from 2 persons ill in the outbreak that occurred in the long-term care facility, which suggested that virus was possibly introduced by the ill passenger from cruise 1. The environmental inspection of the ship identified no major violations.

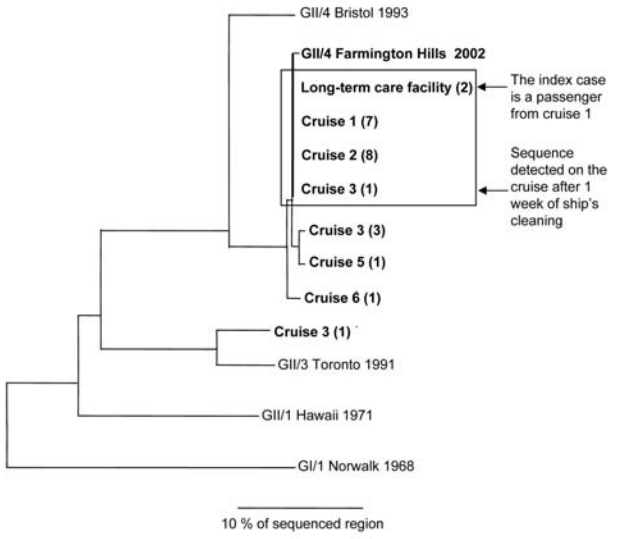

Figure 2. Phylogram of 9 norovirus sequence types detected in outbreaks on ship X, 4 reference sequences from GenBank, and the Farmington Hills virus. The tree is based on a 277-nucleotide region (region C) of the capsid gene and was created using uncorrected distances calculated by the DISTANCES program (Genetics Computer Group, Madison, WI) and was constructed by neighbor-joining using the GROWTREE program version 10.3 (Genetics Computer Group). Numbers in parenthesis indicate number of samples with the identical sequence detected in a given outbreak. Box highlights sequence types belonging to the Farmington Hills virus that are indistinguishable in region C. GenBank accession no. for reference strains include Bristol virus, X76716; Toronto virus, U02030; Hawaii virus, U07611; and Norwalk virus, M87661. Scale bar represents 10% divergence.

Conclusions

We report on a large outbreak of NoV-related gastroenteritis that affected 6 consecutive cruises on 1 ship and recurred despite thorough sanitization after the second

Table. Results of laboratory testing for norovirus in stool specimens, November 2002–January 2003*

Outbreak identification	No. samples			No. sequences detected
	Tested	Positive (%)	Sequenced	
Cruise 1	12	7 (58)	7	1
Cruise 2	14	8 (57)	8	1
Cleaning	1	0 (0)	0	0
Cruise 3	15	8 (53)	5	3
Cruise 4	6	0 (0)	0	0
Cruise 5	3	1 (33)	1	1
Cruise 6	4	1 (25)	1	1
Long-term care facility	2	2 (100)	2	1

*Specimens were collected from ill persons in linked outbreaks of acute gastroenteritis on a cruise ship and in a long-term care facility.

cruise. In the past, investigations of shipboard outbreaks of viral gastroenteritis were limited by the lack of adequate molecular methods for detecting and characterizing viruses (7). In this investigation, epidemiologic analysis suggested an initial foodborne source of infection with subsequent secondary spread from person to person, while molecular analysis provided several new insights into disease transmission. Application of genetic sequencing documented persistence of the same strain onboard between cruises by detecting identical sequences in stool samples from ill passengers before and after 1 week of the vessel's cleaning. Although these findings suggest that environmental contamination may have helped perpetuate the outbreak, infected crew members could have also been a reservoir of infection between cruises. Molecularly fingerprinting of detected viruses confirmed several introductions of new strains aboard, which underscores the difficulty in controlling outbreaks of NoV on cruise ships. Sequence analysis provided evidence that an outbreak of NoV in the care facility was caused by a person returning ill from an outbreak-affected cruise.

Like other outbreaks of viral gastroenteritis on cruise ships (3,6,8–11), this outbreak affected several hundred people, was transmitted by multiple modes, and recurred on subsequent cruises. Multiple routes of NoV transmission have been documented in other reports, such as that of an outbreak of gastroenteritis among football players, in which initial foodborne transmission of virus and secondary person-to-person spread was demonstrated (12). Outbreaks of gastroenteritis aboard cruise ships are similar to those in other closed and crowded settings where identifying and interrupting multiple routes of transmission has proved particularly challenging (2,13,14).

A limitation of this study was that the investigation on cruise 1 started 7 days after the first cases were reported, and because of logistic constraints, surveying was restricted to passengers in a short period before their disembarkation. Thus, we were unable to investigate risk factors for illness among crew and determine if any of the food-handlers were ill. In addition, poor recall of exposures resulted in a lack of complete data for a detailed evaluation of risk factors. We also did not perform a full investigation on the subsequent cruises because the number of ill persons did not exceed 3%.

Our investigation suggests that efforts to control gastroenteritis outbreaks on cruise ships should address all possible modes of NoV transmission, including foodborne, environmental persistence, and person-to-person spread. Such measures should include extensive disinfection, good food and water handling practices, isolating ill persons, providing paid sick leave for ill crew, and promoting handwashing with soap and water among passengers and crew. Developing strategies and incentives to dissuade symptomatic passengers from boarding may also minimize opportunities to introduce new strains aboard. Cruise ship outbreaks with <3% of passengers reporting ill should be considered for investigation because they may contribute substantial information on the transmission and epidemiologic characteristics of NoV, which could be used to develop control strategies and prevent future outbreaks on land and at sea.

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Serologic Evidence of Human and Swine Influenza in Mayan Persons

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Antibodies against influenza viruses were detected in 115 serum samples from indigenous Mayan persons from Kochol, Yucatán. Seropositivity rates were 26.9% to A/Bayern/7/95, 40.8% to A/Sydney/5/97, 1.7% to A/Swine/Wisconsin/238/97, and 79.1% to A/Swine/Minnesota/593/99. This report is the first in Mexico of the prevalence of antibodies to swine influenza virus in humans.

Influenza virus type A has the capacity to infect humans, birds, swine, and other animals. Studies have repeatedly shown that influenza virus can move from 1 species to another. The pig has been proposed as an animal that could play a key intermediary role in interspecies transmission. Pigs are the only domesticated mammalian species that are reared in abundance and are susceptible to both avian and human influenza virus and allow productive viral replication (1,2).

In rural zones in the Mexican state of Yucatán, the "backyard system," a production system in which animals such as pigs, ducks, turkeys, and chickens are all raised in close proximity to humans, is common. This system is a traditional activity of indigenous Mayan persons, as well as other ethnic groups in Mexico, and provides an economical way to produce animals. The animals eat, live, and share space, water sources, and even food with humans; they may even be found inside houses. These activities create health concerns because of potential for the adaptation and reassortment of human and avian viruses.

Despite abundant evidence supporting interspecific transmission and genetic reassortment of influenza virus around the world, little is known about the influenza virus in humans and domesticated animals in Yucatán in southeastern Mexico. We describe serologic evidence of antibodies against influenza strains from humans and pigs in indigenous Mayan persons from Yucatán.

The Study

Kochol is located in east Yucatán, ≈20 km from the municipality of Maxcanu. The 1,207 residents are mostly dedicated to agricultural activities (3). The population has high illiteracy rates, poor environmental health, and crowded and inadequate housing. In Kochol, pigs are found around the town, walking in and out of houses. All pigs are wild or *criollos*. Some families have 1–18 pigs. For this study, serum samples from 115 persons were made available by the health official of Kochol in 2000. Serum samples were from Kochol residents who came to the health service for any medical condition and required laboratory tests.

Samples were treated with receptor-destroying enzyme from *Vibrio cholera* and heated at 56°C in a water bath to inactivate nonspecific inhibitors (4). The following 4 influenza strains were used to detect antibodies: A/Swine/Wisconsin/238/97 (classical swine H1N1), A/Bayern/7/95 (human H1N1), A/Sydney/5/97 (human H3N2), and A/Swine/Minnesota/593/99 (reassortant swine H3N2); all were grown in 10-day-old embryonated chicken eggs. The hemagglutination inhibition tests were performed by using chicken erythrocytes at a concentration of 0.5%. A sample was considered seropositive to H1 and H3 when the HA titer was ≥1:40. Each serum sample was tested against chicken receptor–destroying enzymes in the absence of virus to rule out induction of nonspecific hemagglutination.

Conclusions

As shown in Table 1, reactivity rates were uniformly high to H3 subtype influenza virus. These results agree with previous serologic tests of human serum samples from Yucatán (G. Ayora-Talavera, unpub. data). H1 viruses likely circulate at a lower frequency than H3 viruses. Overall, 31 (26.9%) of 115 samples were positive to H1, whereas 93 (80.8%) of 115 were seropositive to H3. The results indicate that influenza virus infection occurs in a large proportion of persons in this area. In general, Mexican persons are not vaccinated, so we can be sure that the antibodies detected reflect actual infection (5). Samples were divided into 5 age groups (Table 2). By analyzing the percentage of seropositive persons in different age groups, we observed that persons 15–24 years of age were most commonly seropositive. Through virus surveillance in Yucatán, we have also observed a very low circulation of influenza A H1. From ≈1,500 throat swabs collected in 5 years, no sample has been found to contain H1 influenza by immunofluorescence assay, and only 5 viruses have been detected with reverse transcription–polymerase chain reaction (G. Ayora-Talavera, unpub. data).

The highest seropositivity rates across all age groups were detected with the A/Sw/Minnesota virus as antigen. Although this strain was isolated from American pigs, the HA, NA, and PB1 genes are of human origin (6). Taking

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Table 1. Hemagglutination inhibition antibodies to influenza virus, Kochol, Yucatán

			No. (%) positive samples			
No. samples	Titer	Month	A/Bayern/7/97 (H1N1)	A/Sw/Wis/238/97 (H1N1)	A/Sw/Mn/593/99 (H3N2)	A/Sydney/5/97 (H3N2)
73		June	22 (30)	2 (2.7)	59 (80.8)	32 (43.8)
	0		41	67	4	10
	10		6	2	2	12
	20		4	2	8	19
	40		5	2	9	9
	80		6		11	17
	160		7		18	4
	320					2
	640		4		18	
	1,280				3	
35		July	8 (23)	0	26 (74)	14 (40)
	0		18		5	13
	10		6		2	2
	20		3		2	6
	40		3		7	8
	80		3		10	4
	160		1		4	2
	640		1		4	
	1,280				1	
7		August	1 (14)	0	6 (85.7)	1 (14)
	0		4		1	3
	10		1			1
	20		1			3
	40		1		1	1
	160				4	
	320				1	

into consideration the cutoff values of this study, seropositivity to the swine H1 virus was only detected in 2 samples, from persons 43 and 59 years of age. However, lower titers were detected in 4 more persons 33–55 years of age. The weak reactivity to this virus could suggest a past exposure of adult persons to viruses of swine origin, a situation that has not occurred in persons >30 years of age.

The animal population owned by persons in this study consisted of pigs (68.7%), chickens (73%), and ducks (17.3%). Any combination of 2 or 3 species was kept by 54.7%. The range of the number of animals owned was 0–12 (mean 2.9) pigs, 0–60 (mean 7) chickens, and 0–23 (mean 0.93) ducks. Since we did not have avian antigens available, serum samples collected from humans, pigs, chickens, and ducks were not tested for exposure to avian influenza viruses.

The relative risk of being seropositive for H1 or H3 viruses from exposure to pigs was 1.93 with human H1 (95% confidence interval [CI] 1.2–3.0), 0.88 with human H3 (95% CI 0.55–1.4), 0.6 with swine H1 (95% CI 0.08–4.2), and 1.0 with swine H3 (95% CI 0.62–1.6).

Serologic evidence of swine antibodies in persons in contact with pigs has been reported in several studies (7–12). In Mexico, apart from this report, no information about the prevalence of antibodies to swine influenza virus in humans exists. The only information available comes from a study carried out on pig farms in central Mexico, where the subtype H1 is prevalent in 20% of pigs (13) and from a previous study from Yucatán, where the most prevalent subtype in pig farms is H3 (65%) and H1 (20%) (14).

As a result of the Mexican outbreak of HPAI H5N2, the Mexican Ministry of Agriculture (SAGARPA) implemented a national surveillance system in all chicken farms (NOM-044-ZOO-1995). Yucatán is considered a free state for avian influenza virus. Chicken farms are sampled 3 times a year for serologic surveillance, and 10% of the

Table 2. Specific hemagglutination inhibition antibodies by age group, Kochol, Yucatán

		n (%)			
Age group	N	A/Bayern/7/97 (H1N1)	A/Sw/Wis/238/97 (H1N1)	A/Sw/Mn/593/99 (H3N2)	A/Sydney/5/97 (H3N2)
8–14	16	4 (25)	0	14 (87)	9 (56)
15–24	33	13 (39)	0	29 (88)	14 (42)
25–34	28	5 (16)	0	22 (78)	9 (32)
35–44	24	4 (16)	1 (4)	16 (66)	9 (37)
45–53	14	4 (33)	1 (8)	10 (71)	6 (43)

backyard flocks are sampled annually (15). On the other hand, swine influenza is not considered within the SAGARPA priorities, and no surveillance program exists for swine farms, although we found serologic evidence that in Yucatán influenza H3 subtype is highly prevalent (14).

Asia has been considered as an epicenter for the generation of pandemic influenza virus, and some factors are high densities of humans and animals in close contact (1). In Yucatán, the backyard system is a common practice, and human and animal encounters could lead to generation of novel reassortant viruses here as well.

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EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

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

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

Figure. Francisco José de Goya y Lucientes (1746–1828). Lazarillo de Tormes (1819).
Oil on canvas. Private Collection, Giraudon/Bridgeman Art Library/www.bridgeman.co.uk

Death of Seurat

Setu K. Vora*

> "A sudden stupid sickness carried him off in a few hours when he was about to triumph: I curse providence and death."
>
> Art critic Jules Christophe, writing after the death of Seurat in La Plume, September 1, 1891

Born in Paris on December 2, 1859, Georges Pierre Seurat was only 31 when he died on March 29, 1891. In his short but productive life, this renowned painter founded a new art movement, neoimpressionism or pointillism. He is most famous for his work A Sunday on La Grande Jatte (1884), now at The Art Institute of Chicago. Art historian Richard Thompson puts Seurat's success in perspective: the 1880s were recognized by contemporaries as a decade of great excitement and innovation and are regarded today as one of the most salient periods of esthetic change. To have forged a new visual language in such challenging circumstances, while only in one's late 20s, was a remarkable achievement (1).

Seurat's early demise and the 1990s reemergence in Russia and neighboring countries of diphtheria, the infectious disease that probably killed him, invite closer examination of the circumstances that surround his death. The diseases affecting many of Seurat's contemporaries have been well documented, including the neuropsychiatric illness of van Gogh (2), visual impairment of Edgar Degas (3), dwarfism of Toulouse-Lautrec (4), and rheumatoid arthritis of Renoir (5). The circumstances and cause of Seurat's untimely death are not clearly understood and deserve medical scrutiny.

Life and Times

Seurat was his parent's belated third child—a younger brother died at age 5. At age 16, he attended a municipal school of design run by the sculptor Justin Lequien, and in 1878, he joined the École des Beaux-Arts under Henri Lehmann, a student of the famous neoclassical painter Ingres. At age 20, Seurat served for a year with the 119th infantry regimen at Brest. His military recruitment papers describe him as follows: "Brown hair, brown eyes, average forehead, prominent nose, average mouth, round chin, oval face, 5 feet 10½ inch, no distinguishing mark" (6).

His friends, who included fellow artists Paul Signac, Charles Angrand, and Albert Dubois-Pillet, always described him as robust, "a solid being, a grenadier" (7). He smoked cigarettes, but it is not known if he drank alcohol or absinthe in excess. He attended periodic compulsory military training, as mentioned in telegraphic correspondence to Signac in August 1887: "…weather mild. Nobody in the streets in the evening (Province). Slight spleen. From 22 August to 18 September, 28 days…" (1). Slight spleen probably refers to his depressed mood or annoyance at the upcoming military training for 28 days rather than to any splenic problem.

Seurat made summer trips to coastal towns (Le Crotoy, Honfleur, Gravelines) (6). In 1889, he traveled to Belgium, where he exhibited at the Salon des Vingt in Brussels. After returning from this trip, he met Madeleine Knobloch, a 20-year-old model, and started secretly living with her (6). When Madeleine became pregnant with his child, they moved from his studio at 128 bis Boulevard de Clichy to a tiny room measuring 5 meters square in a quiet courtyard off the Passage de l'Elysee-des-Beaux-Arts. Seurat acknowledged the paternity of his son born on February 16, 1890, and entered the name of the child in the civil registers as Pierre Georges. At his exhibition in the Salon des Indépendants the same year, he showed his only known portrait of Madeleine Knobloch: Young Woman Powdering Herself. Yet, his family and close friends were still completely unaware of his mistress and child. According to one biographer, Seurat inherited secretive tendencies and preference for isolation from his father (1). He became distressed at the news of van Gogh's suicide and the death of his friend and follower Dubois-Pillet (8) of smallpox that August.

Madeleine Knobloch was pregnant again at the beginning of 1891. Seurat was hard at work painting The Circus. Art critic and author Arsène Alexandre, who was seeing Seurat regularly at this time, described his comings and goings, the way he climbed up the ladder and down again, how he worked long into the night (9). In early March, Seurat helped arrange the Indépendants exhibition, inspecting the entries and hanging the paintings. As if he

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had a premonition of death, he displayed his unfinished painting The Circus. He hardly noticed an ordinary sore throat that followed a cold (10). On March 26, he suddenly fell ill with fever and weakness. On March 27, Good Friday morning, he moved to his mother's apartment in the boulevard Magenta, supported by a friend and accompanied by pregnant Madeleine and their 13-month-old son. His illness was diagnosed as infectious angina or quinsy, and he was confined to bed. After a short crisis marked by fever and delirium, Seurat "choked to death" on Easter Sunday, March 29, at 6 a.m. (9). His son Pierre George died of a similar illness on April 13, and was buried alongside Seurat in Père-Lachaise cemetery (7). Seurat's father died on May 24, cause unknown. Three generations of the Seurat family died within a span of 2 months.

Cause of Death

"Infectious angina" (6,7) is most widely blamed, but a form of meningitis (1) or pneumonia is also mentioned as the cause of Seurat's death (11). Given the more common current association of angina with the heart and not the throat, it is likely that an art historian may have misinterpreted infectious angina to mean heart infection (12).

Pneumonia, especially pneumococcal pneumonia, can begin as upper respiratory infection followed by fever, weakness, and difficulty breathing. Severe disease leading to sepsis can cause delirium and even meningitis followed by death. Conversely, bacterial meningitis caused by *Neisseria meningitidis* often begins as sore throat, followed by fever and delirium progressing to death. However, in Seurat's case, description of infectious angina and the terminal event of choking to death suggest infectious upper airway obstruction. A variety of infections can produce this syndrome.

In the absence of clinicopathologic data, it is impossible to know the exact infectious cause of Seurat's death. Peritonsillar abscess, also known as quinsy, is the most common deep infection of the head and neck in adults. Peritonsillar abscess is most common in persons 20 to 40 years of age (13); however, it is less likely to cause upper airway obstruction, unless it is bilateral. Acute bacterial epiglottitis, caused primarily by *Haemophilus influenzae* type b, usually affects children (ages 2 to 7 years) but also occurs in adults. Onset is usually explosive, with sore throat, fever, and dyspnea progressing rapidly to dysphagia, pooling of oral secretions, and drooling. Abrupt deterioration occurs within a few hours and, in the absence of adequate treatment, results in death (14). The clinical course of Seurat's illness was over a period of 1 week, and his son died 2 weeks later, which makes acute epiglottitis caused by *H. influenzae* type b a less likely diagnosis.

Eminent biographer Jean Sutter was the first to suggest diphtheria as the cause of Seurat's death (6). Known variously as deadly angina, gangrenous angina, angina suffocant, and malignant angina, diphtheria was epidemic in France in the 19th century (15). Humans are the only known reservoir for *Corynebacterium diphtheriae*. The primary modes of spread are airborne droplets and direct contact with respiratory secretions. Most respiratory tract diphtheria occurs in the colder months in temperate climates and is associated with crowded indoor living conditions (16). After an incubation period of 2 to 4 days, signs and symptoms of inflammation can develop at various sites within the respiratory tract. Although diphtheria mainly affects children, according to William Osler, in his textbook published a year after Seurat's death, adults are also frequently affected (17). Osler also noted that diphtheria epidemics vary in intensity. While in some epidemics infection is mild and rarely fatal, in others it is characterized by wide extension of the pseudomembrane and tends to attack the larynx.

Biographic evidence mounts on the side of pharyngeal-tonsillar diphtheria with toxemia resulting in prostration and stupor. But the immediate cause of Seurat's death was probably extension of the laryngeal membrane, causing acute airway obstruction and asphyxiation.

Contributing Factors

Although clinical details are not available, the death of Seurat's son from a similar illness, within 2 weeks of his death, suggests household transmission. Seurat lived with his mistress and son in very confined quarters. Overcrowding is a known factor in *C. diphtheriae* transmission (18).

The case of Madeleine Knobloch, the only family member who did not succumb to diphtheria, remains enigmatic. We have no details about her life, health, or personal habits (e.g., alcohol abuse), except for the fact that her second child died at or soon after birth. Madeleine herself died of cirrhosis of the liver at age 35 (7). An asymptomatic carrier and "disperser" state for diphtheria has been described (19), and Madeleine might have played such a role in the cold month of March in the confines of their tiny room.

Seurat was working extremely hard just before he became ill with sore throat. On an earlier occasion, art critic and collector Gustave Kahn saw him in the Boulevard de Clichy studio completing a big canvas "with zeal so strong and in a heat so oppressive that he lost pounds before it was finished" (9). Signac recounted that Seurat would often have only a snack for lunch, a croissant and a bar of chocolate, so that he would not waste valuable time (1). The combination of acute severe exertion, poor nutrition, and grueling work could increase susceptibility for upper respiratory infection (20). Signac, the closest friend, follower, and champion of Seurat, was close to the truth

when he sadly concluded, "Our poor friend killed himself by overwork."

Historical Context

Throughout the 19th century, diphtheria was common and had even found its place in contemporary art. Goya's painting Lazarillo de Tormes (1819), also known as el Garrotillo (Figure), shows a child suffering from diphtheria and the man attending him. Diphtheria especially affected children (death rate 40%–50%) (21). Necrosis of the mucosa of the upper respiratory track resulted in the formation of a pseudomembrane, which in turn obstructed the airway, causing asphyxia and death. The technique of tracheotomy evolved during the 19th century, prompted by the need to control diphtheria epidemics (15). In 1807, death of his nephew's son from diphtheria prompted Napoleon Bonaparte to stimulate diphtheria research by offering a grand prize. It was in this context that French physician Pierre Bretonneau (1778–1862) studied the disease and coined the word *la diphtherite* (Greek for leather, describing the pharyngeal membrane). He also formed the clinical case definition and performed the first tracheotomy in 1825 (15). In 1885, New York physician Joseph O'Dwyer introduced tracheal intubation for the treatment of severe diphtheria (22).

Pierre Paul Emile Roux, an assistant of Louis Pasteur, proved that the diphtheria bacterium produced toxin (23). This discovery was instrumental to the development of antitoxin by Emil von Behring in 1890, for which Behring won the first ever Nobel Prize for physiology or medicine. On December 25, 1891, just a few months after Seurat's death, a patient with diphtheria was successfully treated with "immune serum" (24). Not much later, diphtheria was to touch and shape the life of another great artist. Picasso's 8-year-old sister died of the disease. While she was sick, Picasso vowed that if she survived, he would give up art. The relief he felt upon her death for not having to keep his promise left him with lifelong guilt. As his sister lay on her deathbed, she like the "sleeper" in many of his paintings and he like the "watcher," the child prayed to God, begging that she be spared. The event would govern his behavior and paintings for the rest of his life (25).

In the wake of the French Revolution, France had a national system of medical licensing in place by 1803 and the first true national healthcare system (26). Paris was the center of the medical world of France. Hotel Dieu, rebuilt during 1868–1878, and Salpetriere Hospital were among the leading hospitals providing healthcare to Parisians. The Pasteur Institute, established by decree on June 4, 1887, was inaugurated on November 14, 1888. France was leading research and treatment of diphtheria. Joseph Kinyoun, the founder of the Hygienic Laboratory—predecessor of the National Institutes of Health—learned the procedure for preparing diphtheria antitoxin at the Pasteur Institute in Paris. In France, tracheotomy had become the standard management for airway obstruction caused by diphtheria.

Loss to the World

Seurat was not a struggling or impoverished artist who could not afford medical care. At a time when the average industrial worker was paid 150 francs a month, Seurat received a monthly allowance of 400 francs. He wore expensive top hats and black suits, which led Edgar Degas to dub him "le Notaire" (the Notary) (6). In spite of comfortable means and access to medically advanced Paris, Seurat chose to go to his mother's house and die there instead of going to a hospital, where tracheotomy or tracheal intubation might have saved him from asphyxiation. No record is available of Seurat's medical care during his lethal illness, and no autopsy was performed. We will never know what Seurat's achievements might have been if he had received medical treatment and lived to ripe old age, nor will we know if his son could have been a great artist himself.

Dr. Vora is currently a fellow in Pulmonary and Critical Care Medicine with special interest in pulmonary infections, especially after transplantation, and biologic therapies for rheumatologic diseases. He is also interested in intensive care unit preparedness against bioterrorism or emerging infectious diseases. Outside of work, he enjoys investigative medical humanities, poetry, and photography.

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SARS-CoV Sampling from 3 Portals

To the Editor: Wang et al. detected severe acute respiratory syndrome–associated coronavirus (SARS-CoV) from throat wash and saliva specimens and suggested that these specimens have advantages over other specimens, including ease of procurement and safety for medical personnel (1). The virus has been detected with variable success from nasopharyngeal aspirates, nose and throat swabs, and tears (2,3). Advocates of all of these sampling methods emphasize the need for early diagnosis of SARS. The probability for nosocomial transmission to healthcare workers when they obtain specimens from patients has not been adequately addressed. In a study of outbreak control for SARS, Chowell et al. suggest "… the strong sensitivity of R_0 to the transmission rate β indicates that efforts in finding intervention strategies that manage to systematically lower the contact rate of persons of all age groups promise an effective means for lowering R_0" (4).

An important component of a comprehensive strategy to lower the contact rate is improving the safety measures recommended for clinical specimen collection by healthcare workers. Recognizing the importance of obtaining multiple specimens and the difficulties associated with obtaining samples from the 3 usual portals of entry, we devised and tested a novel method of specimen collection, conjunctiva–upper respiratory tract irrigation (5). We coupled our specimen collection method with detailed written instructions to enable the patients themselves to perform the entire procedures. Almost all other specimen collection methods require assistance from healthcare workers or have other limitations, such as inability to sample all 3 portals. The method is not perfect because some persons have difficulty performing the procedure; however, self-instillation of the irrigation into the nostrils, with or without the addition of a throat wash or saliva, is likely to improve the success rate. The data supplied by Loon et al. (3) and Wang et al. (1) confirm that collecting specimens by a method that involves minimal contact between a possible source of infection and susceptible persons is desirable.

The author has shares of a company that owns a patent-pending conjunctiva-upper respiratory tract irrigation system.

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Occupational Health Response to SARS

To the Editor: Severe acute respiratory syndrome (SARS), an occupational disease risk for healthcare workers, warrants an occupational health response, as clearly described by Esswein et al. (1). Occupational health professionals played a role in the assessment of healthcare facilities in Taiwan and many other countries. For example, occupational health professionals were invited to perform audits in at least 2 hospitals in Singapore during the height of the crisis, (2) and to conduct follow-up discussions with the hospital management. In addition to assessment of the industrial hygiene aspects, which included evaluating the ventilation modifications needed for effective infection control, temperature and humidity were significant factors affecting the use of protective gear in a tropical country like Singapore. The occupational health audits included site inspections and reviews of work processes of those areas where actual transmission of SARS had occurred and where triage of febrile patients was taking place. Other issues identified as requiring urgent attention were providing sufficient rest, shower, and changing facilities for staff, monitoring staff sickness absenteeism, and proactively managing staff mental health. Occupational health physicians subsequently served on hospital SARS debriefing committees that reviewed institutional shortcomings and recommended new measures for future outbreaks. An occupational health service unit headed by a trained occupational health physician was formed in 1 hospital.

Other occupational groups, as well as healthcare workers, are also at potential risk. These groups may include the following: 1) food handlers, defined as persons who handle,

kill, or sell food animals, and persons who prepare and serve food. (More than one third of the cases in China with onset of SARS before February 1, 2003, were in food handlers [3].); 2) public transportation workers and airline crew (4); and 3) laboratory workers handling samples or items contaminated with SARS-associated coronavirus (5). In Singapore, 2 taxi drivers were infected after ferrying SARS patients to healthcare facilities, and 1 Singapore Airlines cabin attendant came down with the infection after a flight with infected passengers on board. Occupationally acquired SARS infections have been documented in Singapore, Taiwan, and Beijing. Clearly, occupational health responses are needed in these occupational settings.

The recognition of SARS as an occupational disease has broader implications. Depending on country legislation, persons who contract SARS while performing their work may be eligible for worker's compensation. Employers would be obligated to provide a safe and healthy workplace for their employees.

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Tracing SARS-Coronavirus Variant with Large Genomic Deletion

To the Editor: Severe acute respiratory syndrome (SARS) has been a global public health issue (1). We completed a study on the evolutionary path of the SARS-associated coronavirus (SARS-CoV) during the 2002–2003 epidemic (2). Most human SARS-CoV strains, as exemplified by the Tor2 sequence (GenBank accession no. AY274119) (3), are characterized by the deletion of a 29-nucleotide (nt) segment upstream of the nucleocapsid (N) gene domain when compared with the viral strains isolated from the earliest human SARS patients (2) or from nonhuman mammalian hosts (4). Towards the end of the epidemic, a variant of the SARS-CoV with a deletion of 386 nt flanking the 29-nt site was first demonstrated by complete genomic sequencing in 2 patients in Hong Kong (GenBank accession nos. AY394999, AY395000, AY395001, AY395002) (2). The 386-nt deleted segment corresponds to the genomic region spanning residues 27719 to 28104 of the Tor2 sequence (3). The deletion results in the disruption of a putative open reading frame, orf 9, while eliminating orfs 10 and 11. This deletion variant was first isolated from 2 SARS patients with disease onset in mid-May 2003. Patient A was a 41-year-old female phlebotomist working in North District Hospital, New Territories East Cluster, Hong Kong. Patient B was a 98-year-old woman admitted to ward X of North District Hospital (2).

With this finding late in the epidemic, we studied the prevalence of this SARS-CoV variant to determine its origin. Twenty-one SARS patients with disease onset dates from mid-April were identified. All cases had been confirmed by positive reverse transcription–polymerase chain reaction (RT-PCR) detection of SARS-CoV RNA in clinical specimens or seroconversion. These patients had been admitted with SARS to 4 different hospitals in Hong Kong, including North District Hospital and hospitals A and B, which were located in the same geographic cluster as North District Hospital, as well as hospital C, which was geographically distant from the other 3 hospitals. Clinical specimens were retrieved, and RT-PCR was performed to specifically amplify a genomic segment of SARS-CoV encompassing the deletion site. Specimens with shortened PCR fragments were sequenced to determine the location and precise extent of the deletion.

RT-PCR products were not observed in 2 specimens. Gel electrophoresis of the RT-PCR products for each of the remaining 19 specimens showed a single genomic fragment; 13 of these fragments were shortened. Direct sequencing of the short amplicons showed a deletion of 386 nt identical to that isolated from patients A and B. The patients' histories were reviewed. Patients A, B, and the 13 patients appeared to be epidemiologically related. The epidemiologic relationships and clinical details of the 15 cases are illustrated in the Figure. Most of the cases were part of a documented outbreak at North District Hospital traceable to an

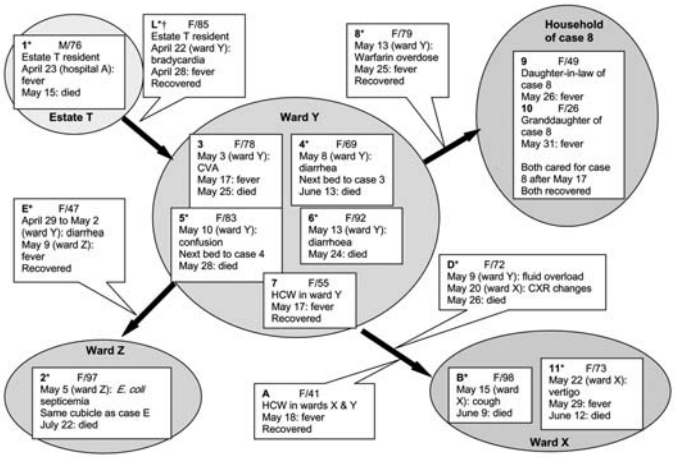

Figure. Schematic illustration of the epidemiologic relationships between patients with the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) variant with the 386-nt deletion. Patients are grouped according to the most probable site where SARS infection was acquired. Blocked arrows indicate the potential epidemiologic relationships between subgroups of patients. Patients who are suspected of being an epidemiologic link between particular subgroups are indicated by their association with the respective blocked arrows. For each patient, "F" denotes female, and "M" denotes male, and age is specified. The date of admission, followed by admission site in parentheses, and the initial complaint are indicated next. Additional noteworthy clinical information and the subsequent outcome of each case are indicated. *Patients with a history of chronic illness. †The viral genotype was not characterized in patient L due to the lack of clinical specimens positive for SARS-CoV by reverse transcription–polymerase chain reaction. CVA, cerebrovascular accident; HCW, healthcare worker; *E. coli*, *Escherichia coli*.

85-year-old woman, L, in whom SARS was not initially suspected but was subsequently confirmed by seroconversion. Patient L had been admitted to ward Y of North District Hospital; subsequently SARS developed in 7 fellow inpatients (patients 3, 4, 5, 6, 8, D, and E) and 2 healthcare workers (patients 7 and A) (Figure). Patient A had been working in both wards X and Y, and patient D was transferred from ward Y to X before symptom onset. Soon afterwards, SARS developed in 2 other inpatients (patients B and 11) in ward X (Figure). Patient E was transferred from ward Y to Z, where symptoms later developed in another inpatient (patient 2) (Figure).

Patients 1, 9, and 10 had not been admitted to North District Hospital but were admitted directly to hospital A. Patients 9 and 10 were household contacts of patient 8 (Figure). Patient 1 had no documented contact with other SARS patients, but coincidentally, patients 1 and L resided in the same estate, T, where a cluster of SARS cases had been documented by the local government (5). The deletion variant was absent in 6 of the studied cases. These case-patients had no identifiable relationship with the cohort of patients illustrated in the Figure and did not reside in the same geographic region as patients L and 1. Three of the patients were admitted to and treated in hospital C. None of the 6 patients had been admitted to North District Hospital.

Therefore, we have isolated a SARS-CoV variant with the largest genomic deletion reported to date in a total of 15 SARS patients, 14 females and 1 male, with ages ranging from 26 to 98 years (median 73 years) (Figure). Nine (60%) of the 15 patients, 8 of whom were known chronic disease patients, died (Figure). This mortality rate is consistent with previous observations where the death rate in patients >65 years generally exceeded 50% (1). Despite the disruption of several putative orfs, as evident from this study, this SARS-CoV variant remained effective in propagating among persons, particularly in the healthcare setting. The predicted orfs 9, 10, and 11 of the SARS-CoV thus may not be functionally important, although further studies are required. We were able to document 3 generations of transmission and traced the first appearance of this deletion variant to mid-April 2003, possibly at Estate T. Investigation on the origin of this enigmatic variant should be continued by studying its prevalence among the earlier human SARS-CoV isolates and potential mammalian hosts.

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Multidrug-resistant *Salmonella* Java

To the Editor: Since 2000, *Salmonella enterica* serovar Paratyphi B variant Java (*S.* Java) with resistance to antimicrobial drugs has been isolated with increasing frequency from patients in Scotland, England, Wales, and the Channel Islands. For England, Wales, and the Channel Islands, drug-resistant *S.* Java was found in humans: 25 in 2000, 36 in 2001, 49 in 2002, and 4 in 2003 (January 1–March 31). These isolates made up 35% of 325 strains of *S.* Java in human infections over the study period (L.R. Ward, unpub. data). A range of drug-resistant spectrums (R-types) have been observed, e.g., ASSpSuTm, ASSpSuTmCp, ASSpSuTTm, ACSSpSuT (A, ampicillin; C, chloramphenicol; S, streptomycin; Sp, spectinomycin; Su, sulphonamides; T, tetracyclines; Tm, trimethoprim; Cp, ciprofloxacin) (1). In general, isolates of *S.* Java of R-types ASSpSuTm, ASSpSuTmCp, and ASSpSuTTm, appear to be associated with imported poultry. In contrast, infections with isolates of R-type ACSSpSuT have not been associated with poultry, and organisms with the ACSSpSuT resistant spectrum have not been isolated from poultry in the United Kingdom (R.H. Davies, pers. comm.).

In England, Wales, and the Channel Islands, *S.* Java of R-type ACSSpSuT was isolated from human patients in 64 instances from 2000 to 2003 (5 in 2000, 22 in 2001, 34 in 2002, and 3 in 2003 [to March 31]). None of these cases were related to eating contaminated foods, and the ACSSpSuT antibiogram has not been isolated from strains of this serotype from foods in the United Kingdom. This resistance pattern corresponds to that of the epidemic clone of *S.* Typhimurium definitive phage type (DT) 104 (DT 104 ACSSpSuT), which caused many infections in humans and food production animals throughout Europe, the United States, and Canada in the 1990s (1). In all isolates of DT104 ACSSpSuT studied, from many different countries, the resistant gene cluster has been chromosomally integrated (1). Resistances have been contained in a 13-kb cluster composed of 2 integrons coding for resistance to SSp (1.0 kb) and ASu (1.2 kb), with the genes for resistance to chloramphenicol and tetracyclines located between these integrons (2,3). To investigate the possibility of the horizontal transfer of the ACSSpSuT gene cluster within *S. enterica*, we have characterized the resistance genes and associated structures in strains of *S.* Java of R-type ACSSpSuT and compared them with those in a strain of DT104 ACSSpSuT

From 2000 to 2002, a total of 20 isolates of *S.* Java of R-type ACSSpSuT from patients in England and Wales (18 isolates) and Scotland (2 isolates) were characterized by phage typing, plasmid profile typing, and pulsed-field gel electrophoresis (PFGE). Pulsed-field profiles of 3 additional isolates of *S.* Java of R-type ACSSpSuT from Scotland were compared with those of isolates from England and Wales by the electronic exchange of tagged image format files (TIFFs) in a Bionumerics database. Resistance genes were identified by polymerase chain reaction (PCR) with primers specific for bla_{TEM} (A), bla_{CARB-2} (A), *cmlA* (chloramphenicol/florfenicol), *catI* (C), *catIII* (C), *aadA2* (SSp), *sul1* (Su), and *tet*G (T) (4). The presence of class 1 integrons was tested with the primers L1 and R1 (2). To identify the complete ACSSpSuT resistance gene cluster, long PCR was used on the basis of amplifying a 10,041-bp fragment of the DT104 isolate H3380 (4). Results were compared with those of standard strain of DT104 ACSSpSuT-P3170700, and DT104 drug-sensitive–P3343110 (4).

Five unrelated phage types, – 1 var 3 (6 isolates), 3b var 2 (5), Dundee (2), Worksop (3), and RDNC (4), and 3 closely-related pulsed-field types, differing by only 1 to 3 of 14 bands in the *Xba*1 PFGE profiles, were identified in the 20 isolates studied; 2 of these pulsed-field types were observed in the electronically transmitted images of the 3 isolates from Scotland. These pulsed-field profile types have been designated SPTJXB001 through SPTJXB003. Of these, SPTJXB002 predominated, being present in 11 of the isolates studied, belonging to 3 phage types. SPTJXB001 was identified in 8 isolates of 3 phage types, and SPTJXB003 in the remaining isolate. PFGE type did not change over time. All isolates were plasmid-free, and resistances were not transferable, either directly or by mobilization after a self-conjugative plasmid was introduced into the strains. By PCR, all isolates possessed bla_{CARB-2}, *cmlA*, *aadA2*, *sul1*, and *tet*G but were negative for bla_{TEM}, *catI*, and *catIII*. These results corresponded to those of the control DT104 ACSSpSuT strain P3170700. When tested for class 1 integrons, all *S.* Java isolates of R-type ACSSpSuT produced characteristic amplicons of 1.0 and 1.2 kb, as did P3170700, but not the drug-

sensitive strain P3343110. When tested by long PCR, all 20 *S.* Java isolates produced a 10,041-bp fragment identical to that produced by P3170700.

PCR was used to determine whether the pentaresistant phenotype was due to the presence of the *Salmonella* genomic island 1 (SGI1) as previously described (5). All 20 strains produced amplicons with primers U7-L12 and LJ-R1 for the left junction and primers 104-RJ and 104-D for the right junction. These results indicate that the SGI1 in the strains of *S.* Java was located in the same chromosomal location as previously described for DT 104 ACSSpSuT but lacks the retronphage found to date only in DT104 strains (6).

These findings demonstrated that the ACSSpSuT resistance gene cluster in *S.* Java isolated from patients in the United Kingdom from 2000 to March 2003 appeared to be chromosomally located and was almost indistinguishable from that found in the epidemic clone of DT104 ACSSpSuT. This resistance gene cluster has also been identified in strains of *S.* Agona from poultry in Belgium (6), in a strain of *S.* Paratyphi B from tropical fish in Singapore (7), and a variant cluster in a strain of *S.* Albany from fish food from Thailand (8). It also appears to be present in isolates of *S.* Paratyphi B of R-type ACSSpSuT from cases of human infection in France in 2003 (F. Xavier-Weill, pers. comm.). The strains of *S.* Paratyphi B from Singapore and France were not tested additionally to identify the Java variant.

The strains of *S.* Java with chromosomally integrated ACSSpSuT resistance identified in the United Kingdom are not those associated with poultry in Germany (9) or the Netherlands (10), which have also caused infections in humans in the United Kingdom (1). The latter strains have different phage types, resistant spectrums, pulsed-field profiles, and they possess plasmid-mediated drug-resistance. However, several *S.* Java infections in the United Kingdom have been associated with tropical fish tanks (L.R. Ward, unpub. data), although the strain has not been isolated from this medium. A substantive increase in multiresistant *S.* Java has also been reported in Australia (D. Lightfoot, pers. comm.). The antibiogram of these isolates are indistinguishable from the isolates of R-type ACSSpSuT made in the United Kingdom.

These results suggest either a common origin of the ACSSpSuT-resistance gene cluster in epidemic multiresistant DT104 and multiresistant *S.* Java or the horizontal transfer of the cluster from DT104 to other *Salmonella* serovars with a worldwide distribution. In either event, the increasing occurrence of the DT104 resistance gene cluster in potentially epidemic serovars other than *S.* Typhimurium DT104 is concerning.

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1990s *Vibrio cholerae* Epidemic, Brazil

To the Editor: We read with interest the letter by Sarkar et al. on new *Vibrio cholerae* phages (1). The description of new *V. cholerae* phages is a welcome tool for epidemiologic

studies of this species. Our main concern about their work is the inaccurate picture that is presented of the cholera epidemic in Brazil. Some of the statements made in the final paragraphs are in disagreement with the official epidemiologic records and the characteristics of the *Vibrio* bacteria that occurred in Brazil during the 1990s epidemic (2).

In 1991, the seventh cholera pandemic reached South America by the Pacific coast, spreading to Brazil in the same year (3). In Brazil, the first cholera cases were reported in the Amazon region bordering Peru; within a few months a large number of cholera cases were recorded in states facing the Atlantic Ocean in the northeastern region (2). According to the official figures of the Brazilian Ministry of Health (2), 168,598 cases of cholera caused by a *V. cholerae* O1 El Tor strain occurred in Brazil from 1991 to 2001. Of these, 155,363 (92.1%) occurred in the northeastern area of the country, with 2,037 deaths. From 2001 to 2003, the number of confirmed cases was 4,756, 734, and 7, respectively.

Sarkar et al. (1) indicate that 60,000 cases occurred from 1991 to 2001 in Rio de Janeiro, a city localized in the southeastern region; the official records report only 349 cases. The statement that "since 1993, no cholera cases caused by O1 have been reported" is also perplexing. From 1994 to 2001, the official records report 68,583 cases of cholera in Brazil (51,324 of these in 1994, the second major year of cholera incidence). Are the authors suggesting that this number of cases was caused by non-O1 *V. cholerae*? The official records state that the cholera epidemic in Brazil was caused by an El Tor O1 strain (4,5).

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Fluoroquinolone-resistant *Salmonella* Paratyphi A

To the Editor: Fluoroquinolones have been the drug of choice for treating typhoid and paratyphoid fever since the beginning of the 1990s. Multidrug-resistant strains began to prevail in disease-endemic areas, and former first-line antimicrobial drugs, such as chloramphenicol, were sometimes ineffective (1). In recent years, however, strains with decreased susceptibility to quinolones have emerged, and clinical treatment failure is a serious concern (2–5).

An 87-year-old woman was referred from a local clinic to Yokohama Municipal Citizen's Hospital in July 2002 because *Salmonella enterica* serovar Paratyphi A was detected in her urine. She had no subjective symptoms such as pain on urination or urinary urgency, and her temperature was normal. She had never had paratyphoid fever, and she had not traveled abroad. No other person in the community had paratyphoid. Before being admitted to the hospital, she had experienced frequent episodes of urinary tract infection and had been empirically treated each time with oral antimicrobial drugs, including ciprofloxacin. She had been given a dose of 600 mg/day for 7 days, 25 times in the last 4 years.

The patient did not display any abnormal findings on physical examination. *S.* Paratyphi A was not detected in the urine but was confirmed in the stool; therefore, the previous report of bacteriuria could have been due to contamination of a urine sample with feces. An ultrasound showed a polyp and multiple stones in her gallbladder. A carrier state was suspected. Bile was obtained by duodenal aspiration and was positive for *S.* Paratyphi A. The patient was considered to be an asymptomatic cholecystic carrier of *S.* Paratyphi A.

On disk diffusion susceptibility testing, the isolate was resistant to nalidixic acid (NA) and to ofloxacin. The MIC of ofloxacin was as high as 256 μg/mL, and the MIC of ciprofloxacin was128 μg/mL (Table). An open cholecystectomy was performed for treatment of the polyp, the stones, and the highly quinolone-resistant bacteria. A routine perioperative intravenous antimicrobial agent, cefmetazole, was administered as surgical prophylaxis. The polyp was malignant, and the operation was

Table. MICs of antimicrobial agents for the isolate of *Salmonella enterica* serovar Paratyphi A*

Antimicrobial agent	MIC (μg/mL)	
	Etest	Broth microdilution
Ampicillin	4	ND
Chloramphenicol	16	ND
Gentamicin	0.06	ND
Kanamycin	1	ND
Streptomycin	0.75	ND
Sulfamethoxazole/trimethoprim	0.5	ND
Tetracycline	8	ND
Cefoperazon	1.5	ND
Cefotaxime	0.38	ND
Ceftriaxone	0.19	ND
Imipenem	0.19	ND
Aztreonam	0.125	ND
Fosfomycin	64	ND
Nalidixic acid	>256	ND
Norfloxacin	>256	1024
Ofloxacin	>32	256
Sparfloxacin	>32	256
Ciprofloxacin	>32	128
Levofloxacin	ND	128
Tosufloxacin	ND	128

*ND, no data.

curative. The patient resumed normal activities, and had no further fecal excretion of *S.* Paratyphi A.

Polymerase chain reaction (PCR) amplification and DNA sequencing were conducted to detect mutations responsible for the fluoroquinolone resistance. Nucleotide sequences of *gyrA*, *gyrB*, *parC*, and *parE* genes were investigated. The primers used for PCR amplification and DNA sequencing have been previously described (6,7). An ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) and an automated sequencer (311A; Perkin-Elmer, Applied Biosystems) were used. The isolated strain possessed triple point mutations. The first 2 mutations were in the *gyrA* gene, which encodes DNA gyrase, at codon 83 (TCC to TTC), which substitutes phenylalanine for serine, and at codon 87 (GAC to AAC), which substitutes asparagine for aspartic acid. The third mutation was in the *parC* gene, which encodes DNA topoisomerase IV, at codon 84 (GAA to AAA), which substitutes lysine for glutamic acid. No mutations were found in *gyrB* and *par E*.

S. Typhi and Paratyphi A with decreased susceptibility to fluoroquinolones emerged on the Indian subcontinent, Southeast Asia, and Central Asia in the mid-1990s (2–5). On disk diffusion testing, these strains were NA-resistant, and susceptible to ofloxacin or ciprofloxacin; however, the MICs of ciprofloxacin increased to 0.25–4 μg/mL, 10- to 100-fold higher than the usual NA–susceptible strains (5,8,9). NA-resistant strains of *S.* Typhi have 1 point mutation at the target site of quinolones, DNA gyrase, in the quinolone resistance–determining region of *gyrA*, either at codon 83 or codon 87 (2,3). Several epidemics of typhoid and paratyphoid fever caused by NA-resistant strains with clinical failure of quinolone treatment have been reported (4,5).

An experimental attempt had been previously made to induce the production of strains with high-level fluoroquinolone resistance by culturing strains of *S.* Typhi and Paratyphi A in ciprofloxacin-supplemented medium (7). One of these in vitro–induced, high-level resistant strains of *S.* Paratyphi A had triple mutations in the *gyrA* gene at codons 83 and 87 and in the *parC* gene at codon 84, which are the same triple mutations as those seen in the current in vivo case.

Full fluoroquinolone resistance has already emerged in the community in nontyphoid *Salmonella* species. In a clinical isolate of *S. enterica* serovar Typhimurium, the MIC of ciprofloxacin was 32 μg/mL, and mutations in both *gyrA* and *gyrB* were noted (10).

Our findings strongly suggest that high-level quinolone resistance was induced through the long-term carrier state of *S.* Paratyphi A under selective pressure of frequent quinolone administration. The resistance is associated with the 2 mutation sites in *gyrA* and 1 site in *parC*, and multiple point mutations are likely related to the acquisition of full resistance. Physicians should be aware of the emergence of *S.* Typhi and Paratyphi A, as well as nontyphoid *Salmonella* species, which are highly quinolone-resistant.

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Pygmy Populations Seronegative for Marburg Virus

To the Editor: A serosurvey was conducted in Durba, a mining village near Watsa, northeastern Democratic Republic of Congo, the epicenter of Marburg hemorrhagic fever (MHF) outbreaks in 1994 and 1998–2000 (1–3). In this survey, Bausch et al. found a prevalence of anti-Marburg immunoglobulin (Ig) G of 0.35% (2 of 565) in the nonmining population, but a prevalence of 3.75% (13 of 347) in miners. Mine work was an independent risk factor for seropositivity for anti-Marburg IgG (1). Given that widespread secondary transmission could not be documented in the seropositive miners, primary transmission from the unknown reservoir likely occurred in the mines where rodent, shrew, bat, and other fauna were abundant. No evidence of Marburg virus (MBGV) infection was found in samples from small mammals, amphibians, and arthropods collected in and around Gorumbwa mine (R. Swanepoel, pers. comm.); the origin of the MHF outbreak remained unknown.

We hypothesized that the MBGV reservoir's habitat might not be limited to gold mines around Durba, but may exist in caves or forests in the wider Watsa area. As hunter-gatherers, pygmies enter caves for shelter and are in frequent contact with wild animals and body fluids of butchered game. Earlier studies found that pygmies were seropositive for filoviruses significantly more often than subsistence farmers (for filoviruses [4,5], for Ebola but not Marburg [6]). We conducted a seroprevalence study to verify whether pygmies living in the Watsa area constitute another population at risk for primary transmission of MBGV.

The Watsa area's population (≈180,000) includes 4,000 pygmies living predominantly in its southern parts (1). The pygmies live seminomadically in the forest, occasionally leaving to exchange goods with the sedentary Bantu population.

We invited the pygmy population to meet with our study representatives at sites 50–90 km from Durba. Three hundred persons volunteered during a 5-day period. After informed verbal consent was obtained, the study participants were interviewed, and a blood sample was taken from each volunteer. For operational reasons, we excluded children <10 years old. According to local customs, men received small quantities of salt and soap and women received an item of second-hand clothing as an appreciation for their efforts. Ethical clearance was obtained by the ethics committee of the Institute of Tropical Medicine in Antwerp and the representative of the Ministry of Health in Watsa.

The study questionnaire was similar to one used in the Durba 1999 survey; we did not maintain a recall period of 1 year for exposures related to medical treatment, as this did not appear to be a meaningful time span for the pygmies. Procedures for collecting and handling blood samples were similar to the Durba survey, and the same laboratory tests were applied. Serum samples were considered positive only if they were positive for Marburg IgG in both enzyme-linked immunosorbent assay and indirect immunofluorescence assay (IFA) (1).

The study participants originated from 39 different settlements. Their median age was 30 years (range 10–75; q1 20, q3 40); half of them were males. Most study participants reported activities (hunting 60%, entering caves 98%) and contacts with wild animals (rodents 79%, bats 78%, monkeys or apes 99%) thought to be risk factors for the primary transmission of filoviruses. Whenever noticeable differences existed between the sexes, men tended to be exposed more frequently than women, often significantly so. Pygmies were significantly more exposed to wild animals than the nonmining general population; the difference was particularly large concerning contact with bats (Table). From one fourth to one third of study participants reported a direct or potential contact with someone with a febrile hemorrhagic syndrome. Women were more frequently exposed to these risk factors for secondary transmission in the household or community than men, sometimes significantly so; pygmies were less exposed to these risk factors than the nonmining general population (Table). Almost all study participants had been exposed at least once in their

life to invasive modern or traditional medical treatment, including injections and scarification, by which an iatrogenic secondary transmission could have occurred.

Thirty-seven percent of the study participants reported having experienced a febrile hemorrhagic syndrome at least once in their life, men more often than women (n = 236; 45% versus 28%, chi-square test: p = 0.006). All serum samples, however, were negative for anti-Marburg IgG; the prevalence of anti-Marburg IgG in the pygmy population (0.0%; exact binomial one-sided 97.5% confidence intervals [CI] 0.00%–1.2%) was similar to that in Durba's nonmining population (0.35%; 95% CI 0.04%–1.3%), significantly lower than in Durba's mining population (3.7%; 95% CI

Table. Frequency of risk factors for Marburg hemorrhagic fever in pygmies and nonmining general population residing in the Watsa Health Zone, Democratic Republic of Congo

Risk factors	Male pygmies (n = 150) (%)	Female pygmies (n = 150) (%)	p*	Pygmy population (n = 300) (%)	Nonmining population (n = 553 to 569)† (%)	p*
Primary transmission risk factors						
Subsistence activities						
Hunting	100	20	< 0.001	60	–	–
Entering caves	98	99	0.7	98	–	–
Contacts with wild animals						
Rodents						
Touched	85	59	< 0.001	72	53	< 0.001
Eaten‡	42	43	0.9	42	34	0.02
Bitten by	33	27	0.3	30	26	0.15
Any contact	88	71	< 0.001	79	65	< 0.001
Bats						
Touched	81	68	0.008	75	16	< 0.001
Eaten‡	59	47	0.04	53	3	< 0.001
Bitten by	23	15	0.06	19	0.9	< 0.001
Any contact	83	72	0.02	78	18	< 0.001
Monkeys, apes						
Touched	99	83	< 0.001	91	59	< 0.001
Eaten‡	97	96	0.8	96	79	< 0.001
Bitten by	6	5	0.6	5	8	0.2
Any contact	99	97	0.1	98	84	< 0.001
Any wild animals	99	98	0.3	99	90	< 0.001
Secondary transmission risk factors						
Contact with FHS§ patient						
In the same household with FHS patient	19	25	0.3	22	25	0.4
In the same room with FHS patient	11	20	0.04	16	22	0.03
Worked with FHS patient	16	25	0.06	20	28	0.02
Participated in funeral of FHS patient	19	25	0.2	22	44	< 0.001
Touched FHS patient	15	23	0.06	19	32	< 0.001
Touched blood, urine, feces of FHS patient	10	13	0.5	11	7	0.03
Touched remains of FHS patient	11	19	0.05	15	10	0.02
Any contact	27	36	0.1	32	58	< 0.001
Any direct contact (touched)	22	31	0.09	26	34	0.02
Invasive medical treatment¶						
Ever received injection	85	90	0.2	88	–	–
Ever received surgical or obstetric care	52	31	< 0.001	41	–	–
Any invasive medical treatment ever	93	93	–	93	–	–
Traditional treatment						
Ever had scarification	99	97	0.4	98	–	–

*Using chi-square test.
†Variation in sample size due to missing data.
‡Bush meat often is smoked, grilled, or cooked; exposure to viable virus may therefore be more likely to happen during preparation of such meat for consumption than during consumption itself.
§FHS (febrile hemorrhagic syndrome): severe illness with high fever and bleeding from the nose, mouth or anus.
¶Includes circumcision, abscess incision, and other minor intervention.

2.0%–6.3%), and as low as, or even lower than, that in other populations in sub-Saharan Africa, where a seroprevalence was found in 0% to 1.7% in 15 studies. Only 2 studies from the Central African Republic and Uganda found a higher seroprevalence (3.2% and 4.5%, respectively; all studies are referenced [1]). In studies conducted before the 1999 Durba survey, the presence of anti-Marburg IgG has been determined by only the less specific IFA; this may explain why we have found a lower prevalence in our study population than reported from certain other locations in sub-Saharan Africa.

We reject our study hypothesis that pygmies residing in the Watsa area are a second population at risk for MHF compared with the nonmining sedentary population. We conclude that the absence of anti-Marburg IgG in the pygmy population reflects the virtual absence of MBGV circulation in the reservoir, the absence of the reservoir in the pygmies' environment, the absence of exposure to the reservoir, or any combination of these. The MHF outbreaks in Durba and Watsa in 1994 and 1998–2000 apparently did not impact the study population. The frequent occurrence of febrile hemorrhagic syndrome was almost certainly due to a different origin than MBGV and may not have been of viral origin at all.

An alternative explanation for the absence of antibodies would be that the case-fatality proportion was higher than observed during the outbreaks in Durba and Watsa (71%) (3). However, there is no reason to assume that pygmies who contract MHF would die more frequently than diseased gold diggers and their family members. Access to basic clinical care is similar in both groups, and this care has a limited effect on the case-fatality proportion.

Another alternative explanation would be that anti-Marburg IgG wanes and becomes undetectable soon after infection. However, all 17 survivors of confirmed MHF in the 1994 and 1998–2000 Durba and Watsa outbreaks with whom we could follow up are still seropositive 22–102 months after onset of disease (M. Borchert, unpub. data).

Our study participants were volunteers who could reach the meeting points along the main road with relative ease. Primary transmission of MBGV may occur more frequently in pygmies living deeper in the forest, but even in those who reached the meeting point and participated in our study, the prevalence of risk factors was very high. Reported exposure patterns correspond to the traditional distribution of tasks such as men hunting and women caring for sick relatives, which lends credibility to our interview data. Gonzalez et al. did not find a significant difference for the risk of filovirus infection between pygmies living in savannah and forest areas (6). That the study used volunteers might also have caused seroprevalence to be underestimated if those who rightfully believed they had had MHF in the past, chose not to take part in the study. However, the proportion of study participants reporting to ever have had a febrile hemorrhagic syndrome was high, and MHF was not stigmatized in the study setting. We therefore believe a selection bias is unlikely.

Despite the MHF epidemics in Durba and Watsa in 1994 and 1998–2000, the prevalence of anti-Marburg IgG in the pygmy population of Watsa was as low as, or lower than, that in Durba's nonmining sedentary population, and that in most other populations in sub-Saharan Africa where serosurveys have been conducted. Infection with MBGV appears to be rare in the pygmy population of the Watsa area. During the 1998–2000 outbreak, primary transmission of MBGV was apparently limited to gold mines around Durba. While the location where primary transmission occurred now appears to be well ascertained, the reservoir species at the origin remains unknown.

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Disseminated Coccidioidomycosis

To the Editor: Coccidioidomycosis, an infection caused by the dimorphic fungus *Coccidioides immitis*, is endemic in the southwestern United States, parts of Mexico, and Central and South America (1). Patients with *C. immitis* infection may have chronic pneumonia, fungemia, and extrapulmonary dissemination to skin, bones, meninges, and other body sites. The clinical features of coccidioidomycosis may mimic those of melioidosis, penicilliosis marneffei, and tuberculosis, which are commonly seen in some southeastern Asian countries, including Taiwan.

A previously healthy, 71-year-old retired gynecologist from Taiwan, visited Los Angeles in August 2003 and traveled to the San Joaquin Valley in November 2003. He had smoked 1 package of cigarettes daily for 50 years. He noted fever 5 days before returning to Taiwan on December 1, 2003. He came to a local hospital on December 4 with a temperature of 39°C and a history of 1 month of night sweats, productive cough, and weight loss of 10 kg. Chest radiograph showed diffuse nodular lung lesions bilaterally (Figure, panel A). His leukocyte count was 16.65 x 10^9/L (neutrophils 85.6%, lymphocytes 6.2%), and C-reactive protein was 21.5 mg/dL (reference value, <0.8 mg/dL). Empiric antimicrobial drugs (amoxicillin/clavulanic acid and ciprofloxacin) and antituberculosis therapy (isoniazid, rifampin, ethambutol, and pyrazinamide) were administered. Blood and sputum specimens were negative for bacteria; HIV antibody test results were negative, but the fever persisted. A follow-up chest film showed a left pleural effusion. The pleural effusion aspirate was exudative with 3.6 x 10^9/L leukocytes (73% neutrophils). Computed tomographic scan of the patient's chest showed collapse of the left lower lung with central necrosis, bilateral pleural effusions, and mediastinal lymphadenopathy. Pleural biopsy by video-assisted thoracoscopic surgery showed no evidence of malignancy, but heavy lymphoplasmacytic infiltration and chronic necrotizing granulomatous inflammation were found (Figure, panel C). On December 17, 2003, 30 mg/day prednisolone orally was prescribed for intermittent fever. Biopsy material and cultures of blood samples taken at admission grew an

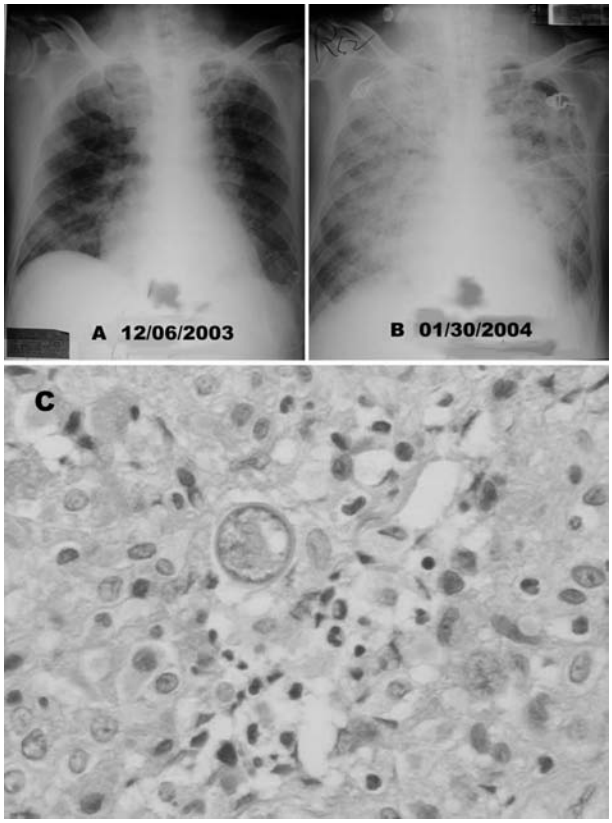

Figure. A) Chest radiograph shows diffuse nodular lesions in both lungs. B) Chest radiographic scan taken 2 months later shows coalescence of nodular shadows and almost complete white-out of bilateral lung fields. C) Hematoxylin and eosin staining of the wound specimen from pleural biopsy site showed spherules of *Coccidioides immitis* and chronic necrotizing granulomatous inflammation (400x).

unidentified mold, which was also isolated from the biopsy wound. The patient was discharged afebrile from the hospital on January 20, 2004. The fever recurred, with a disturbance in consciousness on January 25, 2004. Computed tomographic scan of the brain revealed no obvious organic lesions. He was referred to our hospital on January 26, 2004.

After the patient was admitted, fever persisted and respiratory distress worsened rapidly. He developed severe headache, seizures, and loss of consciousness. He was transferred to the intensive care unit for aggressive management of acute respiratory distress syndrome and deterioration of renal function. Chest radiograph showed coalescence of nodular shadows and almost complete white-out of bilateral lung fields (Figure, panel B). Meropenem, antituberculosis agents, and intravenous voriconazole, 200 mg every 12 hours, were administered.

Both the unidentified mold, which was sent to our hospital for further identification, and a mold cultured from the previous biopsy wound at our hospital were identified as *C. immitis* by their characteristic gross and microscopic morphotypes in standard slide cultures incubated at 28°C for 10 days. Hematoxylin and eosin staining of the biopsied tissue showed many spherules.

Lumbar puncture was performed on January 30, 2004, and showed an elevated opening pressure of 380 cm H_2O and a few destructed large spherules in the cerebrospinal fluid (CSF). However, cultures of CSF were negative for bacteria and fungi. After the diagnosis of disseminated coccidioidomycosis (pneumonia, fungemia, and meningitis), voriconazole was replaced by intravenous fluconazole, 400 mg/day. The patient's intensive care course was complicated by *Pseudomonas* pneumonia and repeated episodes of upper gastrointestinal bleeding. A second lumbar puncture was conducted on February 13, 2004, and also showed an elevated opening pressure (290 cm H_2O). Uncontrolled coccidioidomycosis meningitis was suspected, and intrathecal amphotericin B treatment was planned. Refractory shock with bradycardia developed when an intrathecal catheter was implanted. The patient did not respond to therapy and died on February 16, 2004. The MIC of fluconzole for the *C. immitis* isolate was 48 μg/mL, and the MIC of amphotericin B was found to be 1 μg/mL for by using the Etest (ABiodisk, Solna, Sweden) according to manufacturer's information.

This case is the first to be reported of disseminated coccidioidomycosis with fulminant pneumonia, fungemia, and meningitis reported from Taiwan (2). Review of the patient's travel history and clinical course indicated that the *C. immitis* was acquired in California and that the initial manifestations had begun before the patient returned to Taiwan. Coccidioidomycosis is commonly diagnosed in disease-endemic areas but frequently overlooked in disease-nonendemic areas because of a low index of suspicion among physicians. The interval from onset of symptoms to disease diagnosis was relatively long (3). Our patient had chills, productive cough, weight loss, and night sweats followed by fever as the initial manifestations of this infection. These symptoms had been most frequently reported in previous coccidioidomycosis cases (4). Radiographic scans of the patient initially showed diffuse reticular lesions, followed by pleural effusion and consolidation. This clinical course was also fully compatible with those of previously reported cases (4). However, the clinical manifestations of chronic pneumonia with pleural effusion, the initial partial response to steroid treatment, and the delay in recognizing the mold contributed to delayed diagnosis of this disease.

The isolate was not susceptible to fluconazole (MIC 48 μg/mL). Although the National Committee for Clinical Laboratory Standards does not have a standard susceptibility method and MIC breakpoint of fluconazole for defining susceptibility against *C. immitis*. Fluconazole has been recommended as a drug of choice for treating meningal coccidioidomycosis, particularly in patients with underlying renal disease or with disease-associated renal function deterioration (4,5). Immunocompromise secondary to steroid use, as well as resistance of the isolate to fluconazole, may have contributed to treatment failure in this patient.

With increasing international travel, physicians should consider those diseases that are endemic in regions where their patients have traveled. In addition to tuberculosis, melioidosis, and penicilliosis marneffii, coccidioidomycosis should be included in the differential diagnosis of chronic pneumonia in Taiwan, considering the number of residents who travel. Only then can prompt microbial investigations be conducted to accurately diagnosis and determine the appropriate antifungal treatment.

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Kytococcus schroeteri Endocarditis

To the Editor: Becker et al. recently reported the probable implication of *Kytococcus schroeteri* in a case of acute prosthetic valve endocarditis, on the basis of its recovery from blood cultures drawn at the time of infection (1). *K. schroeteri* was only characterized on that occasion and is a new micrococcal species resistant to penicillins (2). Here, we report the isolation of this organism from prosthetic valve vegetations in a patient who had undergone aortic valve replacement 3 years earlier. The 73-year-old man was admitted with fever (38.8°C) and shortness of breath, which had both increased gradually over the previous 2 months. He had no recent history of intravenous drug administration or catheterization. Laboratory findings showed a leukocyte count of 12 x 10^9/L (90% neutrophils) and a raised C-reactive protein level. Transesophageal echocardiogram revealed several small vegetations on the Carpentier-Edwards aortic bioprosthesis and a voluminous perivalvular abscess. Four sets of blood cultures were drawn before antimicrobial therapy was initiated.

Intravenous vancomycin (2 g twice a day) and gentamicin (240 mg/d) were started empirically. The prosthetic material was replaced promptly and the abscess was debrided extensively. Vegetations from the resected material showed numerous polymorphonuclear neutrophils and gram-positive cocci on microscopic examination. Oral rifampicin (600 mg twice a day) was added to the initial regimen.

The postoperative course was uneventful except for cutaneous intolerance to vancomycin, which was replaced with teicoplanin. The physical condition of the patient improved steadily. Gentamicin and rifampicin were discontinued after 3 weeks. Eight months after completion of the 6-week treatment, the patient had no clinical or biologic evidence of infection, although moderate aortic incompetence persisted.

All blood cultures drawn on admission grew gram-positive cocci after 72 hours and subcultures on Trypticase soy agar yielded convex, muddy-yellow colonies of heterogeneous sizes. The vegetations, pus samples of the abscess, and prosthetic valve cultures grew the same type of colonies. All isolates displayed identical biotype and antimicrobial susceptibility and were considered as a single strain. The causative organism (designated ROG140) was initially identified as *Micrococcus* sp. based on the morphologic features, resistance to nitrofurantoin, and inability to grow anaerobically. Assignment to the genus *Kytococcus* was suggested by the arginine dihydrolase activity and resistance to oxacillin, 2 characteristics that are not shared by other micrococci (3).

The definitive *K. schroeteri* identification was provided by analysis of the fatty acid content, which was similar to that of the type strain (2), and sequencing of the 16S rRNA genes.

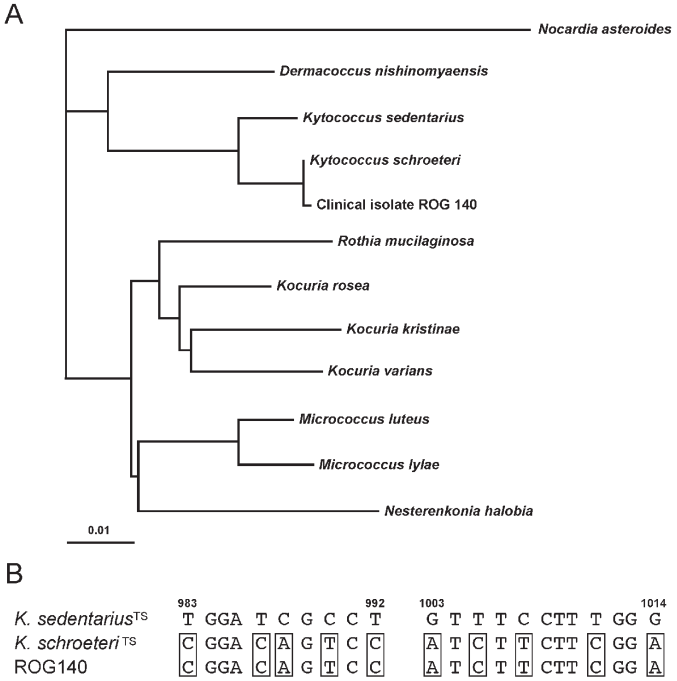

Figure. (A) Phylogenetic tree showing relationships among 16S rDNA sequences of clinical isolate ROG140 and type strains of members of the former *Micrococcus* genus. *Nocardia asteroides* was included as an out-group organism. The scale bar represents 1% differences in nucleotide sequences. (B) Sequence alignment of 16S rDNA nucleotides 983-992 and 1003-1014 of *Kytococcus* sp. type strains (TS) and clinical isolate ROG140. *K. schroeteri* molecular signatures are boxed. Nucleotide numbering refers to the sequence of the 16S rDNA of *E. coli*.

We sequenced a 1,012-bp fragment encompassing the first two thirds of the 16S rDNA of ROG140 (accession no. AY692224). The sequence was compared with those of type strains of all members of the former genus *Micrococcus*, and a phylogenetic tree was deduced by the neighbor–joining method (Figure). The sequences of ROG140 and the *K. schroeteri* type strain only differ by an A-to-G substitution at position 747 (*E. coli* numbering). Among the 21 nucleotide differences between the sequences of *K. schroeteri* and the closely related species *K. sedentarius*, 10 are located on a 30-base stretch and constitute a convenient *K. schroeteri* signature (Figure)

Antimicrobial susceptibility testing performed with the disk diffusion method and Etests (AB Biodisk, Solna, Sweden) indicated that the isolate was resistant to penicillins, cephalosporins, kanamycin, tobramycin, erythromycin, clindamycin, sulfonamides, and fusidic acid, but susceptible to imipenem (MIC, 0.25 μg/mL), gentamicin (MIC, 1 μg/mL), trimethoprim (MIC, 0.25 μg/mL), tetracycline (MIC, 0.12 μg/mL), linezolid (MIC, 0.25 μg/mL), vancomycin (MIC, 0.125 μg/mL), teicoplanin (MIC, 0.06 μg/mL), and rifampicin (MIC, <0.002 μg/mL). Unlike the original isolate reported by Becker et al. (1), isolate ROG140 was resistant to ofloxacin and ciprofloxacin (MICs, 8 μg/mL). Conversely, moxifloxacin displayed excellent in vitro activity (MIC, 0.05 μg/mL). As moxifloxacin was more rapidly microbicidal than vancomycin in an animal model of *Staphylococcus aureus* prosthetic valve endocarditis (4), it might present a potential advantage against infections caused by *K. schroeteri*, especially when the oral route is favored.

The natural habitat of *K. schroeteri* remains unknown. The only isolates of *K. schroeteri* identified so far originated from blood or cardiac material, although *Kytococcus* literally means "a coccus from the skin." Our attempts to recover *K. schroeteri* from the mouth, nose, or skin of our patient were unsuccessful. In a recent study, Szczerba et al. were able to isolate most micrococcal species, including *K. sedentarius* but not *K. schroeteri*, from human skin and mucosa (5). However, at that time the authors may not have been aware of this newly described species. The mode of contamination also remains unclear. In the original description (1), *K. schroeteri* endocarditis had developed in the patient <3 months after she underwent cardiac surgery, which suggested perioperative contamination. Here, we describe a late onset, subacute infection 3 years after surgery, which is more likely to have been caused by hematogenous spread.

Although *Micrococcus*-like organisms cause endocarditis infrequently (6), the description of 2 independent infections due to a new species in a short period is intriguing and suggests a specific pathogenicity, at least on prosthetic heart devices. By demonstrating the presence of the bacteria in the infected site, this report establishes *K. schroeteri* as a genuine pathogen in this clinical setting and should prompt further investigations to identify its natural habitat and virulence determinants. At present, commercial systems are not able to identify *K. schroeteri*. However, gram-positive cocci that are strictly aerobic, oxacillin-resistant, and arginine dihydrolase-positive should be recognized as potential *Kytococcus* species and taken into account when endocarditis is suspected.

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Viral Gastroenteritis in Mongolian Infants

To the Editor: Viral agents of gastroenteritis affect millions of persons of all ages worldwide (1). The major agents include rotavirus, norovirus, sapovirus, astrovirus, and enteric adenovirus. Rotavirus is the most frequent cause of acute sporadic childhood gastroenteritis (1), whereas norovirus infects both adults and children and is mainly associated with

outbreaks of acute gastroenteritis (1). These viruses are commonly transmitted by foodborne, person-to-person, fecal-oral, and environmental routes.

In 1999, the infant death rate was 37.3 per 1,000 live births in Mongolia (2). Bacterial pathogens, such as *Shigella flexneri* and *Salmonella*, are commonly detected in hospitalized patients with gastroenteritis, but no data exist concerning viral agents of gastroenteritis in hospitalized patients or in the general community (2).

This preliminary community-based molecular epidemiologic study was the first to report viral agents of gastroenteritis in Mongolian infants. Stool specimens collected from July to August 2003 from 36 infants belonging to 25 different households from 2 areas in Mongolia were screened for rotavirus, norovirus, sapovirus, astrovirus, and adenovirus. The 2 areas were Tov Province, which included Zuun Mod (provincial center) and Bayanchandmani (provincial district center), and Ulaanbaatar area (capital city), which included Chingeltei, Bayangol, Songinok-harikhan, and Bayanzurkh. A total of 48 stool specimens, which were randomly selected from negative-enterovirus specimens (poliovirus and nonpolio enterovirus (Minako Kuramitsu, unpub. data), were screened. Of the 36 infants in the study, 2 specimens were collected 3 weeks apart from each of 12 infants, and 1 specimen was collected from each of 24 infants. In 10 households, specimens were collected from 2 or 3 siblings. Clinical symptoms were recorded when available.

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) were performed as described elsewhere (3); for norovirus genogroup (G) I (GI), PCR, G1SKF, and G1SKR primers were used, and for norovirus GII PCR, G2SKF, and G2SKR primers were used (4). For sapovirus, a nested PCR approach was used for all human genogroups (5). For the first sapovirus PCR, SV-F11 and SV-R1 primers were used, while for the nested PCR, SV-F21, and SV-R2 primers were used. For astrovirus PCR, Mon244, and 82b primers were used (6). All PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. For rotavirus and adenovirus screening, a rapid dry-spot latex agglutination test, Diarlex Rota-Adeno (Orion Diagnostica, Espo, Finland) was used.

Reverse transcription (RT)–PCR products were excised from the gel and purified by the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1) and determined with the ABI 3100 avant sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were aligned with Clustal X and the distances were calculated by Kimura's 2-parameter method (3). The nucleotide sequence data determined in this study have been deposited in GenBank under accession no. AY590250–AY590262.

Specimens from 12 (33%) of 36 infants were positive for viral agents of gastroenteritis. Specimens from 9 infants were positive for noroviruses, specimens from 2 infants were positive for astroviruses, and a specimen from 1 infant was positive for sapovirus. All specimens were negative for rotavirus and adenovirus. Ten isolated norovirus sequences (9 persons) were classified according to the recent capsid-based sequence scheme of Kageyama et al. (7). Two norovirus sequences belonged to genogroup I/genotype 11 (G1/11), 4 sequences belonged to GII/3, 1 sequence belonged to GII/7, and 3 sequences belonged to GII/6 (Table).

In 1 household, 2 female infants (isolates 213-3 and 214-3, respectively) were infected with a norovirus GI/11 strain that shared 100% nucleotide identity. This strain was

Table. Mongolian infants positive for viral agents of gastroenteritis

Virus	Genogroup/genotype	Specimen*	Symptom†	Age (mo.)	Sex
Norovirus	GI/11	213-3‡	NA	4	F
Norovirus	GI/11	214-3‡	NA	24	F
Norovirus	GII/6	101-1	None	5	F
Norovirus	GII/3	109-1	Diarrhea	6	F
Norovirus	GII/6	205-3	NA	5	F
Norovirus	GII/3	209-1	Diarrhea	3	M
Norovirus	GII/3	317-1§	NA	24	M
Norovirus	GII/6	613-1	None	5	M
Norovirus	GII/7	613-3	NA	5	M
Norovirus	GII/3	609-3§	NA	5	M
Astrovirus	GI¶	121-3	NA	4	M
Astrovirus	GI	201-3	NA	5	M
Sapovirus	GI	217-1	Diarrhea	1	F

*First 3 numbers before the hyphen refer to the infant; number after the hyphen refers to the week the specimen was collected.
†NA, not available.
‡Two siblings from the same household.
§Only 1 of the siblings from this household was infected.
¶Astrovirus GI = serotype 1.

likely the same and suggests a common source of contamination or person-to-person transmission. Strains belonging to this new genotype have only been detected in Japan and Switzerland (7).

In a different household, 2 different norovirus strains were detected 3 weeks apart in a 5-month-old male infant (isolates 613-1 and 613-3, respectively). These 2 isolated norovirus sequences shared 77.5% nucleotide identity and clustered into two different genotypes, GII/6 (isolate 613-1) and GII/7 (isolate 613-3). In spite of this infection, the infant had no symptoms of gastroenteritis during excretion of the first norovirus strain.

In 4 other households, 4 infants (isolates 109-1, 609-3, 317-1, and 209-1) were infected with norovirus strains belonging to GII/3. These 4 isolated sequences shared >98% nucleotide identity to Arg320 sequence (AF190817), which was previously found to be a recombinant norovirus (8). This result suggests these 4 strains are also recombinant noroviruses, though further sequence analyses of other genetic regions are needed to confirm this result.

Astrovirus was detected in 2 male infants from different households. One infant was 4 months of age (isolate 121-3), and the other infant was 5 months of age (isolate 201-3). These 2 isolated astrovirus sequences had 100% nucleotide identity, which suggests a common source of contamination. These isolated astrovirus sequences shared 98% nucleotide identity to astrovirus Oxford virus sequence (genogroup I). Sapovirus was detected in 1 stool specimen (isolate 217-1) from a 1-year-old female with diarrhea. The isolated sapovirus sequence shared 98% nucleotide identity to sapovirus Manchester virus sequence (genogroup I). Rotavirus and adenovirus were not detected in any of these specimens; further studies, including those of hospitalized infants, may be useful since infants with rotavirus infections are commonly admitted to hospitals (9).

Our preliminary findings have shown that norovirus was a common agent of gastroenteritis (9 of 36 persons) in Mongolian infants. In a recent report on norovirus gastroenteritis, the risk of contracting gastroenteritis was high when another household member was infected and slightly higher when that member was a child (10). In our study, we found 2 siblings infected with an identical norovirus strain during the same period. In Mongolia, diarrhea has become a major healthcare problem (2), therefore, general education in sanitation and hygiene practices may help reduce the transmission of these viruses and lessen the frequency of this disease.

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Bordetella pertussis Isolates, Finland

To the Editor: Pertussis, or whooping cough, is a highly contagious respiratory disease in humans caused by *Bordetella pertussis*. Reemergence of pertussis has been observed in many countries with high vaccination coverage. In the United States, reported cases of pertussis in adolescents and adults have increased since the 1980s, despite increasingly high rates of vaccination in infants and children (1). At the same time, clinical *B. pertussis* isolates have become antigenically divergent from vaccine strains (2,3). This observation has raised the question of whether vaccination has caused selection for the variant strains, and whether the reemergence of pertussis in vaccinated populations is due to vaccination not protecting against these antigenic variants as effectively as it protects against vaccine type strains. On the other hand, vaccine-induced immunity wanes over time, and pertussis is not only a childhood disease but also a frequent cause of prolonged illness in adults and adolescents today (4).

In Finland, children are vaccinated with diphtheria-tetanus whole-cell pertussis vaccine at 3, 4, and 5 months, and at 20 to 24 months of age. The whole-cell vaccine contains 2 strains and has remained unchanged since 1976. The vaccine strain 18530 contains fimbriae 3 (Fim3), pertussis toxin S1 subunit D (PtxS1D), and pertactin 1 (Prn1); the other vaccine strain, 1772, contains Fim2,3, PtxS1B, and Prn1. Ninety-six percent of Finland's population has been vaccinated with 4 doses of pertussis vaccine. The incidence of pertussis is highest in infants <1 year of age and in schoolchildren from 6 to 14 years old, although about 30% of the cases occur in adults older than 20 years. In Finland, as in many other countries with large-scale vaccination programs, several outbreaks of pertussis occurred in the 1990s. We studied prospectively 3 pertussis outbreaks in 2 elementary schools and 1 municipality in southwestern Finland (5,6). The aim of the study was to characterize the strains circulating and causing outbreaks and to track the transmission of *B. pertussis* during these outbreaks.

Sample were collected and primary cultures were done as described earlier (5,6). (See online Tables 1–3 at http://www.cdc.gov/ncidod/EID/vol11no01/04-0632.htm#table1, http://www.cdc.gov/ncidod/EID/vol11no01/04-0632.htm#table2, http://www.cdc.gov/ncidod/EID/vol11no01/04-0632.htm#table3) The outbreaks took place in 3 rural municipalities: in 1992, in Paimio with 9,900 inhabitants; in 1995, in Oripää with 1,400 inhabitants; and in 1996, in Rusko with 3,500 inhabitants. The isolates were obtained from schools and local health centers. In addition, 1 isolate was obtained from a household contact (See online Table 3 at http://www.cdc.gov/ncidod/EID/vol11no01/04-0632.htm#table3). Most of the cases occurred in schoolchildren >8 years of age and in adults.

Various DNA fingerprinting techniques, such as IS*1002*-based restriction fragment length polymorphism (IS*1002*-RFLP) and pulsed-field gel electrophoresis (PFGE) have been used to study *B. pertussis* isolates (7–10). DNA polymorphism analysis of *prn* and *ptxS1* has previously been used as a typing method for detecting antigenic shifts (2,3,8). In addition to *prn* and *ptxS1*, only tracheal colonization factor (*tcfA*), a surface–associated protein involved in the adhesion of *B. pertussis* to host cells, has been found to be polymorphic in recent *B. pertussis* isolates (3). The isolates were typed as described earlier (8,10).

Of the 46 isolates, 43 (94%) expressed Fim2, 2 (4%) expressed both Fim2 and Fim3, and 1 (2%) expressed Fim3 (See online Tables). The predominant *prn* allele in all 3 outbreaks was *prn*2, contained by 39 (85%) of the isolates. Six (13%) isolates contained *prn*3 and 1 (2%) isolate contained *prn4*. All isolates contained the *ptxS1A* allele. The predominant *tcfA* allele was *tcfA*2, contained by 42 (91%) of the isolates. Four (9%) isolates contained *tcfA*3. The *tcfA3* allele was observed only in isolates with *prn*3. All but 1 of the 27 isolates subjected to the *IS1002*-RFLP analysis had the same pattern.

Seven PFGE patterns were found among the 46 isolates studied. The isolates were considered to be closely related, as the differences between the patterns were small, differing by 1 or 2 bands. Three PFGE patterns were found in both Paimio and Rusko. A major pattern was circulating in each of the schools A, B, and C, which indicates that pertussis is effectively transmitted in schools. However, in school D, the isolate from the index patient had PFGE pattern 5, whereas the rest of the isolates from patients in school D had pattern 6. In addition, the 1 isolate obtained from a household contact had a distinct PFGE pattern, 7. Similarly, in Paimio, the isolate from the index patient had a distinct PFGE pattern, 1. These findings, as well as the fact that 3 PFGE patterns were found in both Paimio and Rusko, indicate that several *B. pertussis* strains may have been circulating simultaneously in these small communities.

Our results suggest that *ptxS1* is not a useful marker in outbreaks to detect antigenic shifts. IS*1002*-RFLP was less discriminative than *Xba*I PFGE, which agree with results of previous studies (8). Most cases occurred in schoolchildren and adults, confirming epidemiologic findings from other countries with vaccination programs. Our results support the earlier observation that the recent *B. pertussis* isolates are antigenically different from vaccine strains. Several *B. pertussis* strains could circulate

simultaneously even in small communities, and only some strains, possibly with increased fitness, are capable of spreading effectively.

Acknowledgments

We thank Anna Musku and Birgitta Aittanen for technical assistance.

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Correction Vol. 10, No. 12

In the article entitled "Wildlife as Source of Zoonotic Infections" by Hilde Kruse et al., errors occurred in the 2nd paragraph under Factors Influencing the Epidemiology of Zoonoses with a Wildlife Reservoir on page 2068. The first sentence incorrectly identified deer as a main reservoir for *Borrelia burgdorferi*. The corrected sentence appears below:

The spirochete *Borrelia burgdorferi*, which causes Lyme borreliosis, has its main reservoir among small rodents and uses various *Ixodes* species as vectors (13).

Instructions for Authors

Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Real-Time PCR: An Essential Guide

Kirsten Edwards, Julie Logan, and Nick Saunders

**Horizon Bioscience,
Norfolk, United Kingdom
ISBN: 0-954232-7-X
Pages: 346, Price: £95; U.S. $180**

Real-time polymerase chain reaction (PCR) technique has advanced greatly over the past 10 years. This timely, comprehensive publication includes information on currently available instrumentation, fluorescent chemistries, assay design, optimization, and validation strategies. The background chapters are followed by chapters on quantification, single nucleotide polymorphism (SNP), mutation detection, and application of the various chemistries to clinical use in pathogen detection, gene expression, and human genetic testing. All of the chapters are well referenced; many of the contributing authors are recognized as respected experts in the field of real-time PCR.

Following a short overview of real-time PCR, the second chapter covers the various instruments currently on the market with a discussion on what features to look for when considering a purchase. The authors have put together a table of the machines, listing such details as the optics, the mode of detection (charge-coupled device camera or photomultiplier tube), the platform (96-well, glass capillary, etc.), and size and weight. The list contains every instrument except the latest offerings by ABI (7300 and 7500) and MJ (Chromo4). A couple of added features are given for some instruments but not others; for instance, the relative quantification software standard on the Stratagene Mx4000 and Mx3000p was not noted. Similar software is also available from ABI for their instruments. The list of websites after the references, containing general real-time PCR sites as well as those of the manufacturers and newsgroups, is an invaluable resource for both the novice and the veteran of real-time PCR.

Chapter 3 delves into the specific fluorescent chemistries, including intercalating dyes for generic detection of PCR product and template-specific designs such as linear hydrolysis (Taqman) and hybridization probes, and conformational probes (i.e., Molecular Beacons, Scorpions). One design not included was the Lux primers, a trademark design from Invitrogen. There is ample discussion on how the various chemistries work, design parameters, and examples of specific applications. The publisher might consider some changes in the placement of the tables and figures in this chapter. Most of the tables and figures are placed at the front of the chapter but not referred to until much later in the text, making it awkward for the reader to refer to them. Some attention to the font size and type for Table 1, which is hard to read, and the gray scale in many other figures throughout the book would improve the depiction of the illustrations.

The next 3 chapters (4–6) cover assay set-up and optimization, assay validation, and the design and use of controls for quantification. Chapter 4 is a general overview; chapter 5 discusses the use of internal and external controls, with the emphasis on the design and optimization of a synthetic mimic as an internal control. Chapter 6 deals with developing and using a quantitative standard. All 3 chapters address the importance of assay optimization and how this relates to PCR efficiency. They also stress the use of appropriate controls to identify false-positive results; more importantly, these chapters discuss how controls can identify false-negative results and their cause (PCR inhibitors, missing reagent components or test sample, or equipment problems), a must for diagnostic applications.

Chapter 7 deals with gene expression but I recommend that anyone considering real-time PCR read this chapter as a primer for what real-time PCR entails. The information on RNA extraction, reverse transcription, and amplification is extensive and includes discussion of the various enzymes, the master mixes and additives, and how they may or may not enhance recovery from any number of sources. The authors also cover optimization as it relates to reaction efficiency and relative versus absolute quantification. The monitoring of gene expression levels in response to viral load or cancer-producing tumor cells has become a critical part of treatment strategies, and the need for rapid, reliable assays has been effectively addressed with quantitative real-time PCR.

Comparison of how the different probe types (linear hybridization, hydrolysis, and conformational) and the Scorpion-labeled primer work in mutation detection is covered in chapters 8 and 9. Examples are given for how real-time PCR detection can be applied to identify human genetic diseases (Factor V Leiden and cystic fibrosis, for example) or to diagnose drug-resistant bacteria for proper drug therapy. Chapter 9 discusses the application of the ARMS (amplification refractory mutation system) technique for discrimination and selection of low levels of a mutant in a high background of wild-type DNA. Another unique real-time assay, nucleic acid sequence–based amplification (NASBA), is discussed in chapter 10. These isothermic NASBA assays are used most often for RNA detection and/or quantification. The reactions require avian myeloblastosis virus reverse transcriptase, ribonuclease H, T7 RNA polymerase in the master mix, and a thermostatic fluorimeter; the probe of choice is the Molecular Beacon.

The final 2 chapters cover a myriad of applications used in clinical microbiology and the diagnosis of infectious diseases. Even though presented as an overview, the >100 references in chapter 11 illustrate how vast and varied the application of real-time PCR, and the technological advances to support its use, have become in the past decade. This publication would be a good addition to any laboratory as an up-to-date resource for both the novice and the experienced researcher.

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Textbook–Atlas of Intestinal Infection in AIDS

Daniele Dionisio

Springer-Verlag, Italia, Milano, Italy
ISBN: 88-470-0174-9
Pages: 489, Price: U.S. $149.00

Gastrointestinal tract infections are prominent in patients with AIDS. Infections may be caused by a variety of bacterial, fungal, viral, protozoal, and helminthic pathogens, and affect persons in both developing and industrialized countries. The problems are especially acute in resource-limited countries where little or no access to highly active antiretroviral therapy exists; the impact of illnesses associated with HIV is most pronounced in these countries.

Daniele Dionisio, an authority in parasitology, has assembled a new treatise, Textbook–Atlas of Intestinal Infections in AIDS, that directly addresses this topic. In 489 pages, the work includes much background, including a fascinating chapter by Esther Diane on the history of the discovery of intestinal parasites. The work and illustrations of parasitic life cycles by Dionisio and colleagues are illuminating for all students of infectious diseases.

Much of the book addresses particular agents and the diseases they cause. A particular strength is the numerous illustrations. Although varying in quality, they represent an enormous compendium of information about these common problems. The figures on microscopic and ultrastructural pathology are particularly strong.

This volume should be considered as a background text for the pathologist, gastroenterologist, or infectious disease specialist who concentrates on HIV infections. The illustrations and references provide readers access to materials not easily obtained. The sections on clinical management of particular conditions are relatively sparse, and healthcare workers with patients with active problems should consult with a more comprehensive text. In total, this textbook-atlas is a useful addition in the battle against HIV infections and its complications. The editor and multinational group of authors are to be congratulated on their scholarly work.

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Public Health Response to Biological and Chemical Weapons: WHO Guidance

World Health Organization

ISBN: 92-4-154615-8
Pages: 357; Price: U.S. $34.20

In this manual, the World Health Organization (WHO) updates its guidance for governments in preparing for a possible terrorist attack with biological or chemical weapons. The book has something for virtually everyone who may have an interest in this topic, from government officials to clinicians, including information about the history of biological and chemical warfare, applicable international treaties, procedures for requesting WHO technical consultation, fundamentals of public health emergency response, basics of infectious diseases, treatment of patients with specific infectious or toxic exposures, physical properties of various agents, the utility of reconnaissance satellites for detecting weapons development, the management of food and water safety programs, etc.

This ubiquity and ambitiousness underlie the manual's limitations and strengths. At times the guidance is so general that is almost an inventory of truisms (e.g., "If it is found that the [emergency] control measures are not effective, they must be changed or modified."); elsewhere, the manual is a detailed resource. Its utility for different users will depend on their backgrounds and information needs. The core chapter, Public Health Preparedness and Response, may disappoint those seeking more than general principles. Yet these principles merit articulation.

Descriptions of the sarin attack in Tokyo in 1995 and the anthrax attacks

in the United States in 2001 illustrate lessons from governments' recent experiences with chemical and biological terrorism. Both episodes demonstrate that relatively small attacks can have a profound impact and expose weaknesses in public health systems. The anthrax case study lauds the success of laboratory preparations but does not sufficiently address three essential questions: Why did clinicians caring for the initial patients with cutaneous anthrax not establish and report the diagnosis sooner? Why did the Centers for Disease Control and Prevention not recognize earlier that anthrax spores could escape from sealed letters? Why did the federal government stumble initially in its efforts to communicate with the public? For each question, an assessment of systemic hurdles would benefit readers seeking to improve the functioning of the public health system.

The manual generally, but not consistently, avoids bureaucratic lingo. While clearly organized, the book lacks an index, complicating efforts to find information quickly. The appendices on chemical and biological agents offer concise, formatted summaries similar to those available through other resources, but ironically provide relatively little information about the agents' potential as weapons.

This manual will find a home on bookshelves worldwide among government officials and others concerned about the threat of biological and chemical terrorism. For those in countries most in need of this guidance, its scope may be overwhelming. But the book's underlying theme—that public health preparedness for biological or chemical terrorism depends on fundamental capacities to respond to more common health threats—is its most salient message, no matter where the user resides.

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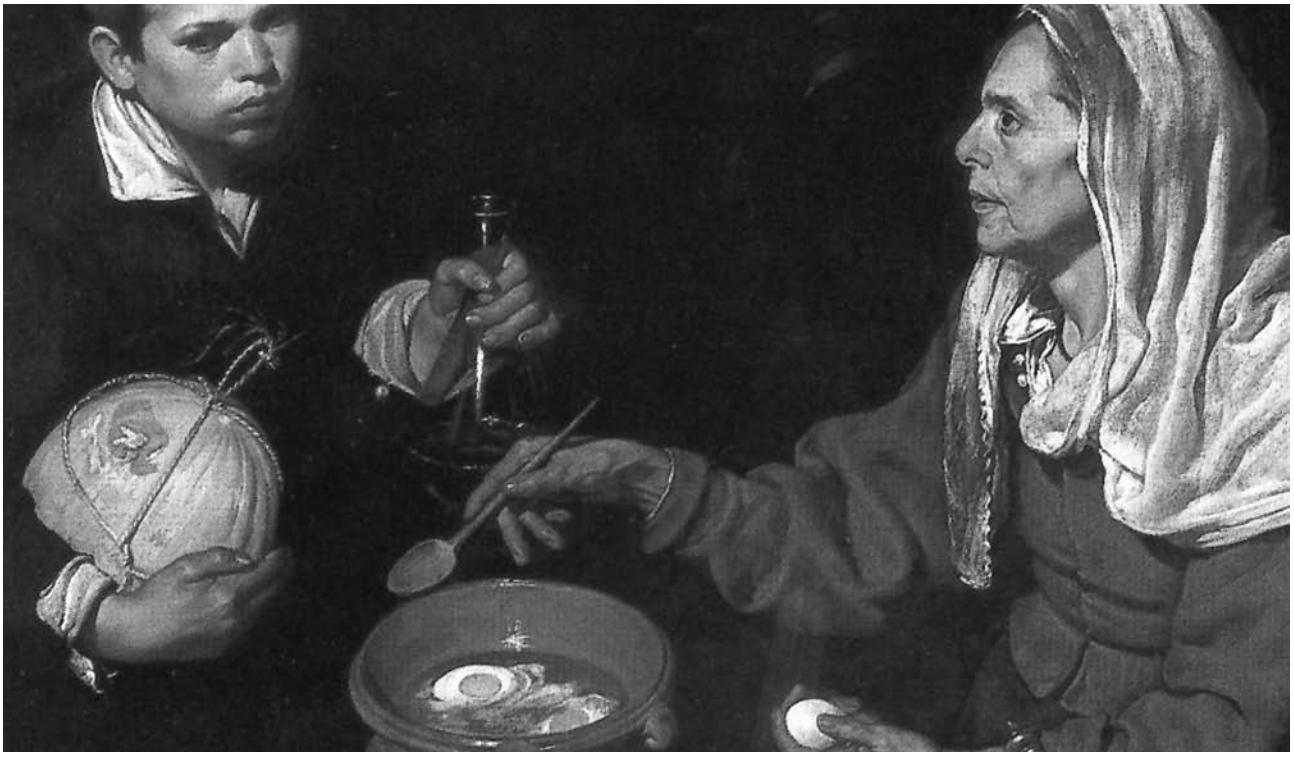

Diego Velázquez (1599-1660). An Old Woman Cooking Eggs (1618)
Oil on canvas (100.5 cm x 119.5 cm). The National Gallery of Scotland, Edinburgh

Genre Painting and the World's Kitchen

Polyxeni Potter*

"Tell me what you eat, I will tell you who you are," boasted famed gastronome Jean Anthelme Brillat-Savarin (1755–1826) (1). A man of many interests, among them archaeology, astronomy, and chemistry, Savarin wrote treatises on economics and history, but his fascination with food was what most informed and entertained readers and followers in his native France and around the world. In nature and on the table, quite apart from its direct link to human survival, food has been an object of intrigue featured prominently in art throughout history. From ancient times and particularly during the development of genre painting in the Middle Ages and later, food—its appearance, abundance, or decay—has been a popular subject.

In 17th-century Spain, genre painting (scenes of everyday life) reached new heights with the work of Diego Velázquez. In a style reminiscent of Caravaggio, Velázquez created and popularized a new genre, the kitchen or tavern scene (bodegón), which showed peasants eating or preparing meals and the objects they used to assemble and serve them. These objects (still life), prominently displayed in realistic terms including their imperfections, assumed a life of their own, introducing a new naturalism in Spanish painting, which had been dominated by the ideal beauty of classical and academic themes (2).

Velázquez grew up in the cosmopolitan climate of Seville, southern Spain, along the banks of the Guadalquivir, an area also home to Cervantes, Lope de Vega, and other luminaries of the Spanish Golden Age. Like the great literature of that era, his art concerned itself with the life, culture, and traditions of the people. He apprenticed with influential biographer, theoretician, and artist Francisco Pacheco, who later wrote about his student: "After five years of education and training, I married him to my daughter, moved by his virtue, integrity, and good parts and by the expectations of his disposition and great talent" (3).

Soon a member of the Seville painters' guild, Velázquez moved from bodegón to portraits and was summoned to

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the court, where he received his first commission to paint King Philip IV, a discerning patron of the arts. He was appointed court painter, a position of great privilege, which gave him access to royal collections including paintings by the Venetian Renaissance master Titian, who greatly influenced the development of his style. The artist led a quiet life, interrupted only by his travels to Italy, sponsored by the king. During his first journey, he traveled with Flemish Baroque master Peter Paul Rubens, who was also influential in his artistic career.

"To go to Madrid to see the Velázquez" was Monet's wish near the end of his life (4). This wish, expressed in a letter to a friend, reflects the mystique associated with Velázquez' work and the breadth of its influence on all modern art schools, even if limited to 100 or so surviving works. His painting showed exceptional mastery of space and light and exuded naturalness and restraint, both in its objectivity and choice of colors, often browns and ochers. Velázquez was gifted with exacting technique and preferred to paint from life. In spite of his meticulous depiction of reality, he seemed more interested in the tensions between reality and appearance than in reality itself (5).

Velásquez painted An Old Woman Cooking Eggs (on this month's cover of Emerging Infectious Diseases) when he was 19 years old. In this kitchen scene, the common utensils used in preparing food (mortar and pestle, pots, ladles, bowl, jugs) have at least as important a place as the preparers themselves. Provocatively in the foreground and along the edges of the painting, these objects seem to contain in their clay, wood, glass, brass, copper, pewter, or other essence the light that defines them against the dark background. The eggshell, the straw of the basket, the skin of the melon and onion, the texture of linen and string, showcase the artist's virtuoso performance in capturing their likeness.

The food preparers, transfixed by some unknown concern, seem removed and distant from the food and from each other. They go through the motions of cooking, but their minds are elsewhere. The boy, cradling a trussed melon, leans forward with a glass cruet containing oil, wine, or some other liquid. The old woman tending the food is staring intently ahead, otherwise preoccupied. On a ceramic heating plate, the pan is tipped forward to show the eggs in various stages of congealing. The curved shadow of the knife over the bowl, the moist surface of the pan above the egg whites, the gleaming copper pot against the shadows of the room confirm the artist's interest in the integrity and dignity of these objects and the people who use them, even if he does not indulge us with their concerns underneath the surface.

These concerns, apart from the underlying threat of decay through the relentless passage of time, a common theme in still-life painting, would be many, even if they were only limited to food. The 17th-century Spanish diet was known for its parsimony. A main concern in the common kitchen was the long-term availability of food. The safety of food, a more modern concern, was probably not on the mind of Velásquez' food preparers. Unlike our contemporary equivalents, they would have known little about the dangers surrounding food. Nor would they have understood Savarin, whose sensitive 18th-century palate might have recoiled at the sight of eggs poaching slowly in oil on a clay stove.

An ancient staple, eggs have run the gamut from plentiful protein to gourmet delicacy. Yet, basic food and epicurean aspirations converge at one point: safety. With high levels of *Salmonella enterica* serovar Enteriditis in shell eggs (6), adequate cooking and proper temperature of the eggs overrule tradition, challenging the consistency of the sauce and the moment of delivery to the table. In our times, safety issues concerning not only eggs but all foods beg a different interpretation of another well-known Savarin aphorism, "The destiny of a nation depends on the manner in which it feeds itself."

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends
Vol. 11, No. 2, February 2005

Upcoming Issue

Look in the February issue for the following topics:

Managing Febrile Respiratory Illnesses during Hypothetical SARS Outbreaks

Human Disease from Influenza A (H5N1), Thailand, 2004

Survey for Bat Lyssaviruses, Thailand

Bacterial Zoonoses and Infective Endocarditis, Algeria

Evidence of Spotted Fever Group and Typhus Group Rickettsioses in Humans, South Korea

Pneumocystis jirovecii in General Population

Cryptosporidiosis Decline after Filtration of Water Supplies, England, 1996–2002

Carbapenemase-producing Enterobacteriaceae, U.S. Rivers

Rickettsial Infection in Ticks, Humans, and Domestic Animals and Brazilian Spotted Fever

Isolation of *Waddlia malaysiensis*, a Novel Intracellular Bacterium, from Fruit Bat (*Eonycteris spelaea*)

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

Upcoming Infectious Disease Activities

January 27–29, 2005
7th International Symposium on Febrile Neutropenia
Barcelo Gran Hotel Renacimiento
Seville, Spain
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

March 16–18, 2005
Focus on Fungal Infections 15
Sheraton Bal Harbour
Miami, Florida, USA
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

April 9–12, 2005
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Los Angeles, California, USA
Contact: 703-684-1006
Web site: http://www.shea-online.org

May 1, 2005
International Society of Travel Medicine (ISTM) offers certificate of knowledge in travel medicine exam (Given prior to the opening of 9th Conference of the ISTM)
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Contact: +49-89-2180-3830
http://www.ISTM.org/

November 13–18, 2005
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EMERGING
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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 http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

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

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

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

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

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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

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

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only (effective January 2005). Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.