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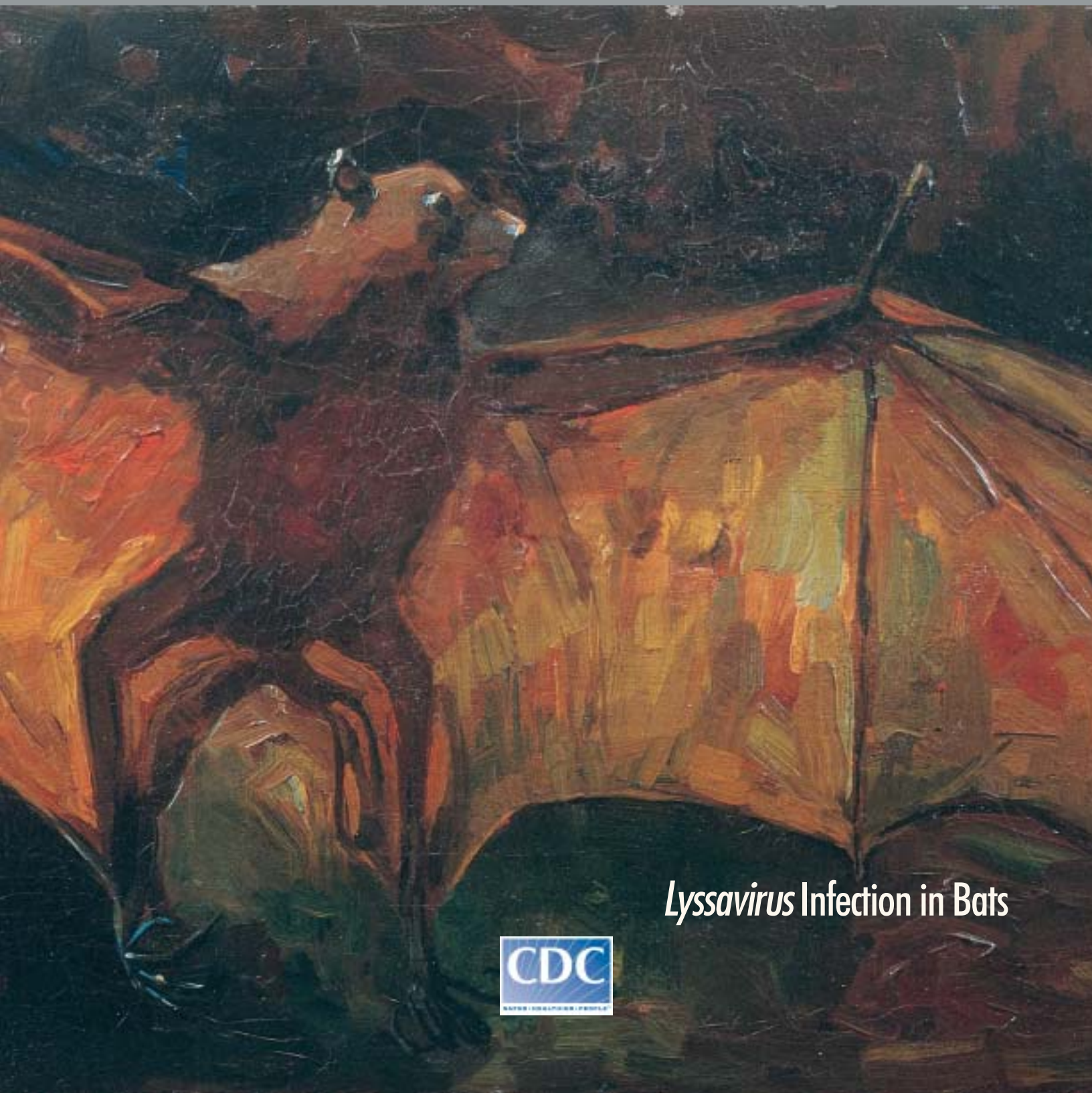

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On the Cover:
Vincent van Gogh, Flying Fox, 1885 (oil on canvas, 41 cm x 79 cm) Courtesy of Van Gogh Museum, Amsterdam (Vincent van Gogh Foundation)

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Letters

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Feasibility of National Surveillance of Health-Care–Associated Infections in Home-Care Settings

Lilia P. Manangan,* Michele L. Pearson,* Jerome I. Tokars,* Elaine Miller,* and William R. Jarvis*

This article examines the rationale and strategies for surveillance of health-care-associated infections in home-care settings, the challenges of nonhospital-based surveillance, and the feasibility of developing a national surveillance system.

Over the past 2 decades, the delivery of health care in the United States has shifted increasingly from hospitals to patients' homes (1-3). Nearly eight million people in the United States received medical care at home in 1996 (4), and an estimated 774,113 (10%) of these patients had at least one indwelling medical device (5). Use of a medical device is the greatest predictor (exogenous) of health-care-associated infection.

Home care is often provided by family members who have little or no formal health-care training, which may place patients at increased risk of health-care-associated infections not typically seen in hospitals. In the home-care setting, patients with open wounds or central venous catheters may undertake activities of daily living (e.g., bathing, exercising, gardening, and playing with pets) that may increase the risk of infections.

Rationale for a National Surveillance System in Home-Care Settings

The epidemiology of health-care-associated infections in home-care settings has not been defined, but infections certainly occur. Outbreaks have been documented in association with use of central venous catheters, parenteral nutrition, bathing practices, educational level of caregivers, and the introduction of new products, such as needleless devices for intravenous infusion (6-8).

Needleless devices are used for connecting and accessing intravenous infusion tubing, replacing traditional needles. These devices are used in both home and hospital settings and are perceived to be safe for patients and effective in reducing needlestick injuries.

From 1993 through 1995, the Hospital Infections Program, now Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention (CDC), investigated three outbreaks of bloodstream infections (BSI) in patients receiving infusion therapy in their homes. In all three outbreaks, needleless devices were associated with BSIs. The first outbreak occurred in Rhode Island in 1993-1994. The endcaps on these devices were changed every 7 days. BSIs were frequent when needleless devices were used to admini-ster total parenteral nutrition (6). The second outbreak, in Oakland, California, during 1992-1994, occurred among pediatric hematology-oncology patients. The BSI rate was higher when needleless devices were used by Asian or Hispanic children but not by white or black children. The racial/ethnic differences were thought to stem from socioeconomic factors or possibly from language barriers that prevented full understanding of instructions on infection control (7). The third outbreak occurred in Houston, Texas, in 1994-1995. The BSI rate was higher when the needleless device endcaps were changed every 7 days and lower when they were changed every 2-3 days. Patients who showered may have had a higher BSI rate than those who took tub baths (8).

These outbreak investigations were, by necessity, retrospective, and some data were difficult to obtain. To better define the epidemiology of BSIs in the home-care setting, in 1995 the Hospital Infections Program conducted a prospective multicenter study of home infusion therapy patients. The objectives were to determine rates of BSI and to identify risk factors, especially the use of needleless devices. The study, which was conducted in Cleveland, Ohio, and Toronto, Canada, involved 827 patients (69,532 catheter-days) (9). The most common underlying diagnoses among this cohort were infections caused by organisms other than HIV (67%), malignancy (24%), nutritional and digestive disease (17%), heart disease (14%), organ transplantation (11%), and HIV infection (7%).

Overall, 7% of these patients had one or more BSIs during a median of 44 days of catheter use (range 1 to 395 catheter days). A multivariate analysis showed that independent risk factors included recent bone marrow transplant, receipt of total parenteral nutrition, receipt of infusion therapy outside the home (e.g., in a clinic or physician's office), use of a multilumen catheter, and having had a previous BSI (9). Needleless devices were not associated with BSI.

Two prevalence surveys of infections among patients of Missouri home health agencies were conducted by CDC in

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collaboration with the Missouri Alliance for Home Care (MAHC) and the Missouri Department of Health, the first during summer (June 1-30, 1999) and the second during winter (February 15-March 15, 2000). Of 5,100 home-care patients enrolled in the summer survey, 16% (793) were reported to have infections; 8% (63) of these infections were reported as being acquired at home, 16% (127) as hospital acquired, 35% (278) as unknown source, and 41% (325) as community acquired. The infection sites reported were urinary tract (214 [27%]), respiratory tract (190 [24%]), skin or soft tissue (190 [24%]), surgical site (95 [12%]), or bloodstream (17 [2%]); 18% (143/793) of infections occurred at other body sites (e.g., gastrointestinal, bone) (10). Of 2,890 patients enrolled in the winter survey, 16% (466) had infections. The prevalence of respiratory tract infections was higher during the winter survey than during the summer survey. These results suggest that an estimated 1.2 million patients receiving home care in the United States have infections annually, supporting a need for surveillance of infections among home-care patients (11).

A nationwide hospital-acquired infection surveillance system and standardized infection definitions have been in existence since the 1970s (12-14). However, no national surveillance or standardized definitions exist for monitoring infections in the home-care setting. The Association for Professionals in Infection Control and Epidemiology, Inc. (APIC) published draft definitions for surveillance of infections in home-care patients (15). However, these definitions have not yet been validated.

National surveillance of health-care-associated infections in home care may potentially decrease infection rates, as has been documented in hospitals by the National Nosocomial Infections Surveillance system (NNIS). This voluntary, hospital-based reporting system was established to monitor hospital-acquired infections and to guide the prevention efforts of infection control practitioners. During 1990-1999, risk-adjusted infection rates in intensive-care units decreased by approximately 40% among hospitals participating in NNIS (16).

A national system for surveillance of health-care-associated infections in home care would not only provide useful data on incidence and types of infections but also simplify identification of risk factors for infection and development of national benchmarks for comparing infection rates. Risk-adjusted rates may assist individual home-care agencies to identify areas for performance and quality improvement and to evaluate the impact of prevention interventions on infection rates.

Challenges to Developing a National Surveillance System

Home-care surveillance poses several unique challenges, including lack of nationally accepted standard definitions and surveillance methods, loss of patient follow-up, lack of trained infection control personnel in home-care settings, difficulty in capturing clinical and laboratory data, and difficulty in obtaining numerator and denominator data.

Lack of Nationally Accepted Definitions and Methods

A cornerstone of surveillance in any setting is development of standardized definitions and methods. Individual home-care agencies have developed surveillance definitions for their own use (17-20), but national definitions of infections in home care do not exist. The draft APIC definitions of home health-care-associated infections have yet to be accepted and implemented nationally. These definitions should be tested to determine their practicality or applicability, given the limited use of laboratory diagnostics in home care. In addition, standard methods of case finding, recording, and calculating rates are also essential. If national benchmark rates are to be established to permit inter- and intra-agency comparisons, consensus definitions of home health-care-associated infections, such as those published by APIC, will have to be implemented.

Loss of Patient Follow-up

Home-care patients often are served by several agencies or are readmitted to the hospital during their illness. Lack of continuity of care hampers detection and reporting of health-care-associated infections. For example, if a home-care patient receiving intravenous therapy has a fever, is admitted to an acute-care facility, and is confirmed to have a BSI, this information may not be communicated to the home-care agency (the same or a different one) when the patient is discharged to continue infusion therapy at home.

Lack of Trained Personnel

Surveillance requires adequately trained infection control personnel, but few home health companies have such employees who are designated to conduct infection control activities, including education, surveillance, and prevention. In a recent survey of home-care agencies in Missouri, only 51 (54%) of 95 had a designated infection control practitioner, and only 27 (53%) of 51 provided ongoing training (21). In most home-care agencies, infection control activities are performed on a volunteer basis with no additional compensation. Successful implementation of surveillance programs and other infection control activities in the home health-care setting will require designated and appropriately trained personnel. Training should include calculation of infection rates, recognition of outbreaks and clusters, providing feedback data to essential personnel, and monitoring compliance of prevention efforts. Educational activities targeted at patients, health-care workers, and other caregivers will also be a necessary part of the infection control program.

Difficulty in Capturing Clinical and Laboratory Data

Many home-care agencies are privately owned and have no hospital or laboratory affiliation; therefore, access to diagnostic services may be limited, and home-care personnel may have difficulties in tracking laboratory results (e.g., contacting out-of-state physician offices or laboratories). Limited access to test results may also encourage home-care personnel to use empiric therapy without documentation of infection or

identification of a causative pathogen. Linkages for sharing clinical and laboratory data among physicians, hospitals, and home-care agencies are essential to optimize patient care in the home.

Difficulty in Obtaining Numerator and Denominator Data

Surveillance for infections in home care will require methods to identify appropriate numerator and denominator data for calculating infection rates for inter- and intra-agency comparison and benchmarks. Collection of numerator data (e.g., BSI or other infectious complications) will require systems that permit data sharing by hospitals and laboratories with home-health agencies.

Capturing appropriate denominator data may even be more difficult (22). For example, to determine device-associated infection rates, device utilization must be measured by monitoring days of use. However, if insertion, care, and removal of the device (e.g., central venous catheter, urinary catheter, tracheostomy tube) are done in different health-care settings, it will be difficult to monitor how many days a device is used. Although infection rates based on device utilization have been shown to be necessary in the acute-care setting, it is not certain that they are necessary in home care.

Another option for denominator is the number of days a patient uses a device during home care only, rather than the total number of days (i.e., from insertion to removal) the device is used. Because all home infusion therapy patients have intravenous catheters, patient days may be substituted for device days as long as they equal one another.

In addition to these challenges, the home-care industry will have to deal with the financial implications of implementing and maintaining a national surveillance system. Data on the cost of a surveillance system and on methods of calculating that cost into the reimbursement systems of health-care payors are very much needed.

Despite cost concerns, patient safety and outcomes are becoming increasingly important in the current health-care environment. Purchasers should base their selection of a home-care agency on patient outcomes and satisfaction rather than cost. Thus, home care agencies must conduct surveillance for adverse events. Without such surveillance systems, it would be very difficult for agencies to know if problems are occurring and whether quality care is being provided.

Progress Toward a National Surveillance System for Health-Care-Associated Infections

Several groups are collecting data on health-care-associated infections in home care and other outpatient areas. These data may prove useful in developing a national home health-care surveillance system.

MAHC is a nonprofit association that provides home care education, advocacy, and information for its 250-member agencies, most of which are located in Missouri. In the early 1990s, MAHC established an infection control committee composed of nurses who provided infection control activities for their agencies. In 1993, the committee implemented the MAHC Infection Surveillance Project (ISP) to monitor infections associated with central venous and urinary catheters. ISP is an active surveillance system that uses standardized criteria and definitions for tracking, aggregating, and reporting urinary infections and BSIs among home-care patients. Currently, 99 home-care agencies from 25 states participate in ISP. Although MAHC has contracted with the Hospital Industry Data Institute, Missouri Hospital Association, to organize and present the ISP data, the results have not yet been published. Although the ISP definitions have not been validated to determine sensitivity and specificity, the data allow participating agencies to compare their infection rates with those of other agencies.

On a broader scale, the Health Care Financing Administration (HCFA), now the Center for Medicare and Medicaid Services, in collaboration with the Center for Health Sciences and Policy Research, has developed the Outcome and Assessment Information Set (OASIS) to measure patient outcomes and improve quality in home care. HCFA requires all Medicare-certified home-care agencies to electronically submit data for their Medicare patients to a central OASIS database in Baltimore, Maryland. The outcomes monitored in OASIS are changes in patient health status, as indicated by need for emergency care or hospitalization, for example. Data collected include patient demographics and medical history, living arrangements, type of wound, urinary tract infection, respiratory devices, medications, emergency care received, transfer to an inpatient facility, and death. Most data items are obtained at start of care, every two calendar months, and at discharge. Since August 1999, more than eight million records have been entered into the OASIS database and information on how to access the OASIS reports can be obtained from http://www.hcfa.gov/medicaid/oasis/osishmp.htm.

Another national and international data source is the Outpatient Parenteral Antimicrobial Therapy (OPAT) registry, which aims to improve delivery of care and outcomes for outpatients receiving parenteral antimicrobial therapy. OPAT provides a broad database for assessing antimicrobial drug-prescribing practices and outcomes among patients with infections treated in outpatient settings. Data collected include patient demographics, diagnosis, pathogen, venous access device, infusion system, adverse events, clinical outcome, and patient satisfaction. Currently, 25 OPAT provider sites from 16 states are participating in the U.S. registry, and 24 provider sites from 6 countries are in the international registry. OPAT data have been presented at scientific conferences (23).

Surveillance methods that are commonly used in hospital programs may not be feasible for home care. Different strategies are needed to make surveillance in the home easier to implement, particularly if adequately trained staff and diagnostic services are limited. For example, the Dialysis Surveillance Network provides a novel way of tracking hospitalization, antimicrobial use, and selected infections in hemodialysis outpatients (24). Episodes of potential infection are identified by a clearly defined sequence of steps that

involves completing an "incident form" for all patients admitted to a hospital or started on intravenous antimicrobial therapy. The presence (or absence) of symptoms indicating infection is recorded rather than the infections themselves, and a computer algorithm determines whether the infection case definitions are met; the data collector is not required to memorize case definitions. The lessons from this surveillance system, in addition to other traditional outpatient systems, may be useful in establishing national surveillance for home health-care-associated infections.

Nearly as many patients receive home care annually as hospital care. With the continued expansion of home health-care delivery and documented infection risk in this setting, a national system for surveillance of health-care-associated infections in the home-care setting is needed. Collaboration between home health-care agencies, state and federal health agencies, private industry, and national or managed-care organizations is essential to make this system feasible and functional. Development and implementation of such a system would foster better understanding of the epidemiology of health-care-associated infections in the home-care setting. Furthermore, this system would provide a means for monitoring the impact of interventions aimed at preventing the emergence of these infections in the home.

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Human Campylobacteriosis in Developing Countries[1]

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Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli.* Research and control efforts on the disease have been conducted more often in developed countries than developing countries. However, because of the increasing incidence, expanding spectrum of infections, potential of HIV-related deaths due to *Campylobacter,* and the availability of the complete genome sequence of *C. jejuni* NCTC 11168, interest in campylobacteriosis research and control in developing countries is growing. We present the distinguishing epidemiologic and clinical features of *Campylobacter* enteritis in developing countries relative to developed countries. National surveillance programs and international collaborations are needed to address the substantial gaps in the knowledge about the epidemiology of campylobacteriosis in developing countries.

Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli* (1). The rate of *Campylobacter* infections worldwide has been increasing, with the number of cases often exceeding those of salmonellosis and shigellosis (2). This increase, as well as the expanding spectrum of diseases caused by the organisms, necessitates a clearer understanding of the epidemiology and control of campylobacteriosis.

Surveillance and control of diseases of public health importance in developing countries have focused on diseases such as malaria, tuberculosis, trypanosomiasis, onchocerciasis, and schistosomiasis (3). Programs for diarrhea and acute respiratory illness also exist (4). These programs have extensive support from the World Health Organization (WHO).

Campylobacter is one of the most frequently isolated bacteria from stools of infants with diarrhea in developing countries—a result of contaminated food or water (5,6). However, national surveillance programs for campylobacteriosis generally do not exist in most developing countries despite the substantial burden of disease. Most data available on campylobacteriosis in developing countries were collected as a result of support provided by WHO to many laboratories in developing countries, including grants for epidemiologic studies and Lior serotyping antisera provided by the Public Health Service of Canada (5,7). The number of reviews and updates on human campylobacteriosis in developed countries (8-11) is greater than that for developing countries (5,6). This disparity may be because *Campylobacter*-associated diarrhea in developing countries is not pathogenic in patients >6 months of age. However, a community-based longitudinal study provided evidence that infection could be pathogenic beyond the first 6 months of life in developing countries (12). Furthermore, the sequencing and publication of the complete genome of *C. jejuni* NCTC 11168 have heralded a renaissance of interest in this organism, offering researchers worldwide, including in developing countries, novel ways to contribute to understanding the organism's biology (13). Thus, to promote research and control of campylobacteriosis in developing countries, review information on human campylobacteriosis in these countries is urgently needed. We present the distinguishing features of campylobacteriosis in developing countries relative to developed countries.

Incidence

Generally, developing countries do not have national surveillance programs for campylobacteriosis; therefore, incidence values in terms of number of cases for a population do not exist. Availability of national surveillance programs in developed countries has facilitated monitoring of sporadic cases as well as outbreaks of human campylobacteriosis (2,8-11). Most estimates of incidence in developing countries are from laboratory-based surveillance of pathogens responsible for diarrhea. *Campylobacter* isolation rates in developing countries range from 5 to 20% (6). Table 1 shows isolation rates for some countries according to WHO regions from studies of diarrhea in children <5 years old (14-25). Despite the lack of incidence data from national surveys, case-control community-based studies have provided estimates of 40,000 to 60,000/100,000 for children <5 years of age (6,12). In contrast, the figure for developed countries is 300/100,000 (8). Estimates in the general population in developing and developed countries are similar, approximately 90/100,000 (5,6,8), confirming the observation that campylobacteriosis is often a

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[1]Portions of this review were presented at the World Health Organization Consultation on the Increasing Incidence of Campylobacteriosis in Humans, Copenhagen, Denmark, November 21-25, 2000. In addition, relevant emerging information from the 11th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, held in Freiburg, Germany, September 1-5, 2001, are included.

Table 1. Isolation rates of *Campylobacter* from diarrhea specimens from <5-year-olds in selected developing countries

WHO region and country	Isolation rate (%)	Reference
Africa		
Algeria	17.7	14
Cameroon	7.7	15
Ethiopia	13.8	16
Nigeria	16.5	17
Tanzania	18.0	18
Zimbabwe	9.3	19
Americas		
Brazil	9.9	20
Guatemala	12.1	21
Eastern Mediterranean		
Egypt	9.0	12
Jordan	5.5	22
Southeast Asia		
Bangladesh	17.4	23
Thailand	13.0	24
Western Pacific		
Laos	12.1	25

WHO = World Health Organization.

pediatric disease in developing countries. The isolation and incidence rates in some developing countries have increased since their initial reports (17). This increase has often been attributed to improved diagnostic methods, but an actual increase in incidence was observed in *Campylobacter*-associated diarrhea in the Caribbean island of Curaçao (26).

Age of Infection

In developing countries, *Campylobacter* is the most commonly isolated bacterial pathogen from <2-year-old children with diarrhea (Table 2). The disease does not appear to be important in adults. In contrast, infection occurs in adults and children in developed countries. Poor hygiene and sanitation and the close proximity to animals in developing countries all contribute to easy and frequent acquisition of any enteric pathogen, including *Campylobacter.* Although infections in infants appear to decline with age (Table 2), a comprehensive community-based cohort study in Egypt has shown that infection could be pathogenic regardless of the age of the child, underscoring the need for strengthening prevention and control strategies for campylobacteriosis (12).

Polymicrobial Infections Involving *Campylobacter*

Campylobacter is isolated relatively frequently with another enteric pathogen in patients with diarrhea in developing countries. In some cases half or more patients with *Campylobacter* enteritis also had other enteric pathogens (23,30). Organisms reported include *Escherichia coli*, *Salmonella*, *Shigella*, *Giardia lamblia*, and *Rotavirus*. Polymicrobial infections involving *Campylobacter* are rare in developed countries (5,6).

Isolation of *Campylobacter* in Healthy Children

The recovery of *Campylobacter* organisms from children without diarrhea is common in developing countries. In some reports the isolation rates for symptomatic and asymptomatic children were not statistically significant. Values as high as 14.9% in controls have been observed (14). Acquisition of the pathogen because of poor sanitation and contact with animals early in life may explain the isolation from healthy children. *Campylobacter* is not frequently recovered from asymptomatic persons in developed countries, as observed in the Netherlands, where a 0.5% isolation rate has been reported (9).

Seasonal Variation

In developing countries, *Campylobacter* enteritis has no seasonal preference; in contrast, in developed countries epidemics occur in summer and autumn (2). Isolation peaks vary from one country to another and also within countries (12,31,32). The lack of seasonal preference may be due to lack of extreme temperature variation as well as lack of adequate surveillance for epidemics (5,6).

Distribution of *Campylobacter* Species

C. jejuni and *C. coli* are the two main species isolated in developing countries. The isolation rate of *C. jejuni* exceeds that of *C. coli*, similar to observations in most developed countries (8,9). Lior biotyping and serotyping methods have been used in developing countries to subtype strains of *C. jejuni* and *C. coli* (5,6). Table 3 shows the distribution of the subtypes from three African countries. Biotype I was the most common, followed by biotype II. The prevalence of specific serotypes only in symptomatic children may indicate virulence traits or treatment, in cases of gastroenteritis (33). Furthermore, correlation between biotypes and serotypes isolated from humans and animals indicates that campylobacteriosis is zoonotic (36). Penner serotyping scheme and DNA-based typing, extensively

Table 2. Age of patients with *Campylobacter* infection in selected developing countries

Countries (ref.)	Age of infection (months)
Nigeria (17)	24
Tanzania (18)	18
China (27)	12-24
Thailand (28)	<12 (18.8%) 12-23 (12.3%) 24-59 (10.3%)
Bangladesh (29)	≤12 (38.8%) >12 (15.9%)
Egypt (12)	0-5 (8%) 6-11 (14%) 12-23 (4%)

Table 3. Distribution of *Campylobacter jejuni* and *C. coli* biotypes and serotypes in three African countries

	Biotypes						
	C. jejuni				*C. coli*		
Countries (ref.)	I	II	III	IV	I	II	Serotypes
Nigeria (33)	52.5	28.7	-	-	9.9	8.9	1, 8, 11, 20, 28, 45
Central African Republic (34)	31.9	11.0	2.4	-	44.0	11.5	-
South Africa (35)	95.4	1.5	-	-	3.1		4, 2, 12, 19, 23, 36

used in developed countries, have been proposed for use in developing countries (37).

Species other than *C. jejuni* and *C. coli*—such as *C. upsaliensis*, *C. concisus*, and aerotolerant campylobacters (*Arcobacter*)—may also be of pathogenic importance; however, diagnostic capacities to determine their distribution are lacking in developing countries (38). These other *Campylobacter* species constitute over 50% of campylobacters isolated at the Red Cross Children's Hospital, Cape Town, South Africa, for example. (A method termed the Cape Town Protocol is used to isolate *Campylobacter* species at this facility [39]). This higher incidence is also supported by a 16% isolation rate of *Arcobacter* species in a 4-month survey from poultry drainage water in Lagos, Nigeria (40).

Antibiotic Resistance in *Campylobacter* Isolates

Campylobacter enteritis is a self-limiting disease, and antimicrobial therapy is not generally recommended. However, antimicrobial agents are recommended for extraintestinal infections and for treating immunocompromised persons. Erythromycin and ciprofloxacin are drugs of choice (10). The rate of resistance to these drugs is increasing in both developed and developing countries, although the incidence is higher in developing countries. Use of these drugs for infections other than gastroenteritis and self-medication are often the causes of resistance in developing countries; in developed countries, resistance is due to their use in food animals and travel to developing countries. The increase in erythromycin resistance in developed countries is often low and stable at approximately 1% to 2%; this is not true for developing countries (41,42). For example, in 1984, 82% of *Campylobacter* strains from Lagos, Nigeria, were sensitive to erythromycin; 10 years later, only 20.8% were sensitive (17). In addition, resistance to another macrolide, azithromycin, was found in 7% to 15% of *Campylobacter* isolates in 1994 and 1995 in Thailand (43). The increasing rate of resistance to the fluoroquinolone, ciprofloxacin limits its clinical usefulness. In Thailand, ciprofloxacin resistance among *Campylobacter* species increased from zero before 1991 to 84% in 1995 (43). Recent data have shown a marked increase in resistance to quinolones in developed countries (41,42,44-46) (Table 4).

Campylobacter as a Cause of Travelers' Diarrhea

Travel to a developing country is a risk factor for acquiring *Campylobacter*-associated diarrhea. The diarrhea is more severe, and strains are associated with antibiotic resistance (47,48). Furthermore, campylobacteriosis acquired abroad contributes to the number of cases reported in developed countries (49). Among Finnish tourists visiting Morocco, the disease was more prevalent in winter months (50).

Clinical Features

The clinical spectrum of *Campylobacter* enteritis ranges from a watery, nonbloody, noninflammatory diarrhea to a severe inflammatory diarrhea with abdominal pain and fever. Disease is less severe in developing countries than in developed countries (5,6). In developed countries, disease is characterized by bloody stool, fever, and abdominal pain that is often more severe than that observed for *Shigella* and *Salmonella* infections. In developing countries the features reported are watery stool, fever, abdominal pain, vomiting, dehydration, and presence of fecal leukocytes; patients are also often underweight and malnourished (12,31,51). In Lagos, Nigeria, *Campylobacter* enteritis is characterized by a history of watery offensive-smelling stool lasting <5 days (51).

Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is an autoimmune disorder of the peripheral nervous system, which is characterized by acute flaccid paralysis. *C. jejuni* infection is the most frequently identified infection preceding GBS (52). In the developing world, sporadic GBS cases associated with *C. jejuni* infection have been reported from Curaçao, China, India, and South Africa (26,53-55). A comparative study between Curaçao and southwest Netherlands indicated that disease in Curaçao was more severe, had a higher incidence of preceding gastroenteritis, and had greater seasonal fluctuation (26). Serotype O:19 is most prevalent worldwide, although other serotypes, such as O:1, O:2, O:57, O:16, O:23, O:37, O:41, and

Table 4. Trends in Resistance to Ciprofloxacin by *Campylobacter jejuni* in selected developed countries up to year 2000

		Resistance strains (%)		
Country	Period	Initial	Year 2000	Ref.
Freiburg, Germany	1992-2000	22	32	41
Styria, Austria	1996-2000	25.2	40.2	42
England and Wales, UK	1993-2000	10	14.8	44
Philadelphia, USA	1995-2000	<10	36	45
Oslo, Norway	1988-2000	6.1	36	46

O:44, have been reported (52). *C. jejuni* strain O:41 appears to be restricted to Cape Town, South Africa, and represents a genetically stable clone (55,56). Detailed studies of the role of GBS in acute flaccid paralysis in developing countries, especially in polio-endemic areas, are needed.

Campylobacter Infection in the Setting of HIV

Campylobacter-associated diarrhea and bacteremia occur in HIV/AIDS patients worldwide. The species isolated include *C. jejuni*, *C. coli*, *C. upsaliensis*, *Arcobacter butzleri*, *Helicobacter fennelliae*, and *H. cinaedii* (57,58). The incidence of clinical manifestations is higher than in HIV-negative patients, with substantial mortality and morbidity. Furthermore, antibiotic resistance and recurrent infections have been observed (59). The incidence of HIV/AIDS is higher in developing countries than in developed countries and contributes substantially to deaths among <5-year-old children in epidemic settings (60). Thus, infants in developing countries are at risk of impaired immunity to *Campylobacter* enteritis. In addition, HIV/AIDS can increase the number of cases of campylobacteriosis in the adult population in these countries. These observations further support the need for improved understanding of the epidemiology of campylobacteriosis in developing countries.

Immunologic Aspects

In developing countries, such as Bangladesh, Thailand, Central African Republic, and Mexico, healthy children and adults are constantly exposed to *Campylobacter* antigens in the environment. As a consequence, serum antibodies to *Campylobacter* species develop very early in life in children in developing countries, and the levels of such antibodies tend to be much higher than those in children in the developed world such as in the United States (61-64). In Nigeria, children who had diarrhea and children who were healthy both had antibodies in their sera that could agglutinate *C. jejuni*; the difference in antibody responses between these groups of children was not statistically significant (65). Thus, antibody responses alone should be interpreted with caution in diagnosing *Campylobacter* infections.

In spite of shortcomings in the use of antibodies for diagnosis, increase in the level of anti-flagellar antibody had an inverse correlation with the rates of *Campylobacter* enteritis in the Central African Republic (63). An age-related relationship in the development of immunity to *Campylobacter* antigens has also been suggested to account for the age-related declines in the case-to-infection ratio and the period of excretion during the convalescent phase (28,66).

Usually, as age increases, level of antibody tends to increase. At the earliest stages in life (first 6 months), immunoglobulin (Ig) A, IgG, and IgM levels in response to *Campylobacter* infection are minimal, but thereafter increases are observed in response to infection. The poor serologic response during the first 6 months of life may be due either to a primary response to *Campylobacter* or to the presence of maternal antibodies via the placenta or breast milk.

Breast-feeding has been reported to play a role in *C. jejuni*-induced diarrhea. It decreases the number of episodes and the duration of diarrhea (67). In Algeria, exclusively breast-fed infants had fewer symptomatic *Campylobacter* infections than infants who were both breast-fed and bottle-fed (14).

Results of experimental observations among Mexican children have also shown that immunity to *Campylobacter* after primary infection may prevent bloody diarrhea from developing and subsequently prevent any disease from manifesting (68). In the developed world, the epidemiology may be different because most cases are usually primary infections with more severe clinical manifestations, greater numbers of people with bloody diarrhea (50%, as opposed to 15% in developing countries), and a more prolonged duration of excretion (approximately 15 days, compared with 7 days in developing countries) (28). The widespread immunity seen among adults in developing countries is absent in adults in developed countries (64).

Sources of Human Campylobacteriosis

Campylobacter infection is hyperendemic in developing countries. The major sources of human infections are environmental contamination and foods. Human-to-human transmission as a result of prolonged convalescent-phase excretion and high population density have also been suggested (5,12), although observations from developed countries show these are less likely factors (2).

Environmental Contamination

Wild birds as well as domestic and companion animals are known reservoirs for *Campylobacter* species, and shedding of the bacteria from them causes contamination of the environment. *C. jejuni* and *C. coli* have been isolated from chickens, goats, sheep, and pigs in developing countries (69,70). Strains isolated from human and chickens were phenotypically and genotypically correlated, confirming that chickens are an important source of human campylobacteriosis in developing countries (36). Poultry is also an important source of campylobacteriosis in developed countries. Extensive epidemiologic investigations have been done in those countries to identify sources of contamination and routes of transmission to humans to facilitate control efforts (71). Risk factors for acquiring campylobacters in developing countries include presence of an animal in the cooking area, uncovered garbage in cooking areas, and lack of piped water (12).

Foods

Campylobacter-contaminated foods—the result of poor sanitation—are an important potential source of infection in humans. For example, campylobacters were isolated from 40% and 77% of retail poultry meat sold in Bangkok, Thailand, and Nairobi, Kenya, respectively (72,73). The serotypes of the organisms isolated in Thailand were similar to those of organisms isolated from humans. In Mexico City, a survey of ready-to-eat roasted chickens showed that they were

contaminated with campylobacters (74). In developed countries, risk factors associated with foods include occupational exposure to farm animals, consumption of raw milk or milk products, and unhygienic food preparation practices (2).

Estimates of Impact of Human Campylobacteriosis in Developing Countries

The Disability Adjusted Life Year (DALY) is the basic unit used in Burden of Disease (BoD) methodology to quantify the impact of disease on a population (75). DALYs have been applied in the Dutch population to measure the mean health burden of *Campylobacter*-associated illness in the period 1990–1995 (76). The mean estimate was 1,400 DALYs per year; the main determinants of health burden were acute gastroenteritis (440 DALYs), gastroenteritis-related mortality (310 DALYs), and residual symptoms of GBS (340 DALYs). Although data on DALYs due to campylobacteriosis in developing countries are not available, diarrhea, which is a clinical manifestation of campylobacteriosis, was one of the top three causes of death and disease in developing countries in 1990 (75). The disease is projected globally to remain one of the top 10 by 2020. (The burden of campylobacteriosis in developing countries may increase by 2020 because HIV is projected to move up to the 10th position from 28th by 2020.) Considering the higher incidence of campylobacteriosis in developing countries, DALYs for the disease in developing countries will likely be higher than those of the Dutch population.

Conclusions

The incidence of human campylobacteriosis is increasing worldwide and has attracted the attention of WHO (http://www1.oecd.org/agr/prog/sum-copenhagen00.htm). Substantial gaps in knowledge about the epidemiology of campylobacteriosis in developing countries still exist. Present reported estimates of incidence are based on isolation rates from laboratory- and community-based studies conducted from 1980 to 1995. When various socioeconomic and health changes in developing countries are taken into account, these values may have changed considerably. Thus, public health awareness about the problem is needed, as are strengthened diagnostic facilities for campylobacteriosis, with a view towards setting up national surveillance programs. Such programs would determine the incidence rates, epidemiologic risk factors, interaction of HIV/AIDS and campylobacteriosis, seasonal variation, current state of resistance to antimicrobial agents, role of species other than *C. jejuni* and *C. coli*, and the role of campylobacteriosis in GBS. Collaboration among researchers in developed and developing countries needs to be strengthened, leading to development of regional centers of excellence. Funding organizations should provide incentives for North-South collaborations in *Campylobacter* research, as is done in other diseases such as malaria and trypanosomiasis that are endemic in some developing countries. All these should contribute to understanding of the global epidemiology of human campylobacteriosis.

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Guidelines for Synopses: 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.

Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Photographs and illustrations are encouraged.

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

Immunization with Heterologous Flaviviruses Protective Against Fatal West Nile Encephalitis

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Prior immunization of hamsters with three heterologous flaviviruses (*Japanese encephalitis virus* [JEV] SA14-2-8 vaccine, wild-type *St. Louis encephalitis virus* [SLEV], and Y*ellow fever virus* [YFV] 17D vaccine) reduces the severity of subsequent *West Nile virus* (WNV) infection. Groups of adult hamsters were immunized with each of the heterologous flaviviruses; approximately 30 days later, the animals were injected intraperitoneally with a virulent New York strain of WNV. Subsequent levels of viremia, antibody response, and deaths were compared with those in nonimmune (control) hamsters. Immunity to JEV and SLEV was protective against clinical encephalitis and death after challenge with WNV. The antibody response in the sequentially infected hamsters also illustrates the difficulty in making a serologic diagnosis of WNV infection in animals (or humans) with preexisting *Flavivirus* immunity.

West Nile virus (WNV) was detected for the first time in North America in summer of 1999, during an outbreak involving humans, equines, and birds in the New York City metropolitan area (1). Persistence of the virus and its spread to other states on the eastern seaboard during 2000 and 2001 suggest that WNV is now endemic in the United States and that its geographic range probably will continue to expand until it extends over much of the continent (2). Although many WNV infections in humans are asymptomatic or unrecognized, some patients have an acute dengue-like illness, and a small percentage have encephalitis or meningoencephalitis (1-5). The latter complication is most common among the elderly, with recent reported case-fatality rates from 4% to 11% (3-9). No specific treatment is available for WNV encephalitis, and no licensed vaccine is available for its prevention.

WNV is a positive-stranded RNA virus; based on its antigenic and genetic characteristics, it is included in the *Japanese encephalitis virus* (JEV) serocomplex of the genus *Flavivirus*, family *Flaviviridae* (10). The JEV serocomplex includes four antigenically related viruses that are important causes of encephalitis in humans: JEV, WNV, *St. Louis encephalitis virus* (SLEV), and *Murray Valley encephalitis virus* (MVEV). In addition to their antigenic and genetic relatedness, these four viruses have many epidemiologic similarities (3,11).

Because of the close antigenic relationships among many viruses in this genus, *Flavivirus* infections are difficult to differentiate by most serologic techniques, especially in persons or animals having a second or sequential *Flavivirus* infection (12-14). Considerable attention has been focused on the immune response in primary and secondary *Flavivirus* infection and the role of immunopathogenesis in the etiology of severe *Flavivirus* disease (11,15,16). In the case of dengue, enhancement of virus replication by heterologous flavivirus antibodies and T-cell activation are thought to occur in some patients during a second or sequential dengue infection, resulting in hemorrhagic fever or shock (15,16). In contrast, animal data indicate that prior infection with a heterologous *Flavivirus* reduces the severity of subsequent challenge with WNV. Results of experimental studies with rodents, monkeys, and pigs (17-21) suggest that heterologous *Flavivirus* antibodies protect against or modify subsequent infection with WNV. This phenomenon could be important in vaccine development against WNV infection and in determining the ultimate geographic distribution and public health importance of WNV if it is introduced into areas of Central and South America where other flaviviruses, such as *Dengue virus* (DENV), Y*ellow fever virus* (YFV), SLEV, and *Ilhéus virus* (ILHV), are endemic.

To determine more precisely the degree of cross-protection among members of the JEV serocomplex and the possibility that this phenomenon could be used to protect against severe WNV infection, a series of experiments was carried out with three heterologous flaviruses and a recently described model (22) of WNV encephalitis. We report the results of these studies, which indicate that prior immunization of hamsters with a JEV vaccine strain and a wild-type SLEV—and to a lesser extent the 17-D YFV vaccine—modify subsequent WNV infection and protect the animals from fatal encephalitis.

Materials and Methods

Four flaviviruses were used in this study: WNV strain 385-99, isolated from a dead snowy owl at the Bronx Zoo during the 1999 epizootic in New York City (23); live attenuated SA14-2-8 vaccine strain of JEV (24,25); 17-D live attenuated vaccine strain of YFV (26); and SLEV strain Be Ar 23379

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(27), originally isolated from mosquitoes (*Sabethes belisarioi*) in Para, Brazil, in 1961.

The hamsters used in our studies were adult (70 g to 100 g) female Syrian golden hamsters (*Mesocricetus auratus*) (Harlan Sprague Dawley, Indianapolis, IN). Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with infected animals was carried out in biosafety level-3 facilities.

All virus titrations were done in cultures of the C6/36 clone of *Aedes albopictus* cells (28), with the presence or absence of viral antigen by immunofluorescence as the endpoint, as described (22,29,30). To determine the quantity of infectious virus in blood samples taken daily after WNV infection, each hamster blood specimen was titrated in 24-well tissue culture plates seeded with C6/36 cells. Serial 10-fold dilutions from 10^{-1} to 10^{-7} were made of each sample in phosphate-buffered saline, pH 7.4 (PBS), containing 10% fetal bovine serum; 0.1 mL of each dilution was added to four wells of a tissue culture plate. Following absorption at 28°C for 2 hours, 1.5 mL of maintenance medium (29) was added to each well, and the plates were incubated at 28°C in a 5% CO_2 atmosphere for 6 days. On day 6, 20 mL of a cell suspension from each well was added to a single spot on 12-spot glass microscope slides (Cell-Line Associates, Inc., Newfield, NJ). After drying at room temperature, the slides were immersed in cold acetone for 10 minutes; the cells were subsequently examined for the presence of WNV antigen by indirect fluorescent antibody test by using a WNV-specific mouse immune ascitic fluid (see below) and a commercially prepared fluorescein-conjugated, goat antimouse immunoglobulin (Sigma, St. Louis, MO). WNV titers were calculated as the tissue culture infectious $dose_{50}$ ($TCID_{50}$) per mL of specimen by the method of Reed and Muench (31).

Experimental Infection of Animals

Hamsters were infected by the intraperitoneal (IP) or subcutaneous (SC) routes, depending on the virulence of the infecting virus for the animals. WNV and YFV were injected IP; JEV and SLEV were administered SC. Infecting doses of the viruses were as follows: WNV $10^{4.0}$ $TCID_{50}$, YFV $10^{6.0}$ $TCID_{50}$, JEV $10^{6.5}$ $TCID_{50}$, and SLEV $10^{6.0}$ $TCID_{50}$.

Immune Reagents

A mouse immune ascitic fluid to WNV was prepared in adult mice. The immunogen was a crude homogenate of brain (10% W/V in PBS) from newborn mice injected intracerebrally (IC) with the B956 prototype strain of WNV (32). The adult immunization schedule consisted of four IP injections of the immunogen mixed with Freund's adjuvant, given at weekly intervals. Sarcoma 180 cells were given after the final injection to induce ascites formation.

Antibody Determinations

Serum antibodies to WNV and the other three flaviviruses were measured by hemagglutination-inhibition (HI) test and to WNV by immunoglobulin (Ig) M antibody capture enzyme immunoassay (MAC-ELISA) (33). Antigens for both serologic tests were prepared from brains of newborn mice injected IC with each of the flaviviruses; the infected brains were treated by the sucrose-acetone extraction method (33). Hamster sera were tested by HI at serial twofold dilutions from 1:20 to 1:5120 at pH 6.6 (WNV, JEV, and SLEV) or 6.4 (YFV) with 4 units of antigen and a 1:200 dilution of goose erythrocytes, following established protocols (33).

For the MAC-ELISA, microtiter plates were coated with a commercial goat anti-rat IgM (capture) antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), diluted 1:500 in carbonate buffer, pH 9.6. All hamster sera were screened at a 1:40 dilution. The WNV antigen was also used at a 1:40 dilution. The secondary (detecter) antibody was a mouse, anti-*Flavivirus*, peroxidase-conjugated monoclonal antibody (6B6C-1) at a dilution of 1:6000. Results were read with a SPECTRA shell reader (SLT Labininstruments, Salzburg, Austria). Specimens wells were recorded as positive when the absorbance values at optical $density_{405}$ nm of the specimen wells exceeded 0.20 after subtraction of average background absorbance of control wells (33).

Results

Infection of Nonimmune Hamsters with WNV

Several groups of *Flavivirus*-naive (control) hamsters were inoculated IP with 10^4 $TCID_{50}$ of WNV to determine the subsequent level and duration of viremia, immune response, and death rate. Table 1 and the Figure show the results of an experiment with a group of 10 hamsters that were bled daily for 6 consecutive days after infection with WNV. Moderate levels of virus were detected in the animals' blood within 24 hours and persisted for 5 or 6 days. The highest blood virus titers were detected on days 2 and 3 after infection (means $10^{5.2}$ and $10^{5.1}$, respectively). HI antibodies were detected in all the animals by day 5, and the titers had increased substantially by day 6. In general, WNV-specific IgM, as detected by MAC-ELISA, appeared at approximately the same time as the HI antibodies (data not shown).

Table 2 shows the results of a second experiment in which 13 hamsters were infected with WNV. All the animals were bled 6 days after injection, and a subset was bled again at 31, 60, and 90 days. Six days after infection, all the animals had specific HI antibodies to WNV antigen and were negative to the other three flaviviral antigens tested (YFV, SLEV, and JEV). At this time, the animals also had a strongly positive IgM antibody response by MAC-ELISA. Thirty-one days after infection, the HI antibody response had become broadly cross-reactive with the four *Flavivirus* antigens, although the highest titer was still to WNV, and the IgM antibody had begun to

Table 1. Pattern of viremia and hemagglutination inhibition (HI) antibody response in 10 adult *Flavivirus*-naïve (control) hamsters, following intraperitoneal inoculation of 10^4 $TCID_{50}$ of *West Nile virus* (WNV)

	Day postinoculation					
Animal No.	D-1	D-2	D-3	D-4	D-5	D-6
8001	4.3[a](0)	5.0 (0)	5.0 (0)	3.3 (0)	1.0 (1:80)	1.0 (1:320)
8002	4.7 (0)	5.5 (0)	5.2 (0)	3.5 (0)	2.5 (1:40)	0 (1:320)
8003	5.3 (0)	5.3 (0)	5.0 (0)	3.5 (0)	2.5 (1:40)	0 (1:320)
8004	2.0 (0)	5.0 (0)	5.0 (0)	4.3 (0)	2.5 (1:40)	1.0 (1:160)
8005	4.0 (0)	5.0 (0)	5.5 (0)	3.7 (0)	1.7 (1:80)	1.0 (1:320)
8006	4.6 (0)	5.2 (0)	5.7 (0)	4.3 (0)	2.7 (1:80)	0 (1:320)
8007	4.3 (0)	5.7 (0)	4.6 (0)	4.0 (0)	2.0 (1:80)	1.0 (1:320)
8008	4.2 (0)	5.8 (0)	4.8 (0)	1.8 (0)	2.0 (1:80)	0 (1:320)
8009	5.2 (0)	5.2 (0)	5.0 (0)	3.2 (0)	2.8 (1:80)	0 (1:320)
8010	4.7 (0)	4.7 (0)	5.5 (0)	3.5 (0)	1.8 (1:80)	0.7 (1:320)
Mean	4.3	5.2	5.1	3.5	2.1	0.5
SD	0.92	0.34	0.34	0.71	0.54	0.50

[a]WNV titer expressed as $\log_{10}$ $TCID_{50}$/mL of blood. 0 ≤0.7. (HI antibody titer; 0 ≤1:20)

decrease. A similar HI antibody pattern was observed at 60 and 90 days after infection, although by 90 days the HI titers were decreasing. Six of the nine WNV-infected hamsters gave a negative reaction in the WNV MAC-ELISA when tested 60 and 90 days after infection.

Five of the 13 hamsters infected in this second experiment died of WNV encephalitis 7 to 14 days after infection (Table 2). Overall, 14 (47%) of 30 adult hamsters injected IP with 10^4 $TCID_{50}$ of WNV died of encephalitis (Table 3). The pathologic reaction of the WNV hamster model has been described (22).

Infection of JEV-Immune Hamsters with WNV

The Figure and Table 4 show the results from another experiment in which 30 adult hamsters were given a single SC injection of approximately $10^{6.4}$ $TCID_{50}$ of the live attenuated JEV SA14-2-8 vaccine strain. Thirty-eight days later, the animals were injected (challenged) IP with 10^4 $TCID_{50}$ of WNV; 10 of the hamsters in this group were bled daily for 6 consecutive days. These blood samples were subsequently titrated to determine the level of WNV viremia. The resulting viremia in the JEV-immune animals was markedly lower than in the naïve hamsters (Figure). Furthermore, the JEV-immune hamsters responded to challenge with WNV by developing a secondary (sequential) type of *Flavivirus* antibody response. Table 4 shows the HI antibody titers to JEV and WNV antigens in sera of 10 of the SA14-2-8 vaccinated hamsters, 30 days after their JEV immunization. At this time the HI antibody titers to JEV and WNV antigens were characteristic of a primary *Flavivirus* infection (13,14). On day 38, the animals were challenged with WNV; 6 days later, their sera were tested for HI and WNV-specific IgM antibodies. The boost in HI antibody titers that was observed 6 days after challenge with WNV was typical of a secondary antibody response to *Flavivirus* infection (13,14). In contrast, IgM antibody response to the second *Flavivirus* (WNV) infection was minimal (Table 4).

All the JEV-immune hamsters (n = 30) survived challenge with WNV (Table 3). Their infection with WNV was confirmed by the presence of low-level viremia (Figure) and the secondary *Flavivirus* antibody response following challenge (Table 4). None of these hamsters appeared clinically ill after infection with WNV, in contrast to the naïve animals. Many of the nonimmune hamsters had clinical signs of acute central nervous system injury (somnolence, muscle weakness, paralysis, tremors, and loss of balance) beginning around day 6 after infection, and approximately half died (22). Thus, prior immunization with JEV vaccine reduced the severity of subsequent WNV infection and prevented death.

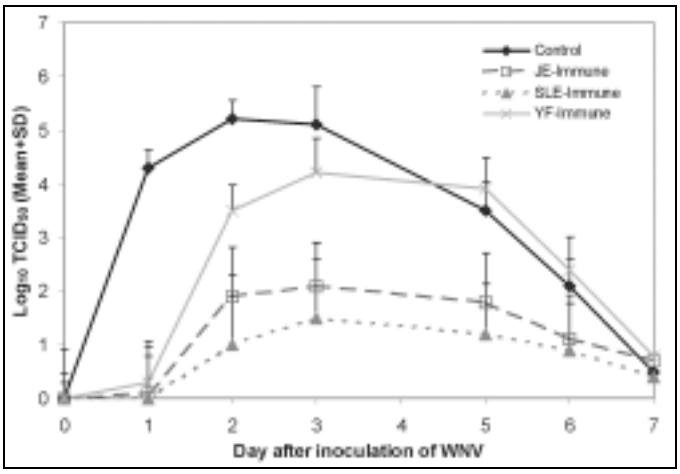

Figure. Summary of mean (±SD) *West Nile virus* (WNV) titers in daily blood samples from four groups of 10 hamsters each (control, *Japanese encephalitis virus* [JEV]-immune, *St. Louis encephalitis virus* [SLEV]-immune, and *Yellow fever virus* [YFV]-immune) after intraperitoneal inoculation of 10^4 tissue culture infective dose $(TCID)_{50}$ of WNV. Mean virus titers are expressed as $\log_{10}$ $TCID_{50}$/mL of blood.

Table 2. Serologic response of adult hamsters to *West Nile virus* (WNV), *Yellow fever virus* (YFV), *St. Louis encephalitis virus* (SLEV), and *Japanese encephalitis virus* (JEV) antigens, at various intervals after intraperitoneal inoculation of $10^{4.0}$ $TCID_{50}$ of WNV

Animal no.	HI antibody titer				WN MAC-ELISA
	WNV	YFV	SLEV	JEV	
Day 6					
8251(D[a])	1:40	0[b]	0	0	0.633[c]
8252	1:80	0	0	0	1.013
8253(D)	1:160	0	0	0	0.878
8254	1:160	0	0	0	1.090
8255	1:80	0	0	0	0.848
8256	1:80	0	0	0	0.840
8257(D)	1:160	0	0	0	1.291
8258	1:80	0	0	0	0.869
8259(D)	1:160	0	0	0	0.939
8260	1:80	0	0	0	0.992
8262	1:80	0	0	0	0.748
8263	1:80	0	0	0	0.797
8264(D)	1:80	0	0	0	0.827
Day 31					
8252	1:1,280	1:320	1:320	1:320	0.401
8254	1:1,280	1:320	1:320	1:320	0.427
8255	1:640	1:160	1:320	1:160	0.488
8256	1:640	1:160	1:160	1:160	0.582
8258	1:640	1:160	1:160	1:160	0.376
8260	1:1,280	1:320	1:320	1:320	0.420
8262	1:1,280	1:320	1:320	1:320	0.246
Day 60					
8252	1:2,560	1:640	1:640	1:640	0.269
8255	1:2,560	1:640	1:640	1:640	0.216
8256	1:640	1:160	1:160	1:160	0.162
8258	1:1,280	1:320	1:320	1:320	0.161
8260	1:320	1:80	1:80	1:40	0.179
8262	1:640	1:160	1:160	1:160	0.181
Day 90					
8260	1:640	1:80	1:80	1:80	0.217
8262	1:640	1:80	1:160	1:160	0.184

[a](D): Hamster died of encephalitis 7 to 14 days after infection. HI = hemagglutination inhibition.
[b]0 ≤1:20.
[c]Optical density value (≥0.200 is positive).

Infection of SLEV-Immune Hamsters with WNV

The Figure and Table 5 summarize the results of another experiment in which 32 adult hamsters were given a single SC injection of approximately 10^6 $TCID_{50}$ of SLEV strain BeAr 23379. This wild-type SLEV strain was selected for immunization, since it is not lethal to hamsters. Thirty-two days after injection with SLEV, the animals were inoculated IP with 10^4 $TCID_{50}$ of WNV. After this WNV challenge, the hamsters were bled daily for 6 consecutive days, as before. Antibody determinations were also done on blood samples taken 6 days after challenge with WNV.

Titration of daily blood samples from the SLEV-immune hamsters gave results similar to those in the JEV-immune animals. After challenge with WNV, 7 of the 10 SLEV-immune hamsters had brief, low-level viremia (Figure). However, three hamsters had no detectable viremia.

Serologic studies on blood samples taken 30 days after SLEV infection indicated that all the tested animals had been infected (Table 5). The HI response at 30 days was characteristic of primary *Flavivirus* infection. Six days after WNV infection, HI antibody titers had increased, indicating a secondary flavivirus antibody response. As with the JEV-immune hamsters, the IgM response of the SLEV-immune animals was minimal following the second flavivirus (WNV) infection (Tables 4,5).

Consistent with the low levels of WNV viremia (Figure), all the SLEV-immune hamsters (n = 32) survived subsequent challenge with WNV (Table 3). These animals did not appear clinically ill. These results indicate that prior immunity to SLEV also protected the hamsters from WNV encephalitis and death.

Infection of YFV-Immune Hamsters with WNV

Based on the results obtained with JEV- and SLEV-immune hamsters, we tested the effect of prior immunization with a non-JEV serocomplex *Flavivirus* on subsequent WNV infection. Accordingly, a group of 30 hamsters was inoculated IP with $10^{6.0}$ $TCID_{50}$ of the live attenuated 17D YFV strain. Thirty days after immunization, nine of the animals were bled and tested for HI antibodies to YFV and WNV (Table 6). Six days later (36 days after 17D vaccination), the hamsters were inoculated IP with 10^4 $TCID_{50}$ of WNV. Ten of these animals were bled daily for 6 consecutive days to determine the level of viremia and subsequent antibody response (Figure) (Table 6).

Following challenge with WNV, YFV-immune hamsters had an intermediate level of viremia (Figure). The mean WNV titers in the YFV-immune hamsters were higher than in the

Table 3. Infection and mortality rates, following intraperitoneal inoculation of 10^4 $TCID_{50}$ of *West Nile virus* (WNV), in nonimmune (control) hamsters, and in hamsters previously immunized with Japanese encephalitis (JE) SA14-2-8 vaccine, *St. Louis encephalitis virus* (SLEV) strain BeAr 23379, or yellow fever (YF) 17D vaccine

Immune group	No. infected with WNV	No. infected (%)[a]	No. died (%)
Nonimmune	30	30 (100)	14 (47)
JEV SA14-2-8	30	30 (100)	0 (0)
SLEV BeAr 23379	32	32 (100)	0 (0)
YFV 17D	30	30 (100)	4 (13)

[a] Total number of animals infected or dead after being infected with WNV.

Table 4. Serologic response of hamsters following immunization with the SA14-2-8 vaccine strain of *Japanese encephalitis virus* (JEV) and subsequent challenge with *West Nile virus* (WNV)

Hamster no.	HI antibody 30 days after JEV immunization		HI antibody 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
	JEV	WNV	JEV	WNV	
8236	1:80	1:80	1:640	1:640	0.166[a]
8237	1:40	1:80	1:320	1:320	0.205
8238	1:80	1:80	1:640	1:640	0.239
8239	1:80	1:80	1:640	1:640	0.173
8240	1:80	1:80	1:640	1:640	0.245
8241	1:80	1:80	1:320	1:640	0.271
8242	1:80	1:80	1:1,280	1:1,280	0.209
8243	1:40	1:80	1:160	1:160	0.205
8244	1:40	1:80	1:320	1:320	0.229
8245	1:80	1:80	1:160	1:160	NT

[a]Optical density value (≥Š0.200 is positive).
HI = hemagglutination inhibition. NT = not tested.

JEV- and SLEV-immune groups, but the titers were lower than in the *Flavivirus*-naïve (control) hamsters. The death rate in the YFV-immune hamsters was also lower; 4 (13%) of 30 YFV-immune hamsters died after challenge with WNV, compared with 47% in the control group (Table 3).

The HI antibody response after vaccination with YFV 17-D virus (Table 6) was less intense than the primary antibody responses to the other three flaviviruses (Tables 1,2,4,5). Monath (26,34) also observed that immunization with 17-D virus induces a weaker HI and complement-fixing antibody response than infection with a wild-type YFV strain. Nonetheless, 6 days after challenge with WNV, the animals previously immunized with 17-D virus demonstrated a strong secondary-type *Flavivirus* antibody response. Interestingly, the 17-D immune animals also had a stronger IgM response to WNV infection. These data indicate that 17-D vaccine gives only partial protection against challenge with WNV.

Table 5. Serologic response of hamsters following infection with *St. Louis encephalitis virus* (SLEV) strain Be Ar 23379 and subsequent challenge with *West Nile virus* (WNV)

Hamster no.	HI antibody 30 days after SLEV infection		HI antibody titer 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
	SLEV	WNV	SLEV	WNV	
8276	NT	NT	1:320	1:640	0.202[a]
8277	NT	NT	1:320	1:640	0.185
8278	1:80	1:80	1:160	1:160	0.141
8279	1:80	1:80	1:160	1:160	0.165
8280	1:80	1:80	1:640	1:640	0.276
8281	1:40	1:20	1:640	1:640	0.555
8282	1:80	1:40	1:160	1:80	0.177
8283	1:80	1:80	1:160	1:160	0.166
8298	1:160	1:80	1:320	1:320	0.139
8299	1:320	1:320	1:640	1:640	0.240

NT = not tested. HI = hemagglutination inhibition.
[a]Optical density value (≥0.200 is positive).

Discussion

The results of these hamster studies provide new information that may be useful in predicting the eventual geographic spread and public health importance of WNV in the Americas, as well as in developing novel methods for its control. The results also demonstrate the difficulty in making a serologic diagnosis of WNV infection in human or animal populations exposed to other flaviviruses.

First, our results clearly demonstrate that prior infection (and immunity) to JEV and SLEV protects hamsters from fatal WNV encephalitis (Table 3) and diminishes the severity of WNV infection (Figure). Other investigators (17-20) have reported similar findings in experimentally infected hamsters, pigs, and monkeys. The SA14-2-8 JEV strain used in our studies is one of several live attenuated JEV vaccines originally derived from the JEV SA14 wild-type parent strain (35,36); two of these vaccine derivatives, SA14-2-8 and SA14-14-2, have been widely used in China to immunize humans, equines, and pigs (24,25,35). Consequently, considerable information is already available on their biological and genetic characteristics, immunogenicity, safety, efficacy, and duration of immunity (24,25,35-37). The SA14 vaccine derivatives were obtained by serial passage (>100 times) in primary hamster kidney (PHK) cell cultures. Because the PHK cell substrate has not been approved by the World Health Organization as a vaccine substrate for use in humans, it is doubtful that the SA14 vaccine derivatives could be used in people in the United States or in other western countries. However, SA14-2-8 live attenuated JEV vaccine has been used successfully in >1 million horses in China (BQ Chen, pers. comm.) (25), and

Table 6. Serologic response of hamsters following immunization with the 17D yellow fever (YF) vaccine and subsequent challenge with *West Nile virus* (WNV)

	HI antibody titer 30 days after YF immunization		HI antibody titer 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
Hamster no.	YF	WNV	YF	WNV	
8226	1:20	1:20	1:320	1:320	0.783[a]
8227	1:40	1:20	1:320	1:320	0.484
8228	1:80	1:40	1:640	1:640	0.378
8229	<1:20	1:20	1:640	1:640	0.311
8230	<1:20	1:20	1:320	1:320	0.694
8231	1:40	1:20	1:640	1:640	0.511
8233	1:40	1:20	1:320	1:320	0.345
8234	1:20	1:20	1:640	1:640	0.418
8235	1:40	1:40	1:320	1:320	0.658

[a]Optical density value (≥0.200 is positive).
HI = hemagglutination inhibition.

potentially it could be used in equines in the United States to protect against WNV encephalitis.

We are testing a commercial inactivated JEV vaccine (JE-VAX) that is already licensed for human use in the United States. If the licensed inactivated JEV vaccine protects hamsters in a manner similar to the SA14-2-8 attenuated vaccine, it might be considered as an interim WNV vaccine for groups of humans at high risk of exposure, such as laboratory workers and veterinarians, to protect against WNV encephalitis until a specific WNV vaccine is available. Several potential human WNV vaccines are now under development (38,39); however, it will probably be years before the testing and approval process is completed and they are licensed for human use.

A second potentially important finding from our hamster studies was that animals previously infected with JEV or SLEV viruses had a much lower viremia on challenge with WNV, compared with nonimmune animals (Table 1) (Figure). If a similar reduction in the level of viremia occurred in JEV- and SLEV-immune animals of other species (i.e., birds and pigs), such animals would probably be inefficient amplifying hosts for WNV virus. Interference from heterologous antibodies to other JEV-serocomplex viruses in birds and other vertebrate hosts may help explain the unique and largely nonoverlapping geographic distribution of the various members of this medically important *Flavivirus* complex (40,41). To date, the spread of WNV in North America has been limited to areas that are largely free of other endemic JEV complex flaviviruses (41-43). However, as WNV moves into South Florida and the Gulf Coast or into the Midwest, regions where SLEV is endemic (43), WNV could be restricted by heterologous antibodies to SLEV in the resident avian population. SLEV is also endemic in tropical America (44), so potentially the spread of WNV into that region might also be restricted for the same reason. It will be interesting to observe how this natural experiment unfolds.

A third important finding of our study concerns the difficulty in making a serologic diagnosis of recent WNV infection. The antigenic cross-reactivity of *Flavivirus* antibodies is well known, especially after a second or sequential *Flavivirus* infection in the same host (11-15). As noted, until now most WNV infections in humans and animals in North America have occurred in areas largely free of SLEV. In the northeastern region of the United States, serologic diagnosis of recent WNV infection has been relatively easy, since most people and animals were experiencing their first *Flavivirus* infection. However, as WNV spreads into geographic regions where people and animals have other preexisting *Flavivirus* antibodies (i.e., SLEV, YFV, DENV), the interpretation of HI, MAC-ELISA, and even neutralization test results will be more difficult. As we have shown (Tables 4, 5, and 6), hamsters with prior immunity to JEV, SLEV, or YFV had a broadly reacting HI antibody response after a second (sequential) WNV infection. Most of the JEV- and SLEV-immune hamsters did not develop specific IgM antibodies after WNV infection. Consequently, the WNV MAC-ELISA also may be of little diagnostic value in such human or animal cases. The HI test and MAC-ELISA are the two serologic tests most commonly used by public health and veterinary diagnostic laboratories in the United States to screen for WNV infection (42). Our data suggest that these tests may give equivocal results in regions where more than one *Flavivirus* is active and that other, more specific diagnostic techniques are needed.

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Associations between Indicators of Livestock Farming Intensity and Incidence of Human Shiga Toxin-Producing *Escherichia coli* Infection

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The impact of livestock farming on the incidence of human Shiga toxin-producing *Escherichia coli* (STEC) infection was assessed by using several livestock density indicators (LDI) that were generated in a systematic approach. A total of 80 LDI were considered suitable proxy measures for livestock density. Multivariate Poisson regression identified several LDI as having a significant spatial association with the incidence of human STEC infection. The strongest associations with human STEC infection were the ratio of beef cattle number to human population and the application of manure to the surface of agricultural land by a solid spreader and by a liquid spreader. This study demonstrates the value of using a systematic approach in identifying LDI and other spatial predictors of disease.

Infection with Shiga toxin-producing *Escherichia coli* (STEC) is associated with a spectrum of illnesses including watery diarrhea, bloody diarrhea, and the hemolytic uremic syndrome, a potentially fatal condition characterized by acute renal failure (1). Although a variety of *E. coli* serotypes have been associated with human illness, the most important among these is O157:H7. Cattle are the principal reservoir for these organisms. Important sources of infection include consumption of undercooked hamburger and other contaminated food products and direct or indirect contact with infected persons (2-4).

Recent studies suggest that direct or indirect exposure to cattle are important potential sources of infection (2,3). Among this evidence is the finding of Michel et al. that the incidence of human STEC infection was higher in rural areas than in urban areas of Ontario, Canada. By using spatial regression analysis, these investigators demonstrated a strong association between the incidence of human STEC infection and cattle density, expressed as total number of cattle per hectare. Potential routes of infection in rural settings include direct contact with cattle (2,5), consumption of raw milk (2,4), contamination of well water with agricultural runoff (6,7), and contamination of locally produced food products (8).

The approach taken by Michel et al. (3) demonstrated the value of spatial analysis for identifying areas at high risk for STEC infection and for elucidating potential risk factors. The objective of the present study was to develop a systematic approach to creating and evaluating spatial measures of livestock density (livestock density indicators, or LDI) as a means of identifying those best suited to assessing the impact of livestock farming activities on the incidence of human STEC infection.

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Methods

Data

Data on 1,276 cases of human STEC infection reported in Ontario from January 1996 to December 1998 were obtained from the Reportable Disease Information System (RDIS) of the Ontario Ministry of Health and Long Term Care. Infection with STEC is notifiable in Ontario, and more than 95% of reported cases are due to *E. coli* O157:H7 (3). Cases were excluded if they were identified as part of a communitywide outbreak resulting from a single source, such as contaminated water supply.

Consolidated census subdivision (CCS) identifiers were added to the database via a software package that links CCS to appropriate postal codes (Postal Code Conversion File; Statistics Canada). The human population distribution of Ontario was obtained from GeoRef 1996 Census (Statistics Canada), which is based on data collected from all households in the province. CCS areas, which are coded in square kilometers, were also extracted from the GeoRef database. Livestock distribution and land use data were collected from the 1996 Census of Agriculture (Statistics Canada), and area units were converted from hectares to square kilometers. Information on soil development and drainage characteristics for Ontario were obtained from the Canadian Soil Information System (CANSIS) website (http://res.agr.ca/cansis/). All data were aggregated to the CCS because this was the most detailed level at which agricultural data were available.

The study area incorporated 435 townships in the southern area of the province. CCS data from the northern portion of the

province were excluded to avoid a potential bias, since this area was sparsely populated and had little agricultural activity. No information was available on confidential records from the 1996 Census of Agriculture.

STEC Incidence Rates

Incidence rates were determined over a 3-year period, from 1996 to 1998, and were expressed as the number of STEC cases per 100,000 population per year in Ontario CCS.

Spatial Analysis

ArcView version 3.1 (ESRI, Redlands, CA, USA) was used to create chloropleth maps based on disease rates and LDI measures. For the purposes of mapping, the method of nested means, as adapted by Michel and colleagues (9), was used to classify incidence rates. Quintile breaks in the data were used to categorize continuous LDI measures (for example, the lower 20% quintile for the ratio of beef cattle to human population is 0.000 to 0.049). ArcView was also used to calculate CCS centroid locations (latitude and longitude) to allow calculation of autocorrelation measures. SpaceStat version 1.9 (Regional Research Institute, West Virginia University, Morgantown, WV, USA) was used to calculate the Euclidean distance between CCS centroids, so that an inverse square distance matrix could be produced. This matrix was used in the calculation of Moran's I and G_i statistics for the 435 townships in the study area.

Soil landscape coverage version 2.2 and hydrological data version 2.2 were downloaded and imported into ArcView. Attributes containing information on the dominant soil type and drainage characteristics of the soil were mapped. ArcView's GeoProcessing extension was used to perform clip overlay analysis to remove areas normally covered with bodies of water.

The Spatial Analyst extension of ArcView was used to perform a cross-tab query to obtain soil type and drainage characteristic in each CCS. Soil typing within each CCS was based on the predominent type of soil; if two or more soil or drainage types occupied equal areas within a township, then the variable was set to a null value. Soil development and drainage characteristics were based on the Canadian system of soil classification (10).

Development of Livestock Density Indicators

For this study, an LDI was defined as a measure of livestock farming intensity that captures information on the number of animals or the amount of their fecal waste relative to various agricultural and environmental factors within a given geographic area. LDIs were created by combining a series of variables that were considered a priori to be potentially spatially associated with human STEC infection, based on a comprehensive analysis of possible sources and pathways of infection. Variables were constrained by their availability in existing databases.

We grouped these variables into "dimensions" and "components" (Table 1). Dimensions included variables related to number of animals, area of manure application, land uses, and human population within a given CCS. Within the dimensions were components that provided further refinement. For example, components within the dimension "animal" included numbers of various animal species within a given CCS, while components within "manure" consisted of specific manure characteristics and methods of application (Table 1).

Dimensions were combined mathematically according to equations denoted as "frames" (Table 2) to form the LDI. Within each frame, all possible combinations of the relevant components were used. For example, Frame 3 (human population/manure) was used to create four separate LDI consisting of the ratios of total human population in a CCS to the area having manure applied either 1) by solid spreader, 2) by irrigation, 3) as liquid on the soil surface, or 4) as liquid by injection into the soil. Each generated LDI was examined for biological and logical plausibility, and those considered inappropriate were discarded.

Statistical Analysis

Data manipulation, merging of data sets, and statistical analyses were conducted by using the Statistical Analysis System for personal computers, version 6.12 (SAS Institute Inc., Cary, NC, USA). Univariate associations between each indicator and the incidence of human STEC infection were examined

Table 1. Dimensions and components used in creating livestock density indicators to predict incidence of human Shiga toxin-producing *Escherichia coli* infection, Ontario

Dimension	Component
Animal	Total no. of cattle in CCS[a]
	Total no. of dairy cattle in CCS
	Total no. of beef cattle in CCS
	Total no. of chickens in CCS
	Total no. of pigs in CCS
Manure	Area of CCS having manure applied via solid spreader
	Area of CCS having manure applied via irrigation system
	Area of CCS having manure applied to soil surface via liquid spreader
	Area of CCS having manure injected into soil via liquid spreader
Land use	Total CCS area
	Absolute or % area of farm land in CCS
	Absolute or % area of pasture land in CCS
	Absolute or % area of crop land in CCS
Human population	Total human population in CCS

[a]CCS = consolidated census subdivision. Area of CCS is in square kilometers; absolute = absolute square kilometers.

Table 2. Frames used developing livestock density indicators for predicting incidence of human Shiga toxin-producing *Escherichia coli* infection, Ontario

Frame No.	Equation	Example
1	$\frac{Animal}{Land\ Use}$	$\frac{Total\ number\ of\ beef\ cattle\ in\ CCS^a}{Absolute\ area\ of\ farm\ land\ in\ CCS}$
2	$\frac{Manure}{Land\ Use}$	$\frac{Area\ of\ CCS\ having\ manure\ applied\ with\ a\ solid\ spreader}{Absolute\ area\ of\ farm\ land\ in\ CCS}$
3	$\frac{Human\ Population}{Manure}$	$\frac{Total\ human\ population\ in\ CCS}{Area\ of\ CCS\ having\ manure\ applied\ with\ a\ solid\ spreader}$
4	$\frac{Animal}{Human\ Population}$	$\frac{Total\ number\ of\ cattle\ in\ CCS}{Total\ human\ population\ in\ CCS}$
5	$Animal \times \frac{Land\ Use}{Total\ CCS\ area}$	$Total\ number\ of\ cattle\ in\ CCS \times \frac{Absolute\ area\ of\ pastureland\ in\ CCS}{Total\ CCS\ area}$
6	$Manure \times \frac{Animal}{Land\ Use}$	$Area\ of\ CCS\ having\ manure\ applied\ with\ solid\ spreaders \times \frac{Absolute\ area\ of\ pastureland\ in\ CCS}{Total\ CCS\ area}$

[a]CCS = consolidated census subdivision.

by using Poisson regression analysis. The GLIMMIX macro in SAS was used, and census division was entered as a repeated effect to induce a correlation structure in an attempt to control for the spatial effects inherent in the data.

Variable selection was performed in several steps. Initially, LDI were grouped along common components in their numerator (e.g., all LDI with dairy cattle in the numerator were combined into a single group). LDI within each group were then entered into a separate multivariate model, and non-significant LDI were removed by backward elimination until a minimum of one variable remained (stage 1 models). This procedure reduced the potential number of variables offered to subsequent models.

Variables thus identified were then offered to a second series of multivariate models (stage 2 models), each of which was subjected to a backward elimination procedure. Variables offered to stage 2 models consisted of all combinations of one variable from each of the models developed in stage 1.

Moran's I and G_i statistics were calculated for STEC incidence rates, which provided a measure of overall and local spatial autocorrelation. For all statistical analyses, a significance level of 5% was used (p=0.05).

Results

Geographic distribution of the yearly incidence of human STEC infection in Ontario between 1996 and 1998 is shown in Figure 1. According to the nested means techniques, STEC incidence rates were classified as very low incidence (0.00 to 0.95 per 100,000) in 204 CCS areas, low (0.96 to 4.54 per 100,000) in 95, average (4.55 to 5.38 per 100,000) in 15, high (5.39 to 15.01 per 100,000) in 78, and very high (15.02 to 77.52 per 100,000) in 41. CCS where the incidence of STEC infection was classified as high or very high were located primarily in the northwestern portion of southern Ontario, with smaller numbers in eastern Ontario.

Moran's I calculation for the incidence of STEC infection indicated a significantly positive autocorrelation (p=0.012). G_i statistics for the CCS areas with the 10 highest and 10 lowest incidence rates were also statistically significant (p<0.003).

A total of 8,316 LDI were generated, of which all but 80 were eliminated on the basis of biological plausibility. Of these 80 variables, 33 had a significant univariate association with the incidence of human STEC infection. Of these 33, 9 (27.3%) were based on the number of beef cattle, the total number of cattle per CCS, and measures of manure application; 4 (12.1%) were based on the number of standardized animal units per CCS; and 1 (3.0%) was based on both the number of dairy cattle and chickens per CCS.

The number of sheep or goats, soil type, or drainage characteristics were not significantly associated with the incidence of human STEC infection in the univariate analysis. The 10 LDI having the highest r^2 values in univariate analyses are shown in Table 3. All of these LDI were based on either the number of cattle, beef cattle, or animal units per CCS.

Multivariate modeling resulted in the creation of 16 unique stage 2 models with r^2 values ranging from 0.0932 to 0.266.

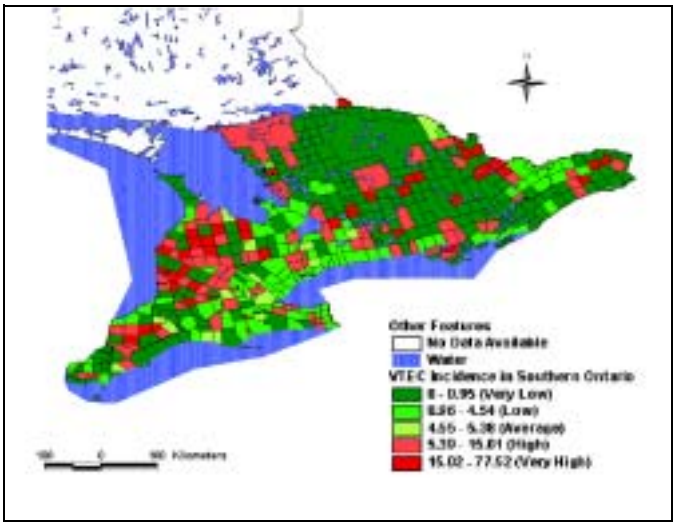

Figure 1. Yearly incidence of shiga toxin-producing *Escherichia coli* infection (per 100,000 population), southern Ontario, 1996–1998.

The multivariate model having the highest r^2 value, shown in Table 4, consisted of HUMBCOW (the ratio of the number of beef cattle to the human population in a CCS), PIGFARM (the total number of swine per km^2 of farm land in a CCS), SMANCCS (the proportion of land in a CCS in which manure is applied by a solid spreader), and LSMANCRP (the proportion of cropland in a CCS in which liquid manure is applied to the soil surface. HUMBCOW, SMANCCS, and LSMANCRP were all positively (and independently) associated with the incidence of human STEC infection, whereas PIGFARM was negatively associated.

Table 4. Multivariable spatial Poission regression models for predicting incidence of human shiga-toxin-producing *Escherichia coli* infection, Ontario, 1996–1998

Variable	Estimate	S.E.	p-value	r^2
Intercept	1.04	0.18	<0.001	0.2655
HUMBCOW[a]	0.65	0.13	<0.001	
PIGFARM[b]	-0.003	0.001	0.04	
SMANCCS[c]	4.19	1.86	0.03	
LSMANCRP[d]	7.82	2.47	0.002	

[a]Ratio of no. beef cattle to human population in a consolidated census subdivision (CCS).
[b]Total no. of swine per km^2 of farmland in a CCS.
[c]Proportion in a CCS having manure applied to land surface via solid spreader.
[d]Proportion in a CCS having manure applied to land surface via liquid spreader.

Discussion

The results of our analyses are consistent with the findings of Michel et al. (3), who demonstrated a higher incidence of human STEC infection in rural areas of Ontario, as opposed to urban areas, and a spatial association between the incidence of human STEC infection and cattle density. These findings are also consistent with other reports in the literature, including outbreaks of STEC infection related to consumption of unpasteurized milk (2) and water from shallow wells, direct contact with cattle (5), and an association between endemic STEC infection and exposure to agricultural environments (2,11).

Table 3. Top competing individual livestock density indicators for predicting the incidence of human shiga toxin-producing *Escherichia coli* infection, Ontario, 1996–1998

Livestock density indicator [a]	Estimate	S.E.	r^2
Ratio of beef cattle to human population	0.6872	0.1384	0.099
Intercept	1.4634	0.1018	
No. of beef cattle per km^2 township area	0.0998	0.0230	0.098
Intercept	1.1645	0.1674	
No. of beef cattle per km^2 weighted farmland (farmland/township area)	0.0004	0.0001	0.076
Intercept	1.3085	0.1430	
No. of beef cattle per km^2 weighted pasture land (pasture land/township area)	0.0014	0.0003	0.060
Intercept	1.4449	0.1182	
Total no. of cattle per km^2 of township area	0.0154	0.0034	0.053
Intercept	1.2217	0.1355	
Total no. of cattle per km^2 weighted pasture land (pasture land/township area)	0.0003	0.0001	0.046
Intercept	1.4165	0.1003	
Total no. of cattle per km^2 farmland	0.0139	0.0036	0.045
Intercept	1.0532	0.1834	
Animal units per km^2 township area	0.0129	0.0030	0.044
Intercept	1.2548	0.1317	
Ratio of total number of cattle to human population	0.1477	0.0344	0.043
Intercept	1.3878	0.1050	
Number of beef cattle	0.0003	0.0001	0.041
Intercept	1.3515	0.1540	

[a]All LDI significant at p>0.001.

To our knowledge, this is the first time the application of manure to land has been identified as a potential risk factor for endemic human STEC infection. Runoff from agricultural land that has been treated with manure has the potential to contaminate local surface water and wells that supply water for human consumption (12).

A relationship between agricultural activities, such as manure spreading, animal density, and elevated fecal bacterial counts in local streams. was demonstrated in 1989 by Meals (13). An outbreak of STEC infection in New York state was associated with contaminated well water used in the preparation of beverages and ice at a county fair (6). It was thought that the well in question became contaminated with manure-laden water as a result of recent heavy rains.

More recently, contamination of a municipal water supply with *E. coli* O157:H7 and *Campylobacter* spp. in Walkerton, Ontario, Canada, resulted in the largest documented outbreak of gastroenteritis caused by multiple pathogens. Strong evidence suggests that contamination of Walkerton's water supply was due to manure runoff from a nearby farm that entered a shallow well supplying the municipal water system (14).

The density of swine within a CCS was negatively associated with the incidence of human STEC infection. This apparent protective effect may simply be the result of a relative absence of cattle in areas where swine are intensively farmed. Although swine commonly harbor STEC within their intestinal tract, they are not considered to be important reservoirs of *E. coli* O157:H7 (15). Past studies have identified sheep and goats as important reservoirs for STEC (15,16), but these animals were not identified as important predictors of human STEC infection in our study. One explanation may be the relatively low numbers of these animals compared with other livestock types.

This study demonstrates the value of using a systematic approach to identifying potential LDI. The approach enabled us to examine a large pool of potential covariates from which appropriate indicators could be assessed and used to evaluate the association between livestock intensity and incidence of human STEC infection. The chosen indicators were biologically plausible and allowed for identification of a previously unreported risk factor.

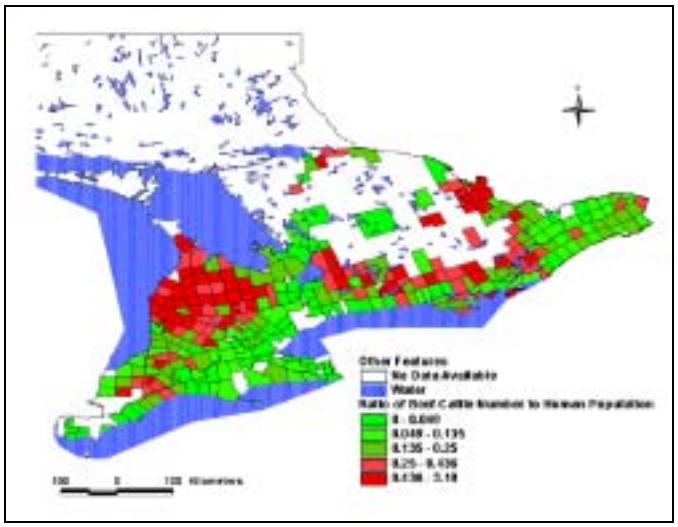

Figure 2. Ratio of beef cattle to human population (number of animals per person), southern Ontario, 1996).

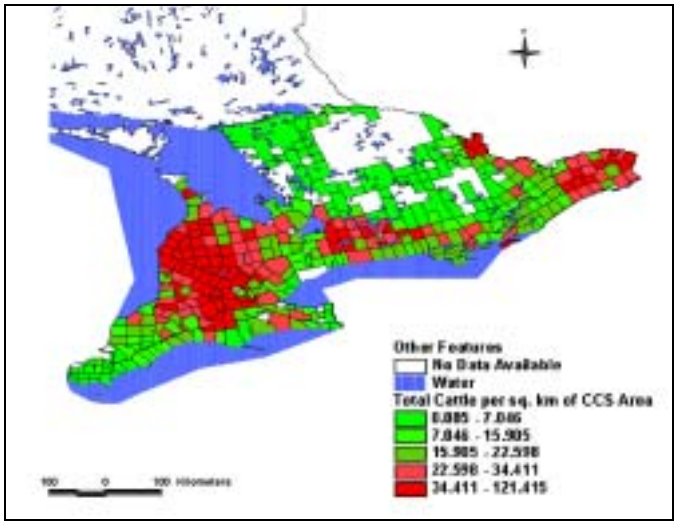

Figure 3. Total number of cattle per square kilometers, southern Ontario, 1996.

By using a systematic construction, we identified LDI that were more strongly associated with the incidence of STEC infection than has been reported previously (3). When modeled at the same geographic scale, the r^2 value for the best model from our investigation (i.e., the ratio of beef cattle to human population as a measure of cattle density) was 0.27 compared with 0.14 for the total cattle density model used in Michel's report (3). These differences in r^2 values may be the result of our selecting beef cattle for the LDI, rather than total number of cattle. It is worth noting that this difference in association is not necessarily evident from maps (compare Figures 2 and 3), because both indicators suggest roughly similar distributions of cattle density, with the greatest concentration in CCS located in south-central and eastern Ontario.

Caution should be exercised when interpreting our study results, however, because not all potential confounding variables (e.g., age or gender of the infected humans) were included in the analysis. Also, systematic errors arising from differential reporting rates may have biased the relationship between the incidence of human STEC infection and the risk factors studied. Since several LDI were investigated, some associations we observed may have arisen from chance alone.

Through linkage of existing data sources, spatial analytic techniques provide a means of identifying populations at high risk and potential risk factors for STEC infection. The approach outlined in this study provides a rational, practical, and powerful tool for public health. As spatial analysis becomes more widely used in epidemiology, we anticipate that the development of such approaches will take on increasing importance.

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Serologic Evidence of *Lyssavirus* Infections among Bats, the Philippines

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Active surveillance for lyssaviruses was conducted among populations of bats in the Philippines. The presence of past or current *Lyssavirus* infection was determined by use of direct fluorescent antibody assays on bat brains and virus neutralization assays on bat sera. Although no bats were found to have active infection with a *Lyssavirus,* 22 had evidence of neutralizing antibody against the *Australian bat lyssavirus* (ABLV). Seropositivity was statistically associated with one species of bat, *Miniopterus schreibersi*. Results from the virus neutralization assays are consistent with the presence in the Philippines of a naturally occurring *Lyssavirus* related to ABLV.

During the past decade, bats have been associated with a number of newly recognized zoonotic agents, including Hendra, Menangle, Nipah, and Ebola viruses and the *Australian bat lyssavirus* (ABLV) (1-5). ABLV and classic *Rabies virus* (RABV) are members of the genus *Lyssavirus.* These viruses are genetically similar and cause indistinguishable clinical syndromes in infected mammals. In the United States, where endemic canine rabies has been eliminated through vaccination and animal control, bat-associated variants of RABV have accounted for 24 (75%) of the 32 cases of human rabies reported since 1990 (6,7). Of the nearly 30,000 laboratory-confirmed cases of animal rabies reported worldwide in 1997, 4% were in bats (8). However, not all countries are included in this survey, and surveillance methods vary between countries included in the compilation. Bat-associated rabies cases in humans are likely underreported in this global surveillance report because not all countries report a history of animal exposure or type the virus variants.

In the Philippines, where approximately 350 cases of human rabies are diagnosed clinically each year, attribution of the animal associated with the exposure is based on history (8). Previous surveys for rabies in Philippine bats conducted in the 1950s and 1960s failed to document active rabies infection in the animals examined (9,10). The increasingly recognized role of bats in the global maintenance and transmission of viral infections, the recent discovery of rabies among bats in Australia, and the unknown proportion of rabies cases in Southeast Asia potentially attributable to bats prompted this initiation of active surveillance for lyssaviruses in Philippine bat populations.

Methods

Collection of Specimens

From June 25 through September 11, 1998, bats were nonrandomly collected from multiple sites on six different islands in the Philippines (Figure). Sites were chosen on the basis of local reports of known bat colonies or after investigation of likely habitats, such as caves, church belfries, or orchards (11,12). Insectivorous and small fruit bats were captured during the day in fine-mesh, long-handled butterfly nets and at night in mist nets. Larger fruit bats were also obtained from hunters. Thick leather gloves were worn when captured bats were transferred into individual muslin pouches for transportation.

Bats were anesthetized by a 0.05- to 0.1-mg intramuscular injection of ketamine hydrochloride and euthanized by intracardiac exsanguination. All blood was transferred from the collecting syringe into serum separator tubes and refrigerated until centrifugation. Serum was decanted into individual screw-topped vials and frozen at –20°C. Bats were identified to species by using a key based on gross morphology (13). The brains of all bats were removed surgically and frozen in individual containers. Additional organs (e.g., liver, spleen, and lungs) were also harvested from each bat and stored either in a freezer at -70°C or in 20% formalin for future studies. Carcasses of representative specimens were stored in formalin for archival purposes.

Direct Fluorescent Antibody (DFA) Testing of Brains

At the Research Institute for Tropical Medicine in Manila, the bat brains were thawed and multiple impressions were prepared for DFA testing (14). Microscope slides were fixed in cold acetone and allowed to dry. Brain impressions were stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabies monoclonal antibodies (Fujirebio Diagnostics, Malvern, PA) and examined under a fluorescent microscope for *Lyssavirus* antigens. This monoclonal antibody preparation reliably detects infection with all known lyssaviruses, including both classic RABV and ABLV (5,15-17).

Serologic Testing for Neutralizing Antibodies

At the Centers for Disease Control and Prevention (CDC), the presence of virus-neutralizing antibodies was determined by a modification of the rapid fluorescent focus inhibition test

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Figure. Collection sites for bats used in active surveillance of lyssaviruses in the Philippines.

(RFFIT) (18). Two different challenge viruses were used: the routine rabies challenge virus standard (CVS-11) and an isolate of ABLV adapted to cell culture. Viruses and bat serum samples were diluted with Eagle minimum essential medium containing 10% fetal calf serum (EMEM10) and antibiotics to reduce bacterial and fungal contamination as described (18). EMEM10 was also used in the growth of murine neuroblastoma (MNA) cells, which were used to propagate and grow sufficient quantities of each virus, and in the modified RFFIT assays. CVS-11 was obtained from stocks at CDC. The ABLV isolate (*Saccolaimus flaviventris* [sm4068]; Australian Animal Health laboratory, Geelong, Australia) was originally obtained from an insectivorous bat in Australia and was amplified by passage in BHK and MNA cells at CDC.

All bat serum samples were thawed and placed in a 56°C water bath for 30 minutes to inactivate complement. Serum samples were then diluted to 1:10 if possible. Samples with insufficient volume were screened at a higher dilution. The RFFIT was conducted by using Lab-Tek 8-well glass slides with covers (Nalge Nunc International, Naperville, IL). Sera were screened for antibody by incubating 100 µL of diluted serum with 100 µL of ABLV or CVS-11 that had been diluted to contain approximately 100 infectious units when incubated for 90 minutes at 37°C in a CO_2 incubator. MNA cells (approximately 75,000 cells/200 µL) were added to each serum-virus mixture, and the incubation was continued. After 48 hours, culture medium was removed, and the slides were fixed in acetone, air-dried, and stained for residual virus with FITC-conjugated anti-rabies monoclonal antibodies. A sample was defined as positive for neutralizing antibody if at least a 90% reduction in infectious centers was observed relative to the positive control. All positive samples were retested at increasing dilutions to estimate endpoint antibody titers. Standard human rabies immune globulin (HRIG) diluted to contain 2 IU/mL antibodies was used as a positive serum control for all tests. The titer of HRIG ranged from 1:125 to 1:625 against both ABLV and CVS-11.

Results from the serologic testing were used to detect patterns in seropositivity by location or type of bat, by using the Chi-square test.

Results

Collection of Specimens

Of the 821 bats collected, all but three were identified to species (Table 1). The collection resulted in 14 different species of both insectivorous and frugivorous bats representing five of the six families of Chiroptera believed to be present in the Philippines. Fifty-one percent of the bats were female, including 22 that were pregnant and 6 that were suckling infants. All bats appeared to be healthy except one with an enlarged spleen and three that appeared to have a mange-like condition. Since some bats died during collection and processing, serum could not be collected from all the bats.

DFA Testing of Brains

Brains from all 821 bats were tested for the presence of RABV antigen by DFA. None of the bats had detectable antigen consistent with an active infection with rabies or a related *Lyssavirus*.

Serologic Testing for Neutralizing Antibodies

Of the 821 bats collected, 231 had sufficient volume and quality of serum to be diluted to 1:10 and successfully

screened at 1:20, after being combined with the challenge virus (Table 2). An additional 43 specimens were screened at higher dilutions. Remaining samples contained insufficient volumes or could not be tested because of hemolysis.

Of the 231 bat sera tested, 22 (9.5%) were positive for neutralizing antibodies against ABLV. Antibody titration studies demonstrated decreasing percent neutralization at progressively higher serum dilutions. Of those 22 bat sera, 8 demonstrated no virus neutralization at the next highest dilution tested; 8 demonstrated some neutralization as dilute as 1:40; 3 had some at 1:80; 2 had some at 1:160; and 1 had evidence of some neutralization at a dilution of 1:320. When the strict definition of 90% to be considered positive was used, only two bat sera remained positive at the 1:40 dilution. This dilution is the equivalent of 0.6 IU/mL antibody. Five of those 22 samples were also positive when tested against CVS-11. Only 1 of the 209 bat sera that was negative when tested against ABLV was positive when tested against CVS-11.

The 22 bats with neutralizing antibodies against ABLV included six different species collected from four islands (Table 3). No location was significantly associated with bat sera that tested positive. Antibody-positive bats were evenly dispersed throughout the collection period (July 5 through September 5). Only 32% of the antibody-positive bat sera were obtained from females. That proportion was not statistically significant. The only significant association in the analysis was that a single species had a statistically greater proportion of samples testing positive. Thirty-six percent of the 11 *Mineopterus schreibersi* (Schreiber's long-fingered bat) tested positive (p=0.01).

The data analysis was repeated with a less strict case definition of 75% reduction in infectious centers relative to the positive control and including the 43 additional samples that could only be screened at higher dilutions. When these criteria were used, 53 (19%) of 274 bat sera tested were positive. The two samples with the highest positive endpoint titers in the initial analysis remained highest, but now at a 1:80 dilution. Although additional species would have been identified as having neutralizing antibodies, *M. schreibersi* remained the only species with a statistically significantly greater proportion of serum samples positive for neutralizing antibody.

Discussion

This study presents evidence of neutralization of ABLV by serum from Philippine bats. This neutralizing activity correlated with the ability to neutralize RABV (CVS-11) and titrated steadily with serial dilutions of the serum. These findings are consistent with the presence of naturally occurring antibodies against a *Lyssavirus* related to ABLV in the Philippine bat populations studied.

Lyssaviruses are classified into groups on the basis of their relative pathogenicity, their binding affinity to specific monoclonal antibodies, and their nucleic acid sequences. There are seven putative genotypes that have been aggregated into two basic groups on the basis of their overall phylogenetic relatedness (19). Phylogroup I includes RABV (genotype 1), *Duvenhage virus* (DUVV) (genotype 4), *European bat lyssavirus* (EBLV) 1 (genotype 5), EBLV-2 (genotype 6), and ABLV (genotype 7). Phylogroup II includes *Lagos bat virus* (LBV) (genotype 2) and *Mokola virus* (MOKV) (genotype 3). Antibodies to viruses within one phylogroup should cross-neutralize viruses of that same phylogroup. The ability to cross-neutralize is directly proportional to the relative nucleotide and amino acid homogeneity between the two viruses being

Table 1. All bats caught on six islands in the Philippines and tested for *Rabies virus* antigen by direct fluorescent-antibody assay, June 25 to September 11, 1998

	Island of origin					
Bat species	Luzon	Bohol	Boracay	Mindanao	Mindoro	Negros
Saccolaimus saccolaimus		53				54
Taphozous melanopogan	96					
Megaderma spasma	16					
Hipposideros diadema		16	1	2		
Rhinolophus spp.	6			3		
Mineopterus schreibersi				14		
Philetor brachypterus				24		
Scotophilus kuhlii	105			1		95
Cynopterus brachyotis	3			12	1	4
Eonycteris spelaea	1		6	2	1	
Macroglossus minimus	4			3	3	
Ptenochirus jagori	36			4	6	
Pteropus hypomelanus			27			
Rousettus amplexicaudatus	1	112	98	6	1	1

Table 2. All bats caught on five islands in the Philippines and screened for neutralizing antibodies against *Australian bat lyssavirus* at a 1:10 serum dilution

	Island of origin				
Bat species	Luzon	Bohol	Boracay	Mindanao	Mindoro
Saccolaimus saccolaimus		23			
Taphozous melanopogan	30				
Megaderma spasma	4				
Hipposideros diadema		4	1	2	
Rhinolophus spp.				2	
Mineopterus schreibersi				11	
Philetor brachypterus				13	
Scotophilus kuhlii	62			1	
Cynopterus brachyotis				1	
Eonycteris spelaea			1		
Macroglossus minimus	1			2	1
Ptenochirus jagori	8				
Pteropus hypomelanus			14		
Rousettus amplexicaudatus		21	28	1	

compared (19). In another study of Nigerian fruit bats, 2 of 50 serum samples had neutralizing antibodies against CVS-11 but failed to neutralize DUVV (20). In the bats in this study, no cases of active *Lyssavirus* infection were discovered from which nucleotide and amino acid sequences could be determined and subsequently compared with ABLV and CVS-11. More samples neutralized ABLV than CVS-11, suggesting that the *Lyssavirus* responsible for the induction of antibodies in these bats might be more similar to ABLV than CVS-11, while still being a member of phylogroup I. Repeating the RFFIT assays with a challenge virus from phylogroup II, such as MOKV or LBV, could have tested this hypothesis further. Although it is possible that we might have been able to demonstrate some cross-reactivity, a finding of greater neutralization activity against MOKV (compared with what was found against ABLV) would not be expected since all known phylogroup II viruses have a rather limited geographic distribution in Africa. In addition to the more widespread distribution of phylogroup I lyssaviruses, the small quantities of available bat sera precluded repeated RFFIT testing with an additional challenge virus such as MOKV.

Table 3. All bats caught on four islands in the Philippines positive for neutralizing antibodies against *Australian bat lyssavirus* at a 1:10 serum dilution

	Island of origin			
Bat species	Luzon	Bohol	Boracay	Mindanao
Taphozous melanopogan	4			
Mineopterus schreibersi				4
Philetor brachypterus				1
Scotophilus kuhlii	4			
Pteropus hypomelanus			3	
Rousettus amplexicaudatus		4	2	

The strict case definition used in the interpretation of the RFFIT assays resulted in the identification of 22 positive bat sera. As noted in the results of the second analysis, for which a lower threshold for positivity was used, additional bat sera that had 75% to 89% neutralization also had progressively decreasing neutralization at increasing dilutions, a finding similar to that for the 22 positive samples that met the strict case definition. Thus, we may have slightly underestimated the actual prevalence of anti-*Lyssavirus* antibody in these bat populations. Reduction in infectious centers by 90% compared with the positive control provided a conservative estimate of the prevalence of anti-*Lyssavirus* neutralizing antibodies. Previous studies have used a cutoff as low as 50% neutralization for the interpretation of data (21). Although the number of positive bat sera more than doubled when the broader case definition was used, no change in the results of the analysis of patterns of seropositivity by location or type of bat was evident. In addition, independent of the cutoff point used, the peak antibody measurement was approximately 0.6 IU/mL. Most of the other positive specimens had approximately 0.3 IU/mL. Many commercial laboratories report serum samples with ≥0.5 IU/mL of antibody as a positive test. However, the 0.5 IU/mL value was established as an arbitrary standard by reference laboratories as evidence above background for the detection of the induction of RABV-neutralizing antibodies in humans after receipt of multiple doses of high-potency rabies vaccines (22). No accepted standard for naturally occurring infections among wildlife exists.

On the basis of the 9.5% prevalence of neutralizing antibodies, it is not surprising that all brain samples studied showed no evidence of RABV antigen by DFA in these

clinically normal bats. Most studies of healthy bats have found a low prevalence of active infection, usually <1% (21). As would be expected, previous surveys of healthy bats in other parts of the world have shown that the prevalence of RABV-neutralizing antibodies is usually considerably higher than the prevalence of active infection, as indicated by positive DFA results for brain tissue. In a study of asymptomatic Mexican free-tailed bats from a single dense cave population in New Mexico, 69% of the bats had neutralizing antibodies, but only 0.5% had active infection demonstrated by DFA (21). A report from the Caribbean described a 40.5% seroprevalence of RABV-neutralizing antibodies among healthy and ill bats, but only 1 bat, which had been submitted as an ill-appearing rabies suspect, had active infection (23). Assuming a ratio of seroprevalence to active infection of approximately 100:1 in healthy populations of bats, based on the seroprevalence of 9.5% demonstrated in this study, one would have needed to test at least 1,052 normal bats to detect one case of active viral infection by DFA. Such further studies should be focused on species of bats such as *M. schreibersi* and in locations with the highest prevalence of neutralizing antibodies. Ideally, if a large stable colony of such bats could be identified, surveillance among sick and dying bats could be conducted. Such a study would increase the likelihood of obtaining a virus isolate and would minimize the potential adverse impact on the bat populations from oversampling large numbers of otherwise healthy bats. Similarly, the routine virus variant typing of human and domestic animal rabies cases in the Philippines and throughout Southeast Asia will provide basic epidemiologic information on the prevalence of different RABV isolates and enhance the likelihood of discovery of any new lyssaviruses affecting the populations in these regions.

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Novel *Cryptosporidium* Genotypes in Sporadic Cryptosporidiosis Cases: First Report of Human Infections with a Cervine Genotype

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In this study, we genotyped parasites from the fecal specimens of sporadic cryptosporidiosis cases in British Columbia from 1995 to 1999. Genotyping was conducted by polymerase chain amplification of the internal transcribed spacer region, a hypervariable region in the 18S rRNA gene and the *Cryptosporidium* oocyst wall protein gene. Subsequent analysis was by restriction fragment length polymorphism and DNA sequencing. We identified two new *Cryptosporidium* genotypes in humans. One of these genotypes has been found recently in deer in New York state. The other genotype has not been identified in humans or animals. These results have important implications for drinking water quality strategies, especially for communities that obtain drinking water supplies from surface sources located in forested regions with deer populations.

In recent studies of cryptosporidiosis cases in North America, South America, Europe, and Australia, various polymorphic gene loci were used to show that two major genotypes of *Cryptosporidium parvum* occur in humans (1-10). Genotype 1, or the human genotype of *C. parvum*, has been isolated almost exclusively from humans and associated mainly with anthroponotic (human-to-human) transmission cycles (1). Experiments to infect animals such as cattle and mice with the human genotype have been unsuccessful, and the only in vivo model that exists for this genotype is a gnotobiotic piglet model (11). So far, the only animals reported to be infected with genotype 1 *C. parvum* are a monkey in the United States (5) and a dugong (*Dugong dugon*) in Australia (12). In contrast, genotype 2 or the calf genotype of *C. parvum* has been isolated from both human and bovine hosts, as well as other livestock and wild animals such as sheep, goats, and deer. Genotype 2 has been associated with zoonotic (animal-to-human) transmission cycles.

Other genotypes of *C. parvum* are found in animals. including the dog, mouse, bear, pig, deer, and marsupial genotypes of *C. parvum*, which have been differentiated by sequence polymorphisms in the small subunit ribosomal RNA (13-15), the acetyl CoA synthetase (15), and heat shock protein 70 (16), as well as the *Cryptosporidium* oocyst wall protein (COWP) (17) genes, and named after the animals from which they were derived. Of these variant *C. parvum* genotypes, three human infections with the dog genotype have been reported—in an HIV patient (4), two Peruvian children (6), and a child in England (18). Aside from *C. parvum*, nine other *Cryptosporidium* species are recognized: *C. felis* (cat), *C. muris* (rodent), *C. andersoni* (cattle), *C. wrairi* (guinea pig), *C. baileyi* (bird), *C. meleagridis* (bird), *C. serpentis* (reptile), *C. surophilum* (lizard), and *C. nasorum* (fish). Although previously thought to be host specific, these other *Cryptosporidium* species have been associated with a few reports of human infections. *C. felis* (4,18,19) and *C. meleagridis* (19) have been found in immunocompromised persons. In addition, *C. felis* (6,18), *C. meleagridis* (6), and possibly *C. muris* (20) infections have been reported in children.

In this study, we genotyped parasites from the fecal specimens of sporadic cryptosporidiosis cases in British Columbia (BC). Genotyping was conducted by polymerase chain reaction (PCR) amplification of the internal transcribed spacer region, a hypervariable region in the 18S rRNA gene (4) and the COWP gene, (21). Subsequent analysis was by restriction fragment length polymorphism (RFLP) and DNA sequencing. We identified two new *Cryptosporidium* genotypes in humans. One of these genotypes has been found recently in deer in New York State (14). The other genotype has not been identified in humans or animals.

Materials and Methods

Cryptosporidiosis Cases and Community Information

C. parvum oocysts were isolated from patients in the Greater Vancouver and Fraser Valley Regional Districts of British Columbia over a 5-year period from 1995 to 1999.

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Demographic data on this geographic area have been described (2). Fecal specimens were collected from patients diagnosed with clinical symptoms consistent with cryptosporidiosis. *Cryptosporidium* oocysts were identified in stool specimens by standard concentration methods, acid-fast staining, and microscopy by the diagnostic parasitology laboratories to which the specimens were submitted. Oocyst-containing specimens were preserved in potassium dichromate solution (2.5% w/v) within 7 days of reception and stored at 4°C. The study was conducted retrospectively on specimens that were coded without personal identifiers. Informed consent from subjects was obtained.

Genomic DNA Extraction

Resuspended stool specimens were strained through cheesecloth. Potassium dichromate was removed by washing the sedimented filtrate 3 times in distilled water. Lipids were then extracted by using ethyl acetate as described (2). *Cryptosporidium* oocysts were disrupted by repeated freezing in a dry ice-ethanol bath and thawing in a boiling water bath in a 20% w/v suspension of Chelex-100 (BioRad Laboratories, Hercules, CA) as described (2). The DNA extracts were stored at –20°C.

PCR Amplification of *C. parvum* Oocyst DNA

Genomic DNA extracts from oocysts were centrifuged at 11,000 rpm (9,000 x *g*) for 20 minutes at 4°C and the supernatants used as template DNA for PCR. The PCR reaction was carried out as described (2) by using the forward primer, cry7, and the reverse primer CP5.8R to amplify the entire internal transcribed spacer 1 (ITS1) region, resulting in a 600-bp product. The amplification procedure using the CPBDIAGF/CPBDIAGR primer pair described by Pieniazek et al. (4) was used to amplify the hypervariable region of the 18S rRNA gene, and the CRY-9/CRY-15 primer pair described by Patel et al. (21) was used for the COWP gene.

In addition, genomic DNA prepared from oocysts that had either been characterized in previous studies (1,2) or were isolated from well-defined sources were included as known genotype controls. Genotype 2 controls included one bovine isolate from a purified batch of Iowa strain oocysts that had been passaged in calves at the University of Arizona; two human isolates from 1996 Cranbrook and 1998 Chilliwack outbreak cases, where animals infected with cryptosporidiosis were found in the watershed area ([2] and Ong et al., unpub. data); and five other human isolates derived from sporadic cases in British Columbia that have been described in a previous study as *C. parvum* genotype 2 isolates (2). Genotype 1 controls included seven isolates from sporadic cases and one isolate from a 1996 Kelowna outbreak case (2), all identified previously as *C. parvum* genotype 1 isolates (2). Deionized water and a culture of a nonpathogenic strain of *Escherichia coli* were used as negative controls.

RFLP Analyses of PCR Products

PCR products were purified by using QIAquick spin columns (Qiagen, Mississauga, ON) according to the manufacturer's instructions before digestion with the restriction endonucleases *Mse* I (New England BioLabs, Mississauga, ON) for the ITS1 locus and *Rsa* I (New England BioLabs) for the COWP gene. Two units of enzyme were added to a final volume of 20 µL containing 15 µL of PCR product and the appropriate dilution of the manufacturer's recommended buffer, and then incubated overnight at 37°C. Restriction fragments were then separated on Metaphor FMC agarose gels (3% for *Mse* I digests of ITS1 products and 3.2% for *Rsa* I digests of COWP products) (Mandel Scientific, Guelph, ON) and stained with ethidium bromide; the patterns were visualized with a UV transilluminator. DNA band sizes were analyzed by using the ProRFLP program version 2.38 (DNA ProScan Inc., Nashville, TN).

DNA Sequencing and Analyses

PCR products from the variable 18S rRNA and COWP gene loci were cleaned by spin column purification using the QIAquick PCR Purification kits (Qiagen). Sequencing reactions were conducted in both directions, i.e., from the 5' and 3'-ends using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and analyzed on an ABI 310 automated DNA analyzer (Applied Biosystems).

Overlapping bidirectional sequences were assembled by using the SeqManII (DNASTAR Inc., Madison WI) sequence analysis software. Consensus sequences obtained were aligned by using the ClustalX program (22), which was also used for calculating the phylogenetic tree by the neighbor-joining method with 1,000 replicates for bootstrap values. Published 18S rRNA gene reference sequences included in the multiple sequence alignment are listed below with their corresponding accession numbers: AF093491 (23) and AF087575 (4) for *C. parvum* genotype 1 human isolates; AF112569 (13) for a *C. parvum* simian isolate; AF087576 (4) and AF093490 genotype 2 isolates from a human and a bovine source, respectively; AF087574 (4) and AF112576 (13) for *C. parvum* "dog" genotype isolates from a human and a canine source, respectively; AF115377 (13), AF247535 (24), and AF112571 (13) for *C. parvum* pig, bear, and mouse genotype isolates, respectively; AF297511 (14), AF297512 (14), and AF297515 (14) for *C. parvum* "deer" genotype isolates; AF297503 (14) for a *C. parvum* muskrat isolate; AF087577 (4) and AF112575 (13) for *C. felis* from a human and a feline source, respectively; AF115378 (13), AF093498 (23), AF093496 (23), AF112574 (13), AF093495 (23), and AF093499 (23) for *C. wrairi*, *C. muris*, *C. andersoni*, *C. meleagridis*, *C. baileyi*, and *C. serpentis*. The phylogenetic tree was displayed visually by using TreeView (25). The *C. muris*, *C. andersoni*, and *C. serpentis* sequences were used in the outgroup, and the tree was rooted with this outgroup.

The 18S rRNA and COWP gene sequences of the 11 patient isolates listed in the table have been submitted to GenBank and assigned accession numbers AY030084 to AY030093 and AF411631 to AF411633. The BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) was used for DNA databases searches.

Results

Cryptosporidium oocysts were isolated from fecal specimens of 150 sporadic cryptosporidiosis cases. Two characteristic restriction profiles were obtained for *Mse* I digests of the 600-bp ITS1 products (Figure 1). The first type of restriction profile (Figure 1, lanes 4 and 15) showed five major bands at approximately 270, 160, 90, 75, and 55 bp. The bovine isolate, patient isolates from the 1996 Cranbrook and the 1998 Chilliwack outbreaks, and 29 (19%) isolates from sporadic cases had this restriction profile. Based on results from previous molecular characterization of a number of these isolates (1,2), this restriction profile was considered to be the *C. parvum* genotype 2 restriction pattern. The second restriction pattern (Figure 1, lanes 5, 6, 14, and 16) with six major bands around 185, 150, 100, 60, 40, and 30 bp was obtained from isolates of 108 (72%) sporadic cases and the one patient from the 1996 Kelowna outbreak. This restriction profile was considered to be the *C. parvum* genotype 1 profile, based on results from previous molecular analyses on the other seven genotype 1 isolates that were included as human genotype controls (2).

Restriction profiles with varying patterns (Figure 1, lanes 7, 9 to 13 and 17) were obtained from 13 (9%) other human isolates. Of these, nine (6%) isolates had identical restriction profiles (Figure 1, lanes 7 and 9 to 12) with five major bands at 150, 85, 70, 45, and 35 bp and were designated cervine genotype isolates. The other four isolates had unique restriction profiles and could be split into two groups of two isolates, based on the similarity of banding patterns. These were CS33 (Figure 1, lane 8) and MH222 (data not shown), which both had restriction fragments at 175 and 50 bp and additional variant bands at 65 and 70 bp, respectively. The remaining two isolates VF383 had bands at 315 and 105 bp (Figure 1, lane 13) and TK348 had bands at 325 and 85bp (Figure 1, lane 17), respectively.

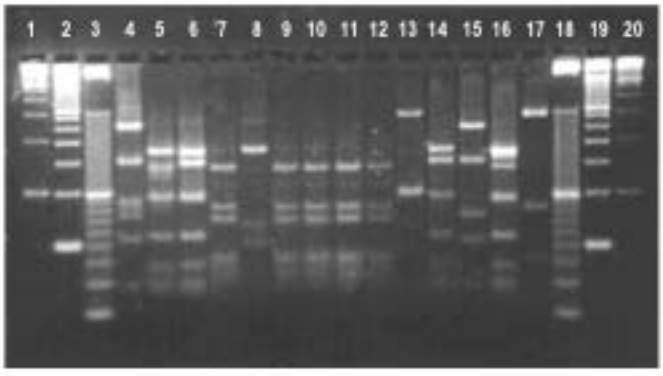

Figure 1. Restriction profiles obtained after digestion of polymerase chain reaction products from the ITS1 locus with *Mse* I. lanes 1- 3-, 100-, 50-, and 10-bp ladder molecular weight markers; lanes 4 and 15, bovine genotype 2 isolates; lanes 5, 6, 14, and 16, human genotype 1 isolates including DE340 (lane 14); lanes 7 and 9–12, cervine genotype isolates including MH205 (lane 7), TK320 (lane 10), and DE302 (lane 11); lane 8, *Cryptosporidium meleagridis* isolate CS33; lanes 13 and 17, other novel genotype isolates such as VF383 (lane 13) and TK348 (lane 17)

Table. Analysis of *Cryptosporidium* isolates collected from 1995 to 1999 from sporadic cases in British Columbia, Canada

Selected isolates	Genotype	RLFP loci	Genes sequenced
TK386	Human	ITS1	18S
TK303	Human	ITS1	18S
TK324	Human	ITS1	18S, COWP
DE340	Human	ITS1	18S
MH205	Cervine	ITS1, COWP	18S, COWP
TK320	Cervine	ITS1	18S, COWP
DE302	Cervine	ITS1	18S
MH222	*C. meleagridis*	ITS1	18S
CS33	*C. meleagridis*	ITS1, COWP	18S
VF383	Other novel	ITS1	18S
TK348	Other novel	ITS1	18S

RFLP = restriction fragment length polymorphism; ITS1 = internal transcribed spacer 1; COWP = *Cryptosporidium* oocyst wall protein.

To identify the *Cryptosporidium* species and genotype of isolates with variant restriction profiles, sequencing of a polymorphic locus on the 18S rRNA gene was carried out. Eleven isolates were selected based on their ITS1 restriction patterns. These included one isolate (TK386) with a characteristic human genotype 1 restriction profile, three isolates (TK324, TK303, DE340) with patterns similar to the human genotype 1 restriction profile but with one restriction fragment shifted slightly in molecular size (e.g., Figure 1, lane 14), three cervine genotype isolates (MH205, TK320, DE302; Figure 1, lanes 7, 10, and 11), and four isolates (CS33, MH222, VF383, TK348) with unique variant profiles (Table). Comparison of the 18S rRNA gene sequences of these isolates with 22 other published reference sequences, derived from a variety of human and animal *Cryptosporidium* isolates by using multiple sequence alignment and phylogenetic analysis (Figure 2), showed that the 11 isolates fell into four main groups. The first group consisted of four isolates (TK386, TK324, TK303, and DE340) that had restriction profiles identical or similar to the characteristic human genotype 1 pattern and all human genotype 1 reference isolates. All human isolates in the genotype 1 group had the characteristic polyT repeat sequence reported previously (10,26) between positions nt 686 and 698. The second group consisted of three human isolates (TK320, DE302, and MH205) and two cervine genotype isolates (Figure 2). The sequences of these three human isolates in the hypervariable 18S rRNA region were identical to that of a genotype 3 deer isolate described by Perz and Le Blancq (14). The third group consisted of two isolates (VF383 and TK348) and a pig genotype isolate (Figure 2). Sequences between the two

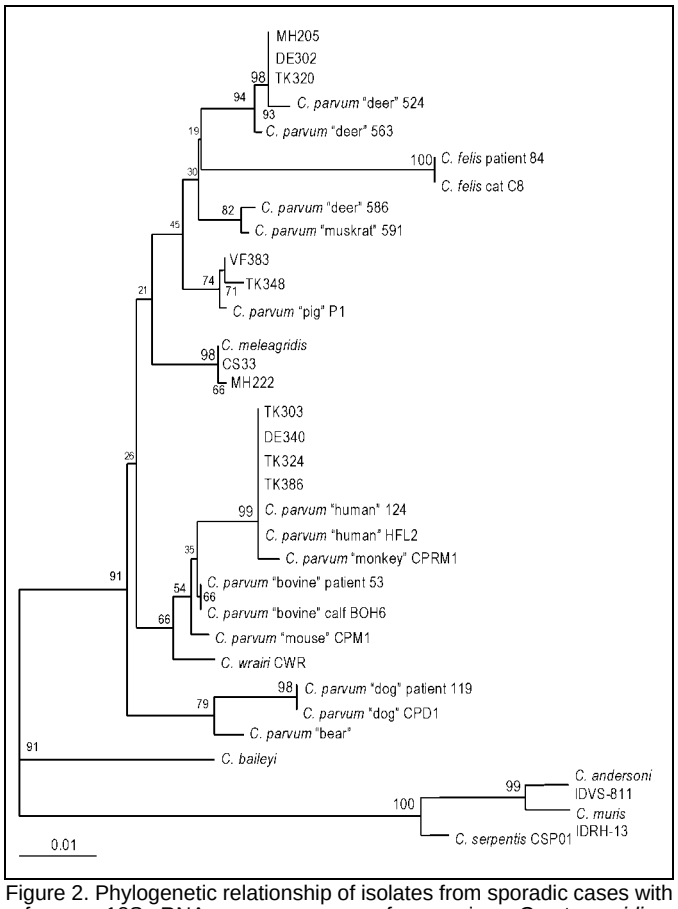

Figure 2. Phylogenetic relationship of isolates from sporadic cases with reference 18S rRNA gene sequences from various *Cryptosporidium* species and genotypes. Bootstrap values that are >95% are shown in larger font. Bar = 0.01 substitution per site.

human isolates were variant from the pig genotype sequence at only two different nucleotide positions between nt 686 and 698. The fourth group consisted of two human isolates (MH222 and CS33) and a *C. meleagridis* isolate (Figure 2).

Twenty-five sporadic isolates were also characterized by using a second locus on the COWP gene. *Rsa* I digests of the 550-bp PCR products (Figure 3) also showed the same dimorphism as the ITS1 locus with two predominant restriction patterns. Of these, 10 (40%) isolates had fragments at approximately 285, 125, 105, and 35 bp, which were characteristic for genotype 1 isolates (Figure 3, lanes 7 and 8). Another six (24%) isolates had the genotype 2 restriction profile with fragments at 410, 105, and 35 bp (Figure 3, Lanes 4 to 6). One isolate (CS33) had a variant restriction profile (Figure 3, Lane 10) with bands at approximately 370, 290, and 150 bp, which were similar in size to fragments reported for a *C. meleagridis* isolate (26). The 18S rRNA gene sequence of this isolate (CS33) was identical to that of *C. meleagridis*. The other isolate (MH205), had a restriction profile that was identical to those obtained from genotype 1 isolates (Figure 3, lane 9). This isolate had an identical ITS1 restriction profile with eight other sporadic human isolates and an 18S rRNA gene sequence that grouped with deer genotype 3 isolates from New York (Figure 2). The COWP gene sequences of MH205 and another cervine genotype isolate TK320 were determined to be identical and novel, sharing only 90% and 91% identity with the COWP gene sequences of the human (AF248741) and bovine (AF248743) alleles, respectively. BLAST analysis showed most alignment (92% identity) with a pig COWP gene sequence (AF266270) (17). However, the cervine allele had identical *Rsa*I restriction sites to the human allele at nt 34, 228, 512, and 618, whereas the bovine allele lacked the site at nt 228. The RFLP patterns could not be determined for the remaining seven isolates as insufficient PCR product was obtained. Over half (51%) of the isolates in this study were derived from pediatric patients <10 years of age, which accounted for seven of the nine cervine genotype infections as well as the two *C. meleagridis* infections.

Discussion

This study describes the discovery of the first zoonotic infections in humans with a novel cervine *Cryptosporidium parvum* genotype. Perz and LeBlancq (14) described this genotype recently after characterizing 111 *Cryptosporidium* isolates from wildlife in New York state. Those researchers did not detect human infections with this genotype in cryptosporidiosis cases in New York City. Other molecular epidemiologic studies in England (8,18) of 1,705 cases also did not identify cervine genotype infections, although rare zoonotic infections in humans with the dog genotype of *C. parvum* as well as other *Cryptosporidium* species such as *C. felis* and *C. meleagridis* were found (27). It is possible that cervine genotype infections in humans were not identified because the novel deer genotype had not been reported at the time of the

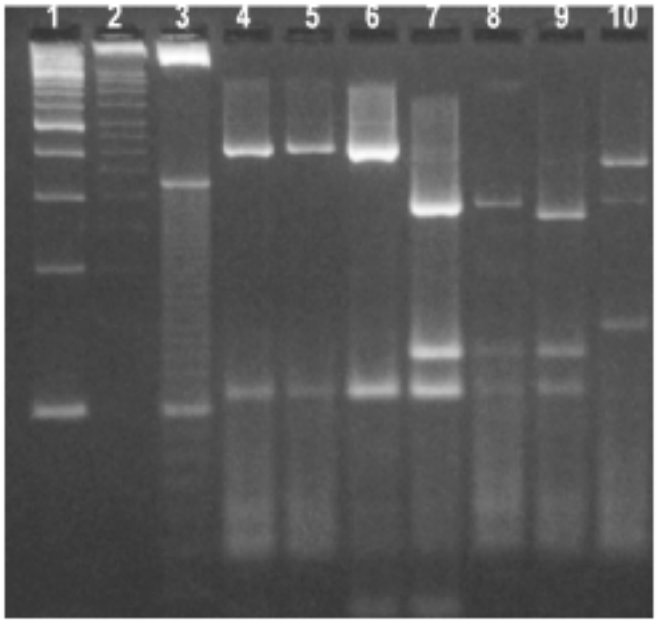

Figure 3. Restriction profiles obtained after digestion of polymerase chain reaction products from the *Cryptosporidium* oocyst wall protein locus with *Rsa* I. Lanes 1- 3-, 100-, 50-, and 10-bp ladder molecular weight markers; lanes 4 and 6, bovine genotype 2 isolates; lanes 7 and 8, human genotype 1 isolates; lane 9, cervine genotype isolate MH205; and lane 10, *C. meleagridis* isolate CS33.

study. As well, the PCR/RFLP profile of the COWP gene from the cervine genotype isolate was identical to that obtained from human genotype 1 isolates. Sequencing of the COWP gene from two cervine genotype isolates confirmed that the human and cervine alleles had identical *Rsa*I restriction sites. Therefore, RFLP analysis using this endonuclease could not differentiate between isolates with these two genotypes.

Xiao et al. (28) have also found this novel cervine genotype in storm water samples collected from a stream in the watershed area of New York State that contributes to the New York City water supply. The transmission of cryptosporidiosis from wildlife to humans in British Columbia is not surprising as many communities are supplied with unfiltered drinking water drawn from surface sources where *Cryptosporidium* spp. oocysts have been detected (29). Many of these watersheds are situated in remote forested areas, where wildlife such as deer are present in abundance. Deer with cryptosporidiosis infections have been identified in these watersheds (Ong et al., unpub. data). Therefore, to have as many as 6% of sporadic cases infected with this novel deer genotype is not an unexpected finding.

The ITS1 and 18S rRNA genes are reportedly multicopy genes with four copies of the Type A and one copy of the Type B rDNA units per haploid genome (30). The sequence divergence found between Type A and Type B units in the ITS1 region was a concern to us initially, as we first characterized the isolates using this locus before this report. However, Morgan et al. (31), who conducted a similar PCR-RFLP analysis of the ITS1 region, found that the restriction profiles were specific for different *C. parvum* genotypes. This study also indicated that intraorganism variation caused by the difference between Type A and Type B rDNA units may not be such a problem. Using primers to amplify the Type B unit, Morgan et al. (31) found that the Type A unit was amplified preferentially for human genotype isolates. To confirm that the observed variation in the ITS1 RLFP patterns was not due to heterogeneous products amplified from different copies of rDNA, further characterization of a select number of sporadic isolates was performed with the 18S rRNA as well as the COWP genes. Results from these additional analyses showed that isolates with distinctly different ITS1 RFLP patterns had different COWP RFLP patterns as well as 18S and COWP gene sequences. Therefore, ITS1 RFLP was useful for generating characteristic fingerprints that could distinguish between different *C. parvum* genotypes and *Cryptosporidium* species.

Using this method of genotyping, we were able to detect two new genotypes of *C. parvum* that had not been reported previously. Nine isolates (including three, MH205, TK320, and DE302, which had been characterized at the 18S locus; and two, MH205 and TK320, which had been characterized at the COWP locus) had the cervine genotype. Two other isolates (VF383 and TK348) had novel genotypes that were most closely related to a pig genotype isolate from Switzerland (13). These results have important implications for drinking water quality strategies, especially for communities that obtain drinking water supplies from surface sources located in forested regions with deer populations.

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Molecular Epidemiology of Adenovirus Type 7 in the United States, 1966–2000[1]

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Genetic variation among 166 isolates of human adenovirus 7 (Ad7) obtained from 1966 to 2000 from the United States and Eastern Ontario, Canada, was determined by genome restriction analysis. Most (65%) isolates were identified as Ad7b. Two genome types previously undocumented in North America were also identified: Ad7d2 (28%), which first appeared in 1993 and was later identified throughout the Midwest and Northeast of the United States and in Canada; and Ad7h (2%), which was identified only in the U.S. Southwest in 1998 and 2000. Since 1996, Ad7d2 has been responsible for several civilian outbreaks of Ad7 disease and was the primary cause of a large outbreak of respiratory illness at a military recruit training center. The appearance of Ad7d2 and Ad7h in North America represents recent introduction of these viruses from previously geographically restricted areas and may herald a shift in predominant genome type circulating in the United States.

Human adenoviruses (Ads) comprise 51 serotypes (1); they are ubiquitous and responsible for a wide range of clinical syndromes. Among recognized serotypes, Ad type 7 (Ad7) (and to a lesser extent Ad type 3) is most often associated with severe disease (2). Although Ad7 infections typically result in mild upper respiratory tract illnesses and conjunctivitis, infections can also lead to more serious lower respiratory tract illnesses, disseminated disease, and death, particularly in infants and persons with underlying immunologic or respiratory compromise (3-7). Ad7 infections have also been associated with diseases of the central nervous system (8,9) and long-term respiratory sequelae that include bronchiectasis and hyperlucent lung or McLeod syndrome (10).

Ad7 accounts for nearly 20% of all Ads reported to the World Health Organization (11), and family clusters and institutional and communitywide outbreaks of Ad7 disease have been extensively documented (5,12-18). Three types of outbreaks have been described (12): i) outbreaks that occur during the winter months among institutionalized infants (<2 years of age) that result in high rates of severe illness and death; ii) periodic nonseasonal communitywide outbreaks involving older children and adults with infrequent serious outcomes; and iii) outbreaks of acute respiratory disease among new military recruits. Outbreaks of acute respiratory disease due primarily to Ad7 and Ad4 were an important cause of illness in new military recruits in the United States until live enteric-coated Ad4 and Ad7 vaccines began to be routinely administered in 1971 (19). The recent cessation of production and administration of these vaccines has resulted in a resumption of Ad-associated acute respiratory disease outbreaks at military recruit training centers throughout the United States (20-22).

To facilitate study of the molecular epidemiology of Ad7, a classification system based on restriction enzyme analysis of Ad genomic DNA was devised by Li and Wadell (23) and later revised by Li et al. (24). Their system uses *Bam*HI as the "type" defining enzyme, with different genome types denoted with a character, e.g., "p" for the Ad7 prototype strain, Gomen; and then "a" through "k." Genome types that are further distinguished by restriction pattern with additional selected enzymes are given an Arabic numeral (e.g., Ad7p, p1, a, a1-6). Their system has been widely used to correlate genome types with geographic distribution and pathogenic potential.

Both globally dispersed and geographically restricted genome types of Ad7 have been identified by restriction analysis, and regional shifts or replacements of predominant genome types have been documented on different continents. Among the 3 Ad7 genome types first distinguished by restriction analysis (25), two shown to be serologically distinct (26) were designated Ad7p (Gomen) and Ad7a (S-1058), and a third, designated Ad7b, was thought to be associated with more severe illness (12). Ad7b eventually spread worldwide (27-30), displacing formerly common genome types (i.e., Ad7p, Ad7a, Ad7a1-6, Ad7c, and others) that are now rarely detected. Exceptions to this pattern have been reported. In the former Soviet Union, a successive shift from Ad7a and Ad7a1-5 to Ad7f1 during 1976-1979 and 1986-1988 was reported (31). In South America, a shift from Ad7c to Ad7h occurred in 1986 (32), and Ad7h has subsequently caused serious respiratory illness in infants and young children in Chile

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and Argentina (33,34). In the early 1980s in China, a new genome type, Ad7d (27), replaced Ad7b as the predominant circulating virus. Recent reports suggest that Ad7d and Ad7h have spread beyond their formerly geographically restricted regions. Ad7d was identified in Japan in 1987 (35), and countrywide epidemics of Ad7 that began in 1995 in Korea (18; Hoan-Jong Lee, pers. comm.) and Japan (15, 36) were attributed to Ad7d and a closely related genomic variant, Ad7d2, respectively. Ad7d2 has emerged as the predominant strain circulating in Israel since 1992 (37). Ad7h was first reported outside South America in 1996, in Japan (36,38).

Beginning in the fall of 1998, an outbreak of Ad7 infection occurred at a pediatric chronic-care facility in Chicago and subsequently spread to a tertiary-care hospital, where staff from two clinic units were infected (17). This multi-site outbreak was associated with considerable illness and death among residents of the chronic-care facility. Isolates from this outbreak were identified by restriction enzyme analysis as Ad7d2. The appearance of this new genome type prompted us to study the temporal and geographic distribution of Ad7 genome types in the United States to better characterize the emergence and spread of this virus.

Materials and Methods

Ads

Of 297 Ad field isolates obtained from the Centers for Disease Control and Prevention (CDC) archives, state public health laboratories, university hospitals, and military training centers, 166 confirmed as Ad7 were selected for genome type analysis (Table 1). Of these, 116 were obtained from 1966 to 2000 from civilians in 25 states and eastern Ontario, Canada; 50 were obtained from February 1997 to May 1998 from military recruits attending training centers in five states (20). Isolates were selected to achieve broad geographic and temporal distribution. Because detailed demographic, epidemiologic, and clinical data from patients were limited, they were not included in this report. Most civilian isolates were obtained from individual cases or family clusters of Ad7 disease, ranging from mild upper respiratory illness to severe lower respiratory tract illness and death. Where civilian outbreaks of Ad7 illness were recognized, only one representative isolate was included in the 166 sample for analysis. Approximately 10% of Ad7 isolates from military recruits with respiratory illness were sampled from all five training sites and were selected to be evenly spaced over the designated time period. Reference strains Gomen and S-1058 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Reference isolates of Ad7d2 were obtained from a postmortem rectal swab from a 4-month-old baby in Israel in 1993, and Ad7h was obtained during a regional outbreak of respiratory illness in Chile in 1998. All Ad isolates were passaged at least once in A549 cells before restriction analysis. Type-specificity of all Ad7 field isolates was confirmed by neutralization or Ad7 type-specific polymerase chain reaction assay (PCR) (39).

Table 1. Human adenovirus 7 (Ad7) field isolates from the United States and Canada, 1966–2000

Location	Isolation year(s)	No. Ad7 isolates
Canada		
Eastern Ontario	1999, 2000	3
United States		
Alabama	1985, 1986	2
Arizona	1995, 1998	3
California[a]	1997	1
California	1981- 4, 1987, 1990, 1992, 1995, 1996	15
Colorado	1987	1
Florida	1986, 1996	2
Georgia	1996	1
Illinois[a]	1996 - 98	28
Illinois	1997	2
Iowa	2000	1
Kansas	1995, 1997	3
Louisiana	1996	2
Maine	1981	1
Maryland	1991, 1993 - 95	16
Massachusetts	1998, 1999	2
Michigan	1986	1
Mississippi	1986	1
Missouri[a]	1997	9
Missouri	1966, 1998, 1999	8
New York	1970, 1985, 1990, 1991, 1993, 1995-97, 1999, 2000	31
Ohio	1993-95, 1997, 1998	10
South Carolina[a]	1997, 1998	11
South Carolina	1998	1
South Dakota	1987	1
Tennessee	1997	2
Texas[a]	1998	1
Texas	1999, 2000	2
Virginia	1985	1
Washington	1996	1
Wisconsin	1996, 1998	3
North America (total)	1966-2000	166

[a]Ad7 isolates obtained from military recruit training centers.

DNA Restriction Analysis

Ad genomic DNA was extracted by a modification of the method of Deryckere and Burgert (40). Briefly, isolates were grown in 75-cm^2 confluent flasks of A549 cells until the 4+ stage of cytopathic effect was attained. The contents of the flask were centrifuged at low speed to remove cells, and the

supernatant was transferred to an ultra-centrifuge tube and centrifuged for 2 hours at 100,000 x *g*. The virus pellet was resuspended in 400 µL of Tris buffer (pH 7.4) with 1% sodium dodecyl sulfate and sequentially digested with DNAse free RNAse A (0.1 mg/mL) and proteinase K (0.5 mg/mL). The digest was extracted twice with equal volumes of phenol and chloroform/isoamylalcohol (24:1) and once with chloroform/isoamylalcohol alone. The purified DNA was then precipitated with absolute ethanol and washed once with 75% ethanol, and the pellet was resuspended in 100 µL of dH_2O. Enzyme digestions were carried out according to manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). DNA from all Ad7 isolates was digested with *Bam*HI and *Sma*I, and selected isolates were also digested with enzymes *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hpa*I, *Hin*dIII,*Sal*I, *Xba*I, and *Xho*I. Enzyme digests were electrophoresed at 100 volts for 5 hours on 0.8% agarose gels, and the DNA bands were visualized by ethidium bromide staining. Restriction fragment size(s) was interpolated from DNA molecular weight standards included in each run. Restriction patterns were compared with previously published profiles (24,37,41,42), and the identification of genome types followed the denomination system of Li et al. (24).

DNA Sequencing

The hypervariable region of the hexon protein gene corresponding to nucleotides 403 to 1356 (Gomen), which have been shown to encode the residues that define Ad serotype, was PCR amplified from selected Ad7 isolates as described (43) and sequenced by using the DyeDeoxy Terminator Cycle Sequencing Kit and ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined for both PCR product strands. Sequence analysis was performed by using the Wisconsin Package ver. 10.0 (Genetics Computer Group, Madison, WI). Hexon gene sequence data for the reference Ad7d2 strain from Israel were submitted to GenBank (accession number AF321311).

Results

Ad7 Genome Types Identified

DNA restriction analysis of the 166 Ad7 field isolates identified 108 (65%) as Ad7b, 46 (28%) as Ad7d2, 4 (2%) as Ad7h, 3 (2%) as Ad7p, 3 (2%) as Ad7a, and 2 (1%) as Ad7a3. Restriction profiles of representative Ad7b, Ad7d2, and Ad7h isolates for selected endonucleases are shown in the Figure. All U.S. (and eastern Ontario, Canada) Ad7d2 isolates and an Ad7d2 reference strain from Israel (37) gave identical restriction patterns for *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hpa*I, *Hin*dIII, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. Identical restriction profiles were also obtained with four U.S. Ad7h isolates and a 1998 isolate of Ad7h from Chile, which were similar to profiles described for Ad7h strains isolated in Argentina and Chile (formerly designated Ad3f) (41,42,44).

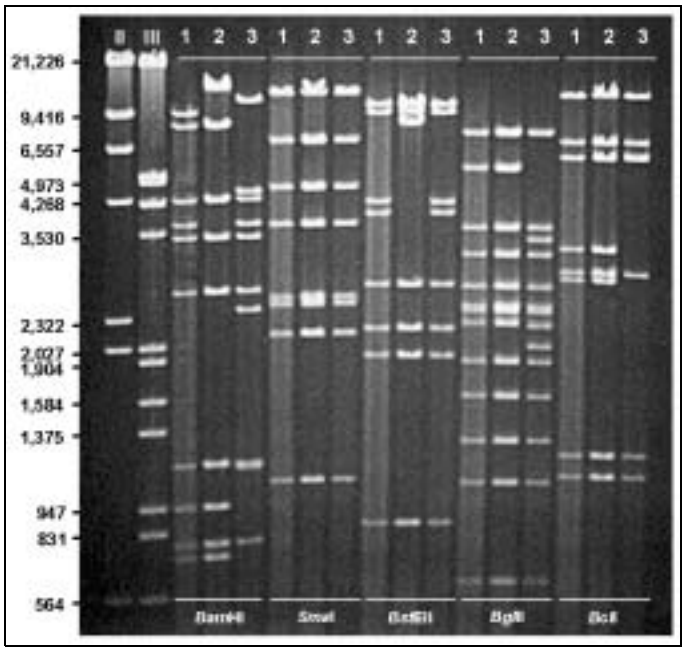

Figure. Restriction profiles of representative human adenovirus (Ad) genome types Ad7b (1), Ad7d2 (2), and Ad7h (3) after digestion with selected enzymes, *Bam*HI, Sma I, *Bst*EII, *Bgl*II, and *Bcl*I. DNA markers II (M *Hin*dIII) and III (M *Hin*dIII/*Eco*RI).

Ad7 Hexon Gene Sequencing

The hypervariable regions of the hexon gene (corresponding to nucleotides 403 to 1356 of the reference strain Gomen) of 24 temporally and geographically diverse Ad7 field isolates (including 11 Ad7b, 10 Ad7d2, and 3 Ad7h and laboratory strains S-1058, 55142 vaccine, and Gomen) were sequenced and compared with published Ad7 hexon sequences available from GenBank (Table 2). Nucleotide and deduced amino acid alignments of these sequences comprised two major genetic clusters as previously described (26,45): cluster 1, Ad7p (Gomen) and Ad7p1; and cluster 2, Ad7a Ad7b, Ad7c, Ad7d, Ad7d2, Ad7g, and Ad7h. Cluster 2 sequences were highly conserved, with over 98% nucleotide identity, and were generally uncorrelated with genome type. However, a unique Gln substitution for Leu (codon CTG > CAG) at amino acid position 443 of loop 2 of the predicted hexon protein was identified in all 10 Ad7d2 isolates from the United States and Israel; this substitution was also present in published hexon sequences of Ad7d isolates from China (45) and Japan (38).

Temporal Distribution of Ad7 Genome Types

The yearly distribution of the 166 Ad7 genome types is shown in Table 3. Ad7b was the only genome type identified from 1970 through 1992 and was the predominant genome type identified through 2000. Ad7d2 first appeared among 1993 isolates and accounted for approximately 28% of all Ad7 isolates obtained from 1993 to 2000. Four epidemiologically unrelated isolates of Ad7h were identified in 1998 and 2000.

Table 2. Human adenovirus 7 (Ad7) field isolates and laboratory strains used for hexon gene sequence comparisons

ID	Genome type	Location	Isolation year	Accession no.	Sequence source[a]
S-1058	7a	USA	1955	af053085	Inada & Mukoyama, direct submission; CDC
55142 vaccine	7a3	USA	1963	af065067	Crawford-Miksza et al. (26); CDC
BC30	7b	China	1958	u75951	Li & Wadell (45)
BC14	7b	China	1965	u77390	Li & Wadell (45)
KCH4	7b	England	1973	u77391	Li & Wadell (45)
v2026	7b	USA, MI	1986		CDC
v2124	7b	USA, SD	1987		CDC
2000017657	7b	USA, MD	1991		CDC
99026790	7b	USA, OH	1993		CDC
2000017667	7b	USA, MD	1994		CDC
2000026630	7b	USA, NY	1996		CDC
Kn T96-0620	7b	USA, CA	1996	af065068	Crawford-Miksza et al. (26)
99018141	7b	USA, MO	1997		CDC
2000016352	7b	USA, IL	1997		CDC
2000016376	7b	USA, SC	1997		CDC
2000016361	7b	USA, MO	1997		CDC
2000016376	7b	USA, SC	1997		CDC
37300	7c	Sweden	1964	u75952	Li & Wadell, (45)
BC3655	7d	China	1981	u77392	Li & Wadell, (45)
BC4492	7d	China	1984	u75953	Li & Wadell, (45)
BC4609	7d	China	1984	u77393	Li & Wadell (45)
BC8488	7d	China	1984	u77394	Li & Wadell (45)
383[b]	7d	Japan	1992	af053086	Hashido et al. (38)
Bal[b]	7d	Japan	1995	af053087	Hashido et al. (38)
2000017663	7d2	USA, MD	1993		CDC
2000026865	7d2	Israel	1993	af321311	CDC
2000017669	7d2	USA, MD	1994		CDC
2000026621	7d2	USA, NY	1995		CDC
99026817	7d2	USA, OH	1995		CDC
2000016333	7d2	USA, IL	1997		CDC
2000016364	7d2	USA, MO	1997		CDC
2000016375	7d2	USA, SC	1997		CDC
98034168	7d2	USA, IL	1998		CDC
2000017983	7d2	USA, WI	1998		CDC
BC25	7g	China	1985	u75954	Li & Wadell (45)
87-922	7h	Argentina	1987	u75956	Li & Wadell (45)
990179044	7h	Chile	1998		CDC
99018196	7h	USA, AZ	1998		CDC
2000016378	7h	USA, TX	1998		CDC
Gomen	7p	USA	1954	z48571	Li et al., direct submission; CDC
BC3423	7p1	China	1981	u75955	Li & Wadell (45)

[a]Sequencing and restriction analysis performed at Centers for Disease Control and Prevention (CDC) or obtained from previously published sources. Published sequences of Ad7 laboratory strains S-1058, Gomen, and 55142 vaccine confirmed at CDC.
[b]Ad7 strains 383 and Bal were originally reported as Ad7d with a "different restriction pattern by *Bst*EII" (38).

Table 3. Yearly distribution of 166 human adenovirus 7 (Ad7) genome types, United States and Canada, 1966–2000

Genome type	1966-1969	1970-1992	1993	1994	1995	1996	1997[a]	1998[a]	1999	2000	Total
7p	0	0	0	0	0	0	3	0	0	0	3
7a	3	0	0	0	0	0	0	0	0	0	3
7a3	2	0	0	0	0	0	0	0	0	0	2
7b	0	31	6	4	14	8	26	4	6	8	107
$7b_{var}$	0	0	0	0	0	0	0	0	1	0	1
7d2	0	0	2	1	3	4	32	2	0	2	46
7h	0	0	0	0	0	0	0	3	0	1	4
Total	5	31	8	5	17	12	61	9	7	11	166

[a]Data include 50 Ad7 isolates collected in 1997 (47 isolates) and 1998 (3 isolates) from military recruit training centers (Table 4).

Geographic Distribution of Ad7 Genome Types

Ad7b was identified among isolates from nearly all states (and eastern Ontario) sampled. Ad7d2 was first identified in isolates from Maryland and New York in 1993 and thereafter primarily from midwestern and northeastern states, including Wisconsin, Illinois, Kansas, Missouri, Louisiana, South Carolina, and Ohio, as well as eastern Ontario. Ad7h was only identified among isolates obtained from Texas and Arizona.

Ad7 Civilian Outbreaks, 1996–2000

During this study, we became aware of five separate outbreaks of Ad7 respiratory illness among civilians (Table 4). Four were institutional outbreaks that involved primarily infants and young children with underlying chronic disease that occurred in the fall or summer months of 1996, 1998, 1999, and 2000. A fifth communitywide outbreak of Ad7 in Tennessee, which occurred during March-July 1997, involved previously healthy children (16).Genome type analysis at CDC identified Ad7d2 in three of the four outbreaks where isolates were available. We attributed one outbreak to a novel *Sma*I restriction variant of Ad7b ($Ad7b_{var}$) that occurred in New York in 1999 (Jennifer Calder, manuscript in preparation).

Ad7 Gnome Types at Military Recruit Training Centers

In anticipation of increased Ad activity following termination of routine vaccination of new military recruits in 1996, the Naval Health Research Center (NHRC) in San Diego, CA, initiated surveillance for new cases of Ad-associated respiratory illness (20) [http://www.nhrc.navy.mil/geis/sites/nhrc.htm]. Clinical specimens collected at five designated training centers (San Diego, CA; San Antonio, TX; St. Robert, MO; Great Lakes, IL; and Columbia, SC) from recruits who reported respiratory illness were submitted to NHRC for identification of viral and bacterial pathogens. Of 50 Ad7 isolates, Ad7d2 was the most common genome type identified (58%), followed by Ad7b (34%), Ad7p (6%), and Ad7h (2%) (Table 5). Most Ad7d2 infections were reported from the Naval Recruit Training Center in Great Lakes, IL, where an outbreak of Ad7 (and Ad3) respiratory illness was documented during the fall of 1997 (21). Over 70% of the Ad7 isolates sampled from the Great Lakes Center from September 1997 to February 1998 were identified as Ad7d2. One of four Ad7h isolates identified in this study was obtained from a new recruit at Lackland Air Force Base, in San Antonio.

Table 4. Recognized civilian outbreaks of human adenovirus 7 (Ad7) respiratory disease, United States, 1996–2000

Location	Date	Setting	No. cases[a]	No. deaths	No. Ad7 isolates	No. restriction	Genome type
Houma, LA[b]	June 1996	Pediatric chronic-care facility	13	7	4	2	7d2
Memphis, TN[c]	Mar 1997	Community acquired	47	1	26	0	nd
Chicago, IL[d]	Nov 1998	Pediatric chronic-care facility	31	8	11	11	7d2
		and tertiary hospital	37	0	6	6	"
New York City, NY[e]	Oct 1999	Chronic-care facility formentally disabled persons	33	7	15	15	$7b_{var}$
Des Moines, IA[f]	Oct 2000	Pediatric chronic-care facility	20	4	9	9	7d2

[a]Suspected and confirmed cases of Ad7 respiratory disease.
[b]Robert Gohd, Children's Hospital, New Oreleans, LA (pers. comm.).
[c]Mitchell et al. (16).
[d]Gerber et al. (17).
[e]Jennifer Calder, The Mailman School of Public Health, Columbia University, New York, NY (manuscript in preparation).
[f]Michael Buley, Iowa Dept of Public Health, Des Moines, IA (pers. comm.).

Table 5. Genome types of 50 human adenovirus 7 (Ad7) isolates obtained from military recruit training centers, Feb 1997–May 1998[a]

Training center	No. Ad isolates	No. Ad typed	No. Ad7 (%)	No. restriction	Dates of isolation	Genome type			
						7b	7d2	7p	7h
Marine Corps Recruit Depot, San Diego, CA	129	128	10 (8)	1	April 1997			1	
Lackland Air Force Base, San Antonio, TX	1	1	1 (100)	1	April 1998				1
Fort Leonard Wood, St. Robert, MO	266	260	29 (11)	9	Feb 1997 to Nov 1997	4	5		
Naval Recruit Training Center, Great Lakes, IL	632	592	396 (67)	28	Sept 1997 to May 1998	8	20		
Fort Jackson, Columbia, SC	786	738	66 (9)	11	June 1997 to April 1998	5	4	2	
Total	1,814	1,719	502 (29)	50		17	29	3	1

[a]Isolation and serotyping of Ads conducted at the Naval Health Research Center, San Diego, CA.

Discussion

Our study represents the most comprehensive survey to date of Ad7 genome types circulating in the United States and provides a basis for future surveillance studies that can better delineate the disease impact of these viruses.

Before this study, the most comprehensive surveys of Ad7 genomic variants in the United States were conducted by Wadell et al. (27) and Adrian et al. (28) with field isolates of Ad7 collected from 1961 to 1985. These authors identified a diverse group of cocirculating Ad7 genome types (Ad7p, Ad7a, Ad7c, and others) that by the late 1960s to early 1970s were replaced by Ad7b, a change that preceded similar shifts to Ad7b seen in other parts of the world. Our data confirm this observation and show a continued dominance of the Ad7b genome type in the United States. Only one genome type from the earlier period, Ad7p, was still identified among currently circulating strains. We also documented the appearance of two new Ad7 genome types: Ad7d2, which was first identified in specimens collected in 1993 and subsequently detected over a wide geographic area in the eastern half of the United States and Canada; and Ad7h, which was first identified in specimens collected in 1998 in the Southwest.

Both epidemiologic and molecular evidence suggests that Ad7d2 entered the United States as part of its recent spread from evolutionarily related Ad7d strains formerly restricted to China. Ad7d2 shows the highest degree of genetic relatedness to Ad7d, differing by only one *Bst*EII restriction site in pairwise comigrating restriction fragment analysis with 12 different endonucleases (24,37); it possesses the unique amino acid substitution in the hexon protein also present in Ad7d isolates from China (45) and Japan (38). Ad7d was identified as early as 1980 in Beijing (24) and 2 years later in Changchin (46), and rapidly displaced Ad7b to become the major genome type circulating in China through 1990. Ad7d was identified in Japan during 1987 to 1992 (35) and in Korea in 1995 (18; Hoan-Jong Lee, pers. comm.), and Ad7d2 was the predominant genome type isolated during the 1995-1998 Ad7 epidemic in Japan (15,36). Ad7d2 was subsequently identified in Israel in 1992 (37) and in the United States in this study in 1993.

The emergence and apparent global spread of Ad7d2 are reminiscent of observations for another genome type of serotype 7, Ad7b. Originally described by Wadell and Varsanyi (25), Ad7b was associated with outbreaks of severe respiratory illness in Europe in the 1970s (12). Although first isolated in 1956 from a Paris orphanage outbreak (12,47), subsequent retrospective studies did not identify Ad7b in Europe again until 1969 (27). Before then, the earliest documented occurrence of Ad7b was in China in 1958 (24), where it was the predominant genome type circulating through the early 1980s (24,46). With the exception of Paris, the first appearance of Ad7b outside China was on the U.S. West Coast in 1962 (27). By 1970, Ad7b was the predominant genome type circulating throughout the United States (28) and eventually throughout many parts of the world.

The mechanism(s) underlying the apparent greater fitness of some Ad7 genome types, as reflected by their capacity to displace other circulating strains, remains speculative. Possible explanations include mutations or recombinations that yield strains with increased pathogenicity and therefore greater chance of causing recognized illness, or biological or antigenic changes that enhance transmission or infection compared with other Ad7 genome types. Although there is no conclusive evidence of differences in pathogenicity between Ad7 genome types, some types appear to be more frequently isolated from healthy carriers (e.g., Ad7p and Ad7a), while others are more often isolated from patients with more serious clinical outcomes (e.g., Ad7b, Ad7c, Ad7d, and Ad7h) (27,34). Some antigenic differences between Ad7 genome types have also been demonstrated; recent studies identified minor differences in neutralization titer between Ad7 prototype strain Gomen (Ad7p) and the vaccine strain 55142 (Ad7a) with rabbit hyperimmune antisera (26). In addition, a unique amino acid substitution in the hexon protein that distinguishes Ad7d/Ad7d2

strains from other genome types is predicted to impart substantial changes in the hydrophilicity of the protein and possibly associated antigenic changes (45).

Although Ad7 can be spread directly by the respiratory route, efficiency of transmission is typically lower than for some other respiratory viruses. Efficient spread usually requires crowding, such as that in closed communities like chronic-care facilities, military barracks, and day-care centers. Widespread community outbreaks of Ad7 can occur but appear to require low levels of herd immunity. For example, in Japan, >95% of persons <40 years of age lacked specific antibodies to Ad7 before the countrywide epidemic of Ad7 that began in 1995 (48,49). The most comprehensive recent seroprevalence data on Ad7 in the United States were obtained in 1992 from 364 military basic trainees attending new recruit training centers (50). Approximately 73% of screened trainees lacked specific antibodies to Ad7. In another study to evaluate the potential for use of Ad vectors in gene therapy for cystic fibrosis, 73.9% of 46 serum specimens collected from 1993 to 1995 from children (median age 4.7 years) were seronegative for Ad7 (51).

To achieve rapid spread, a novel genome type presumably requires an immunologically naive population, greater biological fitness than the indigenous circulating strains, and a means of introduction to the susceptible community. Azar et al. (37) noted that the appearance of Ad7d2 in Israel coincided with the arrival of large numbers of immigrants from the former Soviet Union and Ethiopia during the early 1990s. The global spread of Ad7b in the 1960s and 1970s may have been aided by the movement of unvaccinated U.S. and allied military personnel during the Vietnam War. In our study, the appearance of Ad7h in the U.S. Southwest in 1998 may be explained by the emigration of persons from Ad7h-endemic regions of South America, where communitywide outbreaks of respiratory illness due to Ad7h occurred as recently as 1998 in Chile (Rodrigo Fasce, pers. comm.). However, a more comprehensive survey of Ad7 isolates from Mexico and U.S. states on the Mexican Border would be necessary to substantiate this observation.

The five recognized civilian outbreaks of Ad7 respiratory illness that occurred during 1996-2000, three of which we attributed to genome type 7d2, might have been due to increased reporting as a result of our interest in this study or may represent a real increase in Ad7-associated disease, as occurred in Europe during the early 1970s and in Japan and Korea (12,15,18) beginning in 1995. One unsubstantiated possibility is that the discontinuation of vaccination of U.S. military recruits for Ad4 and Ad7 in 1996 and the subsequent increase in Ad-associated disease at military bases throughout the United States (20-22) provided a new focus for Ad7 dissemination to civilian populations. A possible example of this is the 1998 outbreak of Ad7d2 illness at a Chicago pediatric chronic-care facility described earlier (17). This outbreak occurred within a few miles of the Naval Training Center in Great Lakes, which had had an outbreak of Ad7d2 the preceding year (21). Most cases of Ad infection at military bases since 1996 have been attributed to Ad4 (20,22), but no comparable outbreaks of Ad4 disease among civilians have been reported. Unlike Ad7, which poses a risk to both civilian and military populations, Ad4 has only infrequently been associated with outbreaks of respiratory illness in civilian populations (2).

Although we identified individual cases of severe lower respiratory tract illness and deaths attributed to Ad7d2 and Ad7h in this study, the possibility that these two genome types may be associated with more severe disease is not yet clear. More extensive clinical and epidemiologic study is required to adequately address this question. The limited data from infected military recruits suggest no differences in clinical illness between those infected with Ad7d2 and Ad7b (data not shown). Reports of Ad7d2 infections in Israel (37) and Ad7d infections in China (46) also noted no clear differences in severity of disease. Cases of severe pneumonia and neurologic disease were reported from a recent regional epidemic of Ad7d2 in Japan (15), but there was no evidence that these severe cases were more common than those reported for outbreaks involving other Ad7 genome types. Ad7h, a genetically unique recombinant between Ad7 and Ad3 (42), has been linked to increased illness and death in infants in Chile and Argentina, where it is second only to *Human respiratory syncytial virus* as a cause of severe viral pneumonia in infants and young children (34,52). However, in this study, too few cases of Ad7h infection were identified to assess differences in disease severity.

In conclusion, our study documents the recent appearance in the United States of two new Ad7 genome types, Ad7d2 and Ad7h, and provides additional evidence of the global spread of these formerly geographically restricted viruses. The possibility that these genome types may be associated with more severe disease makes it prudent to monitor their spread and associated disease.

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The Relationship between Antimicrobial Use and Antimicrobial Resistance in Europe

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In Europe, antimicrobial resistance has been monitored since 1998 by the European Antimicrobial Resistance Surveillance System (EARSS). We examined the relationship between penicillin nonsusceptibility of invasive isolates of *Streptococcus pneumoniae* (an indicator organism) and antibiotic sales. Information was collected on 1998-99 resistance data for invasive isolates of *S. pneumoniae* to penicillin, based on surveillance data from EARSS and on outpatient sales during 1997 for beta-lactam antibiotics and macrolides. Our results show that in Europe antimicrobial resistance is correlated with use of beta-lactam antibiotics and macrolides.

Antimicrobial resistance is a growing problem worldwide, requiring international approaches. The World Health Organization (WHO) and the European Commission have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control (1-3). In European countries, antimicrobial resistance has been monitored in selected bacteria from humans since 1998 through the European Antimicrobial Resistance Surveillance System (EARSS). Funded by the European Commission, EARSS is an international network of national surveillance systems intended to collect comparable and reliable resistance data. The purpose of EARSS is to document variations in antimicrobial resistance over time and place and to provide the basis for and assess the effectiveness of prevention programs and policy decisions.

One of the indicator organisms in EARSS is *Streptococcus pneumoniae*. It was included for three reasons: it is of major clinical importance for pneumonia, bacterial meningitis, and otitis media; many countries have reported that its resistance to penicillin is increasing; and *S. pneumoniae* is representative of organisms that are transmitted in the community.

A major risk factor for the development of resistance is thought to be inappropriate use of antimicrobial drugs. Most studies that have investigated the relationship of antimicrobial use and antimicrobial resistance have been undertaken in hospital, multicenter, or country settings (4-7). For infections with penicillin-nonsusceptible *S. pneumoniae* (PNSP), studies have demonstrated that at the individual level, previous use of beta-lactam antibiotics such as penicillin is an important risk factor (8-10). Studies on carriage of PNSP in children have shown that sulfamethoxazole-trimethoprim (co-trimoxazole) and macrolides such as erythromycin have also been associated with selection of PNSP (11,12). Translated to the population level, sales of beta-lactam antibiotics, co-trimoxazole, or macrolides in a given geographic region may be proportional to microbial resistance to penicillin. If on the European level a relationship between antimicrobial resistance and antimicrobial use could be found (as in the case of *S. pneumoniae* and resistance to penicillin), efforts to control antimicrobial use and misuse could be stimulated and monitored in Europe.

We used an ecologic study design to examine the correlation between use of relevant antibiotics in the outpatient setting and the proportion of PNSP among invasive isolates of *S. pneumoniae* in 11 European countries.

Methods

Antimicrobial Resistance Data

The estimated average coverage of the populations of countries participating in EARSS is 52% (range 10% to 90%) (13). Laboratories that participate in EARSS screen invasive *S. pneumoniae* isolates for oxacillin resistance (14). When an isolate is found to be nonsusceptible, the EARSS protocol requests confirmation as intermediate- or high-level resistance to penicillin by determination of MICs. Laboratories perform microbiologic testing and interpret results according to their own standards. National guidelines in Europe differ; isolates of *S. pneumoniae* are considered nonsusceptible to penicillin if the MIC is >0.06 (15-18) or >0.12 (19,20) mg/L. For this report, we use nonsusceptibility and intermediate resistance as synonyms; PNSP isolates are either intermediate or fully

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resistant to penicillin. Only the first invasive isolate per patient per quarter is reported.

To assess the comparability of susceptibility test results, a quality assurance exercise was performed in September 2000 among 482 laboratories from 23 countries participating in EARSS. The concordance (agreement of reported results with intended results) for the detection of penicillin resistance in the three *S. pneumoniae* control strains was 91% (21). Laboratories sent standardized data to the national EARSS data manager, who checks data contents and ensures conformity with the EARSS data format. In collaboration with WHO, an export module from the laboratory-based software WHONET was developed for EARSS (22). Every quarter, data are forwarded to the central database at the National Institute of Public Health and the Environment (RIVM), Bilthoven, Netherlands, where the project is coordinated.

Antimicrobial Use Data

National outpatient sales data for antibiotics from 1997 were purchased from IMS Health Global Services, London, United Kingdom, for 13 of the 15 member states of the European Union. Corresponding data were obtained from the Danish Medicines Agency for Denmark and from the National Corporation of Swedish Pharmacies for Sweden (23). The IMS data were examined and adjusted according to the Anatomic Therapeutic Classification (ATC) system used by WHO (24). The amount in kilograms for an antimicrobial agent was converted to a number of defined daily doses (DDD). The DDD, which is based on the average daily dose used for the main indication of the drug, is appropriate for comparisons of drug use over time and in different geographic areas. For beta-lactam antibiotics, we combined ATC groups J01C (extended- and narrow-spectrum penicillins) and J01D (cephalosporins); macrolides were classified under code J01F. No data were available for the combination of trimethoprim and sulphonamide.

Nonadherence

We considered nonadherence of patients to the physician's prescription in individual countries as a possible confounder of antimicrobial resistance. Branthwaite et al. reported nonadherence levels from a population-based survey in seven countries (25). Data from four of the seven countries (Spain, Belgium, the United Kingdom, and Italy) were also captured in EARSS.

Statistical Analysis

We calculated the proportion of PNSP among all invasive *S. pneumoniae* isolates from each country reported during 1998-99. Because probabilities allow only values between 0 and 1, we modeled the natural logarithm of the odds of PNSP resistance (logodds).

Least-square linear regression analysis was used to assess correlation between antimicrobial use (of beta-lactam antibiotics and macrolides, expressed in DDD per 1,000 population per day) and the logodds of resistance. We correlated nonadherence levels with the logodds of resistance in the same way. We calculated the Spearman coefficient of determination (r-square) and its corresponding p value. For the calculation of the regression lines, we weighted the data points by the inverse of the variance of each data point. We used SAS software (SAS Institute Inc., Release 6.03., Cary, NC).

Results

Antimicrobial Resistance

During 1998-99, 337 laboratories from 11 European Union member states (Belgium, Finland, Germany, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden, and United Kingdom) and one nonmember state (Iceland) reported 4,872 invasive *S. pneumoniae* isolates to EARSS. The proportion of PNSP among isolates of invasive *S. pneumoniae* ranged from 1% to 34% (Table) (Figure 1). Southern European countries reported higher rates than northern European countries.

Antimicrobial Use

Data on outpatient sales of beta-lactam antibiotics and macrolides were available for 1997 from all 15 European Union member countries. Antimicrobial use varied widely between countries. Sales to outpatients ranged from 3.8 to 23.6 DDD per 1,000 inhabitants per day for beta-lactam antibiotics and from 0.97 to 5.98 DDD for macrolides. The three countries with the highest reported use were France, Spain, and Portugal for beta-lactam antibiotics and France, Spain, and Italy for macrolides; the three countries with the lowest use were the Netherlands, Germany, and Austria for beta-lactam antibiotics and Sweden, the Netherlands, and Finland for macrolides.

Correlation

For 11 countries, information was available for both antimicrobial resistance and antimicrobial use. Linear regression of the correlation of use of beta-lactam antibiotics and the logodds of resistance showed an r-square of 0.80 (p=0.0002) (Figure 2). The equation for the regression is

logodds of resistance =(-3.94)+(0.16xDDD).

For the use of macrolides, we calculated an r-square of 0.46. Figure 3 shows the graph for nonadherence to antibiotics and the logodds of resistance. The r-square is 0.8 (p=0.2).

Discussion

We present for the first time Europe-wide, country-specific, representative data on antimicrobial resistance collected by EARSS. Using an ecologic study design, we demonstrate through the correlation with data on antimicrobial use one aspect of the usefulness of surveillance for antimicrobial resistance. The results from 11 European countries show a linear relationship between use of beta-lactam antibiotics and macrolides and the proportion of PNSP among all invasive *S. pneumoniae* isolates.

Table. Number of submitting laboratories, number of isolates of *Streptococcus pneumoniae*, number (#) and percent (%R) of penicillin nonsusceptible *S. pneumoniae* isolates, logodds of resistance (ln(%R/[1-%R]), and outpatient sales of beta-lactam antibiotics and macrolides

Country	No. of laboratories	No. of *S. pneumoniae* isolates	Penicillin nonsusceptible *S. pneumoniae*			Outpatient sales of antibiotics in DDD[a]/1,000 inhabitants/day	
			No.	%R (95% CI)	ln (%R/[1-%R])	Beta-lactam antibiotics	Macrolides
Austria	-	-	-	-	-	6	3.7
Belgium	96	940	131	14 (12-16)	-1.82	14	4.1
Denmark	-	-	-	-	-	7	2
Finland	11	211	8	4 (2-8)	-3.18	8	1.9
France	-	-	-	-	-	24	6.0
Germany	15	222	4	2 (1-5)	-3.89	5	2.5
Iceland	2	54	1	2 (0-11)	-3.89	Not available	Not available
Ireland	12	157	30	19 (13-26)	-1.45	11	2.5
Italy	46	194	26	13 (9-19)	-1.87	15	5.1
Luxembourg	1	11	2	18 (3-52)	-1.52	14	4.7
Netherlands	20	760	8	1 (0-2)	-4.6	4	1.2
Portugal	12	134	25	19 (13-27)	-1.45	16	3.7
Spain	76	1,240	418	34 (31-36)	-0.66	21	5.9
Sweden	24	706	21	3 (2-5)	-3.48	8	1
United Kingdom	22	243	21	9 (6-13)	-2.31	9	3.2

[a]DDD = defined daily doses; CI = confidence interval.

EARSS data show that resistance for PNSP follows a north-south gradient. Southern European countries have higher proportions of PNSP than countries in northern Europe. A possible reason for this observation could be the difference in antimicrobial use, which also tends to be higher in southern European countries. If use of relevant antibiotics (beta-lactam antibiotics and macrolides) and the logodds of resistance are modeled through linear regression, a strong linear and statistically significant relationship is demonstrated.

Our findings agree with those of Austin et al., who modeled the relationship between antimicrobial use and endemic resistance, based on population genetic methods and epidemiologic observations (26). The correlation in Figure 2 is consistent with the model developed by Austin et al. on theoretical grounds.

We correlate antimicrobial sales data for 1997 with antimicrobial resistance data for 1998 and 1999. Others have observed that after a lag time of 1 or more years, changes in antimicrobial use may be followed by changes in antimicrobial resistance (27,28). Therefore, we believe that it is reasonable to correlate antimicrobial sales data in 1997 with antimicrobial resistance data from 1998-99.

We address several limitations in our study. First, because it is an ecologic study, we can make no inferences on the individual level. Second, resistance rates in some countries (Table) are calculated from a relatively limited number of isolates. However, based on communications with EARSS country representatives, our data are consistent with antimicrobial resistance levels derived from other sources (29). Third, an explanation for the differences in antimicrobial resistance could be sampling bias: clinicians in southern European countries may request blood cultures more frequently than their northern European colleagues, who may sample only in case of empirical treatment failure. Fourth, we have not addressed other, potentially important contributing factors for the development of antimicrobial resistance of organisms that are transmitted in the community, particularly nonadherence and over-the-counter sales of antimicrobial agents. Both these factors are difficult to measure. However, in 1993 nonadherence to prescribed antimicrobial agents was assessed in a survey in six

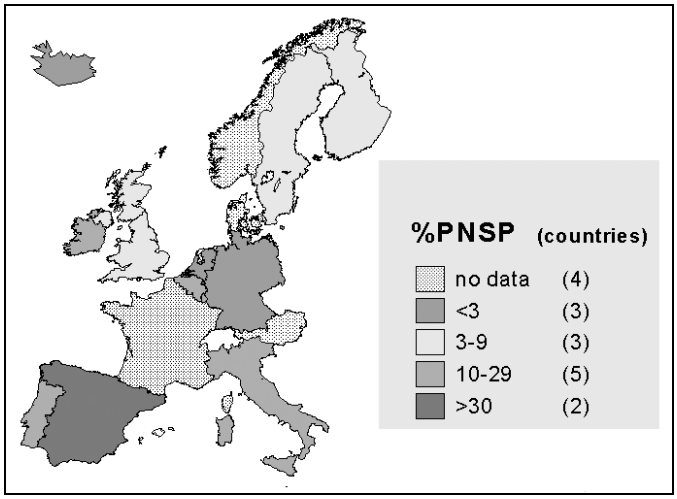

Figure 1. Proportions of invasive isolates of *Streptococcus pneumoniae* resistant to penicillin (PNSP) among 12 European countries, 1998–99.

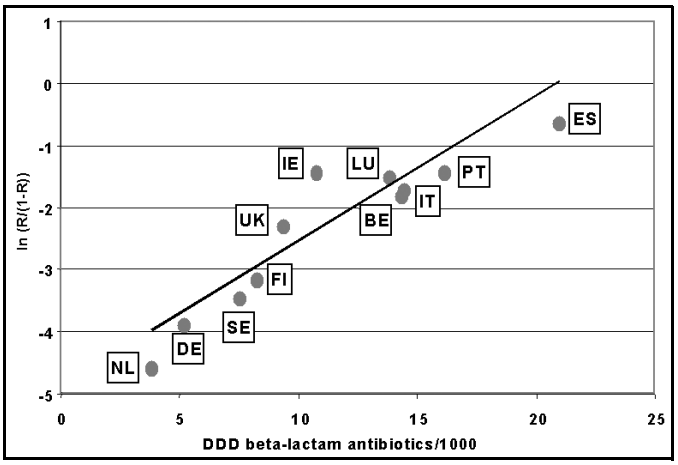

Figure 2. The logodds of resistance to penicillin among invasive isolates of *Streptoccus pneumoniae* (PNSP; ln(R/[1-R])) is regressed against outpatient sales of beta-lactam antibiotics in 11 European countries; antimicrobial resistance data are from 1998 to 1999 and antibiotic sales data are from 1997. DDD = defined daily dose; BE = Belgium; DE = Germany; FI = Finland; IE = Ireland; IT = Italy; LU = Luxembourg; NL = the Netherlands; PT = Portugal; ES = Spain; Se = Sweden; UK = United Kingdom.

European countries (25). Although the number of data points is limited, Figure 2 suggests a direct relationship between nonadherence rates and logodds of resistance. Thus, if nonadherence is also related to sales of antimicrobial agents, it could potentially confound the relationship between use and resistance. Data on the degree of over-the-counter use among European countries are not widely available; we know of one Spanish and one Greek study reporting an estimate of over-the-counter use (30,31). The influence of these and other parameters on the level of resistance should be quantified and understood. Finally, because children are the main reservoir of carriage of *S. pneumoniae*, an age-stratified analysis would be desirable, i.e., a correlation of resistance with antimicrobial use among children. However, this analysis would require more detailed use data, for example, of liquid formulations of antibiotics.

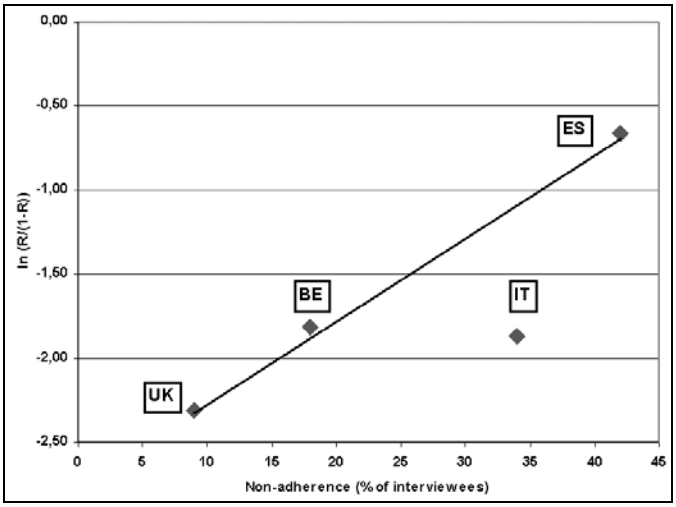

Figure 3. The logodds of resistance of invasive isolates of *Streptococcus pneumoniae* to penicillin (PNSP; ln(R/(1-R))) is regressed against nonadherence rates to antibiotic therapy in four European countries. Nonadherence rates are from 1993; PNSP data are from 1998-99. UK = United Kingdom; BE = Belgium; IT = Italy; ES = Spain.

At least two studies in northern Europe have demonstrated that PNSP rates can be halted or even reversed when physicians avoid the inappropriate prescription of antimicrobial agents (32,33). Our study is timely because it shows that even at the European level a correlation can be observed between antimicrobial resistance (of *S. pneumoniae* to penicillin) and antimicrobial use. In several European countries, national action plans for the appropriate use of antimicrobial agents are being planned or implemented; their effectiveness should be monitored through prospective and continuous surveillance of antimicrobial resistance and antimicrobial sales data (34-38).

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Eastern Equine Encephalomyelitis Virus Infection in a Horse from California

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A yearling quarter horse, which was raised in southern California, received routine vaccinations for prevention of infection by *Eastern equine encephalomyelitis virus* (EEEV). One week later, severe neurologic signs developed, and the horse was humanely destroyed. A vaccine-related encephalomyelitis was later suspected. A final diagnosis of EEEV infection was established on the basis of acute onset of the neurologic signs, histopathologic and serologic testing, and isolation and molecular characterization of EEEV from brain tissue**.** The vaccine was extensively tested for viral inactivation. Nucleotide sequences from the vaccine and the virus isolated in the affected horse were also compared. In California, arboviral encephalomyelitides are rarely reported, and EEEV infection has not previously been documented. This report describes the occurrence of EEEV infection in the horse and the investigation to determine the source of infection, which was not definitively identified.

Eastern equine encephalomyelitis virus (EEEV) is a mosquito-borne virus in the family *Togaviridae*, genus *Alphavirus*. EEEV, *Western equine encephalomyelitis virus* (WEEV), and *Venezuelan equine encephalomyelitis virus* (VEEV) are related but genetically distinct alphaviruses. EEEV and VEEV are lethal in up to 90% of recognized equine cases, whereas WEEV is least virulent in horses, which have a mortality rate of approximately 40% (1). EEEV may also cause fatal encephalitis in humans (mortality rate 50%-75%) (2). In the United States, enzootic EEEV occurs mainly from New England to Florida and along the Gulf Coast, with rare reports of foci as far inland as Michigan and South Dakota (3). In North America, sylvatic populations and the mosquito *Culiseta melanura* maintain the virus in hardwood, salt-water swamp habitats. Large populations of this mosquito allow amplification of the virus by transmission among wild birds (4). In wild birds indigenous to North America, the infection is usually innocuous, whereas in pheasants, cranes, and emus, the disease is often lethal. *C. melanura* feeds almost exclusively on passerine birds; however, spillover of EEEV from the enzootic vector into several other mosquito species (e.g., *Aedes* spp.), which feed on tangential hosts such as humans and equines, may result in large epizootics with high mortality rates (4-6). Our paper describes a sporadic case of EEEV infection in a horse outside the known geographic range of this virus and the ensuing investigation to determine the source of exposure.

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Materials and Methods

Case Report

In April 2000, a 14-month-old gelding quarter horse was seen at a veterinary referral hospital in southern California for sudden onset of quadraparesis and recumbency. The horse had no history of prior neurologic disease. He had been castrated approximately 90 days before the illness without complication. A multidose, multivalent vaccine containing formalin-inactivated EEEV and WEEV, influenza virus, and tetanus toxoid was administered to the affected horse and 27 stable mates 1 week before the onset of illness.

The horse appeared alert and healthy the night before onset of clinical signs. At 6:30 a.m. on April 21, he was found down in his stall and unresponsive to external stimuli. The referring veterinarian found a recumbent, comatose horse with spontaneous nystagmus and flailing, incoordinated movements. Initial therapy included intravenous corticosteroids, fluid therapy (including glucose to treat possible hyperkalemic periodic paralysis), and diazepam for intermittent seizures. The horse did not respond to therapy and was sent to the referral hospital.

On examination at the hospital, the horse was comatose with elevated heart and respiratory rates and a normal rectal temperature. A neurologic exam showed that pupillary light responses were absent bilaterally. Palpebral reflexes were present although weak. No organized motor movements occurred in response to stimuli. Initial emergency treatment consisted of intravenous fluids with dimethyl sulfoxide and

flunixin meglumine. Cervical and skull radiographs were performed and were within normal limits. An atlanto-occipital cerebrospinal fluid tap was also performed, and no abnormalities were seen on gross observation. At this point, diffuse cortical disease was evident. Trauma appeared very unlikely, and an infectious process or toxicosis seemed more probable. Because of the grave prognosis, the owners elected to euthanize the horse. The carcass was sent to the Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, for necropsy.

Results

Pathology

Results of gross necropsy examination were unremarkable except for markedly hemorrhagic bladder mucosa. Histologic examination revealed lesions mainly confined to the cerebral cortex, thalamus, hypothalamus, and anterior portion of the spinal cord (C_1-C_4). Lesions in the brain were characterized by a multifocal to diffuse neutrophilic response with gradual progression to mononuclear cell infiltrates in some areas. Vascular damage and fibrin thrombi were evident (Figure 1). Some blood vessels had swollen endothelium surrounded by a thick layer of mononuclear cells. A mild degree of meningitis was present, with pleocellular response containing mainly mononuclear and neutrophilic infiltrates. The neuropil showed fine vacuolation, indicating edema. Some axons were markedly shrunken. The remaining portion of the spinal cord was unremarkable. The urinary bladder had diffuse submucosal hemorrhages. The lung showed flooding of the alveoli with eosinophilic fluid. The remaining tissues were unremarkable.

Virology

Portions of brain tissue were collected and sent for rabies testing at the local public health laboratory. Results were negative. Fresh, frozen brain tissues and serum were submitted to the National Veterinary Services Laboratories (NVSL). Tests for equine encephalomyelitides included virus isolation and serology for WEEV, EEEV, VEEV, *Equid herpesvirus 1* (EHV-1), and *West Nile virus* (WNV). For virus isolation, a 10% suspension of brain sample was prepared and injected into flasks of RK13, equine dermal, and Vero-MARU cells (Vero M). This cell line was obtained by NVSL at the 135th passage level from the Middle America Research Unit (MARU) as a multipurpose cell line for virus isolation in 1980 and has been maintained by the NVSL since that time. Additional brain suspension was injected intracerebrally into 16 suckling 4-day-old mice (from 2 litters). Cytopathic effects were observed in the RK13 and Vero M cells at 2 days after injection. Examination using electron microscopy of the RK13 cell culture fluids showed particles with morphologic features compatible with alphaviruses.

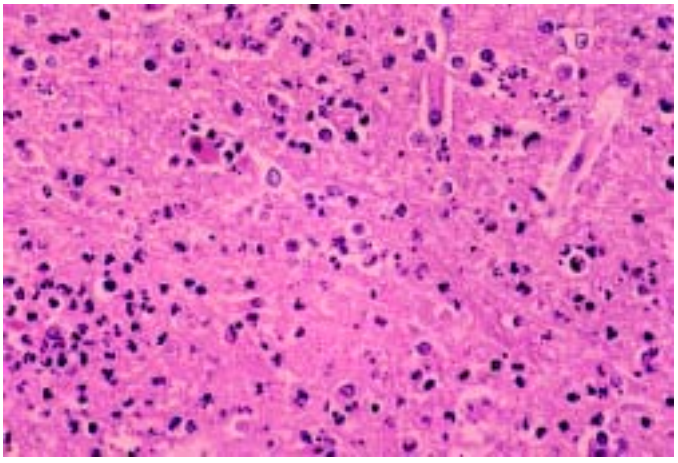

Figure 1. Photomicrograph of a section of the cerebral cortex from horse with *Eastern equine encephalomyelitis virus* infection. Note the dense neutrophilic response, vascular damage, and fibrin thrombi. Hematoxylin and eosin stain.

Virus preparations from both the cell culture supernatant and suckling mouse brains of mice that died were identified as EEEV by a complement fixation test with reference antisera. In that test, virus reacted strongly with EEEV antiserum and weakly or not at all with WEEV and VEEV antisera.

Serum collected from the yearling horse on April 21 before it died was tested for antibodies to EEEV, WEEV, and VEEV by hemagglutination inhibition (HI) and plaque reduction neutralization testing (PRNT). The serum had a HI titer of 20 against both EEEV and WEEV. HI antibodies to VEEV were not detected. In the PRNT, the serum neutralizing antibody titer versus EEEV was ≥100 but was undetectable against either WEEV or VEEV. The serum was also positive in an immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay for EEEV with a titer of ≥1000. Additional tests for antibodies to equine herpesvirus 1 and WNV were negative.

The Center for Vector-Borne Disease Research at the University of California, Davis (CVBDR), U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), and California Department of Health Services (CDHS) also received homogenized brain suspension material. Isolation of EEEV by mouse inoculation and cell culture supported NVSL's findings. The isolate was further characterized by USAMRIID by reverse transcriptase-polymerase chain reaction (RT-PCR) testing and sequencing as described (7,8), but with primers listed in Table 1. A 1,165-nucleotide portion of the viral genome including parts or all of the E2, 6K, and E1 genes was determined. Sequencing of the isolate showed an 18-nucleotide difference (98.5% homology) from the reference PE6 EEEV strain. Comparison with sequences that have been submitted to GenBank indicated that the virus is a North American antigenic variety in subtype 1 of the taxonomic scheme recently proposed by Brault et al. (9).

Field Investigation

Local, state, and federal agencies participated in a joint field and laboratory investigation to determine the source of infection. Four hypotheses were investigated to explain the occurrence of EEEV outside its usual range: 1) imported

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers used to sequence *Eastern equine encephalomyelitis virus* RNA

Title	Sense	Primer	Use	Region
9930	Forward	5´- GCGCTGCTTATTTTGCTGT -3´	RT-PCR	E1
11582	Reverse	5´- ATTATGCGCTGCCTGTAGTGTTTA -3´	RT-PCR	E1
4043	Forward	5´- GTGCGCGGCGACATAACAAAGAG- 3´	RT-PCR	NSP3
4976	Reverse	5´- CCGGGGGTACAGTGCCAGAGAA -3´	RT-PCR	NSP3
10470	Forward	5´- ATGCCAAACTCATCATAGGTCCACT -3´	Sequencing	E1
10938	Forward	5´- GTATAGCCACCGTTGCCTACAAATC -3´	Sequencing	E1
11345	Forward	5´- CAGGCAGTGTATAAGGCTGTCTTAC -3´	Sequencing	E1
T3	Forward	5´- AATTAACCCTCACTAAAGGGA -3´	Sequencing	NSP3

infection from an EEEV-endemic region, 2) autochthonous transmission by locally infected mosquitoes, 3) intentional inoculation of the horse with live EEEV by a person or by purposeful contamination of the vaccine and 4) incomplete inactivation of EEEV in a commercially inactivated viral vaccine.

Travel History

We found no evidence that this case was due to importation from an EEEV-endemic region. The horse was born in northern California and at 6 months of age was moved to southern California for training. The horse traveled as far east as Fort Worth, Texas, in July 1999 for showing purposes. The rest of the 1999 horse show season took place in southern California. The horse was last moved to a new stable (Farm A) in southern California during February 2000. He attended several shows in this area and as far east as Hurricane, Utah. A review of a list of participants at the horse shows recently attended showed no horses from EEEV-endemic areas and no reports of encephalomyelitis among other equine participants.

Surveillance for EEEV in Southern California

No evidence for autochthonous transmission of EEEV by local mosquito populations was found through surveillance in mosquitoes, sentinel chickens, wild birds, horses, and humans from the region. California has an extensive, long-established Arboviral Encephalitis Surveillance Program that is active from April through October each year (10,11). The program includes collection and testing of mosquito pools and sentinel chicken flocks for WEEV and *St. Louis encephalomyelitis virus* and surveillance for encephalomyelitis cases among equids, ratites (e.g., emus, ostriches), and humans. Following recognition of the equine EEEV case, CDHS began including EEEV screening in its routine testing program.

Coincidentally, a sentinel chicken flock was located on Farm A. Sera submitted from this flock in April were retrospectively tested for EEEV antibody by indirect immunoassay and found to be negative. The flock remained seronegative for EEEV from May through October. In addition, mosquitoes were collected at Farm A and within a 5-mile radius with carbon dioxide traps. A total of 74 mosquitoes, including the *Culex* spp. *tarsalis, quinquefasciatus, erythrothorax*, and *stigmatosoma*, and *Culiseta particeps*, were collected in 23 trap nights during May. Only 8 of 74 mosquito species were *Culex tarsalis*, a known vector species of WEEV and a potential vector species of EEEV in California (12). Surveys for resting adult mosquitoes in barns and other buildings yielded no mosquitoes. All mosquito pools were tested and found negative by virus isolation in tissue culture. Routine biweekly testing of sentinel chicken flocks and mosquito pools throughout California until the end of October showed no further evidence of EEEV activity in the state.

Despite enhanced surveillance, additional cases of EEV infection in local animal and human populations were not identified. Surveillance for encephalitis cases in horses and humans was heightened in southern California after the equine case was recognized. Veterinarians were alerted statewide through a newsletter published by the California Department of Food and Agriculture, and the local health department issued a press release. Following the publicity, a veterinarian reported three horses with acute neurologic disease during mid-May at another ranch, Farm B, approximately 50 km from Farm A. Necropsy and serologic testing of these cases performed at the California Animal Health and Food Safety Laboratory System, San Bernardino Branch, showed EHV-1 as the likely cause of the outbreak at Farm B; no evidence of EEEV infection was found. In addition, a brown-headed cowbird (*Molothrus ater*) die-off at another horse ranch, Farm C, approximately 80 km from Farm A, was investigated. No laboratory evidence of EEEV infection was found in three dead cowbirds collected from Farm C, although the causes of their deaths were not determined.

No other horses at Farm A had encephalitis. To further assess potential equine exposures at Farm A, a serosurvey of 10 randomly chosen stable mates of the affected horse were tested for EEEV antibodies. The sample ranged in age from weanlings to elderly horses; each had been vaccinated with the multivalent vaccine against WEEV, EEEV, influenza viruses, and tetanus (Vaccine A) from the same lot on the same day as the case. These horses showed positive neutralizing antibody titers by PRNT ranging from <20 to 320; none had IgM antibodies to EEEV by an ELISA-capture test. Previous vaccination histories were not available for these horses or the case,

but the findings in the stable mates were compatible with recent vaccination or the presence of maternal antibodies in younger horses without natural exposure.

Criminal Mischief

Although an intentional introduction of EEEV seemed highly unlikely, recent concerns about bioterrorism made this an important possibility to consider. We found no evidence of purposeful contamination of the vaccine or intentional inoculation of the horse. EEEV is not readily obtainable. Furthermore, no motive for such an act was found.

Vaccine Studies

An extensive evaluation of the final hypothesis, residual live EEEV in the vaccine, could not eliminate Vaccine A as the source of infection. The farm manager ordered the multivalent EEEV, WEEV, influenza viruses, and tetanus toxoid vaccine by mail from an out-of-state vendor and stored the vials at 6° C in a refrigerator at Farm A. The vaccine was a commercial, four-way, multidose product that was administered intramuscularly by farm personnel. The viruses in the vaccine were formalin-inactivated, adjuvant-type, and of tissue-culture origin.

Virus Isolation

Three unused vials and one partially used vial of Vaccine A were found in the refrigerator at Farm A. CDHS and CVBDR attempted virus isolation by mouse inoculation and cell culture by using the residual vaccine from Farm A. One-day-old mice were inoculated by either intraperitoneal or intracranial injections of the vaccine and were monitored for 18 days. Live EEEV was not isolated from any of the vials. Additionally, the Center for Veterinary Biologics Laboratory conducted safety tests on stored vaccine from the same lot as Vaccine A, which was available because of licensing procedures that require samples from each lot to be retained. Virus isolation attempts on these samples were also negative by cell culture and wet chick inoculations.

Molecular Comparison of Horse and Vaccine Strains

EEE viral RNA was extracted from the horse isolate and passaged once via BHK (baby hamster kidney) cell culture at CVBDR and directly from the residual vaccine and amplified and sequenced in two separate regions of the genome according to previously published protocols (7,8). The structural E1 region was amplified and compared with several other published EEEV E1 sequences in GenBank; 1,100-nucleotide sequences of the horse isolate and Vaccine A strain were compared with each other as well as with the 10 most closely matched published sequences in GenBank. The conclusions from this laboratory's study were very similar to the initial gene sequencing of the horse isolate by USAMRIID. Table 2 illustrates a comparison of each GenBank sequence to the sequence of the horse isolate. The phylogram is depicted in Figure 2.

Table 2. Comparisons of 1,100 nucleotide sequences of the horse virus isolate, Vaccine A virus strain, and selected GenBank isolates of the structural unit E1

GenBank accession no.	Strain/isolate	Mutations	% Match
Horse		0	100
Vaccine		5	99.5
AF159551	LA50	7	99.4
L37662	PE6 vaccine	11	98.9
U01552	Decuir	13	98.5
U01558	Tenbroeck	15	98.6
U01554	NJ/60	15	98.6
AF159556	FL96-14834	17	98.5
X63135	ssp. N. Am. Variant	18	98.4
AF159550	MA38-Mass	19	98.3
U01555	ME771332	19	98.2
U01034	82V2137	19	98.3

The NSP3 (nonstructural) region was also amplified and cloned to check for variability within Vaccine A and in the horse viral isolate, as well as to compare with published EEEV NSP3 sequences in GenBank. Four horse and five vaccine viral RNA clones were sequenced and analyzed. Of the 508 nucleotides in each fragment, only one nucleotide difference was evident among the cloned vaccine sequences, and only one was found among the four sequences from horse isolates. These differences could be a result of taq polymerase errors. The consensus sequences for both the vaccine and the horse EEEVl RNAs were compared with each other and with the only two EEEV GenBank sequences that came up in a BLAST search (Table 3, Figure 3).

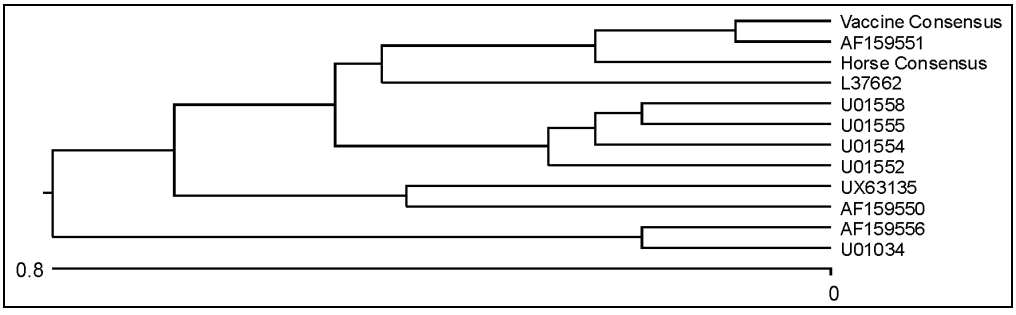

Figure 2. Phylogram based on nucleotide comparison from the E1 region of a horse infected with *Eastern equine encephalomyelitis virus*.

Table 3. Comparisons of 508 nucleotide sequences of the horse virus isolate, Vaccine A virus strain, and selected GenBank isolates of the nonstructural unit NSP3

GenBank accession no.	Strain/isolate	Mutations	% Match
Horse		0	100
Vaccine		0	100
X63135	ssp. N. Am. Variant	5	99.0
U01034	82V2137	5	99.0

Discussion

The identification of EEEV in a horse in California was unprecedented and clearly represented a potential human and animal health threat. In other areas of the country, equine epizootics have been recognized as precursors to human disease (13,14). The rapid recognition and reporting of the case permitted an extensive investigation into the source of exposure.

Several factors must be met to sustain epidemics, including virulent viruses, adequate vectors, neighboring intermediate hosts, and populations of susceptible horses and people (5,15). Such isolated cases as the one mentioned are sure to increase veterinary attention to the possibility of neurologic patients having EEEV infections, as well as elevating public awareness of the disease and methods of prophylaxis.

A diagnosis of EEEV infection was made on the basis of the rapid clinical onset of neurologic signs, compatible histopathologic and serologic findings, and isolation and molecular characterization of EEEV from brain tissue. Several neurologic conditions were considered in the differential diagnosis, including other viral encephalomyelitides (rabies, Aujesky disease, Borna disease, EHV-1 myeloencephalopathy, WEEV, and WNV encephalomyelitis), bacterial meningitis, listeriosis, leukoencephalomalacia, lead poisoning, equine protozoal myeloencephalitis, nigropallidal encephalomalacia, botulism, and verminous encephalitis.

California's Arboviral Encephalitis Surveillance Program is among the most comprehensive in the United States. The jurisdiction where the horse was stabled participated in the program, and a sentinel chicken flock was located adjacent to the farm. In this case, locally infected mosquitoes were apparently not the source of exposure. Furthermore, there was no evidence of spread from the infected horse to the local mosquito populations based on mosquito pool and sentinel chicken flock testing throughout the year. The likelihood of EEEV's having become established in California following this isolated equine case is remote but still important to monitor because of the public health implications. First, the primary vector of EEEV in North America, *C. melanura*, is not known to occur in California (16). In addition, our equine case was diagnosed in April, when mosquito populations are low in southern California; particularly the vector species known to feed on both birds and horses. Second, this case had a rapid clinical course, with euthanasia in <24 hours after onset of clinical signs. Since horses are known to have a short viremia (1 to 3 days' duration) it is unlikely that any mosquitoes acquired the infection from the horse during this short time period. However, if vector abundance were increased, this horse would have had the potential to amplify the virus (5). Incidental infections could have occurred among barn personnel and susceptible horses at Farm A and nearby locations by transmission from mosquitoes that acquired the infection from the case. Of even greater concern, competent vectors could then spread the disease further by feeding on susceptible wild bird populations, potentially establishing an enzootic cycle in southern California.

After we excluded disease by natural infection, bioterrorism, and importation, incomplete formalin inactivation of the EEEV in the vaccine had to be considered a likely possibility. Previous reports of residual virus in formalin-inactivated vaccines exist. Documented outbreaks due to *Poliovirus* (PV*), Foot-and-mouth disease virus,* and VEEV have been directly related to the use of formalin-inactivated vaccines (17-19). Attempts to isolate live EEEV from residual and stored vaccine were unsuccessful. However, this does not eliminate the possibility that the horse received live virus with its immunization. If inactivated viruses existed in the vaccine, they were likely present in undetectable levels during vaccine development and testing. Additionally, the live viruses were probably distributed sporadically throughout the vaccine lot, allowing for only an isolated recognized case. The situation could also be analogous to the 1955 "Cutter inactivated poliovirus incident," when children became infected with PV after vaccination and follow-up investigation disclosed that several lots of Salk PV vaccine contained live PV, despite being produced with formalin inactivation in full compliance with federal regulations (19). In the PV vaccine example, live virus was not uniformly distributed in that vaccine lot (20).

We further explored the hypothesis of residual live virus in the vaccine through molecular epidemiologic studies. Similar studies were used to examine the role of the VEEV vaccine in the 1967-1972 VEEV pandemic in Central America (21). Unfortunately, the North American variety of EEEV is the most genetically homologous of the alphaviruses and therefore the least conducive to molecular comparison of strains (9,22). In our study, the greatest nucleotide homology in the E1 region was among the horse virus isolate, Vaccine A virus, and the

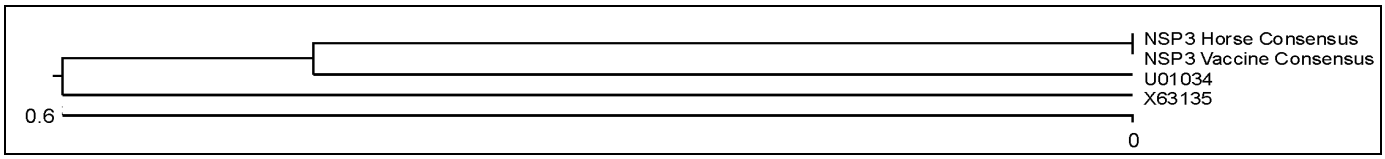

Figure 3. Phlyogram based on nucleotide comparison from the NPS3 (nonstructural) region of a horse infected with *Eastern equine encephalomyelitis virus*.

LA50 virus strain (Figure 2). Differences among sequences from Vaccine A EEEV and the horse viral isolate in the E1 region might represent mutations that occurred when virus passed through various hosts (horse brain/BHK cell culture/1) or genetic variants within the vaccine strain. However, we concluded on the basis of the limited number of clones analyzed that there were few to no other EEEV subclones in the horse viral isolate or vaccine virus. The NSP3 region proved to be more highly conserved and therefore less conclusive. Also, very few EEEV sequences that included the nonstructural regions have been published in GenBank, so comparison was limited. Regardless, the Vaccine A EEEV appears to be closely related to the horse viral isolate; thus, the possibility of live virus in the formalin-treated vaccine infecting the horse remains.

We are unaware of any reports of problems with this vaccine lot, despite notification of the manufacturer and other state veterinarians. If Vaccine A or portions of the lot contained live virus, many exposed horses may not have been susceptible because of previous immunization or presence of maternal antibodies. In addition, cases may have been unrecognized or unreported. If Vaccine A was the source of infection for this case or other cases, it was probably a rare event.

A definitive source of infection may never be revealed in this case. However, the case illustrates the need to maintain awareness that EEEV can occur outside its normal geographic boundaries; it also underscores the importance of prompt diagnosis, reporting, and surveillance for arboviral encephalomyelitides.

Acknowledgments

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Predicting the Risk of Lyme Disease: Habitat Suitability for *Ixodes scapularis* in the North Central United States

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The distribution and abundance of *Ixodes scapularis* were studied in Wisconsin, northern Illinois, and portions of the Upper Peninsula of Michigan by inspecting small mammals for ticks and by collecting questing ticks in state parks and natural areas. Environmental data were gathered at a local level (i.e., micro and meso levels), and a geographic information system (GIS) was used with several digitized coverages of environmental data to create a habitat profile for each site and a grid map for Wisconsin and Illinois. Results showed that the presence and abundance of *I. scapularis* varied, even when the host population was adequate. Tick presence was positively associated with deciduous, dry to mesic forests and alfisol-type soils of sandy or loam-sand textures overlying sedimentary rock. Tick absence was associated with grasslands, conifer forests, wet to wet/mesic forests, acidic soils of low fertility and a clay soil texture, and Precambrian bedrock. We performed a discriminant analysis to determine environmental differences between positive and negative tick sites and derived a regression equation to examine the probability of *I. scapularis* presence per grid. Both analyses indicated that soil order and land cover were the dominant contributors to tick presence. We then constructed a risk map indicating suitable habitats within areas where *I. scapularis* is already established. The risk map also shows areas of high probability the tick will become established if introduced. Thus, this risk analysis has both explanatory power and predictive capability.

Lyme disease, the most common vectorborne disease of humans in the United States, is caused by the spirochete *Borrelia burgdorferi* and transmitted by the blacklegged tick *Ixodes scapularis* (1). The distribution of Lyme disease in the Midwest has been determined largely by mapping the distribution of its vector, *I. scapularis*, which was first detected in northwestern Wisconsin in the late 1960s (2). Its range then expanded southward and eastward (3-6). Even though an isolated established population was discovered in northeastern Wisconsin in Marinette County (7), *I. scapularis* does not appear to have become established in several counties in northeastern Wisconsin. This area is heavily populated with white-tailed deer (*Odocoileus virginianus*) and white-footed mice (*Peromyscus leucopus*) (8), which serve as hosts for *I. scapularis* (1). Since host densities do not appear to be a limiting factor for the tick population (9), the physical environment, both at the macro and micro levels, may affect the tick's ability to survive in this habitat. Moreover, even if establishment is successful, environmental factors may limit tick population densities.

In northwestern Illinois, well-established *I. scapularis* populations were found along the Rock River in Ogle County and in Rock Island and Lee counties since the late 1980s (10-14). Through the early 1990s, Jo Daviess County was the only positive area along the Wisconsin border, and Putnam County was the only positive along the Illinois River. In southern Illinois, no blacklegged ticks were found among white-tailed deer in a survey conducted from 1980 to 1983 (15). Northern Illinois also maintains populations of white-tailed deer and white-footed mice (8), although a large proportion of land is used for agriculture (16).

The phenology of *I. scapularis* has been studied in Michigan (17), Wisconsin (18), and Illinois (19). In the Midwest, adults have both a longer activity period as well as higher peak densities in the spring than in the fall.

Studies of habitat preferences of *I. scapularis*, which have been conducted at various spatial scales (20-22), found environmental factors that are associated with vector and host distribution and densities. *I. scapularis* presence has been correlated with sandy soils (23, 24) and wooded vegetation (25-28). At the macro level, environmental risk factors for Lyme disease have been determined using satellite, climatological, and ecological data to characterize the habitat of the vector tick using geographic information systems (GIS), both in Europe (29-33) and the United States (22-24, 34-36).

The purpose of this study was to determine the distribution of *I. scapularis* in the upper Midwest based on data from Wisconsin, northern Illinois, and the Upper Peninsula of Michigan, and to explain the environmental factors that facilitate or inhibit the establishment of *I. scapularis*. Since host

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abundance is not a limiting factor for the maintenance of tick populations in this area, survival of *I. scapularis* may depend on a combination of several environmental risk factors, resulting in a patchy, discontinuous distribution of this vector. We propose a hierarchic interpretation, starting from the bedrock geology through glacial history and climate patterns, to explain the topography, soil, and vegetation patterns that may directly affect tick survival. By characterizing the habitat preferences of *I. scapularis* using digitized databases (some derived from satellite imagery) and field data integrated into a GIS, the distribution of Lyme disease and other diseases transmitted by the blacklegged tick can be predicted, and the risk of transmission to the human population can be assessed.

Methods

Site Selection

In Wisconsin, a statewide survey of parks and forests was conducted to determine the presence of *I. scapularis*. Sites were selected to represent each region in the state, with 28 of 59 states parks and forests selected. In Michigan, three sites were selected in Menominee County, where *I. scapularis* had previously been identified (7). In Illinois, paired positive and negative sites were sampled in Ogle and Rock Island counties, and additional sites were sampled along the Illinois River. Data are presented separately for each collection site.

Tick Collection

Tick collection was conducted at a total of 138 sites in July and September-October 1996, June 1997, and May-June 1998. The most comprehensive trips were made June 14-26, 1997, and May 27 through June 3, 1998, in the southern part of the study region. In several natural areas, more than one site was dragged, and results for each site were considered separately.

Questing *I. scapularis* ticks were collected in two ways: 1) by dragging a 1-m^2 white flannel cloth through vegetation for a total of at least two hours at each site (timed dragging), or 2) by dragging 1000 m on a grid (distance dragging). Timed dragging was conducted by teams of 4 or 5 persons, with each person dragging for 30 minutes. Distance dragging was also conducted by teams of 4 or 5 persons, which required an estimated 2 to 2.5 hours per grid. Thus, each site was dragged for a total of 2 or 2.5 hours per visit. All calculations of tick numbers are per 1 drag-hour.

Nymphs and adults were maintained alive in plastic vials with moistened cotton balls on ice for *B. burgdorferi* culture. Larvae were placed in vials containing 70% ethanol for later identification.

Vertebrate Collection

Small mammals were trapped overnight during July and October 1996, June 1997, and June 1998 at 13 selected sites in Wisconsin, and at all the Michigan and Illinois sites. Sherman live traps (H.B. Sherman Traps, Inc, Tallahassee, FL) were placed approximately 10 meters apart and baited with bread and peanut butter. Approximately 35 to 50 traps were placed per site, and 0 to 15 mice and 0 to 7 chipmunks were trapped at each site. White-footed mice and chipmunks were anesthetized with the inhalant anesthetic methoxyflourane (Shering-Plough, Inc., Madison, NJ), examined for ticks, and ear-tagged, and their sex and weight were recorded (LACAC animal use protocol # 99099). Ticks were removed and placed in vials containing 70% alcohol for identification.

Site Classification

For each site, the average number of each stage of the deer tick was calculated per hour of dragging. The number of ticks per dragging hour is based on an average of all drags. There was no situation where all or most ticks were found on one drag. The average number of larvae and nymphs was determined per small mammal captured. These data were not pooled with the dragging data because animals were not trapped at all sites.

A site was classified as negative (0) if *I. scapularis* was never found on vegetation or small mammal hosts. There was no case where ticks were found only on small mammals but not on drags. A site was rated 1 if only one stage of the tick was found, regardless of the quantity. A rating of 2 was given if all stages of the tick were found at low density (<10 larvae, <4 nymphs, <2 adults), and a rating of 3 indicated all stages were found at higher density.

We considered several types of classification, including calculating each stage separately and each collection trip separately. Although repeat visits increase the chance that a site will be classified as positive for the presence of ticks, there were no sites where more than one stage was found in only one visit. The finding of only one stage, however, may indicate accidental introduction without establishment. We selected a very conservative and coarse classification to account for the limitations of such an extensive field survey and to allow for differences in weather conditions, time of day, and other variables.

Soil Data

After removing the layer of leaf litter, soil samples were collected at each site from the uppermost 6 inches of topsoil. Data on predominant vegetation, leaf litter thickness, slope, and compass direction were also recorded at each site. Particle size analysis was performed on 10 gm samples of soil (37). The pH and the percentages of sand, silt, and clay were measured for each sample, and the soil texture class was determined from a combination of these percentages. The percentages and classes were compared with site positivity using Spearman rank correlation.

Forest Moisture Index

The classification of forest type was derived from the predominant trees at each site. The number of mature trees (>4 inches in diameter) was counted within a 50-m^2 grid at each site and identified according to species (38). The most

common species were used to classify the forests via a moisture index (38). The sites were divided into five categories: dry, dry/mesic, mesic, wet/mesic, and wet.

Georeferenced Databases

Data Sources

Geographic coordinates of sites were determined by using a Trimble Geoexplorer (Trimble Navigation, Ltd., Sunnyvale, CA) global positioning system (GPS) and exported by using the Trimble Pathfinder software into ARC/INFO and ArcView GIS (ESRI, Redlands, CA). The generated georeferenced database was overlaid on digitized state coverages of environmental data. Land cover and elevation data for Wisconsin were obtained from WISCLAND/GAP (University of Wisconsin and Wisconsin Department of Natural Resources, Madison, WI) at a scale of 1:40,000. WISCLAND/Upper Midwest GAP analysis created land cover classifications based on Landsat Thematic Mapper (TM) data and stratification of the satellite imagery with a hierarchic classification system into wetlands, urban areas, and upland areas. For Illinois, land cover, elevation, and quaternary geologic data were obtained from the Illinois GIS (Department of Natural Resources, 1996, Springfield, IL) at a scale of 1:500,000. Bedrock geology data were obtained from the Digital Geologic Map and Mineral Deposits of Minnesota, Wisconsin, and Michigan (U.S. Geological Survey, Reston, VA) at a scale of 1:1,000,000 for Wisconsin and Michigan, and from the Illinois GIS at a scale of 1:500,000. Soil data, including order, texture, drainage, and quaternary geology, were obtained from STATSGO (U.S. Department of Agriculture, Washington, DC) with a resolution of 2.5 km^2.

Climate data, gathered by the weather station closest to each site, were obtained from the National Oceanographic and Atmospheric Administration (National Climate Data Center, Asheville, NC). Variables included yearly and seasonal precipitation. Landsat TM satellite images were obtained for the entire study area from summer and fall of 1989 through 1993. For each site, average values of TM bands 3 (red), 4 (near-infrared), 5 (mid-infrared), and the normalized difference vegetational index (NDVI) were calculated for the surrounding 3x3 (0.01 km^2), 10x10 (0.1 km^2), and 30x30 (0.9 km^2) pixels. Indices of greenness, brightness, and wetness were obtained through the tasseled-cap transformation (39). Brightness is a measure of reflectance and is correlated to the texture and moisture content of soils, while greenness is a measure of the density of green vegetation present. Wetness is a measure of moisture in soils and vegetation. These remote sensing indices were treated as interval-level data and were associated with tick abundance at each site.

Environmental Variables

Land cover data were grouped into five ordinal categories: agriculture, grasslands, coniferous forest (in which $\geq$75% of trees maintain leaves all year), mixed forest (neither deciduous nor coniferous species make up >75% of land cover), and deciduous forest (at least 75% of trees shed foliage simultaneously in response to seasonal change).

Bedrock geology was classified as Precambrian, which consists of volcanic and metamorphic rocks, and sedimentary deposits from the Silurian, Ordovician, and Devonian eras (40). Quaternary geology information was obtained from the USDA Forest Service North Central Research Station (General Technical Report NC-178). Categories were classified as outwash plains and pitted outwash, lake plain, till plain, ground moraine, loess, and plateau.

Soil orders are defined by amount of organic matter present, pH, and the type of vegetation growing on the soil (40). In Wisconsin and northern Illinois, 8 of 12 soil orders are represented: mollisols (present under prairie), alfisols (deciduous forests), spodosols (coniferous forests), entisols and inceptisols (both of which are associated with poorly developed soils), histosols (peat and muck), and vertisols and paleosols (which represented <1% of the area). These orders were classified into ordinal categories based on increased fertility and decreased acidity: 0 = histosol and spodosol, 1 = entisol, 2 = inceptisol, 3 = mollisol, and 4 = alfisol.

Soil texture (40) was divided into seven groups in order of increasing particle size, ranging from clay (<2 mm) through silt (2 to 50 mm) to sand (0.05 to 2.0 mm). Drainage was divided accordingly into seven categories (STATSGO, Washington, DC), from very poorly drained to well drained. Excessively drained soils were ranked as 0 since they are too dry to support a biotic environment (40).

For each site, yearly and seasonal rainfall averages and average snowfall per year were obtained from the weather station (NOAA) nearest each site. Elevation ranged from 495 m in northern Wisconsin to 197 m in western Illinois. Precipitation, elevation, and remote sensing indices were treated as interval-level data.

Statistical Analysis

All analyses were performed by using SPSS software (SPSS, Chicago, IL). Soil texture classifications of samples from the sites were compared with those listed in STATSGO, the soils database (STATSGO, Washington, DC), and Spearman rank correlation was used to assess correlations between field data and data from the GIS. Univariate analysis was initially performed by using chi square contingency tables to determine significant associations between site positivity and environmental variables coded as previously described. Discriminant analysis was performed by using only the significant ($p<0.25$) environmental variables from the univariate analysis (41). A linear discriminant function was obtained from the combination of variables that best characterized the differences between the groups. A stepwise approach was used to enter variables one at a time until the discriminating power between tick abundance categories ceased to improve.

Analyses were performed by grouping the outcome variables into positive or negative sites and into the four abundance categories described previously.

As mentioned, since a site classified as category 1 (finding only one stage of the tick) could result from introduction into an unsuitable habitat, categories 0 and 1 were combined for additional analysis. Only 112 sites were used in the analysis, with no more than three sites included per natural area where multiple sites were sampled. The resulting classification functions were then used to predict tick abundance categories and assess how well the functions discriminated. Separate discriminant analyses were performed by using the seven indices obtained from the remote sensing data at three spatial scales and the precipitation data.

Logistic regression analysis was performed by using the primary environmental factors as independent variables and the positive and negative sites as outcome variables. Forest moisture index was excluded from the model because this variable was not available as digitized geographic coverage.

To develop a risk map for Lyme disease in the area studied, a grid was created encompassing the states of Wisconsin and Illinois with a resolution of 2.5 km^2 per cell. The grid was overlaid with the selected coverages by using ARC/INFO and ArcView GIS(ESRI, Redlands, CA), and data values corresponding to each layer were assigned to each cell. The Summarize Zones procedure from the ArcView Analysis Menu was used to calculate summary attributes for features by using a grid scheme that divided the entire study area into 2.5-km^2 cells. Each cell was assigned a value for each layer included in the logistic regression based on the most common category. The logistic equation was then used to generate the probability of the presence of *I. scapularis* within each 2.5-km^2 cell of the grid map. The map was generated with probabilities divided into quartiles and deciles.

Results

The locations of the 138 sites that were sampled in Wisconsin, Illinois, and Michigan are shown in Figure 1. Among the four categories, 56 sites were classified as negative, 24 were ranked as 1, 32 as 2, and 26 as 3. Most negative sites were in northeastern Wisconsin. In the southeastern part of Wisconsin, sites were negative except those situated in the Kettle Moraine State Forests (Sheboygan, Fond du Lac, Jefferson, Walworth, and Waukesha counties), which are located on the terminal glacial moraines. Negative sites in Illinois were at Blackhawk Nature Preserve (Rock Island County), located in a suburban area, and White Pines State Park (Ogle County), which has large stands of secondary growth pine forest. In Wisconsin, positive high-density sites were found in the southwestern driftless area and in the central sandy uplands, as well as in the well-recognized northwest part of the state (and across the state line into Minnesota).

In Michigan, where only a small area of the Upper Peninsula was sampled, all sites had very dense tick populations, except for a site that was classified as excessively drained

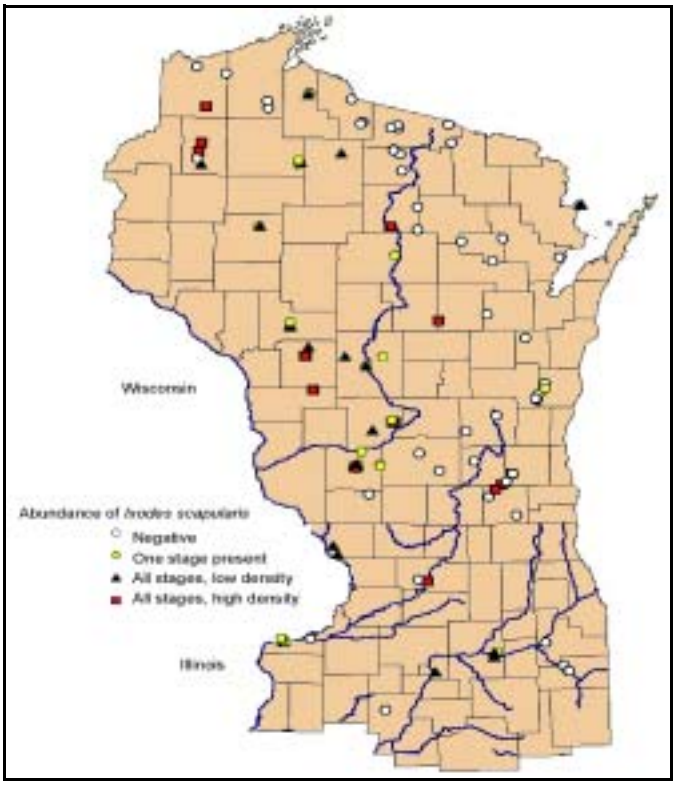

Figure 1. Geographic distribution of study sites ranked by abundance of *Ixodes scapularis* in Wisconsin, northern Illinois, and Menominee County in Michigan.

(>99% sand). The sites classified in the other two abundance categories (1 and 2) did not appear to cluster in any areas. In Illinois, the two parks that have been infested for at least a decade, Castle Rock State Park (Ogle County) and Loud Thunder Forest Preserve (Rock Island County), were classified as having dense tick populations, with lower populations in some sites along the Illinois River.

Particle size analysis, which is a function of the proportions of sand, silt, and clay, was performed at 82 sites (Figure 2). The positive sites were clustered in the sand/loamy sand texture classes. Individual percentages of sand, silt, and clay per sample were not correlated with tick abundance; however, texture class, which is a combination of these three percentages, correlated significantly ($r=0.42$, $p<0.05$) with greater tick densities found in soils with a greater proportion of sand. The soil texture class of samples determined from the soil analysis correlated significantly ($r=0.46$, $p<0.001$) with the soil texture class of each site as obtained from the STATSGO database.

The univariate analysis detected significant associations ($p<0.25$) between tick presence and land cover, soil order, bedrock geology, quaternary geology, soil texture, forest type, spring, summer, fall and winter precipitation, snowfall, and elevation (Figure 3). The results of the discriminant analysis are listed in Table 1. When negative and positive sites were contrasted, the variables forest type, soil order, land cover, soil texture and bedrock were significant. Tick presence was positively associated with deciduous (Figure 3a), dry/mesic and

Table 1. Significant environmental variables to determine favorable habitat for *Ixodes scapularis* using discriminant analysis

	Groups (sample size)						
	0 vs. 1,2,3 (47 vs. 65)		0,1 vs. 2,3 (63 vs. 69)		0 vs. 1 vs. 2 vs. 3 (47 vs. 16 vs. 24 vs. 25)		
Variable	Wilk's lambda	Disc F(x)	Wilk's lambda	Disc F(x)	Wilk's lambda	Disc F(x)1	Disc F(x)2
Forest type	0.784	0.552	0.789	0.789	0.754	0.665	-0.747
Soil order	0.618	0.521	0.699	0.542	0.569	0.633	0.774
Land cover	0.586	0.387					
Soil texture	0.564	0.381					
Bedrock	0.525	0.518					
Eigenvalue	0.904		0.431		0.681		0.045
% correctly classified	85.7		78.6		51.8		78.6
Canonical correlation coefficient	0.689		0.549		0.636		0.207

dry forests (Figure 3b), fertile soils such as alfisols (Figures 3c, 4), sand and loamy/sand soil texture (Figures 2, 3d), and sedimentary bedrock (Figure 3e). There was a negative association with grasslands and conifer forests (Figure 3a), wet and wet/mesic forests (Figure 3b), acidic soils such as spodosols (Figure 3c), clay soil texture (Figure 3d), and Precambrian bedrock (Figure 3e). Elevation was not an important discriminator in the model, nor was Quaternary geology (Figure 3f) important even though sites located on the plateaus and loess-covered areas were all positive. However, the distribution of the sites among the categories of Quaternary deposits was skewed because a large proportion of the state parks were located on terminal glacial moraines. The discriminate model was able to correctly classify 85.7% of the sites. The canonical correlation coefficient was 0.69, and the eigenvalue was close to 1 (0.91), indicative of a strong discriminant function. When the single stage category (1) was included with the negative group, only two variables, forest type and soil order, were significant. Most of the sites (78.6%) were still correctly classified; however, the eigenvalue decreased to 0.43. These same variables were significant when all the groups were considered separately; but the model only correctly classified 51.8% of the sites. Even though only 4/33 in the negative group were misclassified, there was very poor discrimination among the tick positive groups. No significant variables resulted from the discriminant analysis performed using the satellite data. Since all sites were located in forested areas, TM imagery may not have been able to discriminate well among suitable and unsuitable forested habitats. The precipitation variable was also not a significant discriminator between positive and negative sites in the model.

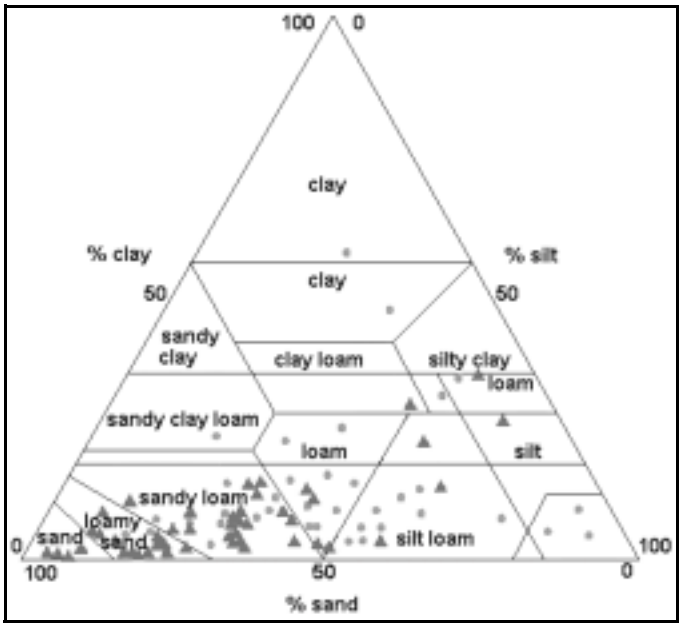

Figure 2. Soil particle size analysis of samples from positive and negative sites. Soil texture is expressed as the sum of percent sand, silt, and clay.

The results of the logistic regression analysis were in agreement with the discriminant analysis model in the positive versus negative group as seen in Table 2. The same variables were significant ($p<0.05$), and the model correctly classified 83.9% of the sites. The predictive risk map generated from the logistic regression model is shown in Figure 5. The higher probabilities indicate increased suitability of habitat for *I. scapularis.* In Wisconsin, the areas of moderate suitability (26%-40%) are located in the western half of the state. Patchy areas of higher probability (60%-100%) are found in the central and northern portion (Juneau, Adams, Waushara, and Marquette counties.) and along the border with Minnesota (Vernon and Crawford counties). In Illinois, the positive sites that were sampled corresponded to areas of increased suitability (60%-100%). Castle Rock State Park, where the highest tick densities are found, had a 90%-100% probability of suitable habitat. The areas bordering the Illinois River appear to be adequate habitat for *I. scapularis*, especially on the western side. Shawnee National Forest in the extreme southern portion of the state also appears to have a high probability (60%-80%), even though *I. scapularis* populations have not been detected (42).

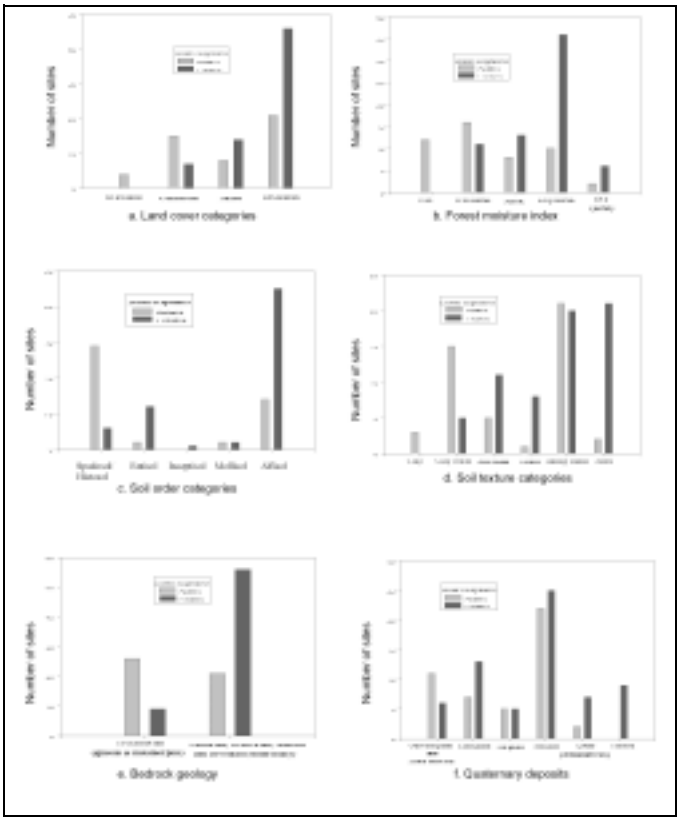

Figure 3. Categories of environmental variables and number of positive and negative sites.

Discussion

Ixodes scapularis may be introduced into new areas by several routes. Adult *I. scapularis* are carried into new areas primarily by deer (43), which are capable of ranging over wide areas, especially along riparian corridors. However, infected adult ticks have limited potential for spreading Lyme disease since transovarial transmission of *B. burgdorferi* is rare. Small mammals are efficient disease reservoirs, and juveniles tend to disperse during the spring and summer when tick larvae and nymphs are questing. However, the potential for long-range dispersal of Lyme disease by rodents is limited, since they occupy much smaller home ranges than deer (44). Birds have a high potential for introducing infected immature stages of *I. scapularis* into distant areas (45-47), especially during spring and fall migration.

To become successfully established in a new area, *I. scapularis* requires available hosts for feeding, which is not a limiting factor in our study area, and a suitable habitat for questing, molting, diapause, and oviposition. The vegetation, soil, topography, and climate are interrelated, and extremes of any one factor may adversely affect the tick's ability to survive.

The environmental characteristics vary throughout the two states, and certain combinations may determine whether introduced *I. scapularis* populations can become established. Tick abundance is an indicator of the suitability of environmental conditions for reproduction and survival. Finding only one stage of the tick may indicate either a poor microenvironment

Table 2. Significant environmental variables in the logistic regression model.

					95% Confidence interval	
Variable	Beta	Standard error	P value	Odds ratio	Lower	Upper
Land cover	0.85	0.40	0.03	2.36	1.08	5.15
Soil order	0.42	0.18	0.02	1.52	1.07	2.16
Bedrock	1.78	0.73	0.01	5.94	1.42	24.78
Soil texture	0.76	0.26	0.004	2.13	1.27	3.57
Constant	-9.06	1.95				

or a recent introduction. Finding all three stages at one site strongly suggests that a population has become established. A less than optimal habitat may account for low density in an established *I. scapularis* population, or it may indicate a recent introduction. Errors in classification may occur in an extensive field survey, as reported here, and a dynamic situation (i.e., the process of invasion of a new site) may mask the occurrence of some positive or potentially positive sites. By including a large number of sites and conducting repeat visits, we have tried to minimize such confounding effects.

Environmental factors such as bedrock geology, quaternary deposits, soils, vegetation, and climate influence each other directly and indirectly to create unique habitats. This is why we included risk factors that are not necessarily independent in a model that is most unbiased. The soil orders in the region (Figure 4) are influenced by the type of underlying bedrock and by quaternary deposits. The soils, in turn, influence the type of vegetation overlying them. Soil texture is the component of soil that influences the extent of drainage. The soil texture classes are independent of soil order and are usually a function of the degree of soil weathering and the parent material (bedrock or quaternary deposit). The tree composition of a forest is determined by a moisture gradient involving soil aeration, soil nutrient supply, and microclimatic features (38), and this gradient functions as a continuum. The forest types classified as dry and dry/mesic have oaks and jack pines as the dominant species that prefer well-drained, sandy soils. Oak forests also have a dense canopy layer that provides protection for the underlying vegetation. Wet and wet/mesic forests are composed of trees that have a high tolerance for very moist soils. The factors interacting at the microclimatic level within the topsoil and leaf litter appear to have an important influence on tick survival. Excessively moist conditions at the soil level were negatively associated with the establishment of *I. scapularis*. Soil texture, in addition to the topography, determines the extent of drainage, and the level of moisture of the ground layer, regardless of the amount of precipitation. However, given the effect of weather on tick abundance (19), associations between tick presence and amount of yearly precipitation or snowfall need to be analyzed further.

Our findings suggest that abiotic factors play a major role in determining whether populations of *I. scapularis* can

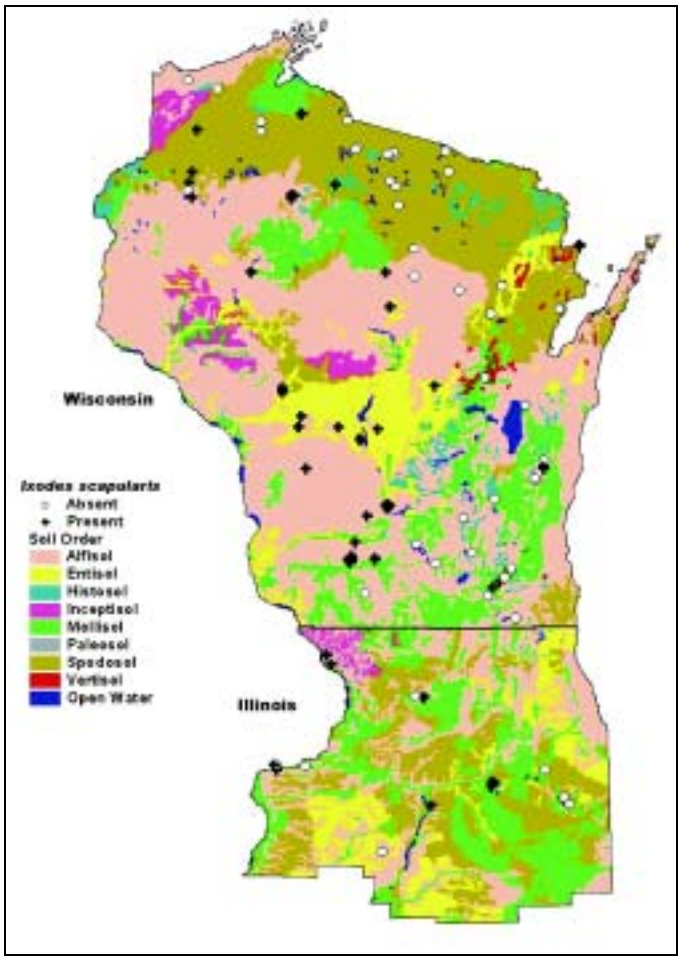

Figure 4. Map of soil orders in Wisconsin and northern Illinois, overlaid with tick study sites.

become established in an environment. Precambrian bedrock of volcanic origin results in the formation of acidic soils that are found mainly under coniferous forests, the forest type least likely to support tick populations. Soils containing increased acidity (spodosols) and a high proportion of clay that can retain excess moisture (48) were also more frequently present in negative sites. Excessive moisture in the soil may be deleterious to tick survival since they overwinter in the topsoil and leaf litter. It may also enhance the growth of organisms, such as fungi and entomophagous nematodes, which may have adverse effects on the tick population (49). Leaf litter is a necessary component for the survival of immature stages of *I. scapularis* (50). However, the type and quantity may determine the densities of ticks in a specific habitat. Tick densities were highest in forests dominated by oak, followed by maple, and lowest in coniferous forests that produce minimal amounts of leaf litter (38). Tick densities were also highest in areas with underlying sedimentary bedrock, which is associated with alfisol and mollisol soil orders and soil textures of increased particle size (38).

The statistically significant risk factors derived from the logistic regression analysis were in agreement with those obtained from the discriminant analysis, and allowed us to quantify and predict the environmental risk for the presence of *I. scapularis*. Several environmental factors must be evaluated simultaneously to assess the combination of factors required for successful establishment. Determining the environmental factors that limit survival can facilitate the development of measures for the control of the tick in the environment.

Using a GIS, we generated a risk map (Figure 5) to predict the presence of the tick vector, *I. scapularis*. The areas of suitable habitat for *I. scapularis* in Wisconsin corresponded to areas of increased incidence of human Lyme disease and known areas of tick endemicity. The extensive area of suitable habitat in the western portion of the state can explain the rapid expansion of the tick from the original northwestern focus to

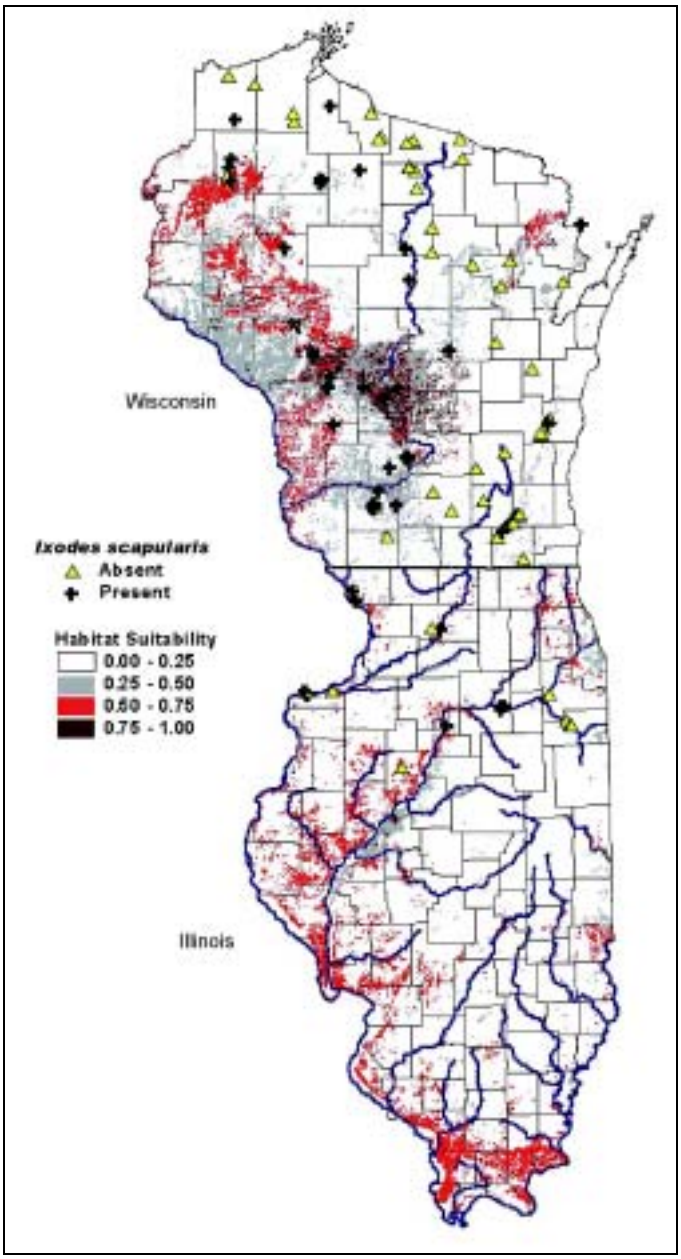

Figure 5: Predictive risk map of habitat suitability for *Ixodes scapularis* in Wisconsin and Illinois.

the southwestern portion of the state (2-5). While initial studies of tick distribution (3) and human granulocytic ehrlichiosis (51) point to the risk of tick-borne disease transmission in Northwest Wisconsin, our study points also to the sandy barrens of Central Wisconsin as most suitable habitats. Indeed, the highest numbers of ticks were collected in Council Ground State Park (Lincoln County), Fort McCoy (Monroe County), Hartman Creek State Park (Waupaca County), and Wildcat Mountain State Park (Vernon County), as well as in sites in the long-recognized Spooner area (Washburn County). Further, the highest prevalence of canine seropositivity to *B. burgdorferi* in northern Illinois and Wisconsin was found in dogs in the west-central counties of Wisconsin (52). Based on the risk map, most of the north-central and northeastern portions of Wisconsin have a <25% probability for tick presence. These are areas where our sampled sites were consistently negative for *I. scapularis*. In the eastern half of the state, the main areas of increased suitability were along the glacial terminal moraines, which is where the positive sites in the Kettle Moraine State Forest were located. There was also a higher probability in the northeastern corner of the state bordering Menominee County, Michigan, where positive sites were located.

In Illinois, areas of increased suitability corresponded to the same areas where the positive sites were located in Ogle, Rock Island, and Jo Daviess counties. The risk map indicated there is adequate habitat for *I. scapularis* populations to become established along the Illinois River, as well as the Mississippi River. However, in Illinois, tick populations may be limited to river corridors since extensive areas are used for agriculture. Where forested habitat is sparse, tick establishment may be restricted, even though geologic and soil factors are favorable. In southern Illinois, where climatic conditions may differ and other reservoir hosts may be present, the inclusion of additional parameters to the model may result in reduced risk probabilities. In contrast, the risk factor model and predictive map may be valid for other north-central areas that have similar environmental characteristics, particularly in parts of Minnesota, Michigan, northern Indiana, and Ohio. The model may be applied to other areas of the United States by using local geographic coverages.

In conclusion, this model can be used to help determine the risk of acquiring Lyme disease and other diseases transmitted by *I. scapularis* by predicting which locations may be currently infested with the tick. It can also be used to assess whether habitats that are currently nonendemic for *I. scapularis* would have the necessary combination of environmental factors to allow new populations of *I. scapularis* to become established. The model can thus be continuously refined based on findings from new areas. The risk of Lyme disease transmission could be predicted in areas capable of sustaining *I. scapularis* populations if ticks harboring *B. burgdorferi* are introduced by migrating deer or birds. The results obtained from these field studies can also form the basis for controlled experimental studies under field and laboratory conditions to further elucidate the preferred microenvironment of *I. scapularis*.

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Molecular Classification of Enteroviruses Not Identified by Neutralization Tests

Hideyuki Kubo,* Nobuhiro Iritani,* and Yoshiyuki Seto*

We isolated six viruses from patients diagnosed with aseptic meningitis or hand, foot, and mouth disease. The cytopathic effect of these viruses on cultured cells was like that of enteroviruses. However, viral neutralization tests against standard antisera were negative. Phylogenetic analysis with the complete VP4 nucleotide sequences of these 6 viruses and 29 serotypes of enteroviruses classified 3 of the viruses as serotype echovirus type 18 (EV18) and 3 as serotype human enterovirus 71 (HEV71). These results were confirmed by remicroneutralization tests with HEV-monospecific antisera or an additional phylogenetic analysis with the complete VP4 nucleotide sequences. Phylogenetic analysis with complete VP4 genes is more useful than neutralization tests with enterovirus serotype-specific antisera in identifying enterovirus serotypes.

The human enterovirus (HEV) genus of the family *Picornaviridae* includes the human pathogens that cause a wide spectrum of acute disease, including hand, foot, and mouth disease (1), aseptic meningitis (2,3), encephalitis (3-6), and neonatal sepsislike disease (7,8). Sixty-four serotypes of HEV have been recognized antigenically by neutralization tests with anti-HEV antibodies (9). HEVs have long been classified on the basis of serotype-specific antisera in virus neutralization tests (1,10), the only method available for serotyping HEVs. However, virus neutralization is both labor- and time-intensive, and antigenic variants in many serotypes of HEV can affect test results (1).

The HEV genome comprises a 5' nontranslated region (NTR), a long open reading frame that encodes a protein of approximately 2,100 amino acid residues, a short 3' NTR, and a polyadenylated tail. The polyprotein is co- and post-translationally cleaved to yield four structural proteins: VP4, VP2, VP3, and VP1 (1). Recently, attempts have been made to classify the HEV serotypes by using the partial nucleotide sequences of the HEV genomes (i.e., the 5' NTR [11-13], the VP4-VP2 junction [14-16], and VP1 [17-20]). Methods for molecular classification of HEVs should not only identify the serotypes rapidly but also detect antigenic variant strains or new serotypes. A new serotype of HEV has recently been identified by comparing the complete VP1 nucleotide sequences; its proposed name is HEV73 (19).

To investigate the HEV serotypes of six HEV-like viruses that were not neutralized by standard HEV typing sera, we determined the complete VP4 nucleotide sequences of these 6 viruses and 21 HEV antigenically defined serotypes, then performed phylogenetic analysis with another 8 HEV serotypes available from GenBank. The classifications of the untypeable viruses were confirmed by using HEV-monospecific antisera or an additional phylogenetic analysis with the VP4 sequences. The molecular classification of HEV with the complete VP4 sequences is useful for identifying the HEV serotypes.

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Methods

Virus Isolation and the Neutralization Test

The clinical specimens were injected into Vero, RD-18S, or MA104 cells to isolate viruses. All cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and maintained in MEM containing 1% to 2% FBS after being added to 48-well plates (Sumitomo Bakelite, Tokyo, Japan). The cells were incubated for 1 week, after which culture fluids were passaged and incubated for another week. Cultured cells showing cytopathic effects were regarded as virus isolation-positive and, together with the culture supernatant, were harvested and stored at -80°C before use. To serotype the viruses, microneutralization tests were performed with antiserum pools of Lim and Benyesh-Melnick (21) (Denka Seiken, Tokyo, Japan) or in-house monospecific immune sera against coxsackie virus A10 (CAV10), CAV16, and HEV71, respectively.

Viruses

Of the six viruses that could not be identified by the neutralization tests described above (Table 1), strains OC/0071, OC/0073, and OC/00272 were isolated from patients diagnosed with aseptic meningitis by using RD-18S cells. OC/00219, OC/00260, and OC/00261 were isolated from patients diagnosed with hand, foot, and mouth disease or aseptic meningitis by using Vero cells. No sera from these patients was available for analysis. Twenty-one serotypes were isolated and identified in our laboratory during 1995-2000 (Table 2); these strains were used in the experiments. For additional investigations of HEV71, we used eight HEV71 strains isolated and identified in our laboratory (Table 3).

Table 1. Unidentified enterovirus strains and patient information, Osaka, Japan, 2000

Strain	Patient age (years)	Specimen	Date of sampling	Clinical symptoms	Isolated cells
OC/0071	2	Stool	5/11/2000	AM[a]	RD-18S
OC/0073	2[b]	CSF	5/11/2000	AM	RD-18S
OC/00219	0	Throat swab	7/7/2000	HFMD	Vero
OC/00260	0	Throat swab	7/18/2000	HFMD, AM	Vero
OC/00261	0[c]	Stool	7/18/2000	HFMD, AM	Vero
OC/00272	6	Stool	7/18/2000	AM	RD-18S

[a]AM = aseptic meningitis; CSF = cerebrospinal fluid; HFMD = hand, foot, and mouth disease.
[b]Same patient as OC/0071.
[c]Same patient as OC/00260.

Table 2. Characteristics of 21 human enterovirus (HEV) serotypes antigenically defined, Osaka, Japan, 1995–2000

HEV serotype	Strain	Age (years)	Specimen	Date of sampling	Isolated cells
PV1	OC/00417	0	Throat swab	10/13/2000	Vero
PV2	OC/00138	0	Stool	6/10/2000	Vero
PV3	OC/99355	0	Nasal mucus	11/8/1999	Vero
EV3	OC/00467	7	Stool	11/13/2000	RD-18S
EV6	OC/99350	0	Stool	11/8/1999	RD-18S
EV7	OC/96221	7	Throat swab	7/22/1996	MA104
EV9	OC/00129	3	CSF[a]	6/8/2000	RD-18S
EV11	OC/98535	3	Stool	9/23/1998	RD-18S
EV16	OC/95378	1	Throat swab	9/11/1995	MA104
EV18	OC/99-Hanasaka	7	Stool	11/8/1999	RD-18S
EV25	OC/00263	0	Stool	7/17/2000	RD-18S
EV30	OC/97633	1	Stool	9/29/1997	RD-18S
CAV9	OC/96234	4	CSF	8/2/1996	RD-18S
CAV16	OC/00351	NA	Throat swab	8/31/2000	Vero
CBV1	OC/00364	0	CSF	9/6/2000	Vero
CBV2	OC/99284	0	Stool	9/11/1999	RD-18S
CBV3	OC/97620	6	CSF	9/19/1997	RD-18S
CBV4	OC/00362	1	Stool	9/8/2000	Vero
CBV5	OC/00223	0	Throat swab	7/7/2000	Vero
CBV6	OC/00325	0	CSF	8/8/2000	Vero
HEV71	OC/00168	2	Throat swab	6/21/2000	Vero

[a] CSF = Cerebrospinal fluid; NA = not available.

RNA Extraction and Reverse Transcription

Viral RNAs were extracted from the cell-culture supernatants by using ISOGEN-LS (Nippon Gene, Tokyo, Japan). cDNAs were synthesized with an Omniscript Reverse Transcriptase Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. The primers used for the synthesis were EVP-2 (5'-CCTCCGGCCCCTGAATGCGGCTAAT-3' relative to nt 444-468 in the genome of Poliovirus (PV) Sabin 1 strain) (22) and OL68-1 (5'-GGTAAYTTCCACCAC-CANCC-3' relative to nt 1178-1197 of Sabin 1), as described (23).

Polymerase Chain Reaction Amplification of cDNAs

Polymerase chain reaction (PCR) was performed by using 2 µL of each cDNA in a 50-µL reaction mixture containing 1.5 U of Taq DNA polymerase (Takara Shuzo, Shiga, Japan), 20 pmol of EVP-2 primer, and 20 pmol of OL68-1 primer. Each reaction was incubated in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) according to the following protocol: 5 minutes at 95°C, 40 cycles of 95°C for 30 seconds, 68°C for 30 seconds, 72°C for 1 minute, and then at 72°C for 5 minutes. After the appearance of approximately 750 bp-specific amplified fragments was confirmed by agarose gel electrophoresis, the amplicons were purified with a QIAquick PCR purification kit (QIAGEN).

DNA Sequence Analysis

Approximately 100 ng of purified amplicon was used in the reaction with the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems), and DNA sequencing was performed by using an ABI PRISM 310 DNA sequencer (Applied Biosystems). All DNA sequencings were performed on both strands using EVP-4 (5'-CTACTTTGGGT-GTCCGTGTT-3' relative to nt 541-560 in the genome of PV Sabin 1 strain) as the forward primer and OL68-1 as the reverse primer (23). Sequencer software (version 3.0; Hitachi Software, Tokyo, Japan) was used to determine the approximately 600-bp nucleotide sequence spanning 5' NTR to one third of VP2 (including all of VP4), translate nucleotide sequence to amino acid sequence, and decide the complete VP4 coding sequence of each virus.

Phylogenetic Analysis

A phylogenetic tree based on the complete VP4 nucleotide sequence was constructed by the neighbor-joining method (24) as implemented with the CLUSTAL X program (version 1.63b, December 1997; http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/). The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. The complete VP4 sequences of eight HEV serotypes not isolated in our laboratory were obtained from GenBank and included in the HEV analysis. Complete VP4 nucleotide sequences of another 18 HEV71 strains were obtained from GenBank and used in the phylogenetic analysis.

Remicroneutralization Tests

According to results of HEV phylogenetic analysis with the complete VP4 nucleotide sequences, remicroneutralization tests using monospecific antiserum against echovirus 18 (EV18; Denka Seiken), or HEV71 (anti-HEV71/BrCr and

Table 3. Characteristics of eight human enterovirus 71 strains, as antigenically defined, Osaka, Japan, 1996-2000

Strain	Age(years)	Specimen	Date of sampling	Clinical symptoms	Isolated cells
OC/9632	NA[a]	Stool	4/11/1996	HFMD	MA104
OC/99-Ikeda	6	Stool	9/11/1999	HFMD, AM	Vero
OC/0078	1	Throat swab	5/18/2000	HFMD, AM	Vero
OC/0080	NA	Stool	5/9/2000	Diarrhea	Vero
OC/0096	5	CSF	5/25/2000	Diarrhea, AM	Vero
OC/00114	0	CSF	5/31/2000	Fever	Vero
OC/00125	6	Throat swab	6/7/2000	Encephalitis	Vero
OC/00168	4	Throat swab	6/21/2000	Herpangina	Vero

[a] NA = Not available; HFMD = Hand, foot, and mouth disease; AM = aseptic meningitis; CSF = cerebrospinal fluid.

anti-HEV71/C7 sera; both supplied by the National Institute of Infectious Diseases, Japan) were performed to confirm the serotype of the untypeable strains from the first microneutralization assay.

Complete VP1 Nucleotide Compared with Deduced Amino Acid Sequences of HEV71 Strains

The complete VP1 nucleotide sequences of HEV71 strains OC/00168, 0C/00219, OC/00260, and OC/00261 were determined by the same procedure described above, except for the primers. The primers used for the analysis of VP1 nucleotide sequence were 71F2399 (5'-AGAAYTTYACCATGAAACTG-3' relative to nt 2380-2399 in the genome of HEV71 MS/7423/87 strain [25]; the nucleotide positions of the following are also relative to this strain: 71F2793 (5'-AGACATAACTG-GYTACGCCAC-3' nt 2774-2793) and 71F3042 (5'-CATGT-CACCYGCGAGCGCTT-3' nt 3023-3042) as the forward, 71R2712 (5'-CTACCAARCCTGCCCTACTG-3' nt 2693-2712), 71R3066 (5'-GGTACCCGTCGTAAAACCAC-3' nt 3047-3066) and 71R3376 (5'-AAGTTGCCCACGTAGAT-GGC-3' nt 3357-3376) as the reverse. The VP1 nucleotide sequence of HEV71 BrCr strain (25) was obtained from GenBank. Sequencer software (version 3.0; Hitachi Software) was used for determination and comparison of the complete VP1 nucleotide and deduced amino acid sequences of these HEV71 strains.

Results

Determination of Complete VP4 Nucleotide Sequences of HEVs

During May to July 2000, six viruses isolated in our laboratory (OC/0071, OC/0073, OC/00219, OC/00260, OC/00261, and OC/00272) could not be neutralized by standard pools of HEV typing sera and three antimonospecific sera (Table 1). However, the cytopathic effects of these viruses on RD-18S or Vero cells were all HEV-like (data not shown). To identify the serotypes of these untypeable HEV-like viruses by a method other than the neutralization assay, we determined the complete VP4 nucleotide sequences of all 6 strains and another 21 HEV serotypes identified in our laboratory over the past 6 years. The 3' end of the VP4 gene of each virus was determined from the deduced amino acid sequences as described (26,27). The complete VP4 nucleotide sequences of all HEV strains used in this study were 207 nt long, and the deduced amino acid sequences of all VP4 proteins were 69 amino acids long (data not shown).

Phylogenetic Analysis of HEVs

A phylogenetic tree was constructed based on the complete VP4 nucleotide sequences of the 6 HEV-like untypeable strains, the 21 HEV serotypes identified in our laboratory as prototype strains, and another 8 HEV serotypes available from the GenBank database (Figure 1). The 29 different HEV serotypes defined antigenically were clustered in four distinct lineages, as described (23). Three of the six untypeable strains (OC/0071, OC/0073, and OC/00272) were classified nearest to EV18. The VP4 nucleotide sequences of strains OC/0071 and OC/0073 were identical. The VP4 gene sequence of OC/00272 was the same as that of OC/99-Hanasaka, which was used as a prototype strain for EV18. The nucleotide sequences of these two clusters differed by 5 nt, but the deduced amino acid sequences were the same (data not shown). The other three untypeable strains (OC/00219, OC/00260, and OC/00261) were classified nearest to HEV71. The VP4 sequence of OC/00219 was the same as that of OC/00168, which was used as a prototype strain for HEV71. The VP4 nucleotide sequences of OC/00260 and OC/00261 were identical. The difference between these two clusters was 11 nt. The deduced amino acid sequences were the same (data not shown).

Remicroneutralization Tests

According to the results of the phylogenetic analysis based on the complete VP4 nucleotide sequences, remicroneutralization tests were performed. Microneutralization tests using the monospecific immune serum for EV18 were done against OC/0071, OC/0073, and OC/00272, and this serum neutralized these viruses. The same tests, using the two species of monospecific immune serum, anti-HEV71/BrCr and anti-HEV71/C7, were performed against OC/00219, OC/00260, and OC/00261, but neither serum neutralized the viruses (Table 4).

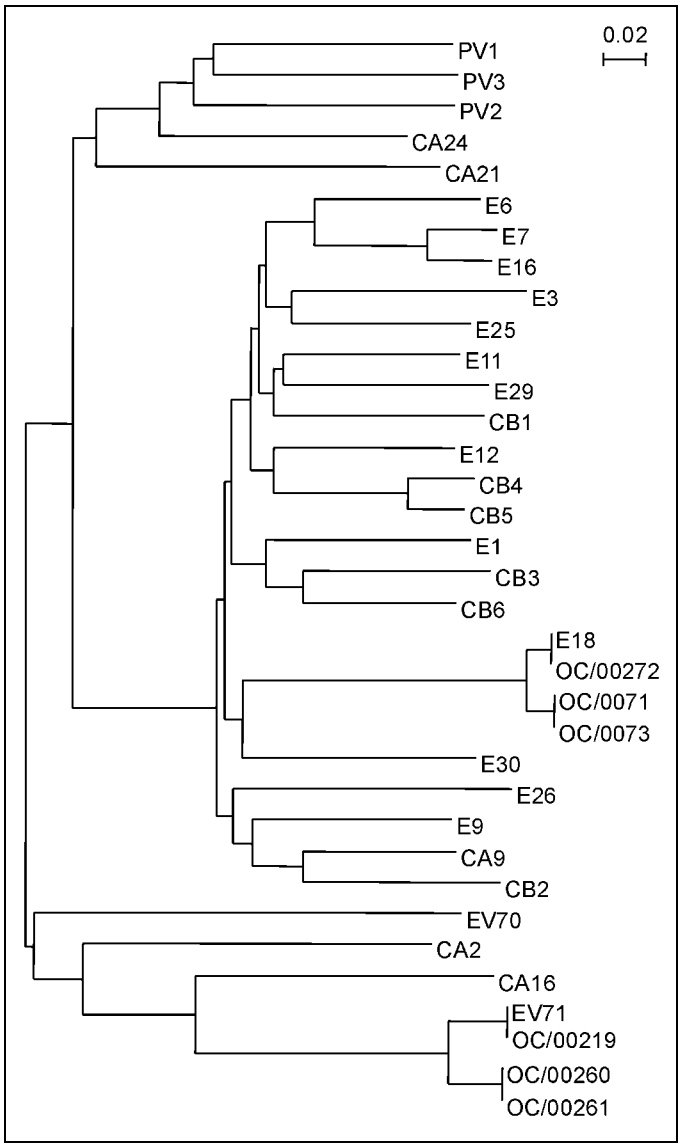

Figure 1. Phylogenetic analysis based on the human enterovirus (HEV) VP4 nucleotide sequences. The phylogenetic tree was constructed by the neighbor-joining method as implemented in CLUSTAL X program (version 1.63b). The marker denotes a measurement of the relative phylogenetic distance. The VP4 sequences of eight HEV serotypes described below are available from GenBank. The strain name and accession number are shown in parentheses: HEV1 (Bryson, AF250874), HEV12 (Travis, NC 001810), echovirus 26 (EV26, Coronel, AF117697), EV29 (JV-10, AF117698), coxsackie virus A2 (CAV2; Epsom/14448/99, AJ2296215), CAV21 (Coe, NC 001428), CAV24 (EH24/70, D90457), and HEV70 (J670/71, D00820).

Phylogenetic Analysis of HEV71 Strains

To establish whether OC/00219, OC/00260, and OC/00261 belong to HEV71, we used another phylogenetic analysis based on the complete VP4 nucleotide sequences of various HEV71 strains (Figure 2). In this analysis, we examined eight HEV71 strains isolated and identified in our laboratory from 1996 to 2000 (Table 3). All these HEV71 strains except for OC/9632 were identified by microneutralization tests with anti HEV71/BrCr serum (data not shown). Of the HEV71 strains available from GenBank, two were isolated in the United States in 1970 and 1987, respectively (25), four in Malaysia in 1997 (15), one in Singapore in 1998 (28), eight in Taiwan in 1998 (15,28), two in the United Kingdom in 1999, and one in China (year unknown). The HEV71 strains were clustered in three distinct genotypes, designated A, B, and C. The genotype nomenclature of HEV71 strains for phylogenetic analyses based on the VP1 (17,25,28) and VP4 (29) nucleotide sequences has been reported, and the results (Figure 2) were consistent with previous findings. Among the HEV71 strains that were identified in our laboratory, only OC/99-Ikeda was classified in genotype C. Seven of eight strains identified in our laboratory by neutralization tests were classified in genotype B; five of these had the same VP4 nucleotide sequence. OC/00219, OC/00260, and OC/00261 were also classified in this genotype. This result demonstrated that strains OC/00219, OC/00260, and OC/00261 were HEV71 serotypes.

Comparison of the Complete VP1 Nucleotide and Deduced Amino Acid Sequences of HEV71 Strains

Strains OC/00219, OC/00260, and OC/00261 were classified in HEV71 by the phylogenetic analysis, although these viruses were not neutralized by monospecific anti-HEV71 sera. Because the VP1 protein contains a number of important neutralization sites (1,30), we determined the complete VP1 nucleotide sequences and compared the deduced amino acid sequences of OC/00219, OC/00260, and OC/00261. OC/00168 used as a prototype strain for HEV71 was also analyzed because this strain was neutralized by anti-HEV71/BrCr serum; moreover, its VP4 gene was the same as that of OC/00219 (Figure 2). The complete VP1 nucleotide sequences of these strains were 891 nt long, and the deduced amino acid sequences were 297 amino acids long. The differences of VP1 nucleotide sequences were 4 to 42 nt (0.4% to 4.7%), and the difference of deduced amino acid sequences was one amino acid (0.3%) among these viruses. The differences of VP1 nucleotide sequences between OC/00168 and OC/00219 were 4 nt (0.4%), and the deduced VP1 amino acid sequences of these strains were the same. The VP1 nucleotide and deduced amino acid sequences of OC/00260 and OC/00261 were

Table 4. Results of re-microneutralization tests with human enterovirus (HEV) monospecific antiserum, Osaka, Japan, 2000

			HEV monospecific antiserum		
Strain	Isolated cells	Predicted HEV Serotype[a]	Anti-EV18	Anti-HEV71/BrCr	Anti-HEV71/C7
OC/0071	RD-18S	EV18[b]	+	ND	ND
OC/0073	RD-18S	EV18	+	ND	ND
OC/00219	Vero	HEV71	ND	--	--
OC/00260	Vero	HEV71	ND	--	--
OC/00261	Vero	HEV71	ND	--	--
OC/00272	RD-18S	EV18	+	ND	ND

[a]Serotypes were predicted from the phylogenetic analysis in Figure 1.
[b] EV18 = echovirus 18; ND = Test not done.

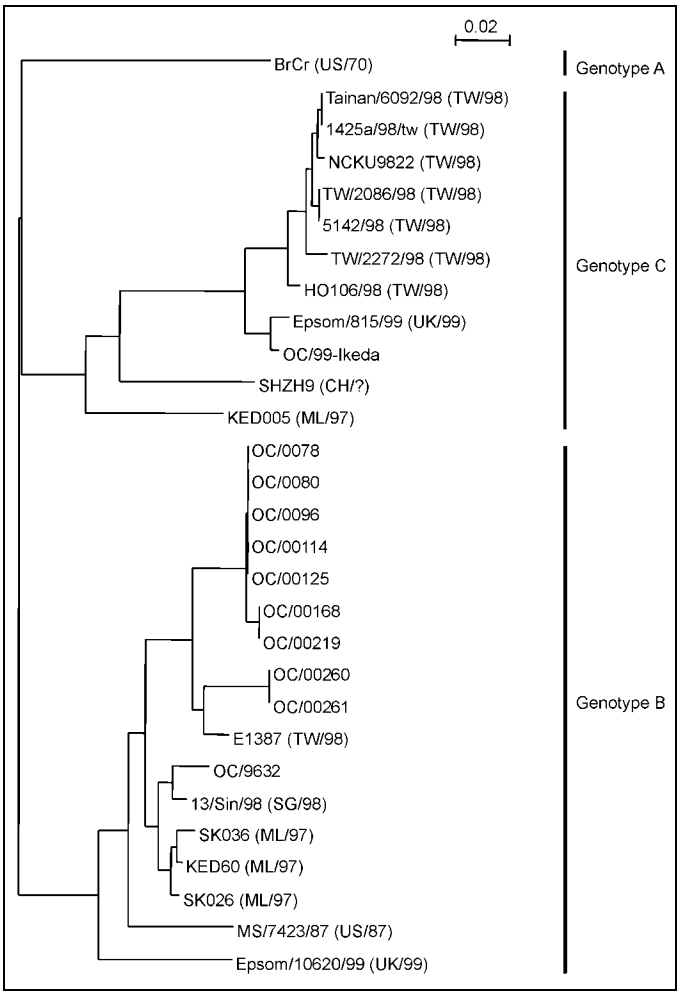

Figure 2. Phylogenetic analysis based on the human enterovirus 71 (HEV71) VP4 nucleotide sequences. The phylogenetic tree was constructed as described in the Figure 1 legend. The genotypes of the HEV71 cluster are denoted on the right. The VP4 sequences of 18 HEV71 strains available from GenBank are denoted by strain name, followed by the country and year isolated. Abbreviations for countries are as follows: US, United States; ML, Malaysia; CH, China; UK, United Kingdom; TW, Taiwan; and SG, Singapore. The accession numbers are as follows: BrCr; U22521, Tainan/6092/98; AF304459, 1425a/98/tw; AF176044, NCKU9822; AF136379, TW/2086/98; AF119796, 5142/98; AB037251, TW/2272/98; AF119795, HO106/98; AB037252, Epsom/815/99; AJ296213, SHZH9; AF302996, KED005; AB051334, E1387; AB051313, 13/Sin/98; AF251358, SK036; AB051333, KED60; AB051335, SK026; AB051332, MS/7423/87; U22522, Epsom/10620/99; AJ296214.

identical (data not shown). The deduced VP1 amino acid sequences of these four strains were compared with that of BrCr (Table 5). There were 18 amino acid (6%) differences between BrCr and other strains. The different amino acid positions of strains OC/00168, OC/00219, OC/00260, and OC/00261 against BrCr were the same. Any mutated residues distinguishable BrCr and OC/00168 from OC/00219, OC/00260, and OC/00261 were not recognized in the VP1 amino acid sequences.

Discussion

The serotype identification of HEVs has been performed by microneutralization tests using standard HEV antiserum pools (1,10). Since >60 serotypes of HEV are known to infect humans (1,19), the HEV serotype is almost impossible to identify by using monospecific antiserum from the first microneutralization test. Furthermore, the neutralization test is labor-intensive and time-consuming, requiring several weeks. As an alternative, identification based on nucleotide sequences has been used successfully in several laboratories (15,19,20, 23,29,31-35). To investigate the serotypes of the six untypeable HEV-like viruses that were not neutralized by the standard HEV antisera, we used phylogenetic analyses based on the complete VP4 nucleotide sequences of HEVs and were able to determine the serotype of each virus in the light of these results. OC/0071, OC/0073, and OC/00272 were thought to be EV18 strains by the phylogenetic analysis (Figure 1) and were neutralized by the monospecific anti-EV18 serum. These results indicate that the phylogenetic analysis based on the VP4 nucleotide sequence is consistent with the result of the microneutralization tests using the serotype-specific sera. OC/00219, OC/00260, and OC/00261 were thought to be HEV71 strains by the same analysis (Figure 1), but these viruses were not neutralized by the two monospecific anti-HEV71 sera. The phylogenetic analysis based on the HEV71 VP4 sequences confirmed that these viruses were HEV71 strains belonging to genotype B (Figure 2). We considered that OC/00219, OC/00260, and OC/00261 were all HEV71 strains not neutralized

Table 5. Differences in deduced VP1 amino acid sequences (aa 1-297) of human enterovirus 71 strains BrCr, OC/00168, OC/00219, OC/00260, and OC/00261

Amino acid position	Strain				
	BrCr[a]	OC/00168	OC/00219	OC/00260	OC/00261
18	Lys	Arg	Arg	Arg	Arg
22	Pro	Gln	Gln	Gln	Gln
30	Pro	Gln	Gln	Gln	Gln
31	Asp	Asn	Asn	Asn	Asn
43	Lys	Glu	Glu	Glu	Glu
58	Ala	Thr	Thr	Thr	Thr
98	Lys	Glu	Glu	Glu	Glu
145	Arg	Glu	Glu	Glu	Glu
164	Asp	Glu	Glu	Glu	Glu
167	Asp	Glu	Glu	Glu	Glu
172	Pro	Gln	Gln	Gln	Gln
183	Ser	Leu	Leu	Leu	Leu
184	Ser	Thr	Thr	Thr	Thr
244	Glu	Lys	Lys	Lys	Lys
246	Ser	Pro	Pro	Pro	Pro
249	Ile	Val	Val	Val	Val
275	Ser	Ala	Ala	Ala	Ala
282	Asp	Asn	Asn	Ser	Ser

[a]VP1 nucleotide sequence was obtained from GenBank (accession no. U22521) and translated into the deduced amino acid sequence.

by anti- HEV71/BrCr and anti-HEV71/C7 sera, both available as standard monospecific anti HEV71 serum in Japan. These results also indicate that phylogenetic analysis with the VP4 sequences of HEVs can identify the serotypes in the same way as neutralization tests with HEV serotype-specific antisera. We are now preparing antiimmune sera against OC/00219, OC/00260, and OC/00261, respectively, to confirm antigenically that these are the prime strains of HEV71 neutralized by anti-HEV71/BrCr serum.

Oberste et al. have shown that HEV VP1 nucleotide sequences correlate with antigenically defined serotypes and have demonstrated the utility of VP1 sequences as a molecular surrogate for antigenic type (19,35). They have also shown that the VP1 sequences have a better correlation with HEV serotypes than the 5' NTR or the VP4-VP2 junction (36). The phylogenetic analysis based on the VP4 sequences we have described also correlates well with HEV serotypings by anti-immune sera. We used 21 HEV serotypes antigenically defined in our laboratory and another 8 strains available from GenBank as prototype strains in this analysis. We do not know whether 29 serotypes are sufficient for the phylogenetic analysis of HEV, as there are >60 serotypes. The good result of HEV phylogenetic classification based on the VP4 sequences might depend on the prototype numbers (29 of 64 serotypes) that we used. Ishiko et al., who performed HEV phylogenetic analyses based on VP4 sequences (23), used 45 HEV serotypes as prototype strains and obtained a phylogenetic tree similar to ours (Figure 1) except for a difference in the prototype strain numbers. Another phylogenetic analysis based on the VP4 sequences in this article was performed against the HEV71 strains (Figure 2). For this analysis, the HEV71 strains were clustered in three distinct genotypes, and the nomenclature was almost the same as for the HEV71 analyses based on the VP1 nucleotide sequences (17,25). Recently, Chu et al. also reported the appropriateness of the phylogenetic analysis with the VP4 sequences for the molecular epidemiology of HEV71 outbreak in Taiwan in 1998 (29). These results suggest that the phylogenetic analysis based on the VP4 nucleotide sequences is also useful as a molecular surrogate for antigenic HEV serotyping. The analysis was more convenient based on the VP4 sequences than the VP1 sequences, since the complete VP4 sequence is 207 nt and the complete VP1 sequences are 834 to 951 nt (35), although the 3' third of the VP1 sequence of 365 nt was used (32).

The VP4 nucleotide sequences of OC/99-Hanasaka and OC/00272 were identical, but the results of neutralization assays were different. OC/99-Hanasaka was easily neutralized by HEV pooled sera against EV18, but OC/00272 was not. The same results were observed for strains OC/00168 and OC/00219 HEV71, i.e., the results of their neutralization tests differed in spite of the VP4 sequence identity. These results indicate that the VP4 nucleotide sequences are highly conserved even though the neutralizable epitopes are antigenic variants. We compared the VP4 nucleotide and deduced amino acid differences of HEV71 strains, BrCr (25), E1387 (15), OC/9632, OC/99-Ikeda, OC/0078, OC/00219, and OC/00260. HEV71 genotypes indicated 1 to 37 nt (0.5% to 17.9%) differences. However, we found no amino acid differences (100% identity) (Table 6). Complete homology of the HEV71 VP4-deduced amino acid sequences has also been described (20,29), and Singh et al. demonstrated amino acid substitutions in the VP2 and VP3 regions, with the greatest variation in VP1 (20). These results indicate that VP4 is the most stable protein; accordingly, VP4 genes will be suitable for the molecular identification of HEV serotypes in the future.

VP4 is not exposed on the outer surface of the capsid, and no neutralizable epitopes appear to exist in VP4. On the other hand, VP1, VP2, and VP3 are outer capsid proteins and contain neutralizable epitopes (37,38). A number of important neutralization epitopes may exist on VP1 (1,30,39). To confirm the important neutralization sites on VP1, we compared the deduced VP1 amino acid sequences of HEV71 strains OC/00168, OC/00219, OC/00260, and OC/00261. OC/00168 was neutralized by anti-HEV71/BrCr serum, while OC/00219, OC/00260, and OC/00261 were not. Comparison of the deduced VP1 amino acid sequences showed that no mutated residues on the VP1 region corresponded to the result of the neutralization tests. This result indicates that either the important neutralization epiotopes for anti-HEV71/BrCr serum do not exist on the VP1 protein, or the epitopes are specifically masked in the cases of OC/00219, OC/00260, and OC/00261. Further analysis against the VP2 and VP3 regions of these strains should allow interpretation of these findings.

Table 6. Number of nucleotide and deduced amino acid differences between the VP4 genes of human enterovirus 71 strains,[a]

	BrCr	E1387	OC/9632	OC/0078	OC/00219	OC/00260	OC/99-Ikeda
BrCr		33	37	36	37	32	34
E1387	0		10	7	8	7	33
OC/9632	0	0		13	14	15	37
OC/0078	0	0	0		1	10	37
OC/00219	0	0	0	0		11	36
OC/00260	0	0	0	0	0		34
OC/99-Ikeda	0	0	0	0	0	0	

[a]Nucleotide numbers are given above the diagonal and amino acid numbers below it.

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Listeria monocytogenes Infection in Israel and Review of Cases Worldwide

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Listeria monocytogenes, an uncommon foodborne pathogen, is increasingly recognized as a cause of life-threatening disease. A marked increase in reported cases of listeriosis during 1998 motivated a retrospective nationwide survey of the infection in Israel. From 1995 to 1999, 161 cases were identified; 70 (43%) were perinatal infections, with a fetal mortality rate of 45%. Most (74%) of the 91 nonperinatal infections involved immunocompromised patients with malignancies, chronic liver disease, chronic renal failure, or diabetes mellitus. The common clinical syndromes in these patients were primary bacteremia (47%) and meningitis (28%). The crude case-fatality rate in this group was 38%, with a higher death rate in immunocompromised patients.

Listeria monocytogenes (Lm) is a ubiquitous pathogen in the environment, capable of causing human and animal infection. Although uncommon in humans, it occurs in sporadic and epidemic forms throughout the world (1-3); a recent multistate outbreak was reported in the United States (4). Most and perhaps all forms of listeriosis in humans result from foodborne transmission (5). In its most severe form, listeriosis is an invasive disease that affects immunocompromised patients and has the highest case-fatality rate of foodborne illnesses (6-10). In immunocompetent persons, it can also cause severe disease (attributed by some investigators to ingestion of high infective doses), as well as outbreaks of benign febrile gastroenteritis (11). Another form of human disease is perinatal infection, which is associated with a high rate of fetal loss (including full-term stillbirths) and serious neonatal disease (12).

Lm infection has been a reportable disease in Israel since 1993. A preliminary report from the Ministry of Health (MOH) claimed a fivefold increase in incidence from 1996 to 1998, but the information was incomplete (13). Our study was undertaken to delineate trends and better characterize the epidemiologic and clinical features of this emerging infection in Israel and to compare these findings with those reported in recent publications worldwide.

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Material and Methods

The Israeli Survey

Of the 24 general (acute-care) hospitals in Israel, 11 are large, with 500-1,200 beds, 8 have 300-499 beds, and 5 have <300 beds. Information on Lm infections was collected by contacting infectious disease specialists in each of the 19 larger hospitals. The specialists were asked to identify retrospectively all patients with listeriosis (as defined below) from the period 1995-1999 in their hospitals and to complete a questionnaire on each. Questionnaires were completed from 17 of the hospitals (11 large, 6 intermediate), and complementary information was retrieved from the MOH passive and active surveillance files on 4 additional hospitals (1 intermediate and 3 small). These 21 hospitals represented approximately 95% of the total acute-care beds in Israel during the study period.

One hundred sixty-one patients with Lm infection were identified. Clinical information was available for all patients except five (3%: two with positive blood cultures and one with a positive vaginal culture who were not hospitalized, and two with positive blood cultures whose hospital charts could not be retrieved).

Lm isolates were identified by standard methods in the microbiology laboratory in each medical center, then sent to the Reference Laboratory for Listeria in Jerusalem for confirmation.

Listeriosis was defined as the growth of Lm (as confirmed at the reference laboratory) from any body site. An infection in a pregnant woman and her fetus or neonate was considered a single perinatal event.

[1]Dr. Lang died November 18, 2001.

Worldwide Review

We conducted a MEDLINE search for studies describing nonselective, population-based surveys of Lm infections in the English language literature of the last decade (1990-2000). All case series describing at least 15 nonperinatal, nongastroenteritis infections were included in the review of nonperinatal listeriosis. All case series describing at least 15 perinatal cases were included in the review of perinatal listeriosis.

Results

The Israeli Survey (1995-1999)

The 161 cases identified during the 5-year study period included 91 (57%) nonperinatal and 70 (43%) perinatal infections. The average annual incidence during the study period was 0.6/100,000 population. The marked increase in 1998 (Figure 1) was exclusively in perinatal cases; the reason for the increase remains unclear. There were no clusters in place during any of these years. Infection occurred throughout the year, but more often during summer and fall, with 70% of cases occurring from May to October (Figure 2).

Nonperinatal Cases

The mean age of the 87 nonperinatal cases with available clinical information was 67 years (range 4-91), 66 (76%) were ≥60 years of age (Figure 3); 56 (64%) were male. Sixty-four patients (74%) had severe immunocompromising conditions (Table 1). Of 45 patients (52%) with malignant disease, most had received chemotherapy, steroid therapy, or both during the month before the Lm infection. Other immunocompromising conditions were chronic renal failure (11 patients, 4 of whom were on hemodialysis), chronic liver disease (10 patients, mostly with cirrhosis), and diabetes mellitus (13 patients). Some of these patients had additional immunocompromising conditions (Table 1). Twenty-three patients (26%) were not immunocompromised. Most (19 [83%] of 23) were ≥63 years of age; some had concomitant conditions not considered to be immunocompromising, including three patients with valvular heart disease, predisposing them to endocarditis. Only four immunocompetent patients were <60 years of age, including a 4-year-old girl and a 38-year-old man with primary bacteremia, a 22-year-old woman with typical pyelonephritis and Lm cultured from blood only, and a 51-year-old man who had gastroenteritis and positive blood cultures (stool was not cultured for Lm).

Clinical syndromes in the 87 nonperinatal cases were primary bacteremia in 41 (47%), meningitis in 24 (28%), bacteremia with a focal infection in 18 (21%), and focal infection without bacteremia in 4 (5%) (Table 2). Six patients with meningitis had Lm isolated from both blood and cerebrospinal fluid (CSF). Two patients with primary bacteremia had symptoms suggestive of meningitis (confusion, aggressiveness), but they died shortly after admission without having a lumbar puncture performed.

The case-fatality rate in the nonperinatal group was 38% (33 of 87). Twelve of the 33 deaths occurred within 48 hours of admission or disease onset. We observed a lower mortality rate (6 [19%] of 31) among persons who received a penicillin (mostly ampicillin) as empiric therapy, compared with those who received a penicillin only after culture results were reported (9 [30%] of 30), but this difference was not statistically significant (p=0.25). The difference in death rates in immunocompromised (28 [44%] of 64) compared with immu-

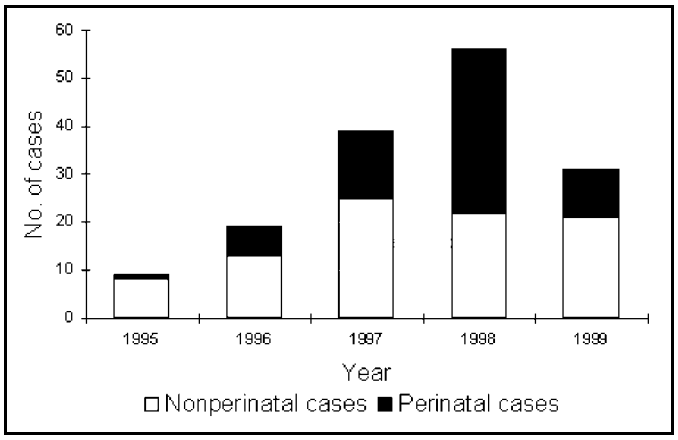

Figure 1. Number of cases of perinatal and nonperinatal *Listeria monocytogenes* infection, Israel, 1995–1999.

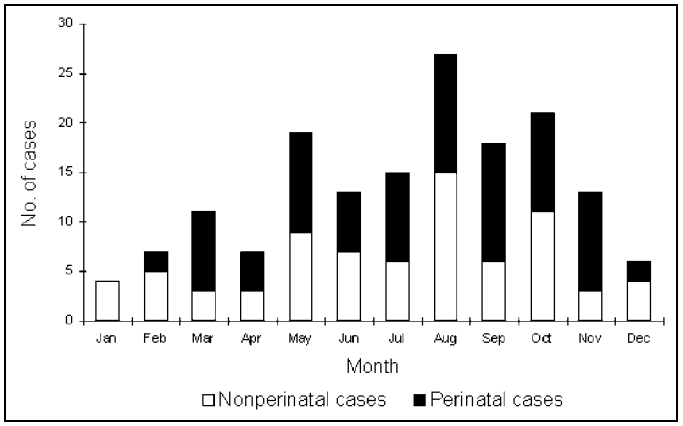

Figure 2. Seasonal occurrence of *Listeria monocytogenes* infection, Israel, 1995–1999.

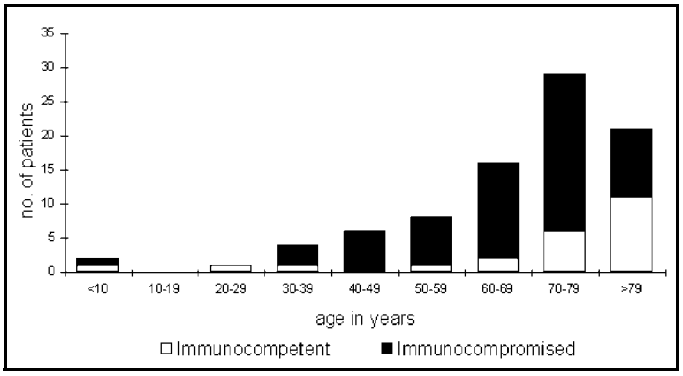

Figure 3. Age distribution of 87 nonperinatal cases of *Listeria monocytogenes* infection by immune-status group, Israel, 1995–1999.

Table 1. Immunocompromising conditions in 64 cases of non-perinatal *Listeria monocytogenes* infection, Israel, 1995–1999

		Additional underlying conditions				
Main underlying illness	No. of cases	Steroids/ chemotherapy	Chronic renal failure	Chronic liver disease	Diabetes mellitus	Others
Hematologic malignancy	23	19	3	2	7	8[a]
Solid malignancy	22	9				
Chronic renal failure	8[b]	1			2	1[c]
Chronic liver disease	8	1			1	2[c]
Diabetes mellitus	3	1				

[a]Splenectomy (2 cases) neutropenia (5) vasculitis (1).
[b]Hemodialysis (4 cases).
[c]Severe congestive heart failure (3 cases).

nocompetent patients (5 [22%] of 23) had borderline statistical significance (p=0.05). There was no correlation between death and age for the whole group; however, all five immunocompetent patients who died were >80 years of age.

Perinatal Cases

Clinical information was available on 69 pregnant women (mean age 28 years; range 21-40 years) and their offspring. Twenty-seven pregnancies (gestational age 9-26 weeks) resulted in intrauterine fetal death and miscarriage, one full-term infant was stillborn, and three others (born at 26, 29, and 39 weeks) died within 24-48 hours of birth (Table 3), for a mortality rate among offspring of 45%. Seventeen (55%) of the 31 infected mothers whose offspring died were bacteremic. For the other 14 mothers, Lm was isolated from other sites, including placenta, amniotic fluid, and fetal tissue (Table 3).

The other 38 mothers gave birth to live infants, 16 of whom had no evidence of Lm infection. Eleven of the 16 uninfected infants were delivered when the mothers had active Lm amnionitis (gestational ages 25 to 40 weeks), and 5 were delivered several weeks after the maternal infection, which occurred at weeks 19, 21, 35, 36, and 37. Twenty-two infants had evidence of Lm infection after birth, 18 within 48 hours of delivery and 4 on days 4-8. Only two (11%) of the 18 infants with early infection had meningitis, compared with all 4 with later onset of infection.

All infected mothers had mild illness and recovered uneventfully; none had meningitis. One mother had an underlying immunocompromising condition (systemic lupus erythematosus).

Table 2. Clinical syndromes in 87 cases of nonperinatal *Listeria monocytogenes* infection, Israel, 1995–99

Clinical syndrome	Immuno-compromised	Immuno-competent	Total
Bacteremia without focus	34 (53%)	7 (30%)	41 (47%)
Meningitis	17[a] (27%)	7 (30%)	24 (28%)
Bacteremia with focus	9[b] (14%)	9[c] (39%)	18 (21%)
A focus without bacteremia	4[d] (6%)	0	4 (5%)
Total	64 (100%)	23 (100%)	87 (100%)

[a] With bacteremia (6 cases), with pneumonia (1 case).
[b] Endocarditis (3 cases), peritonitis (2), pneumonia (4, one with shunt infection).
[c] Endocarditis (3 cases), gastroenteritis (3), pyelonephritis (2), anal abscess (1).
[d] Peritonitis (3 cases), pleuritis (1).

Worldwide Review (1990-2000)

Nine case-series of nonperinatal listeriosis and five case-series of perinatal infection matched the inclusion criteria. These reports and our study provided 1,250 cases of nonperinatal and 494 cases of perinatal listeriosis for analysis (Tables 4 and 5). Nonperinatal infection constituted, on average, 65% (1,025 of 1,583) of cases among studies that supplied this information (Table 4). In total, 1,250 cases of nonperinatal infections were reviewed; information about mortality was provided for 1,129 patients. The patients' ages ranged from <1 year to >90 years, but most were >60 years of

Table 3. Types of infection, sources of cultures, and outcome in 69 cases of perinatal *Listeria monocytogenes* infection, Israel, 1995–99

		Mothers' cultures			Infants' cultures		
Type of mother-infant infection	No. of cases	Blood only	Blood and tissue	Tissue only	Blood only	Blood and tissue	Tissue only
Uninfected mother and infected infant	13 (19%)				4	7 (3)	2 (2)
Infected mother and infected infant	9 (13%)	2	1	6	3	2	4 (1)
Infected mother and uninfected infant	16 (23%)	9	3	4			
Fetal/neonatal death (amnionitis)	31[a] (45%)	4	13	14	1	1 (1)	
Total	69 (100%)		54			24	

[a]Includes 27 intrauterine deaths with abortions, 1 stillbirth, and 3 early neonatal deaths. Numbers in parenthesis are cases of meningitis. Tissue denotes any material that is not blood, such as cerebrospinal fluid, placenta, and amniotic fluid.

age. The mean ages in the different series ranged from 50 to 67 years; 60% were male. Annual incidence rate varied widely (i.e., 0.1-1.1 per 10^5), not only between countries but also between consecutive years in the same setting. Most authors also described seasonal variation, with a peak incidence in summer possibly related to seasonal consumption of specific food products (8) or to more frequent breakdowns in food handling in higher temperatures.

Most (74%) of the persons affected in the reported cases (Table 4) were immunocompromised. Malignancy, chemotherapy, steroid therapy, organ transplantation, alcoholism, liver disease, renal insufficiency, and diabetes mellitus were most commonly reported, with few cases of acquired immunodeficiency syndrome.

With regard to clinical syndromes, the most common (47%) site of infection was the central nervous system (CNS) (Table 4), frequently associated with bacteremia. An additional 48% of patients were bacteremic without CNS involvement.

Perinatal infection constituted 34% (470 of 1,378) of cases among studies that supplied this information (Table 5). In total, 494 cases of perinatal infections were reviewed. Infection during the first two trimesters of pregnancy was almost invariably fatal to the fetus. One hundred six (21%) of the 494 pregnancies reviewed here resulted in intrauterine death.

Two hundred seventy-eight (56%) live-born infants had neonatal listeriosis and survived. Most of this neonatal infection was of early onset (209 cases), but the definition of early onset varied (from ≤5 to ≤7 days), and information concerning day of onset was incomplete in some series (Table 5). Almost all the late-onset infections (69 cases) were of the CNS. An additional 61 infants (12%) with neonatal listeriosis died from the infection in the postnatal period, for an overall intrauterine and postnatal mortality rate of 34% (167 of 494). In 49 (10%) of the affected pregnancies, the infant was born alive and without evidence of listeriosis.

Table 4. Characteristics of nonperinatal listeriosis from 10 recently reported series

	First author, year (ref)										
Characteristic	McLauchlin, 1990 (8)	Gellin, 1991 (6)	Cherubin, 1991 (14)	Skogberg, 1992 (15)	Nolla-Salas, 1993 (16)	Jones, 1994 (17)	Paul, 1994 (18)	Bula, 1995 (10)	Goulet, 1996 (9)	Siegman-Igra, 2001 (present study)	Total or average
Country and scope	England, national	USA, six areas	USA, four centers	Finland, Helsinki	Spain, Barcelona	England, Bristol	Australia, Sydney	Switzerland, western part	France, national	Israel, national	Worldwide
Study period	1967-1985	1986	1982-1999	1971-1989	1990	1983-1992	1983-1992	1983-1997	1992	1995-1999	1967-1999
Total no. of cases	722	246	119	74	31	29	84	122	225[a]	156	1,808
Nonperinatal cases (% of total)	474 (66%)	179 (73%)	54 (45%)	58 (78%)	29 (94%)	16 (55%)	71 (85%)	57 (47%)	225 (NA)	87 (56%)	1,025/1583 (65%) 1,025+225=1,250
Mean age (range) (years)	59 (1-97)	NA (<1-95)	NA	50 (29-66)	58 (17-89)	60 (1-95)	39% (>60)	66 (31-96)	65 (1-101)	67 (4-91)	50-67
Male gender	265 (58%)	101 (56%)	NA	NA	24 (77%)	9 (56%)	NA	33 (58%)	135 (62%)	56 (64%)	623 (60%)
Peak season	Autumn and spring	Late spring to fall	May-Aug	NA	39% in July-Sept	76% in July-Dec	NA	NA	NA	70% in May-Oct	Summer and fall
Annual incidence[b]	NA	0.7	NA	0.09 - 0.65	1.1	0.35	0.3	NA	NA	0.6	0.1-1.1
Immunocompromised	261/337 (77%)	NA	53/ 54(98%)	47/58 (81%)	24/29 (83%)	13/16 (81%)	59/71 (83%)	25/57 (42%)	159/225 (71%)	64/87 (74%)	705/934 (74%)
CNS infection	268/474 (57%)	55/179 (31%)	19/54 (35%)	29/58 (50%)	9/31 (29%)	6/16 (37%)	29/71 (41%)	45/57 (79%)	110/224 (49%)	24/87 (28%)	594/1,251 (47%)
Bacteremia ± focus[c]	183/474 (39%)	119/179 (66%)	35/54 (65%)	24/58 (41%)	20/31 (65%)	10/16 (73%)	40/71 (56%)	12/57 (21%)	97/224 (43%)	59/87 (68%)	599/1,251 (48%)
Focal disease only[d]	9/474 (5%)	5/179 (3%)	-	5/58 (8%)	2/31 (6%)	-	2/71 (3%)	-	17/224 (8%)	4/87 (5%)	44/1,124 (4%)
Mortality	164/371 (44%)	63/ 179(35%)	17/ 54(31%)	15/ 58(26%)	16/31 (52%)	6/16 (37%)	27/71 (38%)	18/57 (32%)	54/225 (24%)	33/87 (38%)	413/1,149 (36%)

[a]Includes nonperinatal cases only.
[b]Estimated annual incidence per 10^5 population.
[c]Bacteremia with or without a non-CNS focus of infection (e.g., pneumonia, endocarditis, urinary tract infection, prostatitis, peritonitis, gastroenteritis, rectal abscess, osteomyelitis, and cellulitis).
[d]For example, peritonitis, pleuritis, arthritis, pericarditis, cholecystitis, appendicitis, and cellulitis.
NA= not available; CNS= central nervous system

Table 5. Characteristics of perinatal listeriosis from six recently reported series

Characteristic	First author, year (ref)						Total or average
	McLauchlin, 1990 (12)	Gellin, 1991 (6)	Cherubin, 1991 (14)	Craig, 1996 (19)	Nolla-Salas, 1998 (20)	Siegman-Igra, 2001 (present study)	
Country and region	England, national	USA, six areas	USA, four centers	Australia, Melbourne	Spain, Barcelona	Israel, national	Worldwide
Study period	1967-1985	1986	1982-1999	1983-1994	1990-1996	1995-1999	1967-1999
Total no. of cases	722	246	119	24[a]	135	156	1,400
Perinatal infection (% of total)	248 (34%)	67 (27%)	65 (55%)	24 (NA)	21 (16%)	69 (44%)	470/1,378 (34%) 470+24=494
Estimated incidence per 10^4 births	NA	0.8-2.4	NA	2	0-4.1	1.4	0.6-4.1
Average maternal age (range) (years)	NA	26 (17-35)	NA	NA (18-39)	30 (26-34)	28 (21-40)	NA (26-30)
Early neonatal infection and survival	114[b] (46%)	31 (46%)	20 (31%)	14[c] (58%)	11 (52%)	19 (28%)	209/494 (42%)
Late neonatal infection and survival	36[d] (15%)	8[e] (12%)	21(32%)		1[d] (5%)	3[d] (4%)	69/494 (14%)
Infected mother and uninfected infant	9 (4%)	13 (19%)	2 (3%)	4 (17%)	5 (23%)	16 (23%)	49/494 (10%)
Intrauterine death	42 (17%)	14 (21%)	15 (23%)	4 (17%)	3 (14%)	28 (41%)	106/494 (21%)
Postnatal death	47 (19%)	1 (1%)	7 (11%)	2 (8%)	1 (5%)	3 (4%)	61/494 (12%)
Gestational age at abortion (weeks)	12-28	11-30	NA	18-29	10-27	9-26	9-29
Immunocompromised mothers	5			1	1	1	8[f]

[a]Includes only perinatal cases.
[b]Including 29 with unknown time of onset.
[c]No differentiation between early and late neonatal infection.
[d]>5 days.
[e]>7 days.
[f]2 diabetes mellitus, 2 renal transplant, 2 systemic lupus erythematosus, 1 Crohn disease and steroids, 1 HIV infection.
NA= not available

Discussion

Ingestion of Lm is a very common occurrence (1,2) since it has been isolated from many food products in Israel (unpub. data, MOH) as well as in many countries worldwide. Development of invasive disease secondary to Lm ingestion is determined primarily by the integrity of the immune system of the host (predominantly cell-mediated immune defects) and possibly also by inoculum size (11). The organism crosses the mucosal barrier of the intestine and invades the bloodstream. It may disseminate to any organ, but it has a clear predilection for the placenta and CNS, thereby determining the main clinical syndromes.

The case-fatality rate in the collected data on perinatal infection was 36% (413 of 1,149 patients for whom this information was available). This high mortality reflects both the severity of Lm infection and the seriousness of the underlying conditions. Higher mortality rates were correlated with older age, presence of CNS infection, and immunodeficiency (5,6,8,15,21). One study reported that deaths in immunocompetent patients occurred exclusively in the elderly (9), a finding that correlates well with our observations.

An unexpected observation in our study was the occurrence of hospital-acquired listeriosis in adults. The presumed hospital acquisition occurred on day 3-67 of hospital stay in 59 (16%) of 369 cases with relevant information, as reported in four studies, including ours (9,16,18). All patients acquiring listeriosis in the hospital (except one) were immunocompromised. No clustering of cases in time or place occurred, and no case had an obvious source for nosocomial acquisition of Lm. Because the incubation period of listeriosis is long (11-70 days) and fecal carriage not uncommon (5%-10%) (1,2), colonization could have been acquired before hospitalization and infection developed in the hospital, possibly even triggered by increased immunosuppression. Another possible explanation is consumption of contaminated food brought in from sources outside the hospital, but this could not be documented. We found only one description of a hospital outbreak of Lm among adults (three cases secondary to an index one), but the method of transmission was not established (22). Hospital transmission among neonates in nurseries was thought to occur more frequently (24%) (12) and was described by several investigators (18,23,24).

Among perinatal infections, we report the highest case-fatality rate (45%). This observation could be related to the frequency of taking cultures from aborted tissues. The diagnosis of Lm can easily be missed if cultures are not routinely

taken from aborted fetal tissues or if blood (and other) cultures are not obtained from febrile pregnant women. The great variability in incidence rates and other epidemiologic features between studies and among medical centers within studies suggests that many cases escaped diagnosis.

Concerning the mothers, all authors described a mild febrile "influenzalike" illness, without maternal deaths. Only one of the 494 mothers had meningoencephalitis with Lm isolated from the cerebrospinal fluid, but underlying condition or maternal and fetal outcomes were not reported (12). Eight mothers (<2%) were immunocompromised (Table 5), but no comparable data are available on the prevalence of these conditions among pregnant women in general.

In conclusion, listeriosis is an emerging zoonosis that constitutes a life-threatening disease for human fetuses and neonates, the elderly, and patients with certain predisposing conditions. Documented cases may not represent the true incidence in the community, especially with regard to perinatal infection. Fetal and maternal cultures should be obtained in every case of spontaneous abortion or stillbirth, to ensure proper diagnosis. Empiric ampicillin therapy should be included in the treatment of neonatal meningitis, sepsis, or meningitis in the elderly and immunocompromised patients and in febrile pregnant women without a source of infection.

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Epidemiologic Features of Four Successive Annual Outbreaks of Bubonic Plague in Mahajanga, Madagascar

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From 1995 to 1998, outbreaks of bubonic plague occurred annually in the coastal city of Mahajanga, Madagascar. A total of 1,702 clinically suspected cases of bubonic plague were reported, including 515 laboratory confirmed by *Yersinia pestis* isolation (297), enzyme-linked immunosorbent assay, or both. Incidence was higher in males and young persons. Most buboes were inguinal, but children had a higher frequency of cervical or axillary buboes. Among laboratory-confirmed hospitalized patients, the case-fatality rate was 7.9%, although all *Y. pestis* isolates were sensitive to streptomycin, the recommended antibiotic. In this tropical city, plague outbreaks occur during the dry and cool season. Most cases are concentrated in the same crowded and insanitary districts, a result of close contact among humans, rats, and shrews. Plague remains an important public health problem in Madagascar, and the potential is substantial for spread to other coastal cities and abroad.

Plague is enzootic in the central highlands of Madagascar, where approximately 200 to 400 bacteriologically confirmed or presumptive cases are reported each year to the World Health Organization; 1,500 to 2,500 clinically suspected cases are reported by the national surveillance system (1,2). In this island, the main reservoir of *Yersinia pestis* is the black rat (*Rattus rattus*) and the main vector the rat flea (*Xenopsylla cheopis)* (3). From the arrival of plague in Madagascar in 1898 until the 1920s, plague occurred in several harbors around the island. It disappeared progressively from the coastal areas as soon as it reached the central highlands where, above an altitude of about 800 m, it found a suitable environment. A period of quiescence in the coastal areas lasted more than 60 years. Then, in August 1991, a sudden outbreak lasting 7 months occurred in the harbor of Mahajanga (4). During the next 3 years, when neither an epizootic nor a human case was reported, the outbreak was considered an isolated epidemiologic incident. However, in 1995 a new epidemic occurred (5,6), which was followed by three others, in 1996, 1997, and 1998. These outbreaks accounted for approximately 30% of the reported cases of plague in Madagascar during the period. We describe the main epidemiologic features of these four urban outbreaks in this exceptional resurgent coastal plague focus.

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Population and Methods

In Madagascar, health workers must report all clinically suspected cases of plague to the national surveillance system. For each patient, a biological sample (bubo aspirate, sputum, or postmortem liver or lung puncture, whenever appropriate) has to be collected and sent by mail to the National Reference Laboratory at the Institut Pasteur de Madagascar in the capital, Antananarivo. The delivery time is frequently 2-3 weeks after the specimen was collected, and the only reliable method to allow bacteriologic diagnosis remains bubo aspiration (as well as sputum and postmortem liver or lung puncture, whenever appropriate) and transportation on a swab in Cary-Blair medium. Blood samples for culture were not adopted in Madagascar since the likelihood of isolating *Y. pestis* is approximately twice as high in buboes as in blood. In August and September 1997, a temporary bacteriology laboratory was established in Mahajanga; thus, all the biological samples collected during these 2 months were processed on site.

The confirmatory diagnosis was based on bacteriologic methods. A case of plague was considered to be confirmed as soon as a strain of *Y. pestis* was isolated by culture or mouse inoculation. A patient was considered to have a presumptive plague case when *Y. pestis* could not be isolated but gram-negative bacillus, with morphologic patterns of *Y. pesti*, could be detected on smear. Microscopy lacks both sensitivity and specificity, and the isolation of *Y. pestis* requires at least 6 days. Prior treatment of patients with antibiotics impedes the culture and may lead to false-negative results. Physicians were asked to collect an acute-phase serum sample before treatment and a convalescent-phase serum sample at least 7 days after the onset of disease. Whenever available, the sera and bubo aspirates were tested for F1-antigen by immunocapture enzyme-linked immunosorbent assay (ELISA) (7,8) and for anti-F1 antibodies by the classical indirect ELISA method (9).

In this study, the bacteriologically confirmed or presumptive patients, the ELISA-positive patients (F1 antigen or antibodies), or both were defined as having laboratory-confirmed cases. Clinical and epidemiologic data were collected on standard forms, after the patients or their families were interviewed. Reporting to the national surveillance system was assumed to be thorough, as confirmed by a seroepidemiologic survey in 1999 (10). In Madagascar all *Y. pestis* isolates are screened for their in-vitro resistance to streptomycin,

gentamicin, chloramphenicol, tetracycline, sulfamethoxazole-trimethoprim, and ampicillin.

The geographic distribution of plague patients was visualized by using a simplified map of Mahajanga. For this purpose, the city was divided into four areas. We defined the boundaries of these areas by aggregating districts that were comparable for population density, sanitation level, and housing type.

Results

In 1995, the first identified case occurred in March, followed by several sporadic cases in May and July. The outbreak proper started by mid-August. From 1995 to 1998, 1,702 clinically suspected bubonic plague cases were reported; 335 were considered confirmed (297) or presumptive (38) cases after bacteriologic testing. None of the *Y. pestis* isolates was recovered from sputum. When either F1 antigen capture or anti-F1 ELISA assays were used, 180 more cases were laboratory confirmed. In all, 515 persons were considered to have contracted plague from January 1, 1995, to December 31, 1998.

For each of the 4 years we studied, a biological result was available for 88.5%, 98.7%, 97.2%, and 99.5% of the suspected patients, respectively. When bacteriologic methods were used, the annual confirmation rates were 22.2%, 14.8%, 30.1%, and 30.3%, respectively. The proportions represented by bacteriologically confirmed cases among the total number of laboratory-confirmed patients were 72.3%, 83.6%, 98.7%, and 88.1%, respectively. Detailed laboratory results are summarized in the Table.

Two of the 297 *Y. pestis* isolates from Mahajanga patients were resistant to one of the tested antibiotics, one to chloramphenicol in 1996 and one to ampicillin in 1998.

The proportion of males (56.1%) was significantly higher among cases than in the general population (p=0.006). The age and sex-distribution of patients with laboratory-confirmed cases remained unchanged during the 4 years (Figure 1). The median age of patients was 15 years, and 75% of patients were <25 years old. Although the highest incidence of the disease was observed in 5-to 19-year-old persons, 59 cases occurred in children <5 years old; 2 were <1 year old.

Among laboratory-confirmed cases, a significantly higher frequency of cervical and axillary buboes occurred in children; by contrast, inguinal buboes represented about 80% of cases in persons ≥20 years of age ($p<10^{-7}$). The distribution of bubo location according to age is shown in Figure 2. Body temperatures were available for 454 of persons with laboratory-confirmed cases: the median temperature was 39.5°C (25th and 75th percentiles were 38.2°C and 40°C). Diarrhea (7.1%), prostration (4.5%), and coma (1%) were the other most frequently reported symptoms.

A total of 507 laboratory-confirmed patients were admitted to hospital; 40 (7.9%) of them died. The case-fatality rate was not significantly different by year (7.1%, 9.3%, 6.7%, and 10.3% in 1995, 1996, 1997, and 1998, respectively). Nor was this rate related to age, sex, bubo location, or delay between onset of disease (as reported by the patients) and initiation of treatment. Lethality was also not correlated with drug susceptibility of *Y. pestis* isolates, since they were all sensitive to streptomycin, the drug recommended by the national program. Only the body temperature at admission to the hospital was significantly higher in the group of deceased patients than in recovered (39.6°C vs. 39.1°C, p=0.01).

Table. Results of bacteriology testing for *Yersinia pestis*, Mahajanga, Madagascar, by year

	1995	1996	1997	1998	Total
Total suspected cases	558	399	539	206	1,702
Laboratory-confirmed[a] cases	117	97	214	87	515
Bacteriology					
Number tested	342	330	501	195	1,368
Number confirmed	55	41	149	52	297
Number presumptive	21	8	2	7	38
Number positive[b]	76	49	151	59	335
Percent positive	22.2	14.8	30.1	30.3	24.5
F1 antigen ELISA					
Number tested	433	335	413	189	1,370
Number positive	38	25	131	35	229
Percent positive	8.8	7.5	31.7	18.5	16.7
Anti-F1 antibodies ELISA					
Number tested	365	344	396	191	1,296
Number positive	68	59	137	48	312
Percent positive	18.6	17.2	34.6	25.1	24.1

[a]Confirmed + presumptive and/or enzyme-linked immunosorbent-assay (ELISA) positive.
[b]Confirmed + presumptive cases

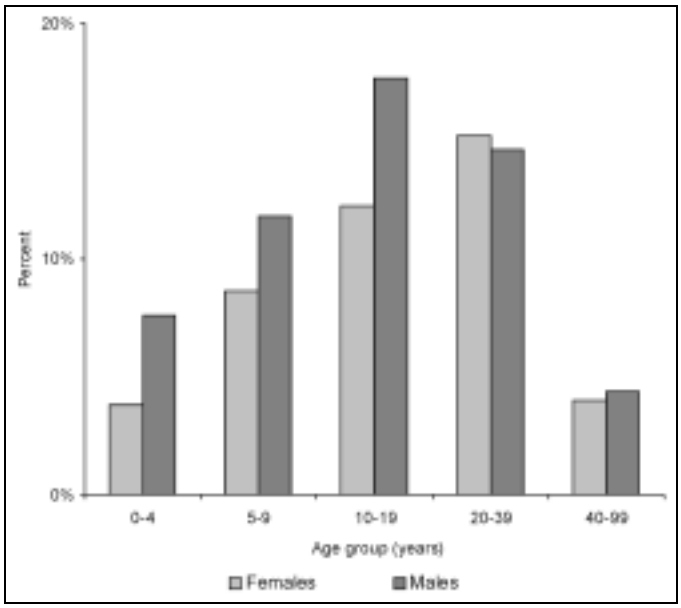

Figure 1. Age and sex-distribution of laboratory-confirmed bubonic plague cases, Mahajanga, Madagascar

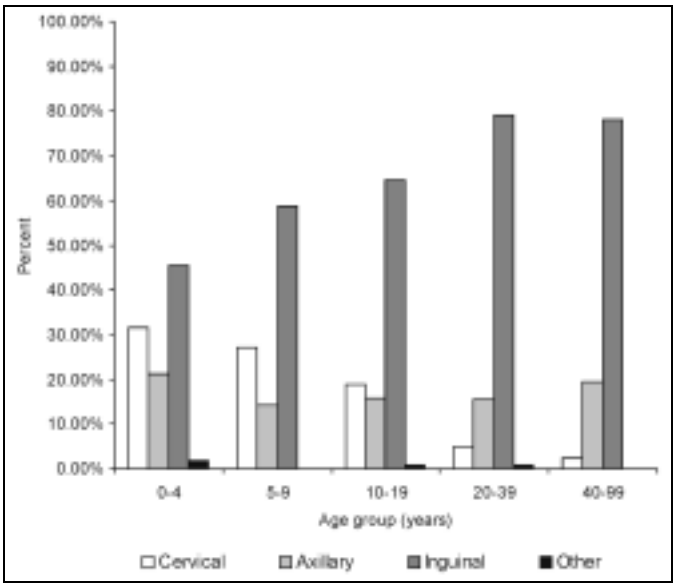

Figure 2. Distribution of bubo location according to age in laboratory-confirmed bubonic plague cases, Mahajanga, Madagascar.

Most (76%) patients were reported during August through October during the dry season, a peak that occurs every year. The temperature patterns in Mahajanga and the monthly distribution of laboratory-confirmed cases are related. The outbreaks used to occur in July, when the temperature is the lowest of the year, and ceased in November, when the temperature rebounds (Figure 3).

Among the 357 laboratory-confirmed plague patients for whom data were available about rat deaths within the 15 days preceding onset of disease, 203 (56.9%) had found dead rats indoors or in the vicinity of their homes; 154 (43.1%) had not noticed dead rats in their surroundings. Of the 203 who had, 117 (57.6%) had found the dead rats inside their homes. The confirmation rate was higher among persons who reported rat deaths in their surroundings than among persons not reporting such deaths (57.7% versus 24.6%, $p<10^{-7}$).

The geographic distribution of plague laboratory-confirmed cases according to districts is shown in Figure 4. The residence of patients could be clearly identified on this map for 473 (91.8%) of the laboratory-confirmed cases. The incidence of plague differed sharply according to districts: most patients (82.9%) lived in Area 1, which pools the most unhealthy and densely populated districts of the town. The southwestern part of the city (Area 2), including the harbor and the old colonial town, had few cases and did not show any trend towards increasing incidence. Areas 3 and 4, greener and less populous suburbs of Mahajanga, showed an intermediate incidence. The increase in cases in 1997, especially in Area 3, was no longer occurring in 1998.

Discussion

World plague foci are mostly restricted to temperate climate highlands such as regions in East Africa, central Asia, and the American Southwest; outbreaks in coastal areas have become rare. Thus, the situation in the harbor of Mahajanga, where plague has found favorable conditions and seems to have established itself, is noteworthy. During 4 successive years, 97 to 214 laboratory-confirmed plague cases were reported annually, raising questions about epidemiologic determinants of this disease's having taken roots in this tropical city. Before its sudden reemergence in 1991 after more than 60 years of quiescence, plague in Madagascar was supposedly restricted to areas above 800 m because of climatic constraints that influence the proliferation of fleas and *Y. pestis* (11). In Mahajanga, as had already been observed in 1907 (12), the plague season starts in July or August, during the dry season, when the air temperature is the lowest. This is in contrast with the central highlands, where most of the cases occur between October and February, during the warmer rainy season (2). Indeed, during the plague season for both the coastal and plateau regions, the minimum temperature is about the same, between 17° and 22°C. Recent studies have shown that the plague season in the central highlands is clearly linked to the abundance both of fleas and the black rat, *R. rattus*, the main reservoir and virtually the only small mammal found in houses (95% of captures in traps) (13). In Mahajanga, ongoing studies have shown that the Asiatic shrew, *Suncus murinus*, accounts for up to 75 % of the trapped animals and is a regular carrier of the rat flea, *X. cheopis* (Duplantier et al., unpub. data). Moreover, the seroprevalence among shrews trapped during the postepidemic period was 43% in 1995. *Y. pestis* strains were also isolated from five shrews in 1996 and 1997 (Chanteau et al., unpub. data). All these findings strongly suggest the determinant role of shrews as a previously

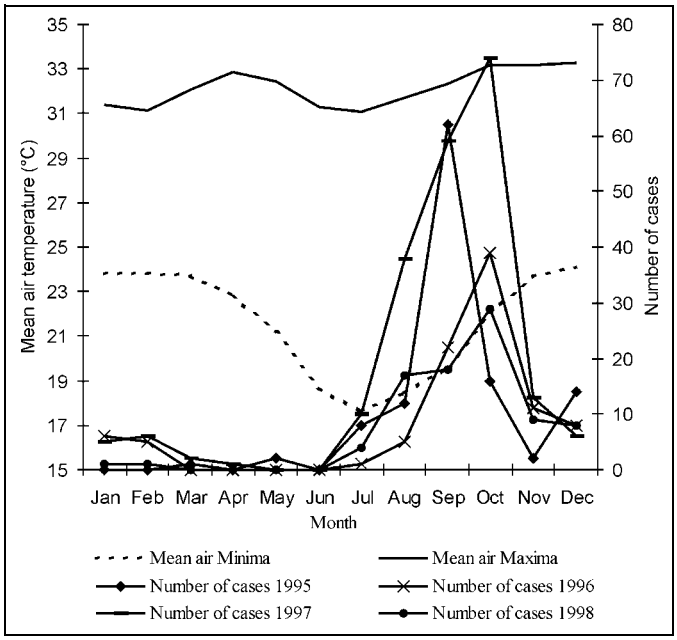

Figure 3. Mean air temperatures and month-distribution of laboratory-confirmed cases of bubonic plague, Mahajanga, Madagascar, since 1995.

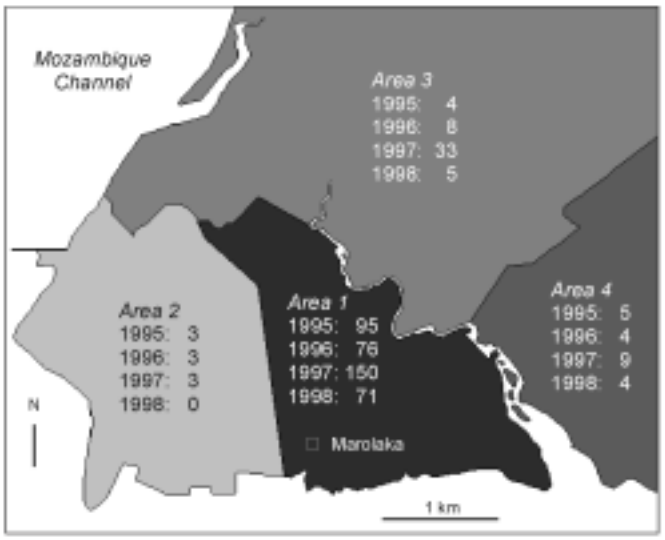

Figure 4. Incidence of laboratory-confirmed bubonic plague cases according to the patients' place of residence, in Mahajanga, Madagascar.

unrecognized host of *Y. pestis* in the epidemiologic cycle of plague in Mahajanga. This new parameter complicates and revises the classical rodent-flea-human triad. In Southeast Asia, the role of *S. murinus* in plague is established (14,15).

The epidemic wave of plague that started in August 1991 and lasted until February 1992 was confined to the neighborhood surrounding the main market in Area 1; this region, and more precisely a place named Marolaka, was considered to be the epicenter of the outbreak (4). The source of the initial contamination was probably inland, due to the trading of agricultural products from the northern plague foci to the marketplace, as suggested by results of the *Y. pestis* genotyping by pulsed-field gel electrophoresis (Buchrieser et al., manuscript in preparation). Although we think that an inland reintroduction of the infection is unlikely to occur for 4 consecutive years under the same pattern, we cannot exclude this hypothesis until molecular analysis of the isolates is available. The epidemic ring did not extend to the other areas of the town, and this quiescence lasted 3 years, during which information about hosts and fleas was scarce. The starting point for the second epidemic wave described here was in exactly the same zone of the marketplace; however, this time, the plague front extended to the other three areas. Over the 4 years, the incidence was higher in Area 1 than in the others, although in 1997 Area 3 was clearly affected. The plague front did not extend further than 10 to 15 km from town.

A geomedical survey (Rakotoarisoa S, unpub. data) concluded that three different types of structures were present in the entire city of Mahajanga**.** Area 2 is almost comparable with a European city with its wide, paved streets, sewer network, store buildings with apartments in the upper floors, and low population density); Areas 3 and 4 are semirural suburbs with low population density. In contrast, Area 1, which was the epicenter for the two waves of the outbreak, is densely populated with very poor people. This area also includes the two largest markets, which generate the town's largest amount of rubbish. Therefore, while no physical barrier separates Area 1 from Area 2, the lower incidence in the latter could be related to the slimmer chance of contact between humans and reservoirs of plague, in short, to better housing conditions.

Clinically, despite some published claims that the clinical diagnosis of bubonic plague is straightforward**,** field data show a bacteriologic confirmation rate no greater than 30% for the best years. This rate improved in 1997 and 1998, compared with 1995 and 1996, suggesting that physicians are becoming more skilled at making this diagnosis. The increased confirmation rate was also due to their increased skill in collecting bubo pus and the progressive shortening of the delay before the samples were analyzed in a laboratory. Except during the 2 months of August and September 1997, bubo samples arrived at the central plague laboratory in Antananarivo as long as 2 or 3 weeks after being collected, which led to false-negative results because they were contaminated or the plague bacillus had died. The use of ELISA methods to detect either F1 antigen in acute-phase bubo samples or antibodies in convalescent-phase serum contributed to the confirmation of 35% of the total laboratory-confirmed cases. However, bacteriology and ELISAs are used as retrospective tools to confirm plague. Only a rapid diagnostic test such as the F1 dipstick assay is a valuable tool for health workers (8).

The higher incidence of bubonic plague in males than females and in young persons rather than in adults is a constant epidemiologic feature in Madagascar, whether in Mahajanga or the highlands (1-3). In published studies, gender differences in incidence rates differ by country (16). In India, females were more frequently infected; in the city of Hai-nan, China, males and females were equally affected; and in Manchuria, the situation was similar to that in Madagascar (16)**.** Despite its being well accepted that incidence is linked to extrinsic more than intrinsic factors, we could not find any link to occupational behavior.

The effects of human age on the relationship between rat fleas and people and of gender on this disease deserve further study. The observation of a relationship between the age and the bubo location is common in Madagascar, although it has not been described elsewhere. As it is widely accepted that the location of the bubo is dependent on the place where the injection of *Y. pestis* occurred, we infer that infective flea bites more often involve the upper extremities in children than in adults. In the urban plague focus of Mahajanga, as in the central highlands, transmission is believed to occur mostly inside houses. Although we do not have any indications that children have specific risks, such as handling dead rodents in play, children do spend more time close to the floor (e.g., games, sleeping) than adults and therefore are closer to fleas.

From the start, the absence of pneumonic plague has been remarkable. The natural course of bubonic plague can lead to secondary pneumonic plague, which can give rise to highly

contagious cases of primary pneumonic plague in contacts, as seen every year in the highlands of Madagascar (17). Yet not a single contact case has been reported, even though several bubonic patients died before being diagnosed and treated and thus contacts remained unidentified who could have benefited from chemoprophylaxis. This observation fits with early studies describing peumonic plague only in temperate places. As far back as 1929, Thiroux pointed out that pneumonic plague outbreaks had never been observed in Madagascar in areas where the absolute minimum temperatures did not remain regularly under 16°C for several consecutive days (18). As early as 1907, in the absence of effective treatment and chemoprophylaxis, only four cases of pneumonic plague had been observed among 72 plague cases during the first epidemic in Mahajanga. The absence of lung infection apparently is not related to a particular strain of the plague bacillus since the *Y. pestis* strain that currently circulates in Mahajanga was likely introduced from the highlands in 1991, as demonstrated by pulsed-field gel electrophoresis genotyping (Buchrieser et al, manuscript in preparation).

The case-fatality rate is somewhat higher than reported in published studies, and it does not show any trend towards decreasing. However, we considered only laboratory-confirmed cases, and technical conditions in Mahajanga hospital are poor. We believe that a major proportion of treatment failures could be avoided if patients did not wait 2 days or more before coming to hospital. Moreover, dates of onset seem to be questionable for some serious cases; apparently families are often reluctant to imply that they have been negligent in managing the patient at home, or they have resorted to traditional healing before referring the patient to a hospital. Up to now, antibiotics used to treat the disease (streptomycin is the recommended treatment in Madagascar) have been totally effective against *Y. pestis* strains from Mahajanga. The discovery of one chloramphenicol-resistant isolate and one ampicillin-resistant isolate requires that the country maintain an efficient bacteriology surveillance system. The appearance and spread of multiresistant *Y. pestis* strains, such as the two isolated in 1995 in the highlands, are cause for concern (19,20).

Plague is still a threat in Madagascar and is no longer restricted to areas in the highlands over 800 m. A bubonic plague outbreak has been reported recently at 500 m altitude (21,22). Such epidemics as we describe may occur in any other coastal city where the shrew *S. murinus* and the flea *X. cheopis* are present. Because trade between the highlands and the ports has intensified, an active program for surveillance and monitoring of plague borders must be maintained. Improved ways to rat-proof structures should also be encouraged.

Dr. Boisier is a physician and specialist in epidemiology, working for the French Army Health Service. His research interests include the epidemiology and control of tropical diseases.

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Rickettsia felis in *Ctenocephalides* spp. Fleas, Brazil

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In June 2000, suspected cases of Brazilian spotted fever (BSF) occurred in Coronel Fabriciano Municipality, Minas Gerais State, Brazil. Pooled fleas collected near two fatal cases contained rickettsial DNA. The nucleotide sequence alignment of the 391-bp segment of the 17-kDa protein gene showed that the products were identical to each other and to the *R. felis* 17-kDa gene, confirming circulation of *R. felis* in Brazil.

The pathogenic rickettsiae are a group of intracellular bacteria responsible for various human diseases. *Rickettsia rickettsii* and *R. typhi* and the diseases they cause—Brazilian spotted fever (BSF), transmitted by the *Amblyomma cajennense* tick, and murine typhus, transmitted by the Oriental rat flea—have been recognized in Brazil since the 1920s (1-3). Molecular methods, including detection by DNA amplification by polymerase chain reaction (PCR) and DNA sequence analysis, are useful in characterizing rickettsial agents in arthropods. This approach has allowed the identification of new species, such as *R. felis* in opossums, fleas (4,5), and blood and skin from ill humans from the United States, Mexico, France, and Brazil (6-9). We report the identification of *R. felis* in *Ctenocephalides* fleas collected during the investigation of an outbreak of spotted fever group rickettsiosis in Brazil.

Material and Methods

In June 2000, fleas and ticks were collected in a periurban area of the city of Coronel Fabriciano, Steel Valley, Minas Gerais State, Brazil (Figure 1). This survey was performed during an outbreak of suspected BSF in which two children died. They were brothers who lived in the same house. The first child who became ill was 12 years old; during the course of his disease he had fever, nausea, vomiting, diarrhea, abdominal pain, headache, myalgia, and edema. Later, renal failure and stupor occurred. The second patient had fever, rash, nausea, vomiting, diarrhea, abdominal pain, headache, myalgia, jaundice, and renal failure. Both patients reported a tick bite a day before the onset of disease. One death was later confirmed as a case of spotted fever group rickettsiosis by immunohistochemical technique in tissues collected at autopsy. PCR was performed on brain, stomach, liver, spleen, and kidney tissues collected at autopsy, preserved in formalin, and sent to the University of Texas Medical Branch at Galveston. Because the DNA was not preserved, the death could not be attributed specifically to *R. rickettsii*, *R. felis*, or other species of *Rickettsia*.

The ticks were collected from three dogs and five horses near the house where the deaths occurred and were stored in 70% ethanol at room temperature. Ticks were separated into 15 pools with three specimens per pool, undifferentiated by life stage or sex. Fleas were also removed from 10 dogs in the home of the child whose death was confirmed as being due to BSF (Galvão et al., unpub. data) and from 3 cats near this residence; fleas were stored at –70°C. The fleas were separated into six pools with five specimens per pool. The ticks and fleas were identified as *A. cajennense* and *Ctenocephalides* spp., respectively.

PCR amplifications were done as previously described (9) with the DNA extracted from pools of ticks and fleas (Figure 2). Each PCR product was cycle sequenced with the primers described above and fluorescein-labeled dideoxynucleotide bases in the Applied Biosystems model (11) DNA sequencing system (ABI, Foster City, CA). Sequences were edited and assembled by using Chromas software (http://www.technelysium.com.au/chromas.html).

To arrive at the most accurate sequence for each PCR product, both forward and reverse sequences were determined. Where differences in nucleotide bases were observed, a predominant base was assigned if most of the sequences contained it. If one base did not predominate, the original chromatographs were consulted to resolve ambiguities.

Figure 1. Map of Brazil and Minas Gerais State, showing Coronel Fabriciano municipality.

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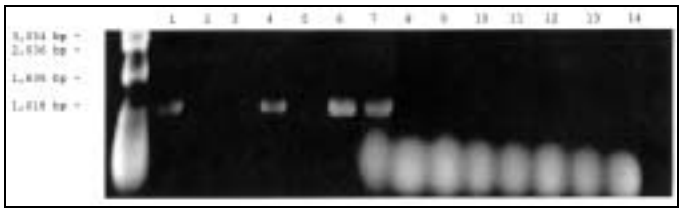

Figure 2. Detection of the *Rickettsia* specific 17-kDa gene by polymerase chain reaction amplification in DNA extracted from ticks and fleas. The vectors were first placed in 1.5-mL microcentrifuge tubes containing 200 µL of 10 mM phosphate-buffered saline, pH 7.4, and were crushed with a micropestle. The suspensions were lysed in 0.5% sodium dodecyl sulfate and incubated with 100 µg/mL proteinase K at 37°C for 1 hour in the case of fleas or overnight in the case of ticks. The lysed suspensions were extracted twice with an equal volume of phenol-chloroform, followed by a single chloroform extraction. The extracted DNA was amplified with primer 1 (5′-GCTCTTGCAACTTCTATGTT-3′) and primer 2 (5′-CATTGTTCGTCAGGTTGGCA-3′) as described by Webb et al. (10) for amplification of a 434-bp fragment from the rickettsial 17-kDa protein gene. PCR was performed at 30 cycles for 1 minute at 94°C, 5 minutes at 48°C, and 2 minutes at 72°C. The PCR products were then separated by electrophoresis in 1% agarose gel and stained with ethidium bromide. Lanes 1-3: DNA from cat fleas, Lanes 4-6: DNA from dog fleas, Lane 7: 17- kDa gene *Rickettsia felis* DNA (Positive Control), Lanes 8-14: DNA from ticks.

Sequences were compared by using the BLAST software program with 17-kDa sequence from other *Rickettsia* species obtained from the GenBank database. These sequences were aligned for maximal homology by using the Multialign software program (12).

Results

Of the 15 samples of pooled ticks and 6 samples of pooled fleas examined, 3 samples of pooled fleas had the 434-bp product expected for a *Rickettsia* (Figure 2). Nucleotide sequence analysis of the aligned 391-bp segment of 17 kDa confirmed that the three PCR products were identical to each other and to the 17-kDa protein gene of *R. felis* in the database.

Discussion

Recent research on rickettsial diseases in Latin America has included tropical Mexico, Andean Peru, and northern Argentina. The investigation in Minas Gerais State, Brazil, added another ecologic zone and geographic region of Latin America to those in which novel rickettsioses and ehrlichioses have been detected and identified.

BSF is the best-recognized rickettsial disease in Brazil; few reports have been published about human cases of other rickettsioses such as murine typhus and Q fever (13). BSF is known to occur in the states of Minas Gerais, São Paulo, Rio de Janeiro, Bahia, and Espírito Santo. Minas Gerais State has a surveillance program for BSF, and since 1990 interest has grown in the study of this disease in areas where residents seeking employment are increasingly exposed to tick-infested habitats. From 1990 to 1994, the incidence of BSF was 0.35 per 100,000 inhabitants, with a higher incidence in the latter half of the year (13). The age range most affected was 5 to 14 years (13), and the case-fatality ratio was 19% during 1993 to 1995 (14).

Our results show that, in addition to *R. rickettsii* and *R. typhi*, *R. felis* is also found in Brazil, as indicated by positive serology in human cases (8). Our data are the first indication by PCR of the presence of *R. felis* in fleas from Brazil. The *Ctenocephalides* spp. flea is proposed as a possible vector of this new rickettsial disease in Brazil.

Because of the complicated differential diagnosis of febrile exanthems, which includes dengue fever and other viral, rickettsial, and bacterial diseases, more attention should be paid to diagnostic laboratory investigation of rickettsial diseases. *R. felis* has been identified as the etiologic agent of a new rickettsiosis wherever it has been investigated: United States (Texas), Mexico, Brazil, and France (8). In Latin America, two reports have been published of human rickettsioses caused by *R. felis* in Mexico and Brazil (7,8). In both these reports, neurologic involvement was described, suggesting a severe clinical course associated with *R. felis* (15).

Although our investigation does not provide evidence for widespread flea infection by *R. felis* in Brazil, we demonstrate for the first time the presence of infection by this bacteria in Brazilian *Ctenocephalides* fleas. The descriptions of *R. felis*-positive human cases (8) in the same area where *R. felis* was identified in *Ctenocephalides* fleas indicate the possibility of this flea's being the vector of human *R. felis* rickettsiosis in Brazil.

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Severe *Ehrlichia chaffeensis* Infection in a Lung Transplant Recipient: A Review of Ehrlichiosis in the Immunocompromised Patient

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We describe a case of human ehrlichiosis in a lung transplant recipient and review published reports on ehrlichiosis in immunocompromised patients. Despite early therapy with doxycycline, our patient had unusually severe illness with features of thrombotic thrombocytopenic purpura. Of 23 reported cases of ehrlichiosis in immunocompromised patients, organ failure occurred in all patients and 6 (25%) died.

Since the discovery in 1987 of *Ehrlichia* as a cause of tick-borne disease in humans (1), ehrlichiosis has been recognized as an increasingly important cause of acute febrile illness (2,3). The two main pathogenic species are *Ehrlichia chaffeensis*, which causes human monocytic ehrlichiosis (HME), and the as-yet-unnamed agent of human granulocytic ehrlichiosis (HGE) (4). A third species, *E. ewingii*, which has been recently described, causes clinical disease indistinguishable from infection caused by *E. chaffeensis* or the agent of human granulocytic ehrlichiosis (5).

Delineation of the epidemiology of human ehrlichiosis has greatly enhanced our understanding of this emerging infection. However, information on the manifestations of ehrlichiosis in immunocompromised patients is limited. We report a case of severe monocytic ehrlichiosis in a lung transplant recipient who had pancytopenia, acute renal failure, and encephalopathy. Despite early diagnosis and treatment with doxycycline, his illness progressed and took on features of thrombotic thrombocytopenic purpura (TTP). A review of reported cases of *Ehrlichia* infection in immunocompromised patients shows that the infection is far more severe in this population and is often fatal.

Case Report

A 38-year-old man with cystic fibrosis had undergone bilateral lung transplantation in 1998 and had been well. In September 2000, he visited a physician with a 3-day history of fever as high as 38.3°C, myalgias, and headache. A resident of Columbia, Missouri, the patient had spent much time outdoors but did not recall tick infestation or recent tick bite. His medications included cyclosporine, mycophenolate, prednisone, diltiazem, trimethoprim-sulfamethoxazole, and valacyclovir.

On physical examination, the patient appeared acutely ill with temperature 38.3° C, blood pressure 140/64, heart rate 110 per minute, and respiratory rate 20 per minute. He was lethargic but could follow commands, and his neurologic exam was unremarkable. Fine bibasilar crackles were present bilaterally, but heart sounds were normal. Examination of the abdomen was negative. Synovitis was not evident, and no cutaneous lesions were found.

The leukoctye count was 3.7×10^9 per L with 68% neutrophils, hemoglobin was 64 g/L, and platelet count was 23,000/L. Serum creatinine was 4.6 mg/dL, aspartate aminotransferase 420 U/L, alanine aminotransferase 96 U/L, and bilirubin 3.2 mg/dL. International normalized prothrombin time ratio (INR) was 1.4. Examination of a peripheral blood smear showed schistocytes and other microangiopathic changes.

Multiple blood cultures were negative. Cytomegalovirus DNA was not detected in peripheral blood. Noncontrast computed tomography of the brain was normal. Chest radiograph showed bilateral infiltrates.

The patient was treated initially with intravenous piperacillin-tazobactam and vancomycin. Cyclosporine and trimethoprim-sulfamethoxazole were discontinued. The next day, his mental status continued to deteriorate. Lumbar puncture was deferred because of thrombocytopenia. Antibiotic therapy was changed to intravenous meropenem. Four days after admission, the bone marrow was examined because of worsening pancytopenia; intracytoplasmic morulae were seen in monocytoid cells, characteristic of monocytic ehrlichiosis (Figure). Leukocytes in a peripheral blood smear also contained morulae. Intravenous doxycycline was begun for treatment of presumed *Ehrlichia* infection. Whole-blood polymerase chain reaction (PCR) (Viromed, Minneapolis, MN) in the first week of illness was subsequently reported positive for *E. chaffeensis*

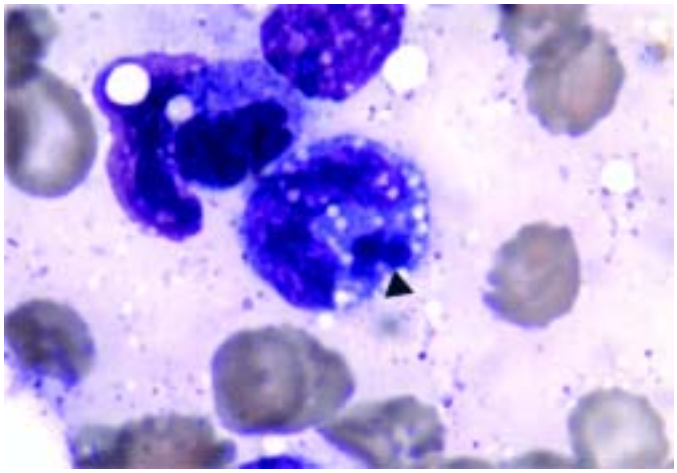

Figure. Bone marrow examination (Wright's stain x1000). Intraleukocytic morulae of *Ehrlichia* can be seen (arrow) within monocytoid cells.

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Table. Reports of immunocompromised patients with *Ehrlichia* infection

Immunocompromised state	Age	Form of ehrlichiosis	Clinical features	Outcome	Reference
Rheumatoid arthritis, on methotrexate	49	*Ehrlichia ewingii*	Fever, headache	Survived	5
Emphysema, on prednisone	65	*E. ewingii*	Fever, headache	Survived	5
Renal transplant	11	*E. ewingii*	Lyphadenopathy, fever	Survived	5
Liver transplant	47	HME[a]	Multiorgan failure	Survived	9
Leukemia	6	HME	Hepatitis, pancytopenia, rash, renal failure	Survived	10
Asplenia	71	HGE	Fever, neurologic dysfunction	Survived	11
Asplenia	30	HGE	Rash, fever	Survived	11
HIV infection (CD4 45/mm^3)	33	HME	Cardiomyopathy, heart failure	Survived	12
Renal transplant	35	HME	Rash, pancytopenia, renal failure	Survived	13
HIV infection (CD4 NS)	38	HME	Multiorgan failure	Died	14
Sickle beta-thalassemia	NS	HME	Respiratory failure	Survived	15
Renal transplant	NS	HME	NS	Survived	15
HIV infection (CD4 164/mm^3)	52	HME	Hepatitis, thrombocytopenia	Died	16
Renal transplant	67	HGE	Pancytopenia, renal failure, hepatitis	Survived	17
HIV infection CD4 (18/mm^3)	36	HME	Hepatitis, renal failure	Died	18
Liver transplant	51	HME	Pancytopenia, shock	Survived	19
Asplenia, chronic lymphocytic leukemia, on steroids	80	HGE	Multiorgan failure	Died	7
HIV infection (CD4 199/mm^3)	37	HME	Fever, pancytopenia, toxic-shock-like illness	Survived	20
Splenectomy	46	HME	Pancreatitis, shock, encephalitis	Survived	20
HIV infection (CD4 64/mm^3)	41	HME	Pancytopenia, pulmonary hemorrhage	Died	21
Asplenia	67	*E. canis*	Renal failure respiratory failure, encephalitis	Died	8
Crohn disease requiring prednisone	57	*E. canis*	Pancytopenia, hepatitis	Survived	22
Bilateral lung transplant	38	HME	Pancytopenia, renal failure, TTP-like illness	Survived	Current case

[a]HME = human monocytic ehrlichiosis; HGE = human granulocytic ehrlichiosis; NS = not specified

DNA. Serology by immunofluorescence antibody testing for both *E. equi* and *E. chaffeensis* performed 2 weeks after onset of illness was negative, with titers <1:40.

Despite treatment with doxycycline, the patient's confusion, thrombocytopenia, and microangiopathic anemia did not improve, and on the fifth hospital day he was transferred to the University of Wisconsin Hospital and Clinics. Physical examination showed blood pressure 144/94 mmHg, heart rate 77/minute, temperature 36.5°C, and respiratory rate 24/minute. Multiple ecchymoses were present on the torso and extremities. Neurologic examination was nonfocal. There were coarse bibasilar crackles in the lungs bilaterally. Examination of the heart and abdomen was unremarkable.

Leukocytes were 2.9 x 10^9/L, hemoglobin 86 g/L, and platelets 30,000/L. Serum creatinine was 6.2 mg/dL (548 mol/L), total bilirubin 2.0 mg/dL, aspartate aminotransferase 105 U/L, and alanine aminotransferase 55 U/L. INR was 1.1, and the activated partial thromboplastin time was 26 seconds. A peripheral blood smear showed numerous fragmented red blood cells. Chest radiograph showed persistence of bilateral infiltrates.

The patient's fever resolved 2 days after doxycycline was started; however, oliguric renal failure necessitated hemodialysis. Hematologic studies showed progressive microangiopathic anemia and thrombocytopenia with a normal INR, suggestive of TTP, presumably secondary to *Ehrlichia* infection. Daily plasmaphereses were begun and continued for 8 weeks. Gradually the hematologic abnormalities and renal function improved, and the patient's mental status returned to normal. Doxycycline was given for 2 weeks.

The patient ultimately made a full recovery with no apparent sequelae. Cyclosporine was not resumed, and he was maintained on sirolimus and prednisone to prevent transplant rejection. No rejection occurred, despite a reduction in immunosuppressive therapy during the treatment of *Ehrlichia* infection.

Review of Published Reports

Ehrlichiosis is a zoonotic illness caused by *Ehrlichia* species, which are pleomorphic, intracellular, rickettsia-like organisms (2-4). The clinical spectrum of ehrlichisis varies from a mild, influenzalike illness to a fulminant sepsis syndrome, but in most patients is self-limiting and not fatal. Death rates of documented ehrlichial infection in large, unselected series have been 1% to 8% (3,6-8). This low rate contrasts sharply with the high death rate of ehrliochiosis in immunocompromised patients (Table).

Cellular immunity represents the most important host defense against rickettsial infection (23). Acute-phase sera of patients with HGE contain elevated levels of interferon gamma, which is associated with the clearance of *Ehrlichia* from peripheral blood (24). In a mouse model of ehrlichiosis, immunocompromised mice have persistent infection, and most eventually die (25). Impairment of cellular immunity, whether from immunosuppresssive therapies or underlying disease, retards recovery, leading to more severe disease and higher death rates.

The population of immunocompromised patients is large and growing; many have asplenia or solid organ or bone marrow transplants (26). An analysis of the published reports of ehrlichial infection shows that the disease in immunocompromised patients is far more severe and prolonged and more likely to be fatal (Table) (5,7-22). Virtually all these patients had signs of organ dysfunction, including pancytopenia (40%), renal failure (24%), respiratory distress (14%), shock (28%), and neurologic dysfunction (18%). Six (25%) of 23 patients died; 4 of the 6 deaths were in HIV-infected patients. Two patients died within 24 hours after coming to medical attention, despite initiation of appropriate antimicrobial therapy; in the third, the diagnosis was not considered until late in the hospital course; and in the fourth, the diagnosis was made postmortem. Two deaths occurred in asplenic patients; in both, *Ehrlichia* infection was not suspected until 1 week after onset of illness.

In a recent series of ehrlichial infection in 21 HIV-infected patients, 6 of which are included in our review, Paddock et al. reported a high frequency (71%) of moderate to severe disease in HIV-infected patients, particularly with *E. chaffeensis* (27). Low CD4 counts were associated with a poor outcome.

Discussion

To our knowledge, this is the first reported case of acute ehrlichiosis in a lung transplant recipient. Our patient had laboratory features typical of *Ehrlichia* infection (thrombocytopenia, leukopenia, and transaminase elevation). However, he also had microangiopathic anemia, renal failure, and neurologic dysfunction characteristic of TTP. Ehrlichiosis with features of TTP has been described in two reports (28,29), one case each of HME and HGE. Both cases were in immunocompetent persons: one was treated with doxycycline and plasmapheresis; in the other, the diagnosis was made postmortem.

Our patient's multiorgan failure and hematologic aberrations persisted, despite doxycycline therapy, until he underwent plasmapheresis. He was receiving cyclosporine, which is a well-known cause of a rare hemolytic uremic syndrome-TTP-like condition that does not respond to plasmapheresis and nearly always proves fatal (30). That our patient's TTP-like illness coincided with *Ehrlichia* infection and responded to doxycycline and plasmapheresis makes it most likely that it was a consequence of acute ehrlichiosis, not cyclosporine.

Neurologic manifestations, ranging from confusion to frank meningitis, have been reported in up to 20% of patients with erhlichiosis (31). Our patient had obtundation and delirium that persisted after doxycycline therapy was initiated and his fever had resolved. The presence of headache and confusion in conjunction with pancytopenia and transaminase elevation should raise suspicion of *Ehrlichia* infection, especially if the patient has had potential tick exposures.

The diagnosis of ehrlichiosis is often delayed because of its nonspecific clinical and laboratory manifestations. In the immunocompromised person, the search for opportunistic infections may further preclude consideration of *Ehrlichia* infection. The empiric antimicrobial regimens used in immunocompromised patients for suspected cryptogenic bacterial and fungal sepsis rarely include a drug or drugs effective against *Ehrlichia*. PCR to detect *Ehrlichia* DNA is invaluable for the diagnosis and has >90% sensitivity and even better specificity (32). This technique is particularly useful in the immunocompromised host in whom rapid diagnosis is of utmost importance. Peripheral blood and bone marrow examinations show intracellular morulae in HME in only 1% to 5% of cases and cannot be relied on diagnostically, unless positive. Serologic testing does not allow rapid diagnosis and may be negative in the immunocompromised patient (21), as was the case with our patient.

The diagnosis of ehrlichiosis should be considered in any patient with fever, transaminase elevations, and new-onset thrombocytopenia or leukopenia who has had potential tick exposures in an endemic area. In the immunocompromised host, clinical manifestations are more severe and can include neurologic deterioration, multiorgan failure, and even a TTP-like illness. Response to appropriate therapy with doxycycline may be delayed. A high index of suspicion, the use of PCR for confirmatory diagnosis and early empiric therapy can be life-saving.

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Enzootic *Angiostrongylus cantonensis* in Rats and Snails after an Outbreak of Human Eosinophilic Meningitis, Jamaica

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After an outbreak in 2000 of eosinophilic meningitis in tourists to Jamaica, we looked for *Angiostrongylus cantonensis* in rats and snails on the island. Overall, 22% (24/109) of rats harbored adult worms, and 8% (4/48) of snails harbored *A. cantonensis* larvae. This report is the first of enzootic *A. cantonensis* infection in Jamaica, providing evidence that this parasite is likely to cause human cases of eosinophilic meningitis.

Angiostrongylus cantonensis is the most common infectious cause of eosinophilic meningitis worldwide (1). Although human infections with *A. cantonensis* are traditionally associated with Southeast Asia and the Pacific Basin, sporadic cases have been reported in several countries outside this region (1,2). In the Caribbean, eosinophilic meningitis has not been commonly reported, although *A. cantonensis* has been found in rats from Cuba, Puerto Rico, and the Dominican Republic (3-5).

A case of eosinophilic meningitis was described in 1994 in an adult Jamaican who had never traveled outside the country (6). In the absence of confirmatory histology or serology, the question of the endemicity of *A. cantonensis* in Jamaica at that time was raised (6). In May 2000, 12 persons in a group of 23 U.S. tourists who visited Jamaica for a week met the clinical definition for eosinophilic meningitis within 6-30 days (median 11) of returning home (7). Nine persons required hospitalization; there were no deaths. There was serologic evidence of exposure to *A. cantonensis* in eight persons who had eaten salad at the same restaurant, a common exposure that might account for all cases (7).

Since *A. cantonensis* has not been documented in Jamaica and many restaurants in Jamaica's tourist areas serve imported vegetables, the source of contamination of the vegetables was not necessarily on the island. We investigated whether *A. cantonensis* occurs naturally in the wild rat and snail populations of Jamaica.

The Ministry of Health collected 109 rats through the rat control program run by the Public Health Department. Rats were collected in eight sites across the island (Table) and sent to the Parasitology Research Laboratories at the University of the West Indies, where the cardiopulmonary system was dissected to determine infection status. In addition, staff from University of the West Indies and the Centers for Disease Control and Prevention (CDC) collected snails from four sites (Table) and examined them for infection.

Adult worms were recovered from the cardiopulmonary systems of 24 rats (20/78 *Rattus norvegicus*; 4/31 *R. rattus*) (Table). These worms had features characteristic of *Angiostrongylus*, including size (males measured 14-15 mm in length; females 24-26 mm in length), body shape, and prominent dark intestine (Figure 1A). The long copulatory spicules in the male worms, which measured approximately 1.2 mm (Figure 1B), are diagnostic for *A. cantonensis*, as the spicules of other species in the genus are generally <0.5 mm long (8).

Overall, 22% of the rats were infected with *A. cantonensis*. Infection rates did not differ significantly between *R. rattus* and *R. norvegicus* (chi square 2.10; p=0.148). The mean number of worms recovered per infected rat was 17±3.5 (range 3-27).

Table. Recovery of *Angiostrongylus cantonensis* from rats and snails, Jamaica, 2000

Location	Host	No. infected/no. examined (%)
Rats		
Freeport	*Rattus norvegicus*	6/10 (60)
	R.rattus	0/0
Mandeville	*R. norvegicus*	10/17 (59)
	R. rattus	0/0
Black River	*R. norvegicus*	2/11 (18)
	R.rattus	1/4 (25)
Kingston	*R. norvegicus*	1/12 (8)
	R.rattus	1/11 (9)
Lucea	*R. norvegicus*	1/15 (7)
	R.rattus	0/0
Montego Bay	*R. norvegicus*	0/0
	R.rattus	1/12 (8)
Port Antonio	*R. norvegicus*	0/8
	R. rattus	0/3
Lime Hall	*R. norvegicus*	0/1
	R.rattus	1/1 (100)
Snails		
Mandeville	*Thelidomus asper*	4/10 (40)
Brown's Town	*Orthalicus jamaicensis*	0/27
	Dentellaria sloaneana	0/2
Yallahs	*Orthalicus jamaicensis*	0/6
Scott's Pass	*Orthalicus jamaicensis*	0/3

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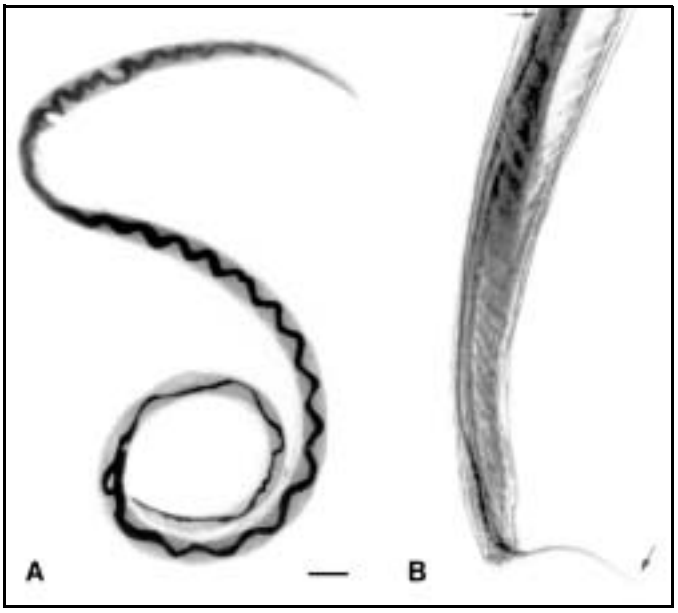

Figure 1. Adult *Angiostrongylus cantonensis* recovered from rat lungs. A. Adult female worm with characteristic barber-pole appearance (anterior end of worm is to the top). Scale bar = 1.5 mm. B. Tail of adult male, showing copulatory bursa and long spicules (arrows). Scale bar = 85 µm.

Land snails (Figure 2) were collected by hand from small farms and residential gardens and sent to the Division of Parasitic Diseases laboratory, CDC, Atlanta. A portion of the muscular head-foot region was excised from each surviving snail, cut into smaller fragments, and placed in separate dishes containing digestion fluid (0.01% pepsin in 0.7% v/v aqueous HCl [9]). Dishes were examined for nematode larvae at 4-5 hours and 24 hours after digestion.

Four of 10 *Thelidomus asper* collected in Mandeville were found positive for *A. cantonensis* larvae, but neither *Orthalicus jamaicensis* (n=36) nor *Dentellaria sloaneana* (n=2) were infected (Table). Living larvae digested from *Thelidomus* were easily recognized and recovered because they retained motility in the digestion fluid. Larvae were examined microscopically, and the morphologic features compared with those in published reports (10) and reference *A. cantonensis* larvae to confirm identification. Two species of lungworm (metastrongyles) larvae were recovered. Most larvae were *Angiostrongylus cantonensis* (375 to 420 [mean 402] µm in length after fixation in hot alcohol), but a small number of *Aelurostrongylus abstrusus* (400 to 440 [mean 427] µm in length after fixation in hot alcohol), a lungworm of cats, were also observed. Typical of lungworm larvae, the two species were similar in size and the presence of characteristic sclerotized rhabdions at the anterior end of the larvae. The larvae were easily distinguished, however, by the shape of the tip of the tail; *A. cantonensis* had a constriction near the end of the tail and ended in a fine point, while *A. abstrusus* terminated in a knob (10,11).

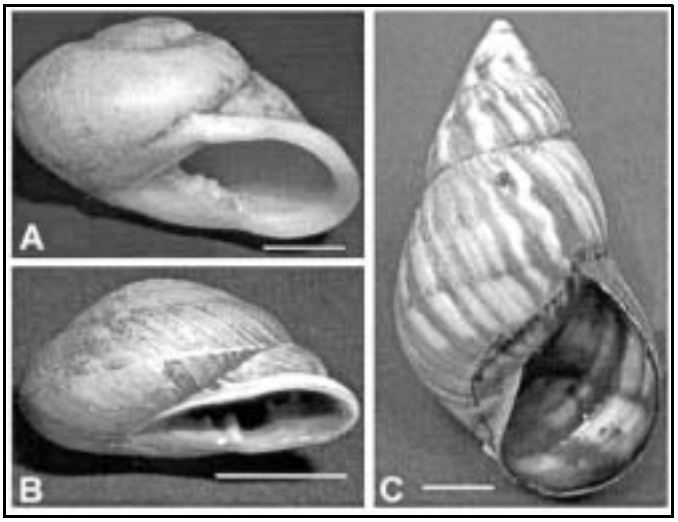

Figure 2. Three species of land snails collected in Jamaica and examined for *Angiostrongylus* larvae. A. *Thelidomus asper.* B. *Orthalicus jamaicensis*. C. *Dentellaria sloaneana*. Scale bar = 1 cm.

This is the first report of enzootic *A. cantonensis* infection in Jamaican rats and snails; our data show that the range of the parasite extends to another Caribbean country outside Cuba, the Dominican Republic, and Puerto Rico (3-5). The occurrence of the parasite at high rates in rats and in specific groups of snails, earlier findings of eosinophilic meningitis in a resident, and the recent outbreak of *A. cantonensis*-associated eosinophilic meningitis in visitors to the island suggest that autochthonous transmission to humans is probable in Jamaica. These studies are being extended to determine the full distribution of the parasite and the species of snails involved in its transmission. Furthermore, serologic tests need to be developed to confirm infections in persons in the Caribbean.

Public health officials, clinical parasitologists, and travel medicine practitioners should consider *A. cantonensis* as a causative agent of eosinophilic meningitis in Jamaican residents and travelers to the island.

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Recent Increase in Meningitis Caused by *Neisseria meningitidis* Serogroups A and W135, Yaoundé, Cameroon

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From 1991 to 1998, *Neisseria meningitidis* serogroups A, B, and C represented 2%-10% of strains isolated from cases of bacterial meningitis in Yaoundé. During 1999 to 2000, the percentage of meningococci reached 17%, a proportion never reported since recordkeeping began in 1984. The increase of serogroup A meningococci and the emergence of W135 strains highlight the need for increased surveillance for better diagnosis and prevention.

Neisseria meningitidis serogroup A causes major epidemics of meningitis in Africa, essentially within the African meningitis belt (1)**.** Epidemics of cerebrospinal meningitis in this belt are often enormous (1). During the first 9 months of 1996 in the World Health Organization (WHO) African Region, 146,166 cases were reported to WHO; 15,783 were fatal. During that year, 42,129 cases occurred in Burkina Faso, 7,244 in Mali, 16,050 in Niger, and 75,069 in Nigeria. These four countries reported 95% of the cases in Africa in 1996, for an overall case-fatality rate of 10.6% (2).

The recommended control practices in Africa involve vaccination with the meningococcal bivalent polysaccharide A/C vaccine in response to epidemics. Efficient public health practice necessitates that epidemics be detected early, stocks of vaccines be set up in target regions, and field vaccination with the bivalent vaccine be rapid, since the quadrivalent ACYW135 vaccine has limited worldwide supply and is more expensive.

The presence of *N. meningitidis* serogroup W135 has been confirmed in Africa for some time. In Burkina Faso in 1980, 1.3% of the meningococcal strains isolated from rhinopharyngeal carriers belonged to serogroup W135. In 1981 and 1982, monitoring of the serogroups responsible for meningococcal meningitis at Dakar (Senegal) and Niamey (Niger) showed that 4% and 3% of strains, respectively, belonged to serogroup W135 (3). In 1984 and 1985**,** 7% of *N. meningitidis* strains isolated from meningitis cases in Gambia belonged to serogroup W135 (4). In 1993 and 1994, two strains of *N. meningitidis* W135 were isolated from patients in Mali; both belonged to the ET-37 complex (5). More recently, in 1994, six strains of serogroup W135 isolated from clinical cases in Gambia were studied; they also belonged to the ET-37 complex. DNA macrorestriction analysis of these strains identified four different profiles in pulsed-field gel electrophoresis (PFGE), indicating that the strains involved were closely related but different (6). W135 strains are often isolated after intensive campaigns of vaccination against meningococci of serogroups A and C (3,4,6).

In spring 2000, an epidemic of *N. meningitidis* W135 infection broke out among Hajj pilgrims (for whom vaccination against meningococci of serogroups A and C is mandatory) and their close contacts. In all, 241 cases were reported in Saudi Arabia and 90 in 13 other countries (7), including the United States (4 cases) (8), the United Kingdom (33 cases), and France (19 cases). All these strains showed markers of the ET-37 complex; had an antigenic formula W135:2a:P1-5,2; a sequence type ST-11; and the same profile on PFGE (9), confirming the clonal origin of the epidemic. Four W135 strains isolated in U.S. patients epidemiologically linked to Hajj pilgrims were further studied. The sequence of the *porA* gene showed that these four strains had variable regions VR1 and VR2 identical to those of the prototype P1.5,2 strain (8).

Apparently, the W135 strains isolated in Africa until 1995 did not cause large epidemics, even if isolated in the countries in the African meningitis belt in which epidemics due to serogroups A meningococci are frequent (e.g., Niger, Mali, Senegal, and Gambia). In Niger in 1981, only one W135 strain of 231 meningococci was isolated from a meningitis case (3). Similarly, W135 accounted for 7 of 42 strains in 1982 in Niger, and 3 of 76 strains in Senegal in 1981 to 1982 (3), 3 of 41 in Guinea in 1984 to 1985 (4), and 2 of 75 strains isolated in 1991 to 1994 in six countries in the African meningitis belt (5). However, available information shows that the case-fatality rate due to W135 strains was relatively high in Africa before 1995, as in Europe during the recent Hajj 2000 epidemic: 6 (35%) of 17 cases in Africa before 1995 (in Senegal, Gambia, and Niger) and 10 (18%) of 56 cases in Europe in 2000 (in the United Kingdom, France, and the Netherlands).

The Study

We report here a sudden increase in the number of meningococcal strains isolated from cerebrospinal fluid (CSF) sent to the Medical Biology Laboratory of the Pasteur Centre of Cameroon (CPC) at Yaoundé in the 1-year period 1999 to 2000 (note that in Cameroon the administrative year begins on July 1). Yaoundé, the capital of Cameroon, is a city of approximately 1,500,000 inhabitants. Located in the forest zone at an altitude of 750 m, about 400 km south of the southern limit of

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the African meningitis belt, it has a humid, tropical climate. The CPC laboratory receives samples from patients admitted to the principal hospitals of Yaounde and, in 1999 to 2000, 91.5% of CSF samples sent to the laboratory were from children <15 years old; 81% were from children <5 years.

From 1984 to 1990, bacteria were isolated from 767 (5.8%) of 13,134 CSF samples; 42 (5.5%) of these were *N. meningitidis.* This proportion was significantly lower than during the 1991 to 2000 period (8.7%; $p<0.05$). We do not know if there were changes in the population of patients using the CPC services that might account for the slow increase in cases of meningococcal disease. Slow improvement of laboratory practices and medical competence might account for slowly increasing proportions of *N. meningitidis* over a 20-year period. However, no major changes in laboratory techniques occurred in 1999 at CPC that could account for the sudden increase observed in 1999 and 2000. Moreover, during that 20-year period, most CSF samples came from the same pediatric wards of the neighboring Central Hospital of Yaoundé and other major children's hospitals.

The table shows the changes in isolation rates of *N. meningitidis* from clinical cases at Yaoundé during 1991-92 to 2000-01. The number of meningococcal strains isolated has remained small for the last 10 years, as would be expected in a zone located at a considerable distance south of the African meningitis belt and one in which pneumococci and *Haemophilus influenzae* are the two most frequent bacterial agents of meningitis. Most meningococcal strains isolated were serogroup A, the most frequent group in Africa. The proportion of meningococci identified in cases of bacterial meningitis varied significantly in this period ($p<0.01$).

In the 2-year period 1998 to 2000, two events occurred. The first was an increase in the number of isolates of *N. meningitidis*; such isolates accounted for >19% of strains isolated from cases of bacterial meningitis in 1999 to 2000, and 21% in 2000 to 2001—two to three times more than normal. We checked records back to 1984 and found that in none of the years in this period was such a large number of meningococci isolated at Yaoundé. The second noteworthy event was the appearance of serogroup W135 strains, which accounted for 9 (19%) of 48 meningococcal strains isolated in 1998 to 2000 versus 0 of 46 in 1991 to 1997 ($p<0.01$).

One W135 case occurred in January 1999 in a 12-year-old boy from Yaoundé who had no known contact with a Hajj pilgrim and no recent history of travel. On the four W135 patients from 1999 to 2000, one was male and three were female. Ages were 2, 3, 29, and 37 years. Onsets of disease were in July 1999 and in May and June 2000, i.e., after the usual meningococcal peak in the dry season, and none had known direct or indirect contact with each other or with a Hajj pilgrim. In 2000 to 2001, four cases occurred, all in males (aged 9, 15, 23, and 40 years); onsets of diseases occurred in January 2001, then in March, May, and June. One of them was in a 23-year-old student, who had been studying in Dakar (Senegal) for 2 years; he became ill while observing holy days in Cameroon. He could have been in indirect contact with Hajj pilgrims, since he was Muslim and Senegal is largely Muslim. Vaccination status was obtained for five of these nine patients: two were vaccinated against meningococcal meningitis, including the student.

The five strains of *N. meningitidis* W135 isolated in 1999 to 2000 were serotyped, subtyped, and studied by molecular biology techniques. All belonged to the ET-37 complex and had the antigenic formula W135:2a:P1.2,5. Two strains were subjected to multilocus sequence typing; both were of sequence type ST-11, typical of isolates of the ET-37 complex (10). These five strains were indistinguishable by multilocus DNA fingerprinting and showed markers of E-37 complex (11). Finally, *Spe*I restriction profiles were determined by PFGE: four of the strains were indistinguishable, and the final strain

Table. Isolation of *Neisseria meningitidis* from meningitis cases at Yaoundé, Cameroon, 1991–2001

Year	No. of CSF samples	No. (%) of cases of bacterial meningitis	No. (%) of cases of meningococcal meningitis	No. of strains of each serogroup
1991-1992	1,246	131 (10.5)	8 (6.1)	6 A; 2 C
1992-1993	1,049	105 (10)	11 (10.5)	11 A
1993-1994	961	88 (9.2)	9 (10.2)	8 A; 1 B
1994-1995	722	69 (9.6)	6 (8.7)	2 A; 4B
1995-1996	998	70 (7)	4 (5.7)	1A; 1B; 1C; 1NT
1996-1997	1,255	97 (7.7)	2 (2.1)	2C
1997-1998	1,282	92 (7.2)	6 (6.5)	4A; 1C; 1NT
1998-1999	1,505	116 (7.7)	8 (6.9)	6A; 1B; 1W135
1999-2000	1,812	120 (6.6)	23 (19.2)	17A; 2B; 4W135
2000-2001	1,612	81 (5)	17 (21)	13A; 4W135
Total	12,442	969 (7.8)	94 (9.7)	68A; 9B; 6C;9W135; 2NT

NT: not serogrouped; CSF: cerebrospinal fluid.

differed by one band only. All these clones differed slightly (by two bands for four isolates and by three bands for one isolate) from the clone isolated from the Hajj pilgrims in 2000.

Conclusions

These results show an increase of serogroup A meningococci in Yaoundé and demonstrate the presence and circulation of at least one indigenous clone of *N. meningitidis* W135 of the ET-37 complex in Central Africa. The clone is very similar to, but differs slightly from, the clone responsible for a meningitis outbreak among Hajj pilgrims in 2000 (8,9). Since none of the patients with W135 meningococci had direct contact with Hajj pilgrims and Cameroonian W135 strains are slightly different by PFGE from the W135 clone isolated in Europe and the United States in 2000, these strains from Cameroon seem to predate the 2000 Hajj-associated outbreak. A larger study of the W135 strains isolated in Africa, Europe, and Asia, from patients with no direct link to the pilgrimage to Mecca (indigenous strains) would make it possible to identify the geographic origin of the strain responsible for the Mecca epidemic in 2000. Such studies would also make it possible to elucidate the role of A and C vaccination in the selection of W135 clones belonging to the ET-37 complex.

We cannot explain with certainty why serogroup A meningococci has increased in Yaoundé, but the finding stresses the importance of continuous surveillance. The circulation of W135 strains in Central Africa raises questions about their epidemic potential and highlights the microbiologic surveillance of meningococcal meningitis. Thus, anti-W135 serogrouping antibodies are necessary for all National Reference Laboratory services. Antigen-detection kits for the diagnosis of meningitis should also contain anti-W135 antibodies. Moreover, the problem of the availability of a quadrivalent vaccine, including the W135 antigen, should be resolved. Strengthening the capacities for epidemiologic and microbiologic surveillance of meningitis in Africa is a prerequisite for prevention and control of meningococcal epidemics.

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HIV Prevalence in a Gold Mining Camp in the Amazon Region, Guyana

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The prevalence of HIV infection among men in a gold mining camp in the Amazon region of Guyana was 6.5%. This high percentage of HIV infection provides a reservoir for the virus in this region, warranting immediate public health intervention to curb its spread. As malaria is endemic in the Amazon Basin (>30,000 cases/year), the impact of coinfection may be substantial.

In Guyana and other South American countries containing large tracts of Amazon jungle, few studies have investigated the prevalence of HIV infection in isolated communities (1). Geographic isolation would lead to low infection rates because of lack of exposure to the disease. In addition, prevalence data on HIV can be negligible even in urban areas. For example, the only reported HIV studies in the Guyanese population have focused on HIV prevalence in commercial sex workers in the capital city of Georgetown (2). No studies have reported the prevalence of HIV in Guyanese men or in the interior Amazon region of the country. We evaluated a group of men living and working in the Amazon region of Guyana to determine the prevalence of HIV infection.

This study was conducted after we obtained Institutional Review Board approvals as well as permission from the Guyanese Ministry of Health and the director of a local Guyanese gold mining camp. Informed consent was obtained from each participant. Typically, men live in gold mining camps for periods of 6 to 8 weeks, working 12-hour days, 7 days per week. At the end of a 6- to 8-week shift, the men rotate out of camp to their homes on the coast for 2 weeks of rest. Mining gold in the Amazon region requires considerable manual labor and long hours working in a hot, humid jungle environment. Salaries paid to gold miners, however, are much better than those of typical jobs in the city. Thus, jobs in the mining industry are attractive, and many men leave their families and work as miners in the jungle for a few years to provide a better standard of living for their families.

The mining camp in this study was approximately 400 km inland from Georgetown, the capital of Guyana, in the heart of the Amazon region of the country. It was typical of many of the mining camps in the jungle (Figure). Men sleep in rows of 20 to 40 hammocks strung underneath a large tarp-like covering. The tarp coverings are not enclosed, but the men usually sleep under mosquito netting, as malaria infection is a constant problem. Pit latrines are available in the camp as are rainwater shower stalls. Water is obtained from a nearby stream, and a generator provides light in the camp in the evenings. The facility is fenced in and heavily guarded by armed patrols, as gold is stored in the camp from daily operations. The camp is a living facility only as all mining occurs outside the camp itself.

We enrolled almost the entire workforce of the mining camp (n = 216) for participation in this study. Only four declined the free HIV test and were excluded from the analysis. All 216 subjects were Guyanese men (age range 18-35 years). Pre- and post-HIV counseling was completed, and informed consent was obtained from all participants. Seven milliliters of venous blood was obtained from each participant after precounseling was completed. Onsite screening for HIV was completed, and serum was stored on ice and transported for confirmatory testing in a laboratory. Onsite HIV testing was by the Determine (Dainabot, Tokyo, Japan) rapid immunochromatographic test for the qualitative detection of HIV-1/2; in previous field work this test yielded 100% sensitivity and specificity (3). The test required 50 µL of serum, with results available for visual interpretation within 15 to 60 minutes. However, most of the positive samples produced a clearly visible red line within 10 minutes. HIV testing by enzyme-linked immunosorbent assay (ELISA) with Western blot confirmation (Abbott, Abbott Park, IL) was completed on all sera on our return from the jungle. Participants were not given results until confirmatory testing was completed. All participants were notified that results were available by a letter from the collaborating local physician, and all were offered personal counseling when they visited him for their results.

Fourteen (6.5%) of the 216 men were found to have HIV infection by results of both the onsite rapid strip test and

Figure. Typical living quarters for miners in the jungle, Amazon Basin, Guyana.

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subsequent ELISA and Western blot tests. Results obtained with the rapid test performed onsite had 100% agreement with those of the laboratory tests performed after our return.

Results of this small HIV screening study, indicating that 6.5% of men living in this remote camp were HIV positive, suggest enormous potential for further transmission of HIV in Guyana, in both jungle and urban environments. Migration of city dwellers into the Amazon jungle region may increase the risk of transmission of HIV to indigenous people. Conversely, gold miners can become infected with HIV during contact with commercial sex workers in small villages near the mining areas. HIV can then be further transmitted to the miner's spouse and unborn children on his return to the city. Whether the miners contracted the infection while living in the jungle or whether they entered the region already infected is unclear; however, the high percentage of HIV infection in this population provides a reservoir for the disease in this region and warrants immediate public health intervention to curb its spread.

Intervention is warranted to increase public awareness of HIV in underserved remote jungle and urban regions in Guyana. Rapid HIV screening tests, which can be completed without equipment or ancillary supplies, may provide an important tool for rapid screening and providing immediate feedback to patients. Initial counseling on risk-reducing behavior can be initiated onsite to provide an immediate intervention strategy to prevent the spread of the disease while follow-up testing with a confirmatory HIV test is completed.

Given the high numbers of malaria cases in the Guyana Amazon region, combined with this new evidence of potentially escalating HIV rates, studies are warranted to measure the impact of HIV/malaria coinfection. Reports showing an average of >30,000 cases of malaria per year over the past decade clearly designate this region as having a high rate of endemic malaria (4). Since T-cell and B-cell function, thought to provide a defense against malaria, are both adversely affected at the early stages of HIV infection and continue to deteriorate, this may contribute to higher rates of malaria mortality or more severe malaria symptoms, as the infected person's impaired immune system is less effective against the invading parasites. Conversely, malaria could exacerbate HIV infection (5), since the already compromised immune system may be overwhelmed by the multiple infection. Thus, HIV/malaria coinfection may contribute to increased rates of illness and death in the Amazon region. Recent studies on HIV/AIDS and malaria in Africa suggest that coinfection with these two diseases has become a concern in Africa (6,7). This problem also merits attention in the Americas so that further research, planning, and interventions will be focused in this region.

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Hajj-Related *Neisseria Meningitidis* Serogroup W135 in Mauritius

Mohammad Iqbal Issack* and Chinien Ragavoodoo†

Meningococcal disease is rare in Mauritius; only one case was reported from 1992 to 1999. However, since June 2000, four cases have occurred. Epidemiologic information and typing results indicate that these recent cases probably followed the introduction of *Neisseria meningitidis* W135 in Mauritius by pilgrims returning from the Hajj in 2000 and 2001.

Mauritius is a small tropical island in the Indian Ocean (population 1.2 million). The country is classified as a middle-income country by the World Bank; its primary commercial links are with Europe, the Indian subcontinent, and southern Africa.

Bacteriologic investigations for government health institutions in Mauritius are conducted in only one laboratory. These centralized results indicate that meningococcal disease is extremely rare in Mauritius. From 1992 to 1999, the only recorded infection occurred in a patient 3 days after he returned from the United Kingdom. However, since June 2000, four cases of meningococcal disease have occurred in Mauritians who have no history of travel outside the island.

Case Reports

Case 1

In June 2000, a 49-year-old Muslim woman was admitted to the hospital with a purpuric rash. She was initially thought to have a hematologic disorder before meningococcal septicemia was suspected. Despite treatment with intravenous penicillin and cefotaxime, she died the following day. *Neisseria meningitidis* was isolated from her blood cultures. A latex agglutination test with polyvalent reagent was positive for groups ACYW135 (Wellcogen bacterial antigen kit, Murex, Dartford, UK), but negative results were obtained with monovalent sera for group A, B, and C antigens (Slidex, Biomérieux, Marcy l'Etoile, France). The isolate was subsequently typed by the World Health Organization (WHO) Collaborating Centre for Meningococcal Infections in Marseilles, where it was confirmed as *N. meningitidis* serogroup W135, type 2a, subtype 1.2,5. It belonged to ST-11, the same sequence type as isolates obtained from English and French pilgrims returning from the Hajj that same year (1). The patient had no history of travel or close contact with a returning pilgrim, according to relatives.

Case 2

In July 2000, a 5-year-old Muslim child was admitted to the hospital with fever, vomiting, and ecchymoses. Initially she was thought to have a bleeding disorder. She had already received intravenous amoxicillin when meningococcal disease was suspected and specimens for microbiologic investigation taken. Her cerebrospinal fluid (CSF) was turbid with a leukocyte count of 11,500 per µL. CSF was positive for *N. meningitidis* antigens, groups ACYW135 with polyvalent serum, and negative for *Haemophilus influenzae* type b, pneumococcus, and meningococcus groups A, B, and C. Cultures of CSF and blood were negative. Cefotaxime was added to her treatment, and she made a good recovery.

The child's father, who returned from the Hajj 3 months earlier, had received the meningococcus A+C bivalent vaccine before travel. He had not been clinically ill during the pilgrimage or after his return.

Case 3

In November 2000, a 27-year-old man was admitted to the hospital with ecchymoses and signs and symptoms of meningitis. He had received antibiotics before investigations were performed. His CSF was turbid, with 26,000 leukocytes and 10,000 red cells per µL. Results of antigen testing on blood and CSF specimens were similar to those of the child in case 2, and cultures were negative. He was treated with cefotaxime and fully recovered. The patient was not Muslim and had not had close contact with a returning pilgrim. However, he and the patient in case 1 lived in the same village (population 10,000) as 22 pilgrims from the 2000 Hajj.

Case 4

In April 2001, a 4-month-old Muslim infant was hospitalized with fever and ecchymoses. Her CSF was turbid with 900 leukocytes per µL, and *N. meningitidis* was confirmed by culture. She was treated with cefotaxime and made a full recovery. Her father had returned from the Hajj 2 weeks before her onset of symptoms, but apart from a cough and cold, he had been clinically well. However, meningococcus was isolated from his oropharynx, as well as from the throat of the patient's 2-year-old brother. Isolates from the patient and her father and brother were positive by agglutination with meningococcus ACYW135 polyvalent reagent and negative with monovalent sera for meningococcus A, B, and C.

The pilgrim's vaccination certificate confirmed that he had received a quadrivalent meningococcal vaccine. All three isolates were sent to the WHO Collaborating Centre, which confirmed meningococcus serogroup W135, type 2a, subtype 1.2,5. They were indistinguishable by pulsed-field gel electrophoresis from isolates obtained from French pilgrims in 2000.

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Close contacts of all four patients with meningococcal disease received antimicrobial prophylaxis within 24 hours of diagnosis: a single 500-mg dose of ciprofloxacin was given to adults, and 2 days of rifampicin (10 mg/kg twice a day) was given to children. Close contacts of the infant (case 4) were also given the quadrivalent meningococcal vaccine. The only meningococcal carriage studies were conducted on some close contacts of this infant. No case secondary to those reported above is known to have occurred.

Two of these patients had meningococcal disease that was shown by typing to have been caused by the same W135 clone isolated from pilgrims returning from the Hajj in 2000 and 2001. Case 2 was culture negative but epidemiologically linked with a returning pilgrim and was probably caused by the same clone. Case 3 was also culture negative, but antigen tests indicated that infection was caused by meningococcus group W135 or group Y.

Conclusions

Cases of W135 meningococcal disease in returning pilgrims and their contacts have been reported in several countries following the Hajj of 2000 (1) and 2001 (2). In many European countries, further cases later occurred in persons with no history of close contact with a returning pilgrim (3).

About 16% of the Mauritian population are Muslims, and approximately 1,800 and 2,200 pilgrims traveled from Mauritius to Saudi Arabia for the Hajj in 2000 and 2001, respectively. In 2000, Mauritian pilgrims received the bivalent A+C meningococcal polysaccharide vaccine, but five Mauritians reportedly died in Saudi Arabia of meningitis of unspecified type. Cases of meningococcal disease in Mauritius itself occurred 3 months after the pilgrims returned, a period that coincided with the annual peak in upper respiratory tract infections (4). Viral upper respiratory tract infections are known to increase the risk of meningococcal disease (5). After the infections were reported in 2000, the Mauritian government decided to import a quadrivalent meningococcal polysaccharide vaccine (Mencevax ACWY, SmithKline Beecham, Genval, Belgium) for pilgrims attending the Hajj in 2001, and almost all of them received that vaccine. Although no case of meningitis was subsequently reported among these pilgrims, case 4 suggests that some have become carriers of meningococcus W135.

Evidence indicates that the quadrivalent meningococcal polysaccharide vaccine may not prevent asymptomatic nasopharyngeal infection with *N. meningitidis* serogroup W135 (6). Prophylactic administration of antibiotics to returning pilgrims may be indicated to reduce the risk for transmission to close contacts. A recent study in the United States showed that 0.8% of 727 returning pilgrims in 2001 were carriers of W135 meningococcus, compared with none on departure (7). All these pilgrims are presumed to have received the quadrivalent meningococcal vaccine. In view of the low carriage rate, administration of chemoprophylaxis to all returning pilgrims was not recommended (7). However, these findings may not be applicable to pilgrims from developing countries. During the Hajj, pilgrims from poorer countries often live in more overcrowded accommodations than those from more affluent regions, which may increase the risk of droplet transmission and result in higher rates of asymptomatic carriage.

Because several countries do not have scheduled flights to Saudi Arabia, many pilgrims travel on chartered airplanes, which would facilitate the administration of prophylaxis at the airport to returning pilgrims. The effectiveness of single-dose oral ciprofloxacin (8) simplifies the task. The disadvantages of ciprofloxacin prophylaxis must, however, be considered, including the small risk for anaphylaxis-like reaction (9). The growing problem of antibiotic-resistant organisms cannot be ignored, as fluoroquinolone resistance in the related species *N. gonorrhoeae* has already emerged in many places (10). Children and pregnant women, who likely represent only a small proportion of pilgrims, could be given intramuscular ceftriaxone, but there is also a risk of anaphylaxis besides the disadvantages of parenteral therapy. The decision to administer chemoprophylaxis to all returning pilgrims should therefore depend on whether transmission of W135 meningococcus during future pilgrimages continues. Information regarding continuing transmission would be useful before pilgrims return to their homes.

During Hajj 2001, many pilgrims, especially from developing countries, were unlikely to have received the quadrivalent vaccine. The Ministry of Health of Saudi Arabia has recently specified that, beginning in 2002, pilgrims must have been vaccinated with a quadrivalent vaccine (2). This requirement may reduce future transmission. However, if cases continue to occur, many countries should consider prophylaxis for returning pilgrims. Clearly, surveillance for meningococcal disease in general and serogroup W135 in particular will remain important in the next few years.

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Contagion on the Internet

To the Editor: Computer viruses are designed to be pests, proliferating in uncontrolled ways and causing severe damage to electronic data. These malignant programs, which amplify between files and computers, are strikingly similar in virulence, modes of spread, and evolutionary pathways over time to the microbes that cause infectious diseases. Both biological viruses and these virtual viruses are transmitted from host to host. Computer viruses are a human invention; however, their development follows a well-recognized biological route. Relatively harmless ancestors gradually or step-by-step evolve into "pathogens;" the host develops adaptive defense mechanisms, which in turn select for new virus "variants;" eventually, equilibrium is reached between infection and host defenses. Comparing "virtual microbes" with their biological counterparts can help us control both.

The term "computer virus" is loosely used to describe computer "malware," an umbrella term that includes the following categories. 1) Viruses. A computer virus is a program that implants a version of itself in any program it can modify. The modified program, once run, attempts to modify other programs directly associated with it. Computer viruses spread by sharing data on infected disks or diskettes. Unlike their biological counterparts (which are fast and very infectious), computer viruses spread slowly and infrequently between computers. 2) Worms. A worm is a self-contained program that replicates itself and sends copies to any connected computer, with little or no user interaction. Unlike biological worms (which spread slowly), computer worms spread rapidly and without much user interaction between computers of a network, including the Internet. (In view of the contagiousness of biological worms and viruses, the terms should have been reversed.) 3) Trojan horses. A Trojan horse is a program concealing harmful code that usually makes a computer or network available to unauthorized users in an appealing or unsuspicious package. A virus, worm, or Trojan horse can be latent (then also called a logic bomb) and become active only after a certain period.

Each class of computer malware has hundreds of variants, and many variants have several slightly modified versions, paralleling microbial diversity. Worms, such as the infamous "ILOVEYOU" worm in 2000, may employ a universal message of gratification to entice users. Their wide dissemination parallels the spread of socially transmitted diseases (e.g., influenza) that have the potential to infect everyone susceptible. In contrast, computer viruses (spread by sharing data on infected diskettes) parallel sexually transmitted diseases, whose spread is related to specific behavioral practices. Viruses or worms that are spread undetected but are activated at a later date (as was the case with the Michelangelo virus, discovered in 1991 and still around) resemble latent microbes, such as HIV. Denial-of-service attacks, which block access to a server by an onslaught of messages, are the equivalent of toxins, since neither can reproduce in their host and are only harmful above a critical concentration. Spam (unwanted but harmless e-mail), the curse of computer users with slow modems through expensive telephone connections, resembles bacterial commensals that can injure the host only under specific conditions.

Biological viruses can mutate rapidly, create novel pathogenic and transmission routes, and develop antigenic variation to evade host immunity. In the computer world, worms exhibit similar behavior. Once a worm has been transmitted successfully, variants quickly emerge. These variants cause damage in similar ways but evade detection and impediments installed to provide "immunity" to the original "strain." Therefore, knowledge of biological infections can be used to predict and anticipate highly virulent computer infections.

Although the computer user has some recourse against computer viruses, the costs may be high. As with biological viruses, good hygienic practice is helpful. Just as they should wash hands frequently, avoid exposure to people with colds, or use condoms to protect against infectious diseases, computer users should mistrust (and thus not open) files received through unexpected channels or with unknown extensions or subject lines, request confirmation from the sender before opening attachments, and regularly back up hard disks to reduce the risk of losing data. The consequences of such actions in terms of time, disk space, and efficiency illustrate a biological truth: immunity has cost. Effective antiviral barriers are impediments to communication. Moreover, virus protection programs are only as good as the last virus recognized, providing only partial protection at best. Computer users have not always taken inconvenient precautions, even in view of serious consequences. ILOVEYOU was a worm that used the same mechanism of spread as Melissa, which had been released a year earlier. Yet, ILOVEYOU turned out to be even more destructive than Melissa.

Biological immune disorders in which host defenses turn against the host and actually cause damage are known as autoimmune diseases. Computer autoimmune disorders parallel their biological counterparts. Recently, a warning (defense mechanism used by computer users) turned out to be a not-so-harmless hoax. The hoax warning stated that certain files were infected by a computer virus. Heeding the warning, unsuspecting computer users removed the affected utility files from their computers' operating systems. The harm mediated by this "host defense" was relatively small in this particular case, resembling the discomfort of allergies, in which immune responses to benign

agents cause limited damage. However, more malignant forms of "auto-mutilating" hoaxes are likely to emerge that could be as devastating to computers as some autoimmune diseases are to humans.

The electronic monoculture that improves communication also increases the risk for contagion. Predominant use of a single operating system has improved communication and sharing of electronic data but has also facilitated ready amplification of virulent programs. As with biological infection, transmission of computer infection depends on susceptibility of the population. Virus producers saw an opportunity in the popular preference worldwide for PCs with Microsoft Windows operating systems. The enormous popularity of these systems, along with their long-recognized inadequate protection against misuse, made computer users susceptible. Virtual viruses able to infect multiple operating systems are rare (as are biological viruses with broad host specificity), and even when infected, computers that run on different operating systems (e.g., Mac, Unix) or other-than-Outlook e-mail programs usually are dead-end hosts for PC viruses.

Pathogens do not reinvent the wheel. Virulence genes are constantly "stolen" and reused. Thus, new combinations of virulence genes can result in new pathogenic strategies, and such combinations frequently accumulate in pathogenicity islands. Reuse and combination of effective (and infective) strategies are also common in computer malware. A recent example demonstrates the value of just the right amount of virulence. A highly dangerous worm called Nimda (Admin in reverse) was released exactly 1 week after the September 11, 2001, terrorist attack in the United States. Nimda combined the most powerful strategies of Code Red and SirCam and spread more rapidly than any previous worm. Clicking on the subject line of an infected e-mail (to delete it, for instance) itself activated the worm. However, because of the immensity of the threat, the Internet community responded extremely rapidly. Within hours after its release, alerts to system administrators on how to block the worm had effectively slowed its spread. Early surveillance and barrier development averted disaster. As in contained epidemics of hemorrhagic fevers, the immense threat of high contagion and lethality prompts effective measures to rapidly recognize outbreaks and prevent pandemics.

The types of measures to be used against computer contagion can be learned from biology. Immune effectors of plants and animals protect against a broad range of pathogens; however, in nature this system evolved over millions of years. Engineering protective computer systems with similar efficacy within a few years is a great challenge. Current protection programs mainly resemble innate immunity, but programs that learn from exposure (thus resembling adaptive immunity) are under development. Vaccination with relatively harmless microbes primes the immune system. Biological hosts also naturally carry protective microflora that compete with pathogens. Could we produce "virtual vaccines" that are beneficial to the computers carrying them (e.g., by blocking preferred sites of entrance for viruses or repairing viral damage automatically) and let these "good" microbes circulate on the Internet just as malignant viruses do? Crude versions of such vaccines have already been developed. Using "good" microbes would have its costs: occupation of Internet capacity and consequent slowdown of data transmission and presence of malicious worms disguised as beneficial ones to elude detection.

Knowledge of infectious diseases may help control computer contagion. Conversely, study of computer malware may help curb infectious disease emergence. Internet contagion illustrates how pathogens emerge and spread in our increasingly small world. The speed of virtual pathogen evolution makes it possible to follow the process of mutation and selection in real-time. With countless interlinked computers, the risk for virtual contagion is so great that urgent steps are needed to avoid catastrophe. How many pandemics will it take before we accept the risks and costs of computer immunity? Similarly, to protect against emerging pathogens, we must use all tools available, including virtual pandemics. A task force to collect data on the epidemiology of virtual infections as a model for infectious diseases might be an important first step.

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Emergence and Rapid Spread of Tetracycline-Resistant *Vibrio cholerae* Strains, Madagascar

To the Editor: The Indian Ocean was free of cholera for decades, until January 1998, when an outbreak was detected in Comoros Islands (1). On March 23, 1999, the Malagasy Epidemiological Surveillance System reported the first case of cholera in Mahajanga, a harbor on the northwest coast (2). In May 1999, the Malagasy sanitary authorities set up sanitary barricades at the borders of the two provinces—Mahajanga and Antananarivo—affected by the epidemic. Oral doxycycline was systematically given to all the travelers crossing the barricades. In addition, doctors in hospitals and dispensaries in these two provinces gave doxycycline to patients

with acute diarrhea. Despite these measures, cholera had reached all six provinces of the island 10 months later. In June 1999, a specific cholera surveillance system was established in every Malagasy province with close collaboration between the Malagasy Ministry of Health and the Institut Pasteur de Madagascar.

The first strain isolated in Mahajanga was *Vibrio cholerae* serogroup O1, serotype Ogawa, biotype El Tor. Its antibiotype showed resistance to trimethoprim-sulfamethoxazole, sulfonamides, trimethoprim, chloramphenicol, streptomycin, and vibriostatic agent O129 (a molecule naturally active against *V. cholerae* and used for identification). Susceptibility was conserved for tetracycline, ampicillin, cephalotin, and pefloxacin (2). This strain showed a rRNA gene restriction pattern similar to those of African and Comorian strains isolated since 1994 and 1998, respectively (2,3).

From July 1999 to March 2001, we monitored the tetracycline resistance of *V. cholerae* isolated from the stool samples sent to the Institut Pasteur de Madagascar in Antananarivo, using the standard disk-diffusion method (4). Stool samples were collected in sterile containers, on Whatman paper, or on rectal swabs. Isolation of *V. cholerae* was carried out immediately after reception. Every *V. cholerae* strain identified belonged to serogroup O1, biotype El Tor. All the tetracycline-resistant *V. cholerae* isolated and 60 randomly selected tetracycline-susceptible strains were tested for sensitivity to the following drugs: ampicillin, cephalotin, doxycycline, sulfonamide, trimethoprim, trimethoprim-sulfamethoxazole, chloramphenicol, streptomycin, spectinomycin, neomycin, kanamycin, nalidixic acid, pefloxacin, erythromycin, rifampicin, and nitrofurantoin, as well as to vibriostatic agent 0129.

During the study period, we isolated 351 (46.1%) *V. cholerae* strains from 761 stool samples analyzed. The provinces of Antananarivo, Mahajanga, and Toliary accounted for 85.9% of the stool samples sent to our laboratory. From these provinces, we isolated 288 strains; by contrast, from the three other provinces (Antsiranana, Fianarantsoa, and Toamasina, located on the east coast), 63 strains were isolated. Rates of isolation, tested by a chi-square test, did not differ significantly between the six provinces ($p=0.32$).

Fifty five (15.7%) of the 351 strains isolated were found to be tetracycline resistant (cross-resistance with doxycycline) but had the same resistance pattern as the index strain isolated in Mahajanga for the other antibiotics tested. During the first rainy season following the epidemic (November 1999 to March 2000), a unique tetracycline-resistant strain

was isolated (in February 2000), in the capital Antananarivo; it was also resistant to ampicillin, nalidixic acid, and nitrofurantoin. During the dry season (from April to October 2000), five (13.2%) of 38 *V. cholerae* new tetracycline-resistant strains were found. However, during the last rainy season (November 2000 to March 2001), 49 (69 %) of 71 strains isolated were tetracycline resistant. They were mainly from the city and suburbs of Antananarivo (95.3%, 41/43 strains). The eight other resistant strains came from the provinces of Antananarivo, Toliary, and Fianarantsoa.

As observed in Tanzania (5), the extensive prophylactic use of tetracycline may have triggered the rapid emergence and spread of tetracycline-resistant strains in Madagascar. The high rate of resistance in Antananarivo, where the major Malagasy hospitals are located, could be due to easier access to drugs in the capital than in the other provinces.

Of the 60 randomly selected tetracycline-susceptible strains, 56 had the original antibiotype; four became susceptible to vibriostatic agent O129 and to all the antibiotics tested, except trimethoprim. Four (3.5%) of the 115 strains tested (55 tetracycline-resistant and 60 tetracycline-susceptible strains) on a large panel of antibiotics were susceptible to trimethoprim-sulfamethoxazole. As usually observed in other African cholera-endemic countries (6), only a small proportion of the strains were susceptible to trimethoprim-sulfamethoxazole, one of the most frequently dispensed drugs.

Faced with this first emergence of cholera in Madagascar and its rapid spread, medical authorities reacted immediately by using doxycycline as chemoprophylaxis (contrary to World Health Organization recommendations [7]), probably because of its easy availability**.**

Our study demonstrates that 2 years after the epidemic began, neither trimethoprim-sulfamethoxazole nor tetracycline, the two first-line drugs used in Madagascar, can be recommended any longer for treating severe cases of cholera. This may represent a critical public health problem in the country, especially as most of the population cannot afford more effective but expensive antibiotics.

Therefore, Malagasy medical authorities should a) abandon any systematic chemoprophylaxis, b) advise only oral rehydration therapy for mild-to-moderate cases, and c) reserve antibiotic therapy for severe illness (7). These measures against the cholera epidemic should be accompanied by general reinforcement of microbiologic surveillance to monitor antibiotic resistance so that the island can respond effectively to any future bacterial epidemics.

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Fifth Annual Conference on New and Re-Emerging Infectious Diseases

University of Illinois,
Urbana-Champaign, IL
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The Fifth Annual Conference on New and Re-Emerging Infectious Diseases will be held April 18-19, 2002. On April 18, 2002 the conference will be held at 4:00 p.m. at the University of Illinois at Urbana-Champaign, Bevier Hall Auditorium.

The keynote speakers are Dr. Beatrice H. Hahn, Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL (SIV reservoirs and human zoonotic risk) and Dr. Dr. Matthew K. Waldor, Howard Hughes Medical Institute, Division of Geographic Medicine/Infectious Diseases, New England Medical Center and Tufts University School of Medicine, Boston, MA, (Cholera toxin). On April 19, 2002, morning and afternoon sessions will be held at Hawthorne Suites Hotel, Champaign from 8:15 am to 6:00 p.m. Invited speakers and topics include: Dr. Edward M. Eitzen, Commander, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland (Biological weapons); Dr. John Donelson, Department of Biochemistry, College of Medicine, University of Iowa (The genome of African trypanosomes); Dr. Daniel Goldberg, Howard Hughes Medical Institute, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, (Genome-wide analysis of malaria parasites); Dr. Barry N. Kreiswirth, Director, Public Health Research Institute Tuberculosis Center, New York, N.Y. (Global dissemination of the multidrug resistant Mycobacterium tuberculosis).

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Participants include health-care practitioners, public health officials, and allied health professionals who are actively involved and/or interested in research or management of Lyme borreliosis and other tick-borne diseases. Major program topics include Lyme borreliosis: state of the art and future research directions; diversity of Borrelia —clinical, pathogenetic, and diagnostic implications and impact on vaccine development; genetics of *Borrelia burgdorferi;* laboratory diagnosis; and strategies for prevention of Lyme borreliosis and other tick-borne diseases.

The deadline for abstracts is May 10, 2002. For further information, contact: Heather Drew, Imedex, 70 Technology Drive, Alpharetta, GA 30005; tel: + 1 770-751-7552; fax: +1 770-751-7334, e-mail: h.drew @imedex.com or online at www.imedex.com/infectiousdisease.htm

About the Cover

Flying Fox

Vincent van Gogh, 1885
(oil on canvas, 41 cm x 79 cm)

Courtesy of Van Gogh Museum, Amsterdam
(Vincent van Gogh Foundation)

According to the Van Gogh Museum in Amsterdam, the origins of Flying Fox are not well documented. Van Gogh probably saw a flying fox in a museum or private collection in Brabant, Antwerp, or Paris. The dark brown background colors in the painting are similar to those in other works of his Nuenen period. The brighter colors and rough brushstrokes in the wings are more avant garde and suggest techniques used in his later paintings.

Flying foxes like the one that captured van Gogh's imagination are very large fruit-eating bats (order Chiroptera, suborder Megachiroptera). These mammals are found in tropical and subtropical regions between Africa and the South Pacific, including the Philippines, where there are 70 species of bats. Flying foxes can weigh as much as 1.5 kg and have a wingspan of up to 1.8 m. Occasionally, they are hunted and used as a food source (1).

Worldwide, bats are a major predator of night-flying insects and farm pests. Throughout the tropics, they are vital to the survival of the rain forest through their seed dispersal and pollination activities. Studies of bats have contributed to medical advances, including the development of navigational aids for the blind.

Paul Arguin

Centers for Disease Control
and Prevention, Atlanta, Georgia

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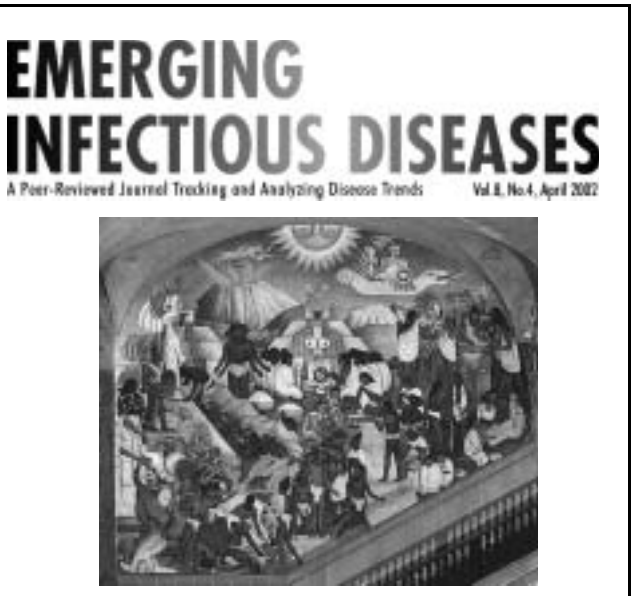

In the next issue

Feline Host Range of Canine Parvovirus (CPV): Recent Emergence of New Antigenic Types of CPV in Cats

Megadrought and Megadeath in 16th Century Mexico

Experimental Infection of Horses with West Nile Virus and Evaluation of Their Potential to Infect Mosquitoes and Serve as Amplifying Hosts

Outcomes of Persons with Treated Human Granulocytic Ehrlichiosis

Nine Years of Survey of European Bat Lyssavirus Infection in Spanish Bat Populations

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

Editorial Policy and Call for Articles

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

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

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

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

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). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Arial. Mac files should be sent in MS Word or RTF formats.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in 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 in full. List the first six authors followed by "et al."

Tables and figures. Create tables within MS Word's table tool. Do not format as columns or tabs; do not submit tables in Excel. For figures, use color as needed. Send digital files (see above) or camera-ready slides, photographs, or laser prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or 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: 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. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles 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. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Another Dimension: Thoughtful essays on philosophical issues related to science and human health.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.8, No.3, March 2002

On the Cover:
Vincent van Gogh, Flying Fox, 1885 (oil on canvas, 41 cm x 79 cm) Courtesy of Van Gogh Museum, Amsterdam (Vincent van Gogh Foundation)

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.8, No.3, March 2002

Letters

Guidelines for Letters. Letters discussing a recent Emerging Infectious Diseases article (400-500 words, 5-10 references) should be received within 4 weeks of the article's publication. Letters reporting preliminary data (500-1,000 words, 10 references) should not duplicate other material published or submitted for publication, should not be divided into sections, and should avoid figures or tables. All letters have the same authorship, financial disclosure, and acknowledgment requirements as full articles and should include a word count. For more guidance on manuscript preparation, see Emerging Infectious Diseases Instructions to Authors. Send letters to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA, or e-mail: eideditor@cdc.gov .

Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

Feasibility of National Surveillance of Health-Care–Associated Infections in Home-Care Settings

Lilia P. Manangan,* Michele L. Pearson,* Jerome I. Tokars,* Elaine Miller,* and William R. Jarvis*

This article examines the rationale and strategies for surveillance of health-care-associated infections in home-care settings, the challenges of nonhospital-based surveillance, and the feasibility of developing a national surveillance system.

Over the past 2 decades, the delivery of health care in the United States has shifted increasingly from hospitals to patients' homes (1-3). Nearly eight million people in the United States received medical care at home in 1996 (4), and an estimated 774,113 (10%) of these patients had at least one indwelling medical device (5). Use of a medical device is the greatest predictor (exogenous) of health-care-associated infection.

Home care is often provided by family members who have little or no formal health-care training, which may place patients at increased risk of health-care-associated infections not typically seen in hospitals. In the home-care setting, patients with open wounds or central venous catheters may undertake activities of daily living (e.g., bathing, exercising, gardening, and playing with pets) that may increase the risk of infections.

Rationale for a National Surveillance System in Home-Care Settings

The epidemiology of health-care-associated infections in home-care settings has not been defined, but infections certainly occur. Outbreaks have been documented in association with use of central venous catheters, parenteral nutrition, bathing practices, educational level of caregivers, and the introduction of new products, such as needleless devices for intravenous infusion (6-8).

Needleless devices are used for connecting and accessing intravenous infusion tubing, replacing traditional needles. These devices are used in both home and hospital settings and are perceived to be safe for patients and effective in reducing needlestick injuries.

From 1993 through 1995, the Hospital Infections Program, now Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention (CDC), investigated three outbreaks of bloodstream infections (BSI) in patients receiving infusion therapy in their homes. In all three outbreaks, needleless devices were associated with BSIs. The first outbreak occurred in Rhode Island in 1993-1994. The endcaps on these devices were changed every 7 days. BSIs were frequent when needleless devices were used to admini-ster total parenteral nutrition (6). The second outbreak, in Oakland, California, during 1992-1994, occurred among pediatric hematology-oncology patients. The BSI rate was higher when needleless devices were used by Asian or Hispanic children but not by white or black children. The racial/ethnic differences were thought to stem from socioeconomic factors or possibly from language barriers that prevented full understanding of instructions on infection control (7). The third outbreak occurred in Houston, Texas, in 1994-1995. The BSI rate was higher when the needleless device endcaps were changed every 7 days and lower when they were changed every 2-3 days. Patients who showered may have had a higher BSI rate than those who took tub baths (8).

These outbreak investigations were, by necessity, retrospective, and some data were difficult to obtain. To better define the epidemiology of BSIs in the home-care setting, in 1995 the Hospital Infections Program conducted a prospective multicenter study of home infusion therapy patients. The objectives were to determine rates of BSI and to identify risk factors, especially the use of needleless devices. The study, which was conducted in Cleveland, Ohio, and Toronto, Canada, involved 827 patients (69,532 catheter-days) (9). The most common underlying diagnoses among this cohort were infections caused by organisms other than HIV (67%), malignancy (24%), nutritional and digestive disease (17%), heart disease (14%), organ transplantation (11%), and HIV infection (7%).

Overall, 7% of these patients had one or more BSIs during a median of 44 days of catheter use (range 1 to 395 catheter days). A multivariate analysis showed that independent risk factors included recent bone marrow transplant, receipt of total parenteral nutrition, receipt of infusion therapy outside the home (e.g., in a clinic or physician's office), use of a multilumen catheter, and having had a previous BSI (9). Needleless devices were not associated with BSI.

Two prevalence surveys of infections among patients of Missouri home health agencies were conducted by CDC in

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collaboration with the Missouri Alliance for Home Care (MAHC) and the Missouri Department of Health, the first during summer (June 1-30, 1999) and the second during winter (February 15-March 15, 2000). Of 5,100 home-care patients enrolled in the summer survey, 16% (793) were reported to have infections; 8% (63) of these infections were reported as being acquired at home, 16% (127) as hospital acquired, 35% (278) as unknown source, and 41% (325) as community acquired. The infection sites reported were urinary tract (214 [27%]), respiratory tract (190 [24%]), skin or soft tissue (190 [24%]), surgical site (95 [12%]), or bloodstream (17 [2%]); 18% (143/793) of infections occurred at other body sites (e.g., gastrointestinal, bone) (10). Of 2,890 patients enrolled in the winter survey, 16% (466) had infections. The prevalence of respiratory tract infections was higher during the winter survey than during the summer survey. These results suggest that an estimated 1.2 million patients receiving home care in the United States have infections annually, supporting a need for surveillance of infections among home-care patients (11).

A nationwide hospital-acquired infection surveillance system and standardized infection definitions have been in existence since the 1970s (12-14). However, no national surveillance or standardized definitions exist for monitoring infections in the home-care setting. The Association for Professionals in Infection Control and Epidemiology, Inc. (APIC) published draft definitions for surveillance of infections in home-care patients (15). However, these definitions have not yet been validated.

National surveillance of health-care-associated infections in home care may potentially decrease infection rates, as has been documented in hospitals by the National Nosocomial Infections Surveillance system (NNIS). This voluntary, hospital-based reporting system was established to monitor hospital-acquired infections and to guide the prevention efforts of infection control practitioners. During 1990-1999, risk-adjusted infection rates in intensive-care units decreased by approximately 40% among hospitals participating in NNIS (16).

A national system for surveillance of health-care-associated infections in home care would not only provide useful data on incidence and types of infections but also simplify identification of risk factors for infection and development of national benchmarks for comparing infection rates. Risk-adjusted rates may assist individual home-care agencies to identify areas for performance and quality improvement and to evaluate the impact of prevention interventions on infection rates.

Challenges to Developing a National Surveillance System

Home-care surveillance poses several unique challenges, including lack of nationally accepted standard definitions and surveillance methods, loss of patient follow-up, lack of trained infection control personnel in home-care settings, difficulty in capturing clinical and laboratory data, and difficulty in obtaining numerator and denominator data.

Lack of Nationally Accepted Definitions and Methods

A cornerstone of surveillance in any setting is development of standardized definitions and methods. Individual home-care agencies have developed surveillance definitions for their own use (17-20), but national definitions of infections in home care do not exist. The draft APIC definitions of home health-care-associated infections have yet to be accepted and implemented nationally. These definitions should be tested to determine their practicality or applicability, given the limited use of laboratory diagnostics in home care. In addition, standard methods of case finding, recording, and calculating rates are also essential. If national benchmark rates are to be established to permit inter- and intra-agency comparisons, consensus definitions of home health-care-associated infections, such as those published by APIC, will have to be implemented.

Loss of Patient Follow-up

Home-care patients often are served by several agencies or are readmitted to the hospital during their illness. Lack of continuity of care hampers detection and reporting of health-care-associated infections. For example, if a home-care patient receiving intravenous therapy has a fever, is admitted to an acute-care facility, and is confirmed to have a BSI, this information may not be communicated to the home-care agency (the same or a different one) when the patient is discharged to continue infusion therapy at home.

Lack of Trained Personnel

Surveillance requires adequately trained infection control personnel, but few home health companies have such employees who are designated to conduct infection control activities, including education, surveillance, and prevention. In a recent survey of home-care agencies in Missouri, only 51 (54%) of 95 had a designated infection control practitioner, and only 27 (53%) of 51 provided ongoing training (21). In most home-care agencies, infection control activities are performed on a volunteer basis with no additional compensation. Successful implementation of surveillance programs and other infection control activities in the home health-care setting will require designated and appropriately trained personnel. Training should include calculation of infection rates, recognition of outbreaks and clusters, providing feedback data to essential personnel, and monitoring compliance of prevention efforts. Educational activities targeted at patients, health-care workers, and other caregivers will also be a necessary part of the infection control program.

Difficulty in Capturing Clinical and Laboratory Data

Many home-care agencies are privately owned and have no hospital or laboratory affiliation; therefore, access to diagnostic services may be limited, and home-care personnel may have difficulties in tracking laboratory results (e.g., contacting out-of-state physician offices or laboratories). Limited access to test results may also encourage home-care personnel to use empiric therapy without documentation of infection or

identification of a causative pathogen. Linkages for sharing clinical and laboratory data among physicians, hospitals, and home-care agencies are essential to optimize patient care in the home.

Difficulty in Obtaining Numerator and Denominator Data

Surveillance for infections in home care will require methods to identify appropriate numerator and denominator data for calculating infection rates for inter- and intra-agency comparison and benchmarks. Collection of numerator data (e.g., BSI or other infectious complications) will require systems that permit data sharing by hospitals and laboratories with home-health agencies.

Capturing appropriate denominator data may even be more difficult (22). For example, to determine device-associated infection rates, device utilization must be measured by monitoring days of use. However, if insertion, care, and removal of the device (e.g., central venous catheter, urinary catheter, tracheostomy tube) are done in different health-care settings, it will be difficult to monitor how many days a device is used. Although infection rates based on device utilization have been shown to be necessary in the acute-care setting, it is not certain that they are necessary in home care.

Another option for denominator is the number of days a patient uses a device during home care only, rather than the total number of days (i.e., from insertion to removal) the device is used. Because all home infusion therapy patients have intravenous catheters, patient days may be substituted for device days as long as they equal one another.

In addition to these challenges, the home-care industry will have to deal with the financial implications of implementing and maintaining a national surveillance system. Data on the cost of a surveillance system and on methods of calculating that cost into the reimbursement systems of health-care payors are very much needed.

Despite cost concerns, patient safety and outcomes are becoming increasingly important in the current health-care environment. Purchasers should base their selection of a home-care agency on patient outcomes and satisfaction rather than cost. Thus, home care agencies must conduct surveillance for adverse events. Without such surveillance systems, it would be very difficult for agencies to know if problems are occurring and whether quality care is being provided.

Progress Toward a National Surveillance System for Health-Care-Associated Infections

Several groups are collecting data on health-care-associated infections in home care and other outpatient areas. These data may prove useful in developing a national home health-care surveillance system.

MAHC is a nonprofit association that provides home care education, advocacy, and information for its 250-member agencies, most of which are located in Missouri. In the early 1990s, MAHC established an infection control committee composed of nurses who provided infection control activities for their agencies. In 1993, the committee implemented the MAHC Infection Surveillance Project (ISP) to monitor infections associated with central venous and urinary catheters. ISP is an active surveillance system that uses standardized criteria and definitions for tracking, aggregating, and reporting urinary infections and BSIs among home-care patients. Currently, 99 home-care agencies from 25 states participate in ISP. Although MAHC has contracted with the Hospital Industry Data Institute, Missouri Hospital Association, to organize and present the ISP data, the results have not yet been published. Although the ISP definitions have not been validated to determine sensitivity and specificity, the data allow participating agencies to compare their infection rates with those of other agencies.

On a broader scale, the Health Care Financing Administration (HCFA), now the Center for Medicare and Medicaid Services, in collaboration with the Center for Health Sciences and Policy Research, has developed the Outcome and Assessment Information Set (OASIS) to measure patient outcomes and improve quality in home care. HCFA requires all Medicare-certified home-care agencies to electronically submit data for their Medicare patients to a central OASIS database in Baltimore, Maryland. The outcomes monitored in OASIS are changes in patient health status, as indicated by need for emergency care or hospitalization, for example. Data collected include patient demographics and medical history, living arrangements, type of wound, urinary tract infection, respiratory devices, medications, emergency care received, transfer to an inpatient facility, and death. Most data items are obtained at start of care, every two calendar months, and at discharge. Since August 1999, more than eight million records have been entered into the OASIS database and information on how to access the OASIS reports can be obtained from http://www.hcfa.gov/medicaid/oasis/osishmp.htm.

Another national and international data source is the Outpatient Parenteral Antimicrobial Therapy (OPAT) registry, which aims to improve delivery of care and outcomes for outpatients receiving parenteral antimicrobial therapy. OPAT provides a broad database for assessing antimicrobial drug-prescribing practices and outcomes among patients with infections treated in outpatient settings. Data collected include patient demographics, diagnosis, pathogen, venous access device, infusion system, adverse events, clinical outcome, and patient satisfaction. Currently, 25 OPAT provider sites from 16 states are participating in the U.S. registry, and 24 provider sites from 6 countries are in the international registry. OPAT data have been presented at scientific conferences (23).

Surveillance methods that are commonly used in hospital programs may not be feasible for home care. Different strategies are needed to make surveillance in the home easier to implement, particularly if adequately trained staff and diagnostic services are limited. For example, the Dialysis Surveillance Network provides a novel way of tracking hospitalization, antimicrobial use, and selected infections in hemodialysis outpatients (24). Episodes of potential infection are identified by a clearly defined sequence of steps that

involves completing an "incident form" for all patients admitted to a hospital or started on intravenous antimicrobial therapy. The presence (or absence) of symptoms indicating infection is recorded rather than the infections themselves, and a computer algorithm determines whether the infection case definitions are met; the data collector is not required to memorize case definitions. The lessons from this surveillance system, in addition to other traditional outpatient systems, may be useful in establishing national surveillance for home health-care-associated infections.

Nearly as many patients receive home care annually as hospital care. With the continued expansion of home health-care delivery and documented infection risk in this setting, a national system for surveillance of health-care-associated infections in the home-care setting is needed. Collaboration between home health-care agencies, state and federal health agencies, private industry, and national or managed-care organizations is essential to make this system feasible and functional. Development and implementation of such a system would foster better understanding of the epidemiology of health-care-associated infections in the home-care setting. Furthermore, this system would provide a means for monitoring the impact of interventions aimed at preventing the emergence of these infections in the home.

Lilia Manangan is a registered nurse with a Masters of Public Health from the University of Hawaii, with special interest in surveillance. She is an epidemiologist in the Surveillance Section, Surveillance and Epidemiology Branch, Division of Tuberculosis Elimination, Centers for Disease Control and Prevention.

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Human Campylobacteriosis in Developing Countries[1]

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Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli.* Research and control efforts on the disease have been conducted more often in developed countries than developing countries. However, because of the increasing incidence, expanding spectrum of infections, potential of HIV-related deaths due to *Campylobacter,* and the availability of the complete genome sequence of *C. jejuni* NCTC 11168, interest in campylobacteriosis research and control in developing countries is growing. We present the distinguishing epidemiologic and clinical features of *Campylobacter* enteritis in developing countries relative to developed countries. National surveillance programs and international collaborations are needed to address the substantial gaps in the knowledge about the epidemiology of campylobacteriosis in developing countries.

Campylobacteriosis is a collective description for infectious diseases caused by members of the bacterial genus *Campylobacter*. The only form of campylobacteriosis of major public health importance is *Campylobacter* enteritis due to *C. jejuni* and *C. coli* (1). The rate of *Campylobacter* infections worldwide has been increasing, with the number of cases often exceeding those of salmonellosis and shigellosis (2). This increase, as well as the expanding spectrum of diseases caused by the organisms, necessitates a clearer understanding of the epidemiology and control of campylobacteriosis.

Surveillance and control of diseases of public health importance in developing countries have focused on diseases such as malaria, tuberculosis, trypanosomiasis, onchocerciasis, and schistosomiasis (3). Programs for diarrhea and acute respiratory illness also exist (4). These programs have extensive support from the World Health Organization (WHO).

Campylobacter is one of the most frequently isolated bacteria from stools of infants with diarrhea in developing countries—a result of contaminated food or water (5,6). However, national surveillance programs for campylobacteriosis generally do not exist in most developing countries despite the substantial burden of disease. Most data available on campylobacteriosis in developing countries were collected as a result of support provided by WHO to many laboratories in developing countries, including grants for epidemiologic studies and Lior serotyping antisera provided by the Public Health Service of Canada (5,7). The number of reviews and updates on human campylobacteriosis in developed countries (8-11) is greater than that for developing countries (5,6). This disparity may be because *Campylobacter*-associated diarrhea in developing countries is not pathogenic in patients >6 months of age. However, a community-based longitudinal study provided evidence that infection could be pathogenic beyond the first 6 months of life in developing countries (12). Furthermore, the sequencing and publication of the complete genome of *C. jejuni* NCTC 11168 have heralded a renaissance of interest in this organism, offering researchers worldwide, including in developing countries, novel ways to contribute to understanding the organism's biology (13). Thus, to promote research and control of campylobacteriosis in developing countries, review information on human campylobacteriosis in these countries is urgently needed. We present the distinguishing features of campylobacteriosis in developing countries relative to developed countries.

Incidence

Generally, developing countries do not have national surveillance programs for campylobacteriosis; therefore, incidence values in terms of number of cases for a population do not exist. Availability of national surveillance programs in developed countries has facilitated monitoring of sporadic cases as well as outbreaks of human campylobacteriosis (2,8-11). Most estimates of incidence in developing countries are from laboratory-based surveillance of pathogens responsible for diarrhea. *Campylobacter* isolation rates in developing countries range from 5 to 20% (6). Table 1 shows isolation rates for some countries according to WHO regions from studies of diarrhea in children <5 years old (14-25). Despite the lack of incidence data from national surveys, case-control community-based studies have provided estimates of 40,000 to 60,000/100,000 for children <5 years of age (6,12). In contrast, the figure for developed countries is 300/100,000 (8). Estimates in the general population in developing and developed countries are similar, approximately 90/100,000 (5,6,8), confirming the observation that campylobacteriosis is often a

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Table 1. Isolation rates of *Campylobacter* from diarrhea specimens from <5-year-olds in selected developing countries

WHO region and country	Isolation rate (%)	Reference
Africa		
Algeria	17.7	14
Cameroon	7.7	15
Ethiopia	13.8	16
Nigeria	16.5	17
Tanzania	18.0	18
Zimbabwe	9.3	19
Americas		
Brazil	9.9	20
Guatemala	12.1	21
Eastern Mediterranean		
Egypt	9.0	12
Jordan	5.5	22
Southeast Asia		
Bangladesh	17.4	23
Thailand	13.0	24
Western Pacific		
Laos	12.1	25

WHO = World Health Organization.

pediatric disease in developing countries. The isolation and incidence rates in some developing countries have increased since their initial reports (17). This increase has often been attributed to improved diagnostic methods, but an actual increase in incidence was observed in *Campylobacter*-associated diarrhea in the Caribbean island of Curaçao (26).

Age of Infection

In developing countries, *Campylobacter* is the most commonly isolated bacterial pathogen from <2-year-old children with diarrhea (Table 2). The disease does not appear to be important in adults. In contrast, infection occurs in adults and children in developed countries. Poor hygiene and sanitation and the close proximity to animals in developing countries all contribute to easy and frequent acquisition of any enteric pathogen, including *Campylobacter.* Although infections in infants appear to decline with age (Table 2), a comprehensive community-based cohort study in Egypt has shown that infection could be pathogenic regardless of the age of the child, underscoring the need for strengthening prevention and control strategies for campylobacteriosis (12).

Polymicrobial Infections Involving *Campylobacter*

Campylobacter is isolated relatively frequently with another enteric pathogen in patients with diarrhea in developing countries. In some cases half or more patients with *Campylobacter* enteritis also had other enteric pathogens (23,30). Organisms reported include *Escherichia coli*, *Salmonella*, *Shigella*, *Giardia lamblia*, and *Rotavirus*. Polymicrobial infections involving *Campylobacter* are rare in developed countries (5,6).

Isolation of *Campylobacter* in Healthy Children

The recovery of *Campylobacter* organisms from children without diarrhea is common in developing countries. In some reports the isolation rates for symptomatic and asymptomatic children were not statistically significant. Values as high as 14.9% in controls have been observed (14). Acquisition of the pathogen because of poor sanitation and contact with animals early in life may explain the isolation from healthy children. *Campylobacter* is not frequently recovered from asymptomatic persons in developed countries, as observed in the Netherlands, where a 0.5% isolation rate has been reported (9).

Seasonal Variation

In developing countries, *Campylobacter* enteritis has no seasonal preference; in contrast, in developed countries epidemics occur in summer and autumn (2). Isolation peaks vary from one country to another and also within countries (12,31,32). The lack of seasonal preference may be due to lack of extreme temperature variation as well as lack of adequate surveillance for epidemics (5,6).

Distribution of *Campylobacter* Species

C. jejuni and *C. coli* are the two main species isolated in developing countries. The isolation rate of *C. jejuni* exceeds that of *C. coli*, similar to observations in most developed countries (8,9). Lior biotyping and serotyping methods have been used in developing countries to subtype strains of *C. jejuni* and *C. coli* (5,6). Table 3 shows the distribution of the subtypes from three African countries. Biotype I was the most common, followed by biotype II. The prevalence of specific serotypes only in symptomatic children may indicate virulence traits or treatment, in cases of gastroenteritis (33). Furthermore, correlation between biotypes and serotypes isolated from humans and animals indicates that campylobacteriosis is zoonotic (36). Penner serotyping scheme and DNA-based typing, extensively

Table 2. Age of patients with *Campylobacter* infection in selected developing countries

Countries (ref.)	Age of infection (months)
Nigeria (17)	24
Tanzania (18)	18
China (27)	12-24
Thailand (28)	<12 (18.8%) 12-23 (12.3%) 24-59 (10.3%)
Bangladesh (29)	≤12 (38.8%) >12 (15.9%)
Egypt (12)	0-5 (8%) 6-11 (14%) 12-23 (4%)

Table 3. Distribution of *Campylobacter jejuni* and *C. coli* biotypes and serotypes in three African countries

	Biotypes						
	C. jejuni				*C. coli*		
Countries (ref.)	I	II	III	IV	I	II	Serotypes
Nigeria (33)	52.5	28.7	-	-	9.9	8.9	1, 8, 11, 20, 28, 45
Central African Republic (34)	31.9	11.0	2.4	-	44.0	11.5	-
South Africa (35)	95.4	1.5	-	-	3.1		4, 2, 12, 19, 23, 36

used in developed countries, have been proposed for use in developing countries (37).

Species other than *C. jejuni* and *C. coli*—such as *C. upsaliensis*, *C. concisus*, and aerotolerant campylobacters (*Arcobacter*)—may also be of pathogenic importance; however, diagnostic capacities to determine their distribution are lacking in developing countries (38). These other *Campylobacter* species constitute over 50% of campylobacters isolated at the Red Cross Children's Hospital, Cape Town, South Africa, for example. (A method termed the Cape Town Protocol is used to isolate *Campylobacter* species at this facility [39]). This higher incidence is also supported by a 16% isolation rate of *Arcobacter* species in a 4-month survey from poultry drainage water in Lagos, Nigeria (40).

Antibiotic Resistance in *Campylobacter* Isolates

Campylobacter enteritis is a self-limiting disease, and antimicrobial therapy is not generally recommended. However, antimicrobial agents are recommended for extraintestinal infections and for treating immunocompromised persons. Erythromycin and ciprofloxacin are drugs of choice (10). The rate of resistance to these drugs is increasing in both developed and developing countries, although the incidence is higher in developing countries. Use of these drugs for infections other than gastroenteritis and self-medication are often the causes of resistance in developing countries; in developed countries, resistance is due to their use in food animals and travel to developing countries. The increase in erythromycin resistance in developed countries is often low and stable at approximately 1% to 2%; this is not true for developing countries (41,42). For example, in 1984, 82% of *Campylobacter* strains from Lagos, Nigeria, were sensitive to erythromycin; 10 years later, only 20.8% were sensitive (17). In addition, resistance to another macrolide, azithromycin, was found in 7% to 15% of *Campylobacter* isolates in 1994 and 1995 in Thailand (43). The increasing rate of resistance to the fluoroquinolone, ciprofloxacin limits its clinical usefulness. In Thailand, ciprofloxacin resistance among *Campylobacter* species increased from zero before 1991 to 84% in 1995 (43). Recent data have shown a marked increase in resistance to quinolones in developed countries (41,42,44-46) (Table 4).

Campylobacter as a Cause of Travelers' Diarrhea

Travel to a developing country is a risk factor for acquiring *Campylobacter*-associated diarrhea. The diarrhea is more severe, and strains are associated with antibiotic resistance (47,48). Furthermore, campylobacteriosis acquired abroad contributes to the number of cases reported in developed countries (49). Among Finnish tourists visiting Morocco, the disease was more prevalent in winter months (50).

Clinical Features

The clinical spectrum of *Campylobacter* enteritis ranges from a watery, nonbloody, noninflammatory diarrhea to a severe inflammatory diarrhea with abdominal pain and fever. Disease is less severe in developing countries than in developed countries (5,6). In developed countries, disease is characterized by bloody stool, fever, and abdominal pain that is often more severe than that observed for *Shigella* and *Salmonella* infections. In developing countries the features reported are watery stool, fever, abdominal pain, vomiting, dehydration, and presence of fecal leukocytes; patients are also often underweight and malnourished (12,31,51). In Lagos, Nigeria, *Campylobacter* enteritis is characterized by a history of watery offensive-smelling stool lasting <5 days (51).

Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is an autoimmune disorder of the peripheral nervous system, which is characterized by acute flaccid paralysis. *C. jejuni* infection is the most frequently identified infection preceding GBS (52). In the developing world, sporadic GBS cases associated with *C. jejuni* infection have been reported from Curaçao, China, India, and South Africa (26,53-55). A comparative study between Curaçao and southwest Netherlands indicated that disease in Curaçao was more severe, had a higher incidence of preceding gastroenteritis, and had greater seasonal fluctuation (26). Serotype O:19 is most prevalent worldwide, although other serotypes, such as O:1, O:2, O:57, O:16, O:23, O:37, O:41, and

Table 4. Trends in Resistance to Ciprofloxacin by *Campylobacter jejuni* in selected developed countries up to year 2000

		Resistance strains (%)		
Country	Period	Initial	Year 2000	Ref.
Freiburg, Germany	1992-2000	22	32	41
Styria, Austria	1996-2000	25.2	40.2	42
England and Wales, UK	1993-2000	10	14.8	44
Philadelphia, USA	1995-2000	<10	36	45
Oslo, Norway	1988-2000	6.1	36	46

O:44, have been reported (52). *C. jejuni* strain O:41 appears to be restricted to Cape Town, South Africa, and represents a genetically stable clone (55,56). Detailed studies of the role of GBS in acute flaccid paralysis in developing countries, especially in polio-endemic areas, are needed.

Campylobacter Infection in the Setting of HIV

Campylobacter-associated diarrhea and bacteremia occur in HIV/AIDS patients worldwide. The species isolated include *C. jejuni*, *C. coli*, *C. upsaliensis*, *Arcobacter butzleri*, *Helicobacter fennelliae*, and *H. cinaedii* (57,58). The incidence of clinical manifestations is higher than in HIV-negative patients, with substantial mortality and morbidity. Furthermore, antibiotic resistance and recurrent infections have been observed (59). The incidence of HIV/AIDS is higher in developing countries than in developed countries and contributes substantially to deaths among <5-year-old children in epidemic settings (60). Thus, infants in developing countries are at risk of impaired immunity to *Campylobacter* enteritis. In addition, HIV/AIDS can increase the number of cases of campylobacteriosis in the adult population in these countries. These observations further support the need for improved understanding of the epidemiology of campylobacteriosis in developing countries.

Immunologic Aspects

In developing countries, such as Bangladesh, Thailand, Central African Republic, and Mexico, healthy children and adults are constantly exposed to *Campylobacter* antigens in the environment. As a consequence, serum antibodies to *Campylobacter* species develop very early in life in children in developing countries, and the levels of such antibodies tend to be much higher than those in children in the developed world such as in the United States (61-64). In Nigeria, children who had diarrhea and children who were healthy both had antibodies in their sera that could agglutinate *C. jejuni*; the difference in antibody responses between these groups of children was not statistically significant (65). Thus, antibody responses alone should be interpreted with caution in diagnosing *Campylobacter* infections.

In spite of shortcomings in the use of antibodies for diagnosis, increase in the level of anti-flagellar antibody had an inverse correlation with the rates of *Campylobacter* enteritis in the Central African Republic (63). An age-related relationship in the development of immunity to *Campylobacter* antigens has also been suggested to account for the age-related declines in the case-to-infection ratio and the period of excretion during the convalescent phase (28,66).

Usually, as age increases, level of antibody tends to increase. At the earliest stages in life (first 6 months), immunoglobulin (Ig) A, IgG, and IgM levels in response to *Campylobacter* infection are minimal, but thereafter increases are observed in response to infection. The poor serologic response during the first 6 months of life may be due either to a primary response to *Campylobacter* or to the presence of maternal antibodies via the placenta or breast milk.

Breast-feeding has been reported to play a role in *C. jejuni*-induced diarrhea. It decreases the number of episodes and the duration of diarrhea (67). In Algeria, exclusively breast-fed infants had fewer symptomatic *Campylobacter* infections than infants who were both breast-fed and bottle-fed (14).

Results of experimental observations among Mexican children have also shown that immunity to *Campylobacter* after primary infection may prevent bloody diarrhea from developing and subsequently prevent any disease from manifesting (68). In the developed world, the epidemiology may be different because most cases are usually primary infections with more severe clinical manifestations, greater numbers of people with bloody diarrhea (50%, as opposed to 15% in developing countries), and a more prolonged duration of excretion (approximately 15 days, compared with 7 days in developing countries) (28). The widespread immunity seen among adults in developing countries is absent in adults in developed countries (64).

Sources of Human Campylobacteriosis

Campylobacter infection is hyperendemic in developing countries. The major sources of human infections are environmental contamination and foods. Human-to-human transmission as a result of prolonged convalescent-phase excretion and high population density have also been suggested (5,12), although observations from developed countries show these are less likely factors (2).

Environmental Contamination

Wild birds as well as domestic and companion animals are known reservoirs for *Campylobacter* species, and shedding of the bacteria from them causes contamination of the environment. *C. jejuni* and *C. coli* have been isolated from chickens, goats, sheep, and pigs in developing countries (69,70). Strains isolated from human and chickens were phenotypically and genotypically correlated, confirming that chickens are an important source of human campylobacteriosis in developing countries (36). Poultry is also an important source of campylobacteriosis in developed countries. Extensive epidemiologic investigations have been done in those countries to identify sources of contamination and routes of transmission to humans to facilitate control efforts (71). Risk factors for acquiring campylobacters in developing countries include presence of an animal in the cooking area, uncovered garbage in cooking areas, and lack of piped water (12).

Foods

Campylobacter-contaminated foods—the result of poor sanitation—are an important potential source of infection in humans. For example, campylobacters were isolated from 40% and 77% of retail poultry meat sold in Bangkok, Thailand, and Nairobi, Kenya, respectively (72,73). The serotypes of the organisms isolated in Thailand were similar to those of organisms isolated from humans. In Mexico City, a survey of ready-to-eat roasted chickens showed that they were

contaminated with campylobacters (74). In developed countries, risk factors associated with foods include occupational exposure to farm animals, consumption of raw milk or milk products, and unhygienic food preparation practices (2).

Estimates of Impact of Human Campylobacteriosis in Developing Countries

The Disability Adjusted Life Year (DALY) is the basic unit used in Burden of Disease (BoD) methodology to quantify the impact of disease on a population (75). DALYs have been applied in the Dutch population to measure the mean health burden of *Campylobacter*-associated illness in the period 1990–1995 (76). The mean estimate was 1,400 DALYs per year; the main determinants of health burden were acute gastroenteritis (440 DALYs), gastroenteritis-related mortality (310 DALYs), and residual symptoms of GBS (340 DALYs). Although data on DALYs due to campylobacteriosis in developing countries are not available, diarrhea, which is a clinical manifestation of campylobacteriosis, was one of the top three causes of death and disease in developing countries in 1990 (75). The disease is projected globally to remain one of the top 10 by 2020. (The burden of campylobacteriosis in developing countries may increase by 2020 because HIV is projected to move up to the 10th position from 28th by 2020.) Considering the higher incidence of campylobacteriosis in developing countries, DALYs for the disease in developing countries will likely be higher than those of the Dutch population.

Conclusions

The incidence of human campylobacteriosis is increasing worldwide and has attracted the attention of WHO (http://www1.oecd.org/agr/prog/sum-copenhagen00.htm). Substantial gaps in knowledge about the epidemiology of campylobacteriosis in developing countries still exist. Present reported estimates of incidence are based on isolation rates from laboratory- and community-based studies conducted from 1980 to 1995. When various socioeconomic and health changes in developing countries are taken into account, these values may have changed considerably. Thus, public health awareness about the problem is needed, as are strengthened diagnostic facilities for campylobacteriosis, with a view towards setting up national surveillance programs. Such programs would determine the incidence rates, epidemiologic risk factors, interaction of HIV/AIDS and campylobacteriosis, seasonal variation, current state of resistance to antimicrobial agents, role of species other than *C. jejuni* and *C. coli*, and the role of campylobacteriosis in GBS. Collaboration among researchers in developed and developing countries needs to be strengthened, leading to development of regional centers of excellence. Funding organizations should provide incentives for North-South collaborations in *Campylobacter* research, as is done in other diseases such as malaria and trypanosomiasis that are endemic in some developing countries. All these should contribute to understanding of the global epidemiology of human campylobacteriosis.

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Guidelines for Synopses: 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.

Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Photographs and illustrations are encouraged.

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

Immunization with Heterologous Flaviviruses Protective Against Fatal West Nile Encephalitis

Robert B. Tesh,* Amelia P.A. Travassos da Rosa,* Hilda Guzman,* Tais P. Araujo,* and Shu-Yuan Xiao*

Prior immunization of hamsters with three heterologous flaviviruses (*Japanese encephalitis virus* [JEV] SA14-2-8 vaccine, wild-type *St. Louis encephalitis virus* [SLEV], and Y*ellow fever virus* [YFV] 17D vaccine) reduces the severity of subsequent *West Nile virus* (WNV) infection. Groups of adult hamsters were immunized with each of the heterologous flaviviruses; approximately 30 days later, the animals were injected intraperitoneally with a virulent New York strain of WNV. Subsequent levels of viremia, antibody response, and deaths were compared with those in nonimmune (control) hamsters. Immunity to JEV and SLEV was protective against clinical encephalitis and death after challenge with WNV. The antibody response in the sequentially infected hamsters also illustrates the difficulty in making a serologic diagnosis of WNV infection in animals (or humans) with preexisting *Flavivirus* immunity.

West Nile virus (WNV) was detected for the first time in North America in summer of 1999, during an outbreak involving humans, equines, and birds in the New York City metropolitan area (1). Persistence of the virus and its spread to other states on the eastern seaboard during 2000 and 2001 suggest that WNV is now endemic in the United States and that its geographic range probably will continue to expand until it extends over much of the continent (2). Although many WNV infections in humans are asymptomatic or unrecognized, some patients have an acute dengue-like illness, and a small percentage have encephalitis or meningoencephalitis (1-5). The latter complication is most common among the elderly, with recent reported case-fatality rates from 4% to 11% (3-9). No specific treatment is available for WNV encephalitis, and no licensed vaccine is available for its prevention.

WNV is a positive-stranded RNA virus; based on its antigenic and genetic characteristics, it is included in the *Japanese encephalitis virus* (JEV) serocomplex of the genus *Flavivirus*, family *Flaviviridae* (10). The JEV serocomplex includes four antigenically related viruses that are important causes of encephalitis in humans: JEV, WNV, *St. Louis encephalitis virus* (SLEV), and *Murray Valley encephalitis virus* (MVEV). In addition to their antigenic and genetic relatedness, these four viruses have many epidemiologic similarities (3,11).

Because of the close antigenic relationships among many viruses in this genus, *Flavivirus* infections are difficult to differentiate by most serologic techniques, especially in persons or animals having a second or sequential *Flavivirus* infection (12-14). Considerable attention has been focused on the immune response in primary and secondary *Flavivirus* infection and the role of immunopathogenesis in the etiology of severe *Flavivirus* disease (11,15,16). In the case of dengue, enhancement of virus replication by heterologous flavivirus antibodies and T-cell activation are thought to occur in some patients during a second or sequential dengue infection, resulting in hemorrhagic fever or shock (15,16). In contrast, animal data indicate that prior infection with a heterologous *Flavivirus* reduces the severity of subsequent challenge with WNV. Results of experimental studies with rodents, monkeys, and pigs (17-21) suggest that heterologous *Flavivirus* antibodies protect against or modify subsequent infection with WNV. This phenomenon could be important in vaccine development against WNV infection and in determining the ultimate geographic distribution and public health importance of WNV if it is introduced into areas of Central and South America where other flaviviruses, such as *Dengue virus* (DENV), Y*ellow fever virus* (YFV), SLEV, and *Ilhéus virus* (ILHV), are endemic.

To determine more precisely the degree of cross-protection among members of the JEV serocomplex and the possibility that this phenomenon could be used to protect against severe WNV infection, a series of experiments was carried out with three heterologous flaviruses and a recently described model (22) of WNV encephalitis. We report the results of these studies, which indicate that prior immunization of hamsters with a JEV vaccine strain and a wild-type SLEV—and to a lesser extent the 17-D YFV vaccine—modify subsequent WNV infection and protect the animals from fatal encephalitis.

Materials and Methods

Four flaviviruses were used in this study: WNV strain 385-99, isolated from a dead snowy owl at the Bronx Zoo during the 1999 epizootic in New York City (23); live attenuated SA14-2-8 vaccine strain of JEV (24,25); 17-D live attenuated vaccine strain of YFV (26); and SLEV strain Be Ar 23379

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(27), originally isolated from mosquitoes (*Sabethes belisarioi*) in Para, Brazil, in 1961.

The hamsters used in our studies were adult (70 g to 100 g) female Syrian golden hamsters (*Mesocricetus auratus*) (Harlan Sprague Dawley, Indianapolis, IN). Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with infected animals was carried out in biosafety level-3 facilities.

All virus titrations were done in cultures of the C6/36 clone of *Aedes albopictus* cells (28), with the presence or absence of viral antigen by immunofluorescence as the endpoint, as described (22,29,30). To determine the quantity of infectious virus in blood samples taken daily after WNV infection, each hamster blood specimen was titrated in 24-well tissue culture plates seeded with C6/36 cells. Serial 10-fold dilutions from 10^{-1} to 10^{-7} were made of each sample in phosphate-buffered saline, pH 7.4 (PBS), containing 10% fetal bovine serum; 0.1 mL of each dilution was added to four wells of a tissue culture plate. Following absorption at 28°C for 2 hours, 1.5 mL of maintenance medium (29) was added to each well, and the plates were incubated at 28°C in a 5% CO_2 atmosphere for 6 days. On day 6, 20 mL of a cell suspension from each well was added to a single spot on 12-spot glass microscope slides (Cell-Line Associates, Inc., Newfield, NJ). After drying at room temperature, the slides were immersed in cold acetone for 10 minutes; the cells were subsequently examined for the presence of WNV antigen by indirect fluorescent antibody test by using a WNV-specific mouse immune ascitic fluid (see below) and a commercially prepared fluorescein-conjugated, goat antimouse immunoglobulin (Sigma, St. Louis, MO). WNV titers were calculated as the tissue culture infectious $dose_{50}$ ($TCID_{50}$) per mL of specimen by the method of Reed and Muench (31).

Experimental Infection of Animals

Hamsters were infected by the intraperitoneal (IP) or subcutaneous (SC) routes, depending on the virulence of the infecting virus for the animals. WNV and YFV were injected IP; JEV and SLEV were administered SC. Infecting doses of the viruses were as follows: WNV $10^{4.0}$ $TCID_{50}$, YFV $10^{6.0}$ $TCID_{50}$, JEV $10^{6.5}$ $TCID_{50}$, and SLEV $10^{6.0}$ $TCID_{50}$.

Immune Reagents

A mouse immune ascitic fluid to WNV was prepared in adult mice. The immunogen was a crude homogenate of brain (10% W/V in PBS) from newborn mice injected intracerebrally (IC) with the B956 prototype strain of WNV (32). The adult immunization schedule consisted of four IP injections of the immunogen mixed with Freund's adjuvant, given at weekly intervals. Sarcoma 180 cells were given after the final injection to induce ascites formation.

Antibody Determinations

Serum antibodies to WNV and the other three flaviviruses were measured by hemagglutination-inhibition (HI) test and to WNV by immunoglobulin (Ig) M antibody capture enzyme immunoassay (MAC-ELISA) (33). Antigens for both serologic tests were prepared from brains of newborn mice injected IC with each of the flaviviruses; the infected brains were treated by the sucrose-acetone extraction method (33). Hamster sera were tested by HI at serial twofold dilutions from 1:20 to 1:5120 at pH 6.6 (WNV, JEV, and SLEV) or 6.4 (YFV) with 4 units of antigen and a 1:200 dilution of goose erythrocytes, following established protocols (33).

For the MAC-ELISA, microtiter plates were coated with a commercial goat anti-rat IgM (capture) antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), diluted 1:500 in carbonate buffer, pH 9.6. All hamster sera were screened at a 1:40 dilution. The WNV antigen was also used at a 1:40 dilution. The secondary (detecter) antibody was a mouse, anti-*Flavivirus*, peroxidase-conjugated monoclonal antibody (6B6C-1) at a dilution of 1:6000. Results were read with a SPECTRA shell reader (SLT Labininstruments, Salzburg, Austria). Specimens wells were recorded as positive when the absorbance values at optical $density_{405}$ nm of the specimen wells exceeded 0.20 after subtraction of average background absorbance of control wells (33).

Results

Infection of Nonimmune Hamsters with WNV

Several groups of *Flavivirus*-naive (control) hamsters were inoculated IP with 10^4 $TCID_{50}$ of WNV to determine the subsequent level and duration of viremia, immune response, and death rate. Table 1 and the Figure show the results of an experiment with a group of 10 hamsters that were bled daily for 6 consecutive days after infection with WNV. Moderate levels of virus were detected in the animals' blood within 24 hours and persisted for 5 or 6 days. The highest blood virus titers were detected on days 2 and 3 after infection (means $10^{5.2}$ and $10^{5.1}$, respectively). HI antibodies were detected in all the animals by day 5, and the titers had increased substantially by day 6. In general, WNV-specific IgM, as detected by MAC-ELISA, appeared at approximately the same time as the HI antibodies (data not shown).

Table 2 shows the results of a second experiment in which 13 hamsters were infected with WNV. All the animals were bled 6 days after injection, and a subset was bled again at 31, 60, and 90 days. Six days after infection, all the animals had specific HI antibodies to WNV antigen and were negative to the other three flaviviral antigens tested (YFV, SLEV, and JEV). At this time, the animals also had a strongly positive IgM antibody response by MAC-ELISA. Thirty-one days after infection, the HI antibody response had become broadly cross-reactive with the four *Flavivirus* antigens, although the highest titer was still to WNV, and the IgM antibody had begun to

Table 1. Pattern of viremia and hemagglutination inhibition (HI) antibody response in 10 adult *Flavivirus*-naïve (control) hamsters, following intraperitoneal inoculation of 10^4 $TCID_{50}$ of *West Nile virus* (WNV)

	Day postinoculation					
Animal No.	D-1	D-2	D-3	D-4	D-5	D-6
8001	4.3[a](0)	5.0 (0)	5.0 (0)	3.3 (0)	1.0 (1:80)	1.0 (1:320)
8002	4.7 (0)	5.5 (0)	5.2 (0)	3.5 (0)	2.5 (1:40)	0 (1:320)
8003	5.3 (0)	5.3 (0)	5.0 (0)	3.5 (0)	2.5 (1:40)	0 (1:320)
8004	2.0 (0)	5.0 (0)	5.0 (0)	4.3 (0)	2.5 (1:40)	1.0 (1:160)
8005	4.0 (0)	5.0 (0)	5.5 (0)	3.7 (0)	1.7 (1:80)	1.0 (1:320)
8006	4.6 (0)	5.2 (0)	5.7 (0)	4.3 (0)	2.7 (1:80)	0 (1:320)
8007	4.3 (0)	5.7 (0)	4.6 (0)	4.0 (0)	2.0 (1:80)	1.0 (1:320)
8008	4.2 (0)	5.8 (0)	4.8 (0)	1.8 (0)	2.0 (1:80)	0 (1:320)
8009	5.2 (0)	5.2 (0)	5.0 (0)	3.2 (0)	2.8 (1:80)	0 (1:320)
8010	4.7 (0)	4.7 (0)	5.5 (0)	3.5 (0)	1.8 (1:80)	0.7 (1:320)
Mean	4.3	5.2	5.1	3.5	2.1	0.5
SD	0.92	0.34	0.34	0.71	0.54	0.50

[a]WNV titer expressed as $\log_{10}$ $TCID_{50}$/mL of blood. 0 ≤0.7. (HI antibody titer; 0 ≤1:20)

decrease. A similar HI antibody pattern was observed at 60 and 90 days after infection, although by 90 days the HI titers were decreasing. Six of the nine WNV-infected hamsters gave a negative reaction in the WNV MAC-ELISA when tested 60 and 90 days after infection.

Five of the 13 hamsters infected in this second experiment died of WNV encephalitis 7 to 14 days after infection (Table 2). Overall, 14 (47%) of 30 adult hamsters injected IP with 10^4 $TCID_{50}$ of WNV died of encephalitis (Table 3). The pathologic reaction of the WNV hamster model has been described (22).

Infection of JEV-Immune Hamsters with WNV

The Figure and Table 4 show the results from another experiment in which 30 adult hamsters were given a single SC injection of approximately $10^{6.4}$ $TCID_{50}$ of the live attenuated JEV SA14-2-8 vaccine strain. Thirty-eight days later, the animals were injected (challenged) IP with 10^4 $TCID_{50}$ of WNV; 10 of the hamsters in this group were bled daily for 6 consecutive days. These blood samples were subsequently titrated to determine the level of WNV viremia. The resulting viremia in the JEV-immune animals was markedly lower than in the naïve hamsters (Figure). Furthermore, the JEV-immune hamsters responded to challenge with WNV by developing a secondary (sequential) type of *Flavivirus* antibody response. Table 4 shows the HI antibody titers to JEV and WNV antigens in sera of 10 of the SA14-2-8 vaccinated hamsters, 30 days after their JEV immunization. At this time the HI antibody titers to JEV and WNV antigens were characteristic of a primary *Flavivirus* infection (13,14). On day 38, the animals were challenged with WNV; 6 days later, their sera were tested for HI and WNV-specific IgM antibodies. The boost in HI antibody titers that was observed 6 days after challenge with WNV was typical of a secondary antibody response to *Flavivirus* infection (13,14). In contrast, IgM antibody response to the second *Flavivirus* (WNV) infection was minimal (Table 4).

All the JEV-immune hamsters (n = 30) survived challenge with WNV (Table 3). Their infection with WNV was confirmed by the presence of low-level viremia (Figure) and the secondary *Flavivirus* antibody response following challenge (Table 4). None of these hamsters appeared clinically ill after infection with WNV, in contrast to the naïve animals. Many of the nonimmune hamsters had clinical signs of acute central nervous system injury (somnolence, muscle weakness, paralysis, tremors, and loss of balance) beginning around day 6 after infection, and approximately half died (22). Thus, prior immunization with JEV vaccine reduced the severity of subsequent WNV infection and prevented death.

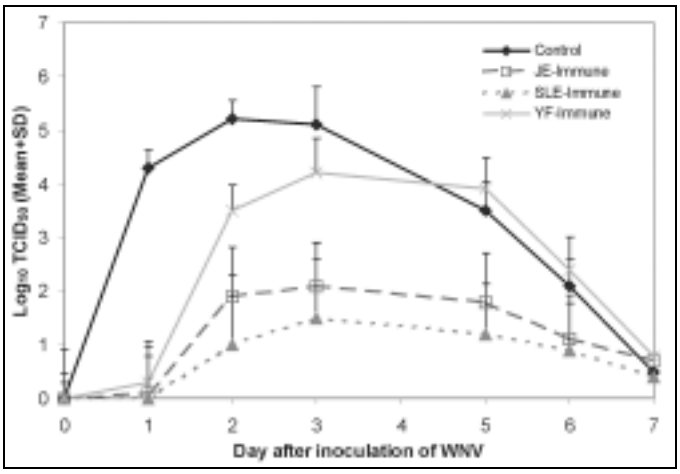

Figure. Summary of mean (±SD) *West Nile virus* (WNV) titers in daily blood samples from four groups of 10 hamsters each (control, *Japanese encephalitis virus* [JEV]-immune, *St. Louis encephalitis virus* [SLEV]-immune, and *Yellow fever virus* [YFV]-immune) after intraperitoneal inoculation of 10^4 tissue culture infective dose $(TCID)_{50}$ of WNV. Mean virus titers are expressed as $\log_{10}$ $TCID_{50}$/mL of blood.

Table 2. Serologic response of adult hamsters to *West Nile virus* (WNV), *Yellow fever virus* (YFV), *St. Louis encephalitis virus* (SLEV), and *Japanese encephalitis virus* (JEV) antigens, at various intervals after intraperitoneal inoculation of $10^{4.0}$ $TCID_{50}$ of WNV

Animal no.	HI antibody titer				WN MAC-ELISA
	WNV	YFV	SLEV	JEV	
Day 6					
8251(D[a])	1:40	0[b]	0	0	0.633[c]
8252	1:80	0	0	0	1.013
8253(D)	1:160	0	0	0	0.878
8254	1:160	0	0	0	1.090
8255	1:80	0	0	0	0.848
8256	1:80	0	0	0	0.840
8257(D)	1:160	0	0	0	1.291
8258	1:80	0	0	0	0.869
8259(D)	1:160	0	0	0	0.939
8260	1:80	0	0	0	0.992
8262	1:80	0	0	0	0.748
8263	1:80	0	0	0	0.797
8264(D)	1:80	0	0	0	0.827
Day 31					
8252	1:1,280	1:320	1:320	1:320	0.401
8254	1:1,280	1:320	1:320	1:320	0.427
8255	1:640	1:160	1:320	1:160	0.488
8256	1:640	1:160	1:160	1:160	0.582
8258	1:640	1:160	1:160	1:160	0.376
8260	1:1,280	1:320	1:320	1:320	0.420
8262	1:1,280	1:320	1:320	1:320	0.246
Day 60					
8252	1:2,560	1:640	1:640	1:640	0.269
8255	1:2,560	1:640	1:640	1:640	0.216
8256	1:640	1:160	1:160	1:160	0.162
8258	1:1,280	1:320	1:320	1:320	0.161
8260	1:320	1:80	1:80	1:40	0.179
8262	1:640	1:160	1:160	1:160	0.181
Day 90					
8260	1:640	1:80	1:80	1:80	0.217
8262	1:640	1:80	1:160	1:160	0.184

[a](D): Hamster died of encephalitis 7 to 14 days after infection. HI = hemagglutination inhibition.
[b]0 ≤1:20.
[c]Optical density value (≥0.200 is positive).

Infection of SLEV-Immune Hamsters with WNV

The Figure and Table 5 summarize the results of another experiment in which 32 adult hamsters were given a single SC injection of approximately 10^6 $TCID_{50}$ of SLEV strain BeAr 23379. This wild-type SLEV strain was selected for immunization, since it is not lethal to hamsters. Thirty-two days after injection with SLEV, the animals were inoculated IP with 10^4 $TCID_{50}$ of WNV. After this WNV challenge, the hamsters were bled daily for 6 consecutive days, as before. Antibody determinations were also done on blood samples taken 6 days after challenge with WNV.

Titration of daily blood samples from the SLEV-immune hamsters gave results similar to those in the JEV-immune animals. After challenge with WNV, 7 of the 10 SLEV-immune hamsters had brief, low-level viremia (Figure). However, three hamsters had no detectable viremia.

Serologic studies on blood samples taken 30 days after SLEV infection indicated that all the tested animals had been infected (Table 5). The HI response at 30 days was characteristic of primary *Flavivirus* infection. Six days after WNV infection, HI antibody titers had increased, indicating a secondary flavivirus antibody response. As with the JEV-immune hamsters, the IgM response of the SLEV-immune animals was minimal following the second flavivirus (WNV) infection (Tables 4,5).

Consistent with the low levels of WNV viremia (Figure), all the SLEV-immune hamsters (n = 32) survived subsequent challenge with WNV (Table 3). These animals did not appear clinically ill. These results indicate that prior immunity to SLEV also protected the hamsters from WNV encephalitis and death.

Infection of YFV-Immune Hamsters with WNV

Based on the results obtained with JEV- and SLEV-immune hamsters, we tested the effect of prior immunization with a non-JEV serocomplex *Flavivirus* on subsequent WNV infection. Accordingly, a group of 30 hamsters was inoculated IP with $10^{6.0}$ $TCID_{50}$ of the live attenuated 17D YFV strain. Thirty days after immunization, nine of the animals were bled and tested for HI antibodies to YFV and WNV (Table 6). Six days later (36 days after 17D vaccination), the hamsters were inoculated IP with 10^4 $TCID_{50}$ of WNV. Ten of these animals were bled daily for 6 consecutive days to determine the level of viremia and subsequent antibody response (Figure) (Table 6).

Following challenge with WNV, YFV-immune hamsters had an intermediate level of viremia (Figure). The mean WNV titers in the YFV-immune hamsters were higher than in the

Table 3. Infection and mortality rates, following intraperitoneal inoculation of 10^4 $TCID_{50}$ of *West Nile virus* (WNV), in nonimmune (control) hamsters, and in hamsters previously immunized with Japanese encephalitis (JE) SA14-2-8 vaccine, *St. Louis encephalitis virus* (SLEV) strain BeAr 23379, or yellow fever (YF) 17D vaccine

Immune group	No. infected with WNV	No. infected (%)[a]	No. died (%)
Nonimmune	30	30 (100)	14 (47)
JEV SA14-2-8	30	30 (100)	0 (0)
SLEV BeAr 23379	32	32 (100)	0 (0)
YFV 17D	30	30 (100)	4 (13)

[a] Total number of animals infected or dead after being infected with WNV.

Table 4. Serologic response of hamsters following immunization with the SA14-2-8 vaccine strain of *Japanese encephalitis virus* (JEV) and subsequent challenge with *West Nile virus* (WNV)

Hamster no.	HI antibody 30 days after JEV immunization		HI antibody 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
	JEV	WNV	JEV	WNV	
8236	1:80	1:80	1:640	1:640	0.166[a]
8237	1:40	1:80	1:320	1:320	0.205
8238	1:80	1:80	1:640	1:640	0.239
8239	1:80	1:80	1:640	1:640	0.173
8240	1:80	1:80	1:640	1:640	0.245
8241	1:80	1:80	1:320	1:640	0.271
8242	1:80	1:80	1:1,280	1:1,280	0.209
8243	1:40	1:80	1:160	1:160	0.205
8244	1:40	1:80	1:320	1:320	0.229
8245	1:80	1:80	1:160	1:160	NT

[a]Optical density value (≥Š0.200 is positive).
HI = hemagglutination inhibition. NT = not tested.

JEV- and SLEV-immune groups, but the titers were lower than in the *Flavivirus*-naïve (control) hamsters. The death rate in the YFV-immune hamsters was also lower; 4 (13%) of 30 YFV-immune hamsters died after challenge with WNV, compared with 47% in the control group (Table 3).

The HI antibody response after vaccination with YFV 17-D virus (Table 6) was less intense than the primary antibody responses to the other three flaviviruses (Tables 1,2,4,5). Monath (26,34) also observed that immunization with 17-D virus induces a weaker HI and complement-fixing antibody response than infection with a wild-type YFV strain. Nonetheless, 6 days after challenge with WNV, the animals previously immunized with 17-D virus demonstrated a strong secondary-type *Flavivirus* antibody response. Interestingly, the 17-D immune animals also had a stronger IgM response to WNV infection. These data indicate that 17-D vaccine gives only partial protection against challenge with WNV.

Table 5. Serologic response of hamsters following infection with *St. Louis encephalitis virus* (SLEV) strain Be Ar 23379 and subsequent challenge with *West Nile virus* (WNV)

Hamster no.	HI antibody 30 days after SLEV infection		HI antibody titer 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
	SLEV	WNV	SLEV	WNV	
8276	NT	NT	1:320	1:640	0.202[a]
8277	NT	NT	1:320	1:640	0.185
8278	1:80	1:80	1:160	1:160	0.141
8279	1:80	1:80	1:160	1:160	0.165
8280	1:80	1:80	1:640	1:640	0.276
8281	1:40	1:20	1:640	1:640	0.555
8282	1:80	1:40	1:160	1:80	0.177
8283	1:80	1:80	1:160	1:160	0.166
8298	1:160	1:80	1:320	1:320	0.139
8299	1:320	1:320	1:640	1:640	0.240

NT = not tested. HI = hemagglutination inhibition.
[a]Optical density value (≥0.200 is positive).

Discussion

The results of these hamster studies provide new information that may be useful in predicting the eventual geographic spread and public health importance of WNV in the Americas, as well as in developing novel methods for its control. The results also demonstrate the difficulty in making a serologic diagnosis of WNV infection in human or animal populations exposed to other flaviviruses.

First, our results clearly demonstrate that prior infection (and immunity) to JEV and SLEV protects hamsters from fatal WNV encephalitis (Table 3) and diminishes the severity of WNV infection (Figure). Other investigators (17-20) have reported similar findings in experimentally infected hamsters, pigs, and monkeys. The SA14-2-8 JEV strain used in our studies is one of several live attenuated JEV vaccines originally derived from the JEV SA14 wild-type parent strain (35,36); two of these vaccine derivatives, SA14-2-8 and SA14-14-2, have been widely used in China to immunize humans, equines, and pigs (24,25,35). Consequently, considerable information is already available on their biological and genetic characteristics, immunogenicity, safety, efficacy, and duration of immunity (24,25,35-37). The SA14 vaccine derivatives were obtained by serial passage (>100 times) in primary hamster kidney (PHK) cell cultures. Because the PHK cell substrate has not been approved by the World Health Organization as a vaccine substrate for use in humans, it is doubtful that the SA14 vaccine derivatives could be used in people in the United States or in other western countries. However, SA14-2-8 live attenuated JEV vaccine has been used successfully in >1 million horses in China (BQ Chen, pers. comm.) (25), and

Table 6. Serologic response of hamsters following immunization with the 17D yellow fever (YF) vaccine and subsequent challenge with *West Nile virus* (WNV)

	HI antibody titer 30 days after YF immunization		HI antibody titer 6 days after WNV challenge		WN MAC-ELISA 6 days after WNV challenge
Hamster no.	YF	WNV	YF	WNV	
8226	1:20	1:20	1:320	1:320	0.783[a]
8227	1:40	1:20	1:320	1:320	0.484
8228	1:80	1:40	1:640	1:640	0.378
8229	<1:20	1:20	1:640	1:640	0.311
8230	<1:20	1:20	1:320	1:320	0.694
8231	1:40	1:20	1:640	1:640	0.511
8233	1:40	1:20	1:320	1:320	0.345
8234	1:20	1:20	1:640	1:640	0.418
8235	1:40	1:40	1:320	1:320	0.658

[a]Optical density value (≥0.200 is positive).
HI = hemagglutination inhibition.

potentially it could be used in equines in the United States to protect against WNV encephalitis.

We are testing a commercial inactivated JEV vaccine (JE-VAX) that is already licensed for human use in the United States. If the licensed inactivated JEV vaccine protects hamsters in a manner similar to the SA14-2-8 attenuated vaccine, it might be considered as an interim WNV vaccine for groups of humans at high risk of exposure, such as laboratory workers and veterinarians, to protect against WNV encephalitis until a specific WNV vaccine is available. Several potential human WNV vaccines are now under development (38,39); however, it will probably be years before the testing and approval process is completed and they are licensed for human use.

A second potentially important finding from our hamster studies was that animals previously infected with JEV or SLEV viruses had a much lower viremia on challenge with WNV, compared with nonimmune animals (Table 1) (Figure). If a similar reduction in the level of viremia occurred in JEV- and SLEV-immune animals of other species (i.e., birds and pigs), such animals would probably be inefficient amplifying hosts for WNV virus. Interference from heterologous antibodies to other JEV-serocomplex viruses in birds and other vertebrate hosts may help explain the unique and largely nonoverlapping geographic distribution of the various members of this medically important *Flavivirus* complex (40,41). To date, the spread of WNV in North America has been limited to areas that are largely free of other endemic JEV complex flaviviruses (41-43). However, as WNV moves into South Florida and the Gulf Coast or into the Midwest, regions where SLEV is endemic (43), WNV could be restricted by heterologous antibodies to SLEV in the resident avian population. SLEV is also endemic in tropical America (44), so potentially the spread of WNV into that region might also be restricted for the same reason. It will be interesting to observe how this natural experiment unfolds.

A third important finding of our study concerns the difficulty in making a serologic diagnosis of recent WNV infection. The antigenic cross-reactivity of *Flavivirus* antibodies is well known, especially after a second or sequential *Flavivirus* infection in the same host (11-15). As noted, until now most WNV infections in humans and animals in North America have occurred in areas largely free of SLEV. In the northeastern region of the United States, serologic diagnosis of recent WNV infection has been relatively easy, since most people and animals were experiencing their first *Flavivirus* infection. However, as WNV spreads into geographic regions where people and animals have other preexisting *Flavivirus* antibodies (i.e., SLEV, YFV, DENV), the interpretation of HI, MAC-ELISA, and even neutralization test results will be more difficult. As we have shown (Tables 4, 5, and 6), hamsters with prior immunity to JEV, SLEV, or YFV had a broadly reacting HI antibody response after a second (sequential) WNV infection. Most of the JEV- and SLEV-immune hamsters did not develop specific IgM antibodies after WNV infection. Consequently, the WNV MAC-ELISA also may be of little diagnostic value in such human or animal cases. The HI test and MAC-ELISA are the two serologic tests most commonly used by public health and veterinary diagnostic laboratories in the United States to screen for WNV infection (42). Our data suggest that these tests may give equivocal results in regions where more than one *Flavivirus* is active and that other, more specific diagnostic techniques are needed.

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Associations between Indicators of Livestock Farming Intensity and Incidence of Human Shiga Toxin-Producing *Escherichia coli* Infection

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The impact of livestock farming on the incidence of human Shiga toxin-producing *Escherichia coli* (STEC) infection was assessed by using several livestock density indicators (LDI) that were generated in a systematic approach. A total of 80 LDI were considered suitable proxy measures for livestock density. Multivariate Poisson regression identified several LDI as having a significant spatial association with the incidence of human STEC infection. The strongest associations with human STEC infection were the ratio of beef cattle number to human population and the application of manure to the surface of agricultural land by a solid spreader and by a liquid spreader. This study demonstrates the value of using a systematic approach in identifying LDI and other spatial predictors of disease.

Infection with Shiga toxin-producing *Escherichia coli* (STEC) is associated with a spectrum of illnesses including watery diarrhea, bloody diarrhea, and the hemolytic uremic syndrome, a potentially fatal condition characterized by acute renal failure (1). Although a variety of *E. coli* serotypes have been associated with human illness, the most important among these is O157:H7. Cattle are the principal reservoir for these organisms. Important sources of infection include consumption of undercooked hamburger and other contaminated food products and direct or indirect contact with infected persons (2-4).

Recent studies suggest that direct or indirect exposure to cattle are important potential sources of infection (2,3). Among this evidence is the finding of Michel et al. that the incidence of human STEC infection was higher in rural areas than in urban areas of Ontario, Canada. By using spatial regression analysis, these investigators demonstrated a strong association between the incidence of human STEC infection and cattle density, expressed as total number of cattle per hectare. Potential routes of infection in rural settings include direct contact with cattle (2,5), consumption of raw milk (2,4), contamination of well water with agricultural runoff (6,7), and contamination of locally produced food products (8).

The approach taken by Michel et al. (3) demonstrated the value of spatial analysis for identifying areas at high risk for STEC infection and for elucidating potential risk factors. The objective of the present study was to develop a systematic approach to creating and evaluating spatial measures of livestock density (livestock density indicators, or LDI) as a means of identifying those best suited to assessing the impact of livestock farming activities on the incidence of human STEC infection.

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Methods

Data

Data on 1,276 cases of human STEC infection reported in Ontario from January 1996 to December 1998 were obtained from the Reportable Disease Information System (RDIS) of the Ontario Ministry of Health and Long Term Care. Infection with STEC is notifiable in Ontario, and more than 95% of reported cases are due to *E. coli* O157:H7 (3). Cases were excluded if they were identified as part of a communitywide outbreak resulting from a single source, such as contaminated water supply.

Consolidated census subdivision (CCS) identifiers were added to the database via a software package that links CCS to appropriate postal codes (Postal Code Conversion File; Statistics Canada). The human population distribution of Ontario was obtained from GeoRef 1996 Census (Statistics Canada), which is based on data collected from all households in the province. CCS areas, which are coded in square kilometers, were also extracted from the GeoRef database. Livestock distribution and land use data were collected from the 1996 Census of Agriculture (Statistics Canada), and area units were converted from hectares to square kilometers. Information on soil development and drainage characteristics for Ontario were obtained from the Canadian Soil Information System (CANSIS) website (http://res.agr.ca/cansis/). All data were aggregated to the CCS because this was the most detailed level at which agricultural data were available.

The study area incorporated 435 townships in the southern area of the province. CCS data from the northern portion of the

province were excluded to avoid a potential bias, since this area was sparsely populated and had little agricultural activity. No information was available on confidential records from the 1996 Census of Agriculture.

STEC Incidence Rates

Incidence rates were determined over a 3-year period, from 1996 to 1998, and were expressed as the number of STEC cases per 100,000 population per year in Ontario CCS.

Spatial Analysis

ArcView version 3.1 (ESRI, Redlands, CA, USA) was used to create chloropleth maps based on disease rates and LDI measures. For the purposes of mapping, the method of nested means, as adapted by Michel and colleagues (9), was used to classify incidence rates. Quintile breaks in the data were used to categorize continuous LDI measures (for example, the lower 20% quintile for the ratio of beef cattle to human population is 0.000 to 0.049). ArcView was also used to calculate CCS centroid locations (latitude and longitude) to allow calculation of autocorrelation measures. SpaceStat version 1.9 (Regional Research Institute, West Virginia University, Morgantown, WV, USA) was used to calculate the Euclidean distance between CCS centroids, so that an inverse square distance matrix could be produced. This matrix was used in the calculation of Moran's I and G_i statistics for the 435 townships in the study area.

Soil landscape coverage version 2.2 and hydrological data version 2.2 were downloaded and imported into ArcView. Attributes containing information on the dominant soil type and drainage characteristics of the soil were mapped. ArcView's GeoProcessing extension was used to perform clip overlay analysis to remove areas normally covered with bodies of water.

The Spatial Analyst extension of ArcView was used to perform a cross-tab query to obtain soil type and drainage characteristic in each CCS. Soil typing within each CCS was based on the predominent type of soil; if two or more soil or drainage types occupied equal areas within a township, then the variable was set to a null value. Soil development and drainage characteristics were based on the Canadian system of soil classification (10).

Development of Livestock Density Indicators

For this study, an LDI was defined as a measure of livestock farming intensity that captures information on the number of animals or the amount of their fecal waste relative to various agricultural and environmental factors within a given geographic area. LDIs were created by combining a series of variables that were considered a priori to be potentially spatially associated with human STEC infection, based on a comprehensive analysis of possible sources and pathways of infection. Variables were constrained by their availability in existing databases.

We grouped these variables into "dimensions" and "components" (Table 1). Dimensions included variables related to number of animals, area of manure application, land uses, and human population within a given CCS. Within the dimensions were components that provided further refinement. For example, components within the dimension "animal" included numbers of various animal species within a given CCS, while components within "manure" consisted of specific manure characteristics and methods of application (Table 1).

Dimensions were combined mathematically according to equations denoted as "frames" (Table 2) to form the LDI. Within each frame, all possible combinations of the relevant components were used. For example, Frame 3 (human population/manure) was used to create four separate LDI consisting of the ratios of total human population in a CCS to the area having manure applied either 1) by solid spreader, 2) by irrigation, 3) as liquid on the soil surface, or 4) as liquid by injection into the soil. Each generated LDI was examined for biological and logical plausibility, and those considered inappropriate were discarded.

Statistical Analysis

Data manipulation, merging of data sets, and statistical analyses were conducted by using the Statistical Analysis System for personal computers, version 6.12 (SAS Institute Inc., Cary, NC, USA). Univariate associations between each indicator and the incidence of human STEC infection were examined

Table 1. Dimensions and components used in creating livestock density indicators to predict incidence of human Shiga toxin-producing *Escherichia coli* infection, Ontario

Dimension	Component
Animal	Total no. of cattle in CCS[a]
	Total no. of dairy cattle in CCS
	Total no. of beef cattle in CCS
	Total no. of chickens in CCS
	Total no. of pigs in CCS
Manure	Area of CCS having manure applied via solid spreader
	Area of CCS having manure applied via irrigation system
	Area of CCS having manure applied to soil surface via liquid spreader
	Area of CCS having manure injected into soil via liquid spreader
Land use	Total CCS area
	Absolute or % area of farm land in CCS
	Absolute or % area of pasture land in CCS
	Absolute or % area of crop land in CCS
Human population	Total human population in CCS

[a]CCS = consolidated census subdivision. Area of CCS is in square kilometers; absolute = absolute square kilometers.

Table 2. Frames used developing livestock density indicators for predicting incidence of human Shiga toxin-producing *Escherichia coli* infection, Ontario

Frame No.	Equation	Example
1	$\frac{Animal}{Land\ Use}$	$\frac{Total\ number\ of\ beef\ cattle\ in\ CCS^a}{Absolute\ area\ of\ farm\ land\ in\ CCS}$
2	$\frac{Manure}{Land\ Use}$	$\frac{Area\ of\ CCS\ having\ manure\ applied\ with\ a\ solid\ spreader}{Absolute\ area\ of\ farm\ land\ in\ CCS}$
3	$\frac{Human\ Population}{Manure}$	$\frac{Total\ human\ population\ in\ CCS}{Area\ of\ CCS\ having\ manure\ applied\ with\ a\ solid\ spreader}$
4	$\frac{Animal}{Human\ Population}$	$\frac{Total\ number\ of\ cattle\ in\ CCS}{Total\ human\ population\ in\ CCS}$
5	$Animal \times \frac{Land\ Use}{Total\ CCS\ area}$	$Total\ number\ of\ cattle\ in\ CCS \times \frac{Absolute\ area\ of\ pastureland\ in\ CCS}{Total\ CCS\ area}$
6	$Manure \times \frac{Animal}{Land\ Use}$	$Area\ of\ CCS\ having\ manure\ applied\ with\ solid\ spreaders \times \frac{Absolute\ area\ of\ pastureland\ in\ CCS}{Total\ CCS\ area}$

[a]CCS = consolidated census subdivision.

by using Poisson regression analysis. The GLIMMIX macro in SAS was used, and census division was entered as a repeated effect to induce a correlation structure in an attempt to control for the spatial effects inherent in the data.

Variable selection was performed in several steps. Initially, LDI were grouped along common components in their numerator (e.g., all LDI with dairy cattle in the numerator were combined into a single group). LDI within each group were then entered into a separate multivariate model, and non-significant LDI were removed by backward elimination until a minimum of one variable remained (stage 1 models). This procedure reduced the potential number of variables offered to subsequent models.

Variables thus identified were then offered to a second series of multivariate models (stage 2 models), each of which was subjected to a backward elimination procedure. Variables offered to stage 2 models consisted of all combinations of one variable from each of the models developed in stage 1.

Moran's I and G_i statistics were calculated for STEC incidence rates, which provided a measure of overall and local spatial autocorrelation. For all statistical analyses, a significance level of 5% was used (p=0.05).

Results

Geographic distribution of the yearly incidence of human STEC infection in Ontario between 1996 and 1998 is shown in Figure 1. According to the nested means techniques, STEC incidence rates were classified as very low incidence (0.00 to 0.95 per 100,000) in 204 CCS areas, low (0.96 to 4.54 per 100,000) in 95, average (4.55 to 5.38 per 100,000) in 15, high (5.39 to 15.01 per 100,000) in 78, and very high (15.02 to 77.52 per 100,000) in 41. CCS where the incidence of STEC infection was classified as high or very high were located primarily in the northwestern portion of southern Ontario, with smaller numbers in eastern Ontario.

Moran's I calculation for the incidence of STEC infection indicated a significantly positive autocorrelation (p=0.012). G_i statistics for the CCS areas with the 10 highest and 10 lowest incidence rates were also statistically significant (p<0.003).

A total of 8,316 LDI were generated, of which all but 80 were eliminated on the basis of biological plausibility. Of these 80 variables, 33 had a significant univariate association with the incidence of human STEC infection. Of these 33, 9 (27.3%) were based on the number of beef cattle, the total number of cattle per CCS, and measures of manure application; 4 (12.1%) were based on the number of standardized animal units per CCS; and 1 (3.0%) was based on both the number of dairy cattle and chickens per CCS.

The number of sheep or goats, soil type, or drainage characteristics were not significantly associated with the incidence of human STEC infection in the univariate analysis. The 10 LDI having the highest r^2 values in univariate analyses are shown in Table 3. All of these LDI were based on either the number of cattle, beef cattle, or animal units per CCS.

Multivariate modeling resulted in the creation of 16 unique stage 2 models with r^2 values ranging from 0.0932 to 0.266.

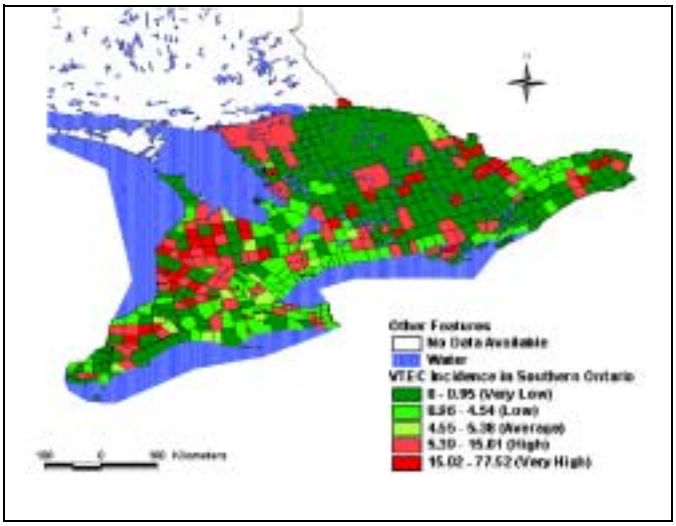

Figure 1. Yearly incidence of shiga toxin-producing *Escherichia coli* infection (per 100,000 population), southern Ontario, 1996–1998.

The multivariate model having the highest r^2 value, shown in Table 4, consisted of HUMBCOW (the ratio of the number of beef cattle to the human population in a CCS), PIGFARM (the total number of swine per km^2 of farm land in a CCS), SMANCCS (the proportion of land in a CCS in which manure is applied by a solid spreader), and LSMANCRP (the proportion of cropland in a CCS in which liquid manure is applied to the soil surface. HUMBCOW, SMANCCS, and LSMANCRP were all positively (and independently) associated with the incidence of human STEC infection, whereas PIGFARM was negatively associated.

Table 4. Multivariable spatial Poission regression models for predicting incidence of human shiga-toxin-producing *Escherichia coli* infection, Ontario, 1996–1998

Variable	Estimate	S.E.	p-value	r^2
Intercept	1.04	0.18	<0.001	0.2655
HUMBCOW[a]	0.65	0.13	<0.001	
PIGFARM[b]	-0.003	0.001	0.04	
SMANCCS[c]	4.19	1.86	0.03	
LSMANCRP[d]	7.82	2.47	0.002	

[a]Ratio of no. beef cattle to human population in a consolidated census subdivision (CCS).
[b]Total no. of swine per km^2 of farmland in a CCS.
[c]Proportion in a CCS having manure applied to land surface via solid spreader.
[d]Proportion in a CCS having manure applied to land surface via liquid spreader.

Discussion

The results of our analyses are consistent with the findings of Michel et al. (3), who demonstrated a higher incidence of human STEC infection in rural areas of Ontario, as opposed to urban areas, and a spatial association between the incidence of human STEC infection and cattle density. These findings are also consistent with other reports in the literature, including outbreaks of STEC infection related to consumption of unpasteurized milk (2) and water from shallow wells, direct contact with cattle (5), and an association between endemic STEC infection and exposure to agricultural environments (2,11).

Table 3. Top competing individual livestock density indicators for predicting the incidence of human shiga toxin-producing *Escherichia coli* infection, Ontario, 1996–1998

Livestock density indicator [a]	Estimate	S.E.	r^2
Ratio of beef cattle to human population	0.6872	0.1384	0.099
Intercept	1.4634	0.1018	
No. of beef cattle per km^2 township area	0.0998	0.0230	0.098
Intercept	1.1645	0.1674	
No. of beef cattle per km^2 weighted farmland (farmland/township area)	0.0004	0.0001	0.076
Intercept	1.3085	0.1430	
No. of beef cattle per km^2 weighted pasture land (pasture land/township area)	0.0014	0.0003	0.060
Intercept	1.4449	0.1182	
Total no. of cattle per km^2 of township area	0.0154	0.0034	0.053
Intercept	1.2217	0.1355	
Total no. of cattle per km^2 weighted pasture land (pasture land/township area)	0.0003	0.0001	0.046
Intercept	1.4165	0.1003	
Total no. of cattle per km^2 farmland	0.0139	0.0036	0.045
Intercept	1.0532	0.1834	
Animal units per km^2 township area	0.0129	0.0030	0.044
Intercept	1.2548	0.1317	
Ratio of total number of cattle to human population	0.1477	0.0344	0.043
Intercept	1.3878	0.1050	
Number of beef cattle	0.0003	0.0001	0.041
Intercept	1.3515	0.1540	

[a]All LDI significant at p>0.001.

To our knowledge, this is the first time the application of manure to land has been identified as a potential risk factor for endemic human STEC infection. Runoff from agricultural land that has been treated with manure has the potential to contaminate local surface water and wells that supply water for human consumption (12).

A relationship between agricultural activities, such as manure spreading, animal density, and elevated fecal bacterial counts in local streams. was demonstrated in 1989 by Meals (13). An outbreak of STEC infection in New York state was associated with contaminated well water used in the preparation of beverages and ice at a county fair (6). It was thought that the well in question became contaminated with manure-laden water as a result of recent heavy rains.

More recently, contamination of a municipal water supply with *E. coli* O157:H7 and *Campylobacter* spp. in Walkerton, Ontario, Canada, resulted in the largest documented outbreak of gastroenteritis caused by multiple pathogens. Strong evidence suggests that contamination of Walkerton's water supply was due to manure runoff from a nearby farm that entered a shallow well supplying the municipal water system (14).

The density of swine within a CCS was negatively associated with the incidence of human STEC infection. This apparent protective effect may simply be the result of a relative absence of cattle in areas where swine are intensively farmed. Although swine commonly harbor STEC within their intestinal tract, they are not considered to be important reservoirs of *E. coli* O157:H7 (15). Past studies have identified sheep and goats as important reservoirs for STEC (15,16), but these animals were not identified as important predictors of human STEC infection in our study. One explanation may be the relatively low numbers of these animals compared with other livestock types.

This study demonstrates the value of using a systematic approach to identifying potential LDI. The approach enabled us to examine a large pool of potential covariates from which appropriate indicators could be assessed and used to evaluate the association between livestock intensity and incidence of human STEC infection. The chosen indicators were biologically plausible and allowed for identification of a previously unreported risk factor.

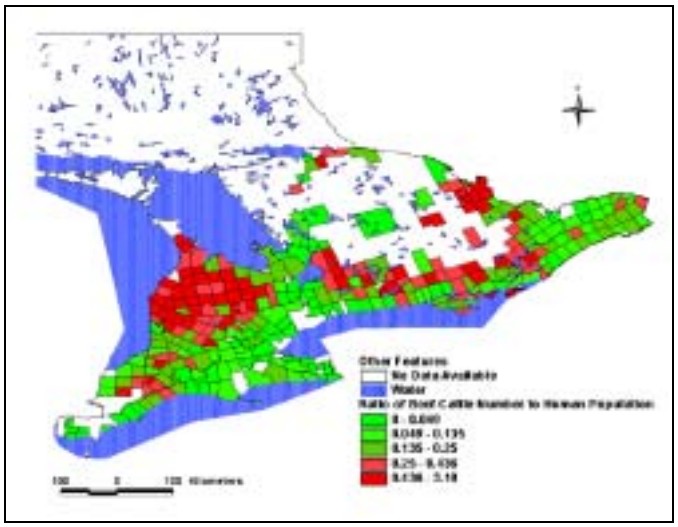

Figure 2. Ratio of beef cattle to human population (number of animals per person), southern Ontario, 1996).

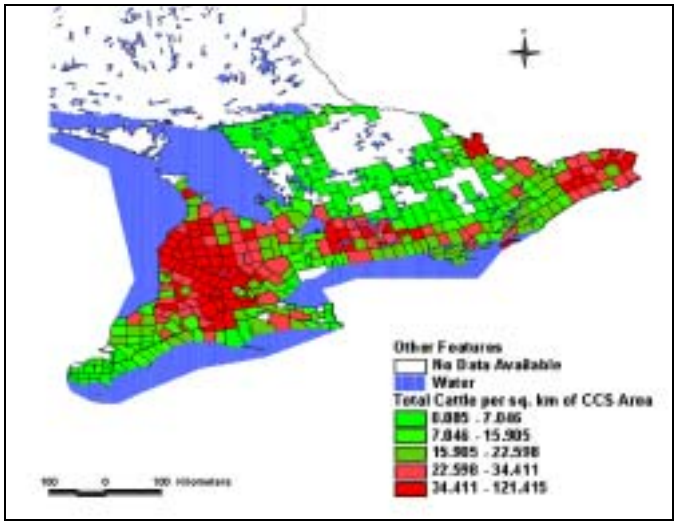

Figure 3. Total number of cattle per square kilometers, southern Ontario, 1996.

By using a systematic construction, we identified LDI that were more strongly associated with the incidence of STEC infection than has been reported previously (3). When modeled at the same geographic scale, the r^2 value for the best model from our investigation (i.e., the ratio of beef cattle to human population as a measure of cattle density) was 0.27 compared with 0.14 for the total cattle density model used in Michel's report (3). These differences in r^2 values may be the result of our selecting beef cattle for the LDI, rather than total number of cattle. It is worth noting that this difference in association is not necessarily evident from maps (compare Figures 2 and 3), because both indicators suggest roughly similar distributions of cattle density, with the greatest concentration in CCS located in south-central and eastern Ontario.

Caution should be exercised when interpreting our study results, however, because not all potential confounding variables (e.g., age or gender of the infected humans) were included in the analysis. Also, systematic errors arising from differential reporting rates may have biased the relationship between the incidence of human STEC infection and the risk factors studied. Since several LDI were investigated, some associations we observed may have arisen from chance alone.

Through linkage of existing data sources, spatial analytic techniques provide a means of identifying populations at high risk and potential risk factors for STEC infection. The approach outlined in this study provides a rational, practical, and powerful tool for public health. As spatial analysis becomes more widely used in epidemiology, we anticipate that the development of such approaches will take on increasing importance.

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Serologic Evidence of *Lyssavirus* Infections among Bats, the Philippines

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Active surveillance for lyssaviruses was conducted among populations of bats in the Philippines. The presence of past or current *Lyssavirus* infection was determined by use of direct fluorescent antibody assays on bat brains and virus neutralization assays on bat sera. Although no bats were found to have active infection with a *Lyssavirus,* 22 had evidence of neutralizing antibody against the *Australian bat lyssavirus* (ABLV). Seropositivity was statistically associated with one species of bat, *Miniopterus schreibersi*. Results from the virus neutralization assays are consistent with the presence in the Philippines of a naturally occurring *Lyssavirus* related to ABLV.

During the past decade, bats have been associated with a number of newly recognized zoonotic agents, including Hendra, Menangle, Nipah, and Ebola viruses and the *Australian bat lyssavirus* (ABLV) (1-5). ABLV and classic *Rabies virus* (RABV) are members of the genus *Lyssavirus.* These viruses are genetically similar and cause indistinguishable clinical syndromes in infected mammals. In the United States, where endemic canine rabies has been eliminated through vaccination and animal control, bat-associated variants of RABV have accounted for 24 (75%) of the 32 cases of human rabies reported since 1990 (6,7). Of the nearly 30,000 laboratory-confirmed cases of animal rabies reported worldwide in 1997, 4% were in bats (8). However, not all countries are included in this survey, and surveillance methods vary between countries included in the compilation. Bat-associated rabies cases in humans are likely underreported in this global surveillance report because not all countries report a history of animal exposure or type the virus variants.

In the Philippines, where approximately 350 cases of human rabies are diagnosed clinically each year, attribution of the animal associated with the exposure is based on history (8). Previous surveys for rabies in Philippine bats conducted in the 1950s and 1960s failed to document active rabies infection in the animals examined (9,10). The increasingly recognized role of bats in the global maintenance and transmission of viral infections, the recent discovery of rabies among bats in Australia, and the unknown proportion of rabies cases in Southeast Asia potentially attributable to bats prompted this initiation of active surveillance for lyssaviruses in Philippine bat populations.

Methods

Collection of Specimens

From June 25 through September 11, 1998, bats were nonrandomly collected from multiple sites on six different islands in the Philippines (Figure). Sites were chosen on the basis of local reports of known bat colonies or after investigation of likely habitats, such as caves, church belfries, or orchards (11,12). Insectivorous and small fruit bats were captured during the day in fine-mesh, long-handled butterfly nets and at night in mist nets. Larger fruit bats were also obtained from hunters. Thick leather gloves were worn when captured bats were transferred into individual muslin pouches for transportation.

Bats were anesthetized by a 0.05- to 0.1-mg intramuscular injection of ketamine hydrochloride and euthanized by intracardiac exsanguination. All blood was transferred from the collecting syringe into serum separator tubes and refrigerated until centrifugation. Serum was decanted into individual screw-topped vials and frozen at –20°C. Bats were identified to species by using a key based on gross morphology (13). The brains of all bats were removed surgically and frozen in individual containers. Additional organs (e.g., liver, spleen, and lungs) were also harvested from each bat and stored either in a freezer at -70°C or in 20% formalin for future studies. Carcasses of representative specimens were stored in formalin for archival purposes.

Direct Fluorescent Antibody (DFA) Testing of Brains

At the Research Institute for Tropical Medicine in Manila, the bat brains were thawed and multiple impressions were prepared for DFA testing (14). Microscope slides were fixed in cold acetone and allowed to dry. Brain impressions were stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabies monoclonal antibodies (Fujirebio Diagnostics, Malvern, PA) and examined under a fluorescent microscope for *Lyssavirus* antigens. This monoclonal antibody preparation reliably detects infection with all known lyssaviruses, including both classic RABV and ABLV (5,15-17).

Serologic Testing for Neutralizing Antibodies

At the Centers for Disease Control and Prevention (CDC), the presence of virus-neutralizing antibodies was determined by a modification of the rapid fluorescent focus inhibition test

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Figure. Collection sites for bats used in active surveillance of lyssaviruses in the Philippines.

(RFFIT) (18). Two different challenge viruses were used: the routine rabies challenge virus standard (CVS-11) and an isolate of ABLV adapted to cell culture. Viruses and bat serum samples were diluted with Eagle minimum essential medium containing 10% fetal calf serum (EMEM10) and antibiotics to reduce bacterial and fungal contamination as described (18). EMEM10 was also used in the growth of murine neuroblastoma (MNA) cells, which were used to propagate and grow sufficient quantities of each virus, and in the modified RFFIT assays. CVS-11 was obtained from stocks at CDC. The ABLV isolate (*Saccolaimus flaviventris* [sm4068]; Australian Animal Health laboratory, Geelong, Australia) was originally obtained from an insectivorous bat in Australia and was amplified by passage in BHK and MNA cells at CDC.

All bat serum samples were thawed and placed in a 56°C water bath for 30 minutes to inactivate complement. Serum samples were then diluted to 1:10 if possible. Samples with insufficient volume were screened at a higher dilution. The RFFIT was conducted by using Lab-Tek 8-well glass slides with covers (Nalge Nunc International, Naperville, IL). Sera were screened for antibody by incubating 100 µL of diluted serum with 100 µL of ABLV or CVS-11 that had been diluted to contain approximately 100 infectious units when incubated for 90 minutes at 37°C in a CO_2 incubator. MNA cells (approximately 75,000 cells/200 µL) were added to each serum-virus mixture, and the incubation was continued. After 48 hours, culture medium was removed, and the slides were fixed in acetone, air-dried, and stained for residual virus with FITC-conjugated anti-rabies monoclonal antibodies. A sample was defined as positive for neutralizing antibody if at least a 90% reduction in infectious centers was observed relative to the positive control. All positive samples were retested at increasing dilutions to estimate endpoint antibody titers. Standard human rabies immune globulin (HRIG) diluted to contain 2 IU/mL antibodies was used as a positive serum control for all tests. The titer of HRIG ranged from 1:125 to 1:625 against both ABLV and CVS-11.

Results from the serologic testing were used to detect patterns in seropositivity by location or type of bat, by using the Chi-square test.

Results

Collection of Specimens

Of the 821 bats collected, all but three were identified to species (Table 1). The collection resulted in 14 different species of both insectivorous and frugivorous bats representing five of the six families of Chiroptera believed to be present in the Philippines. Fifty-one percent of the bats were female, including 22 that were pregnant and 6 that were suckling infants. All bats appeared to be healthy except one with an enlarged spleen and three that appeared to have a mange-like condition. Since some bats died during collection and processing, serum could not be collected from all the bats.

DFA Testing of Brains

Brains from all 821 bats were tested for the presence of RABV antigen by DFA. None of the bats had detectable antigen consistent with an active infection with rabies or a related *Lyssavirus*.

Serologic Testing for Neutralizing Antibodies

Of the 821 bats collected, 231 had sufficient volume and quality of serum to be diluted to 1:10 and successfully

screened at 1:20, after being combined with the challenge virus (Table 2). An additional 43 specimens were screened at higher dilutions. Remaining samples contained insufficient volumes or could not be tested because of hemolysis.

Of the 231 bat sera tested, 22 (9.5%) were positive for neutralizing antibodies against ABLV. Antibody titration studies demonstrated decreasing percent neutralization at progressively higher serum dilutions. Of those 22 bat sera, 8 demonstrated no virus neutralization at the next highest dilution tested; 8 demonstrated some neutralization as dilute as 1:40; 3 had some at 1:80; 2 had some at 1:160; and 1 had evidence of some neutralization at a dilution of 1:320. When the strict definition of 90% to be considered positive was used, only two bat sera remained positive at the 1:40 dilution. This dilution is the equivalent of 0.6 IU/mL antibody. Five of those 22 samples were also positive when tested against CVS-11. Only 1 of the 209 bat sera that was negative when tested against ABLV was positive when tested against CVS-11.

The 22 bats with neutralizing antibodies against ABLV included six different species collected from four islands (Table 3). No location was significantly associated with bat sera that tested positive. Antibody-positive bats were evenly dispersed throughout the collection period (July 5 through September 5). Only 32% of the antibody-positive bat sera were obtained from females. That proportion was not statistically significant. The only significant association in the analysis was that a single species had a statistically greater proportion of samples testing positive. Thirty-six percent of the 11 *Mineopterus schreibersi* (Schreiber's long-fingered bat) tested positive (p=0.01).

The data analysis was repeated with a less strict case definition of 75% reduction in infectious centers relative to the positive control and including the 43 additional samples that could only be screened at higher dilutions. When these criteria were used, 53 (19%) of 274 bat sera tested were positive. The two samples with the highest positive endpoint titers in the initial analysis remained highest, but now at a 1:80 dilution. Although additional species would have been identified as having neutralizing antibodies, *M. schreibersi* remained the only species with a statistically significantly greater proportion of serum samples positive for neutralizing antibody.

Discussion

This study presents evidence of neutralization of ABLV by serum from Philippine bats. This neutralizing activity correlated with the ability to neutralize RABV (CVS-11) and titrated steadily with serial dilutions of the serum. These findings are consistent with the presence of naturally occurring antibodies against a *Lyssavirus* related to ABLV in the Philippine bat populations studied.

Lyssaviruses are classified into groups on the basis of their relative pathogenicity, their binding affinity to specific monoclonal antibodies, and their nucleic acid sequences. There are seven putative genotypes that have been aggregated into two basic groups on the basis of their overall phylogenetic relatedness (19). Phylogroup I includes RABV (genotype 1), *Duvenhage virus* (DUVV) (genotype 4), *European bat lyssavirus* (EBLV) 1 (genotype 5), EBLV-2 (genotype 6), and ABLV (genotype 7). Phylogroup II includes *Lagos bat virus* (LBV) (genotype 2) and *Mokola virus* (MOKV) (genotype 3). Antibodies to viruses within one phylogroup should cross-neutralize viruses of that same phylogroup. The ability to cross-neutralize is directly proportional to the relative nucleotide and amino acid homogeneity between the two viruses being

Table 1. All bats caught on six islands in the Philippines and tested for *Rabies virus* antigen by direct fluorescent-antibody assay, June 25 to September 11, 1998

	Island of origin					
Bat species	Luzon	Bohol	Boracay	Mindanao	Mindoro	Negros
Saccolaimus saccolaimus		53				54
Taphozous melanopogan	96					
Megaderma spasma	16					
Hipposideros diadema		16	1	2		
Rhinolophus spp.	6			3		
Mineopterus schreibersi				14		
Philetor brachypterus				24		
Scotophilus kuhlii	105			1		95
Cynopterus brachyotis	3			12	1	4
Eonycteris spelaea	1		6	2	1	
Macroglossus minimus	4			3	3	
Ptenochirus jagori	36			4	6	
Pteropus hypomelanus			27			
Rousettus amplexicaudatus	1	112	98	6	1	1

Table 2. All bats caught on five islands in the Philippines and screened for neutralizing antibodies against *Australian bat lyssavirus* at a 1:10 serum dilution

	Island of origin				
Bat species	Luzon	Bohol	Boracay	Mindanao	Mindoro
Saccolaimus saccolaimus		23			
Taphozous melanopogan	30				
Megaderma spasma	4				
Hipposideros diadema		4	1	2	
Rhinolophus spp.				2	
Mineopterus schreibersi				11	
Philetor brachypterus				13	
Scotophilus kuhlii	62			1	
Cynopterus brachyotis				1	
Eonycteris spelaea			1		
Macroglossus minimus	1			2	1
Ptenochirus jagori	8				
Pteropus hypomelanus			14		
Rousettus amplexicaudatus		21	28	1	

compared (19). In another study of Nigerian fruit bats, 2 of 50 serum samples had neutralizing antibodies against CVS-11 but failed to neutralize DUVV (20). In the bats in this study, no cases of active *Lyssavirus* infection were discovered from which nucleotide and amino acid sequences could be determined and subsequently compared with ABLV and CVS-11. More samples neutralized ABLV than CVS-11, suggesting that the *Lyssavirus* responsible for the induction of antibodies in these bats might be more similar to ABLV than CVS-11, while still being a member of phylogroup I. Repeating the RFFIT assays with a challenge virus from phylogroup II, such as MOKV or LBV, could have tested this hypothesis further. Although it is possible that we might have been able to demonstrate some cross-reactivity, a finding of greater neutralization activity against MOKV (compared with what was found against ABLV) would not be expected since all known phylogroup II viruses have a rather limited geographic distribution in Africa. In addition to the more widespread distribution of phylogroup I lyssaviruses, the small quantities of available bat sera precluded repeated RFFIT testing with an additional challenge virus such as MOKV.

Table 3. All bats caught on four islands in the Philippines positive for neutralizing antibodies against *Australian bat lyssavirus* at a 1:10 serum dilution

	Island of origin			
Bat species	Luzon	Bohol	Boracay	Mindanao
Taphozous melanopogan	4			
Mineopterus schreibersi				4
Philetor brachypterus				1
Scotophilus kuhlii	4			
Pteropus hypomelanus			3	
Rousettus amplexicaudatus		4	2	

The strict case definition used in the interpretation of the RFFIT assays resulted in the identification of 22 positive bat sera. As noted in the results of the second analysis, for which a lower threshold for positivity was used, additional bat sera that had 75% to 89% neutralization also had progressively decreasing neutralization at increasing dilutions, a finding similar to that for the 22 positive samples that met the strict case definition. Thus, we may have slightly underestimated the actual prevalence of anti-*Lyssavirus* antibody in these bat populations. Reduction in infectious centers by 90% compared with the positive control provided a conservative estimate of the prevalence of anti-*Lyssavirus* neutralizing antibodies. Previous studies have used a cutoff as low as 50% neutralization for the interpretation of data (21). Although the number of positive bat sera more than doubled when the broader case definition was used, no change in the results of the analysis of patterns of seropositivity by location or type of bat was evident. In addition, independent of the cutoff point used, the peak antibody measurement was approximately 0.6 IU/mL. Most of the other positive specimens had approximately 0.3 IU/mL. Many commercial laboratories report serum samples with ≥0.5 IU/mL of antibody as a positive test. However, the 0.5 IU/mL value was established as an arbitrary standard by reference laboratories as evidence above background for the detection of the induction of RABV-neutralizing antibodies in humans after receipt of multiple doses of high-potency rabies vaccines (22). No accepted standard for naturally occurring infections among wildlife exists.

On the basis of the 9.5% prevalence of neutralizing antibodies, it is not surprising that all brain samples studied showed no evidence of RABV antigen by DFA in these

clinically normal bats. Most studies of healthy bats have found a low prevalence of active infection, usually <1% (21). As would be expected, previous surveys of healthy bats in other parts of the world have shown that the prevalence of RABV-neutralizing antibodies is usually considerably higher than the prevalence of active infection, as indicated by positive DFA results for brain tissue. In a study of asymptomatic Mexican free-tailed bats from a single dense cave population in New Mexico, 69% of the bats had neutralizing antibodies, but only 0.5% had active infection demonstrated by DFA (21). A report from the Caribbean described a 40.5% seroprevalence of RABV-neutralizing antibodies among healthy and ill bats, but only 1 bat, which had been submitted as an ill-appearing rabies suspect, had active infection (23). Assuming a ratio of seroprevalence to active infection of approximately 100:1 in healthy populations of bats, based on the seroprevalence of 9.5% demonstrated in this study, one would have needed to test at least 1,052 normal bats to detect one case of active viral infection by DFA. Such further studies should be focused on species of bats such as *M. schreibersi* and in locations with the highest prevalence of neutralizing antibodies. Ideally, if a large stable colony of such bats could be identified, surveillance among sick and dying bats could be conducted. Such a study would increase the likelihood of obtaining a virus isolate and would minimize the potential adverse impact on the bat populations from oversampling large numbers of otherwise healthy bats. Similarly, the routine virus variant typing of human and domestic animal rabies cases in the Philippines and throughout Southeast Asia will provide basic epidemiologic information on the prevalence of different RABV isolates and enhance the likelihood of discovery of any new lyssaviruses affecting the populations in these regions.

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Novel *Cryptosporidium* Genotypes in Sporadic Cryptosporidiosis Cases: First Report of Human Infections with a Cervine Genotype

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In this study, we genotyped parasites from the fecal specimens of sporadic cryptosporidiosis cases in British Columbia from 1995 to 1999. Genotyping was conducted by polymerase chain amplification of the internal transcribed spacer region, a hypervariable region in the 18S rRNA gene and the *Cryptosporidium* oocyst wall protein gene. Subsequent analysis was by restriction fragment length polymorphism and DNA sequencing. We identified two new *Cryptosporidium* genotypes in humans. One of these genotypes has been found recently in deer in New York state. The other genotype has not been identified in humans or animals. These results have important implications for drinking water quality strategies, especially for communities that obtain drinking water supplies from surface sources located in forested regions with deer populations.

In recent studies of cryptosporidiosis cases in North America, South America, Europe, and Australia, various polymorphic gene loci were used to show that two major genotypes of *Cryptosporidium parvum* occur in humans (1-10). Genotype 1, or the human genotype of *C. parvum*, has been isolated almost exclusively from humans and associated mainly with anthroponotic (human-to-human) transmission cycles (1). Experiments to infect animals such as cattle and mice with the human genotype have been unsuccessful, and the only in vivo model that exists for this genotype is a gnotobiotic piglet model (11). So far, the only animals reported to be infected with genotype 1 *C. parvum* are a monkey in the United States (5) and a dugong (*Dugong dugon*) in Australia (12). In contrast, genotype 2 or the calf genotype of *C. parvum* has been isolated from both human and bovine hosts, as well as other livestock and wild animals such as sheep, goats, and deer. Genotype 2 has been associated with zoonotic (animal-to-human) transmission cycles.

Other genotypes of *C. parvum* are found in animals. including the dog, mouse, bear, pig, deer, and marsupial genotypes of *C. parvum*, which have been differentiated by sequence polymorphisms in the small subunit ribosomal RNA (13-15), the acetyl CoA synthetase (15), and heat shock protein 70 (16), as well as the *Cryptosporidium* oocyst wall protein (COWP) (17) genes, and named after the animals from which they were derived. Of these variant *C. parvum* genotypes, three human infections with the dog genotype have been reported—in an HIV patient (4), two Peruvian children (6), and a child in England (18). Aside from *C. parvum*, nine other *Cryptosporidium* species are recognized: *C. felis* (cat), *C. muris* (rodent), *C. andersoni* (cattle), *C. wrairi* (guinea pig), *C. baileyi* (bird), *C. meleagridis* (bird), *C. serpentis* (reptile), *C. surophilum* (lizard), and *C. nasorum* (fish). Although previously thought to be host specific, these other *Cryptosporidium* species have been associated with a few reports of human infections. *C. felis* (4,18,19) and *C. meleagridis* (19) have been found in immunocompromised persons. In addition, *C. felis* (6,18), *C. meleagridis* (6), and possibly *C. muris* (20) infections have been reported in children.

In this study, we genotyped parasites from the fecal specimens of sporadic cryptosporidiosis cases in British Columbia (BC). Genotyping was conducted by polymerase chain reaction (PCR) amplification of the internal transcribed spacer region, a hypervariable region in the 18S rRNA gene (4) and the COWP gene, (21). Subsequent analysis was by restriction fragment length polymorphism (RFLP) and DNA sequencing. We identified two new *Cryptosporidium* genotypes in humans. One of these genotypes has been found recently in deer in New York State (14). The other genotype has not been identified in humans or animals.

Materials and Methods

Cryptosporidiosis Cases and Community Information

C. parvum oocysts were isolated from patients in the Greater Vancouver and Fraser Valley Regional Districts of British Columbia over a 5-year period from 1995 to 1999.

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Demographic data on this geographic area have been described (2). Fecal specimens were collected from patients diagnosed with clinical symptoms consistent with cryptosporidiosis. *Cryptosporidium* oocysts were identified in stool specimens by standard concentration methods, acid-fast staining, and microscopy by the diagnostic parasitology laboratories to which the specimens were submitted. Oocyst-containing specimens were preserved in potassium dichromate solution (2.5% w/v) within 7 days of reception and stored at 4°C. The study was conducted retrospectively on specimens that were coded without personal identifiers. Informed consent from subjects was obtained.

Genomic DNA Extraction

Resuspended stool specimens were strained through cheesecloth. Potassium dichromate was removed by washing the sedimented filtrate 3 times in distilled water. Lipids were then extracted by using ethyl acetate as described (2). *Cryptosporidium* oocysts were disrupted by repeated freezing in a dry ice-ethanol bath and thawing in a boiling water bath in a 20% w/v suspension of Chelex-100 (BioRad Laboratories, Hercules, CA) as described (2). The DNA extracts were stored at –20°C.

PCR Amplification of *C. parvum* Oocyst DNA

Genomic DNA extracts from oocysts were centrifuged at 11,000 rpm (9,000 x *g*) for 20 minutes at 4°C and the supernatants used as template DNA for PCR. The PCR reaction was carried out as described (2) by using the forward primer, cry7, and the reverse primer CP5.8R to amplify the entire internal transcribed spacer 1 (ITS1) region, resulting in a 600-bp product. The amplification procedure using the CPBDIAGF/CPBDIAGR primer pair described by Pieniazek et al. (4) was used to amplify the hypervariable region of the 18S rRNA gene, and the CRY-9/CRY-15 primer pair described by Patel et al. (21) was used for the COWP gene.

In addition, genomic DNA prepared from oocysts that had either been characterized in previous studies (1,2) or were isolated from well-defined sources were included as known genotype controls. Genotype 2 controls included one bovine isolate from a purified batch of Iowa strain oocysts that had been passaged in calves at the University of Arizona; two human isolates from 1996 Cranbrook and 1998 Chilliwack outbreak cases, where animals infected with cryptosporidiosis were found in the watershed area ([2] and Ong et al., unpub. data); and five other human isolates derived from sporadic cases in British Columbia that have been described in a previous study as *C. parvum* genotype 2 isolates (2). Genotype 1 controls included seven isolates from sporadic cases and one isolate from a 1996 Kelowna outbreak case (2), all identified previously as *C. parvum* genotype 1 isolates (2). Deionized water and a culture of a nonpathogenic strain of *Escherichia coli* were used as negative controls.

RFLP Analyses of PCR Products

PCR products were purified by using QIAquick spin columns (Qiagen, Mississauga, ON) according to the manufacturer's instructions before digestion with the restriction endonucleases *Mse* I (New England BioLabs, Mississauga, ON) for the ITS1 locus and *Rsa* I (New England BioLabs) for the COWP gene. Two units of enzyme were added to a final volume of 20 µL containing 15 µL of PCR product and the appropriate dilution of the manufacturer's recommended buffer, and then incubated overnight at 37°C. Restriction fragments were then separated on Metaphor FMC agarose gels (3% for *Mse* I digests of ITS1 products and 3.2% for *Rsa* I digests of COWP products) (Mandel Scientific, Guelph, ON) and stained with ethidium bromide; the patterns were visualized with a UV transilluminator. DNA band sizes were analyzed by using the ProRFLP program version 2.38 (DNA ProScan Inc., Nashville, TN).

DNA Sequencing and Analyses

PCR products from the variable 18S rRNA and COWP gene loci were cleaned by spin column purification using the QIAquick PCR Purification kits (Qiagen). Sequencing reactions were conducted in both directions, i.e., from the 5' and 3'-ends using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and analyzed on an ABI 310 automated DNA analyzer (Applied Biosystems).

Overlapping bidirectional sequences were assembled by using the SeqManII (DNASTAR Inc., Madison WI) sequence analysis software. Consensus sequences obtained were aligned by using the ClustalX program (22), which was also used for calculating the phylogenetic tree by the neighbor-joining method with 1,000 replicates for bootstrap values. Published 18S rRNA gene reference sequences included in the multiple sequence alignment are listed below with their corresponding accession numbers: AF093491 (23) and AF087575 (4) for *C. parvum* genotype 1 human isolates; AF112569 (13) for a *C. parvum* simian isolate; AF087576 (4) and AF093490 genotype 2 isolates from a human and a bovine source, respectively; AF087574 (4) and AF112576 (13) for *C. parvum* "dog" genotype isolates from a human and a canine source, respectively; AF115377 (13), AF247535 (24), and AF112571 (13) for *C. parvum* pig, bear, and mouse genotype isolates, respectively; AF297511 (14), AF297512 (14), and AF297515 (14) for *C. parvum* "deer" genotype isolates; AF297503 (14) for a *C. parvum* muskrat isolate; AF087577 (4) and AF112575 (13) for *C. felis* from a human and a feline source, respectively; AF115378 (13), AF093498 (23), AF093496 (23), AF112574 (13), AF093495 (23), and AF093499 (23) for *C. wrairi*, *C. muris*, *C. andersoni*, *C. meleagridis*, *C. baileyi*, and *C. serpentis*. The phylogenetic tree was displayed visually by using TreeView (25). The *C. muris*, *C. andersoni*, and *C. serpentis* sequences were used in the outgroup, and the tree was rooted with this outgroup.

The 18S rRNA and COWP gene sequences of the 11 patient isolates listed in the table have been submitted to GenBank and assigned accession numbers AY030084 to AY030093 and AF411631 to AF411633. The BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) was used for DNA databases searches.

Results

Cryptosporidium oocysts were isolated from fecal specimens of 150 sporadic cryptosporidiosis cases. Two characteristic restriction profiles were obtained for *Mse* I digests of the 600-bp ITS1 products (Figure 1). The first type of restriction profile (Figure 1, lanes 4 and 15) showed five major bands at approximately 270, 160, 90, 75, and 55 bp. The bovine isolate, patient isolates from the 1996 Cranbrook and the 1998 Chilliwack outbreaks, and 29 (19%) isolates from sporadic cases had this restriction profile. Based on results from previous molecular characterization of a number of these isolates (1,2), this restriction profile was considered to be the *C. parvum* genotype 2 restriction pattern. The second restriction pattern (Figure 1, lanes 5, 6, 14, and 16) with six major bands around 185, 150, 100, 60, 40, and 30 bp was obtained from isolates of 108 (72%) sporadic cases and the one patient from the 1996 Kelowna outbreak. This restriction profile was considered to be the *C. parvum* genotype 1 profile, based on results from previous molecular analyses on the other seven genotype 1 isolates that were included as human genotype controls (2).

Restriction profiles with varying patterns (Figure 1, lanes 7, 9 to 13 and 17) were obtained from 13 (9%) other human isolates. Of these, nine (6%) isolates had identical restriction profiles (Figure 1, lanes 7 and 9 to 12) with five major bands at 150, 85, 70, 45, and 35 bp and were designated cervine genotype isolates. The other four isolates had unique restriction profiles and could be split into two groups of two isolates, based on the similarity of banding patterns. These were CS33 (Figure 1, lane 8) and MH222 (data not shown), which both had restriction fragments at 175 and 50 bp and additional variant bands at 65 and 70 bp, respectively. The remaining two isolates VF383 had bands at 315 and 105 bp (Figure 1, lane 13) and TK348 had bands at 325 and 85bp (Figure 1, lane 17), respectively.

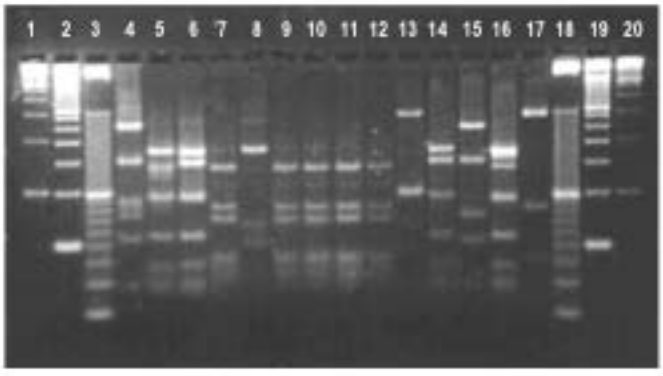

Figure 1. Restriction profiles obtained after digestion of polymerase chain reaction products from the ITS1 locus with *Mse* I. lanes 1- 3-, 100-, 50-, and 10-bp ladder molecular weight markers; lanes 4 and 15, bovine genotype 2 isolates; lanes 5, 6, 14, and 16, human genotype 1 isolates including DE340 (lane 14); lanes 7 and 9–12, cervine genotype isolates including MH205 (lane 7), TK320 (lane 10), and DE302 (lane 11); lane 8, *Cryptosporidium meleagridis* isolate CS33; lanes 13 and 17, other novel genotype isolates such as VF383 (lane 13) and TK348 (lane 17)

Table. Analysis of *Cryptosporidium* isolates collected from 1995 to 1999 from sporadic cases in British Columbia, Canada

Selected isolates	Genotype	RLFP loci	Genes sequenced
TK386	Human	ITS1	18S
TK303	Human	ITS1	18S
TK324	Human	ITS1	18S, COWP
DE340	Human	ITS1	18S
MH205	Cervine	ITS1, COWP	18S, COWP
TK320	Cervine	ITS1	18S, COWP
DE302	Cervine	ITS1	18S
MH222	*C. meleagridis*	ITS1	18S
CS33	*C. meleagridis*	ITS1, COWP	18S
VF383	Other novel	ITS1	18S
TK348	Other novel	ITS1	18S

RFLP = restriction fragment length polymorphism; ITS1 = internal transcribed spacer 1; COWP = *Cryptosporidium* oocyst wall protein.

To identify the *Cryptosporidium* species and genotype of isolates with variant restriction profiles, sequencing of a polymorphic locus on the 18S rRNA gene was carried out. Eleven isolates were selected based on their ITS1 restriction patterns. These included one isolate (TK386) with a characteristic human genotype 1 restriction profile, three isolates (TK324, TK303, DE340) with patterns similar to the human genotype 1 restriction profile but with one restriction fragment shifted slightly in molecular size (e.g., Figure 1, lane 14), three cervine genotype isolates (MH205, TK320, DE302; Figure 1, lanes 7, 10, and 11), and four isolates (CS33, MH222, VF383, TK348) with unique variant profiles (Table). Comparison of the 18S rRNA gene sequences of these isolates with 22 other published reference sequences, derived from a variety of human and animal *Cryptosporidium* isolates by using multiple sequence alignment and phylogenetic analysis (Figure 2), showed that the 11 isolates fell into four main groups. The first group consisted of four isolates (TK386, TK324, TK303, and DE340) that had restriction profiles identical or similar to the characteristic human genotype 1 pattern and all human genotype 1 reference isolates. All human isolates in the genotype 1 group had the characteristic polyT repeat sequence reported previously (10,26) between positions nt 686 and 698. The second group consisted of three human isolates (TK320, DE302, and MH205) and two cervine genotype isolates (Figure 2). The sequences of these three human isolates in the hypervariable 18S rRNA region were identical to that of a genotype 3 deer isolate described by Perz and Le Blancq (14). The third group consisted of two isolates (VF383 and TK348) and a pig genotype isolate (Figure 2). Sequences between the two

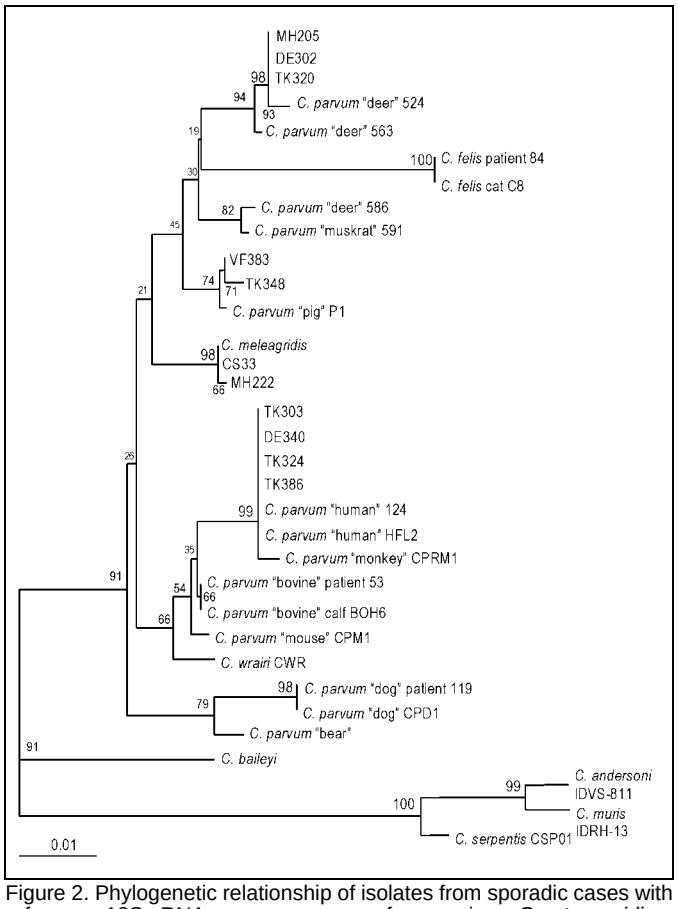

Figure 2. Phylogenetic relationship of isolates from sporadic cases with reference 18S rRNA gene sequences from various *Cryptosporidium* species and genotypes. Bootstrap values that are >95% are shown in larger font. Bar = 0.01 substitution per site.

human isolates were variant from the pig genotype sequence at only two different nucleotide positions between nt 686 and 698. The fourth group consisted of two human isolates (MH222 and CS33) and a *C. meleagridis* isolate (Figure 2).

Twenty-five sporadic isolates were also characterized by using a second locus on the COWP gene. *Rsa* I digests of the 550-bp PCR products (Figure 3) also showed the same dimorphism as the ITS1 locus with two predominant restriction patterns. Of these, 10 (40%) isolates had fragments at approximately 285, 125, 105, and 35 bp, which were characteristic for genotype 1 isolates (Figure 3, lanes 7 and 8). Another six (24%) isolates had the genotype 2 restriction profile with fragments at 410, 105, and 35 bp (Figure 3, Lanes 4 to 6). One isolate (CS33) had a variant restriction profile (Figure 3, Lane 10) with bands at approximately 370, 290, and 150 bp, which were similar in size to fragments reported for a *C. meleagridis* isolate (26). The 18S rRNA gene sequence of this isolate (CS33) was identical to that of *C. meleagridis*. The other isolate (MH205), had a restriction profile that was identical to those obtained from genotype 1 isolates (Figure 3, lane 9). This isolate had an identical ITS1 restriction profile with eight other sporadic human isolates and an 18S rRNA gene sequence that grouped with deer genotype 3 isolates from New York (Figure 2). The COWP gene sequences of MH205 and another cervine genotype isolate TK320 were determined to be identical and novel, sharing only 90% and 91% identity with the COWP gene sequences of the human (AF248741) and bovine (AF248743) alleles, respectively. BLAST analysis showed most alignment (92% identity) with a pig COWP gene sequence (AF266270) (17). However, the cervine allele had identical *Rsa*I restriction sites to the human allele at nt 34, 228, 512, and 618, whereas the bovine allele lacked the site at nt 228. The RFLP patterns could not be determined for the remaining seven isolates as insufficient PCR product was obtained. Over half (51%) of the isolates in this study were derived from pediatric patients <10 years of age, which accounted for seven of the nine cervine genotype infections as well as the two *C. meleagridis* infections.

Discussion

This study describes the discovery of the first zoonotic infections in humans with a novel cervine *Cryptosporidium parvum* genotype. Perz and LeBlancq (14) described this genotype recently after characterizing 111 *Cryptosporidium* isolates from wildlife in New York state. Those researchers did not detect human infections with this genotype in cryptosporidiosis cases in New York City. Other molecular epidemiologic studies in England (8,18) of 1,705 cases also did not identify cervine genotype infections, although rare zoonotic infections in humans with the dog genotype of *C. parvum* as well as other *Cryptosporidium* species such as *C. felis* and *C. meleagridis* were found (27). It is possible that cervine genotype infections in humans were not identified because the novel deer genotype had not been reported at the time of the

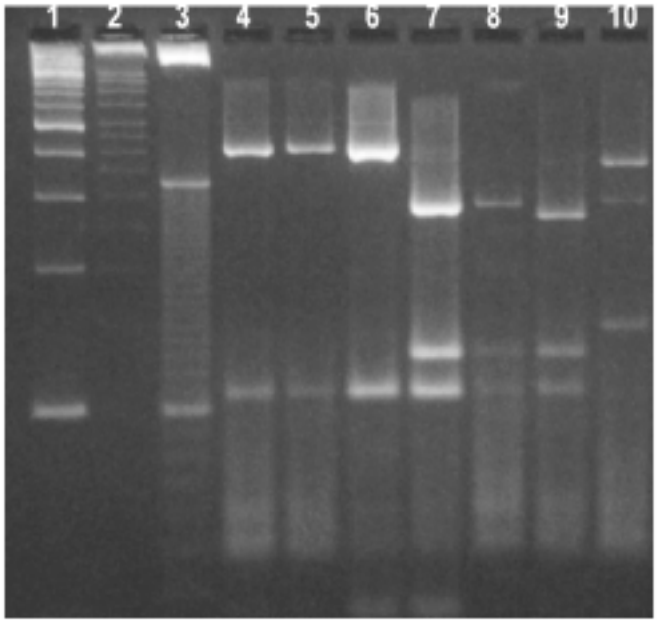

Figure 3. Restriction profiles obtained after digestion of polymerase chain reaction products from the *Cryptosporidium* oocyst wall protein locus with *Rsa* I. Lanes 1- 3-, 100-, 50-, and 10-bp ladder molecular weight markers; lanes 4 and 6, bovine genotype 2 isolates; lanes 7 and 8, human genotype 1 isolates; lane 9, cervine genotype isolate MH205; and lane 10, *C. meleagridis* isolate CS33.

study. As well, the PCR/RFLP profile of the COWP gene from the cervine genotype isolate was identical to that obtained from human genotype 1 isolates. Sequencing of the COWP gene from two cervine genotype isolates confirmed that the human and cervine alleles had identical *Rsa*I restriction sites. Therefore, RFLP analysis using this endonuclease could not differentiate between isolates with these two genotypes.

Xiao et al. (28) have also found this novel cervine genotype in storm water samples collected from a stream in the watershed area of New York State that contributes to the New York City water supply. The transmission of cryptosporidiosis from wildlife to humans in British Columbia is not surprising as many communities are supplied with unfiltered drinking water drawn from surface sources where *Cryptosporidium* spp. oocysts have been detected (29). Many of these watersheds are situated in remote forested areas, where wildlife such as deer are present in abundance. Deer with cryptosporidiosis infections have been identified in these watersheds (Ong et al., unpub. data). Therefore, to have as many as 6% of sporadic cases infected with this novel deer genotype is not an unexpected finding.

The ITS1 and 18S rRNA genes are reportedly multicopy genes with four copies of the Type A and one copy of the Type B rDNA units per haploid genome (30). The sequence divergence found between Type A and Type B units in the ITS1 region was a concern to us initially, as we first characterized the isolates using this locus before this report. However, Morgan et al. (31), who conducted a similar PCR-RFLP analysis of the ITS1 region, found that the restriction profiles were specific for different *C. parvum* genotypes. This study also indicated that intraorganism variation caused by the difference between Type A and Type B rDNA units may not be such a problem. Using primers to amplify the Type B unit, Morgan et al. (31) found that the Type A unit was amplified preferentially for human genotype isolates. To confirm that the observed variation in the ITS1 RLFP patterns was not due to heterogeneous products amplified from different copies of rDNA, further characterization of a select number of sporadic isolates was performed with the 18S rRNA as well as the COWP genes. Results from these additional analyses showed that isolates with distinctly different ITS1 RFLP patterns had different COWP RFLP patterns as well as 18S and COWP gene sequences. Therefore, ITS1 RFLP was useful for generating characteristic fingerprints that could distinguish between different *C. parvum* genotypes and *Cryptosporidium* species.

Using this method of genotyping, we were able to detect two new genotypes of *C. parvum* that had not been reported previously. Nine isolates (including three, MH205, TK320, and DE302, which had been characterized at the 18S locus; and two, MH205 and TK320, which had been characterized at the COWP locus) had the cervine genotype. Two other isolates (VF383 and TK348) had novel genotypes that were most closely related to a pig genotype isolate from Switzerland (13). These results have important implications for drinking water quality strategies, especially for communities that obtain drinking water supplies from surface sources located in forested regions with deer populations.

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Molecular Epidemiology of Adenovirus Type 7 in the United States, 1966–2000[1]

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Genetic variation among 166 isolates of human adenovirus 7 (Ad7) obtained from 1966 to 2000 from the United States and Eastern Ontario, Canada, was determined by genome restriction analysis. Most (65%) isolates were identified as Ad7b. Two genome types previously undocumented in North America were also identified: Ad7d2 (28%), which first appeared in 1993 and was later identified throughout the Midwest and Northeast of the United States and in Canada; and Ad7h (2%), which was identified only in the U.S. Southwest in 1998 and 2000. Since 1996, Ad7d2 has been responsible for several civilian outbreaks of Ad7 disease and was the primary cause of a large outbreak of respiratory illness at a military recruit training center. The appearance of Ad7d2 and Ad7h in North America represents recent introduction of these viruses from previously geographically restricted areas and may herald a shift in predominant genome type circulating in the United States.

Human adenoviruses (Ads) comprise 51 serotypes (1); they are ubiquitous and responsible for a wide range of clinical syndromes. Among recognized serotypes, Ad type 7 (Ad7) (and to a lesser extent Ad type 3) is most often associated with severe disease (2). Although Ad7 infections typically result in mild upper respiratory tract illnesses and conjunctivitis, infections can also lead to more serious lower respiratory tract illnesses, disseminated disease, and death, particularly in infants and persons with underlying immunologic or respiratory compromise (3-7). Ad7 infections have also been associated with diseases of the central nervous system (8,9) and long-term respiratory sequelae that include bronchiectasis and hyperlucent lung or McLeod syndrome (10).

Ad7 accounts for nearly 20% of all Ads reported to the World Health Organization (11), and family clusters and institutional and communitywide outbreaks of Ad7 disease have been extensively documented (5,12-18). Three types of outbreaks have been described (12): i) outbreaks that occur during the winter months among institutionalized infants (<2 years of age) that result in high rates of severe illness and death; ii) periodic nonseasonal communitywide outbreaks involving older children and adults with infrequent serious outcomes; and iii) outbreaks of acute respiratory disease among new military recruits. Outbreaks of acute respiratory disease due primarily to Ad7 and Ad4 were an important cause of illness in new military recruits in the United States until live enteric-coated Ad4 and Ad7 vaccines began to be routinely administered in 1971 (19). The recent cessation of production and administration of these vaccines has resulted in a resumption of Ad-associated acute respiratory disease outbreaks at military recruit training centers throughout the United States (20-22).

To facilitate study of the molecular epidemiology of Ad7, a classification system based on restriction enzyme analysis of Ad genomic DNA was devised by Li and Wadell (23) and later revised by Li et al. (24). Their system uses *Bam*HI as the "type" defining enzyme, with different genome types denoted with a character, e.g., "p" for the Ad7 prototype strain, Gomen; and then "a" through "k." Genome types that are further distinguished by restriction pattern with additional selected enzymes are given an Arabic numeral (e.g., Ad7p, p1, a, a1-6). Their system has been widely used to correlate genome types with geographic distribution and pathogenic potential.

Both globally dispersed and geographically restricted genome types of Ad7 have been identified by restriction analysis, and regional shifts or replacements of predominant genome types have been documented on different continents. Among the 3 Ad7 genome types first distinguished by restriction analysis (25), two shown to be serologically distinct (26) were designated Ad7p (Gomen) and Ad7a (S-1058), and a third, designated Ad7b, was thought to be associated with more severe illness (12). Ad7b eventually spread worldwide (27-30), displacing formerly common genome types (i.e., Ad7p, Ad7a, Ad7a1-6, Ad7c, and others) that are now rarely detected. Exceptions to this pattern have been reported. In the former Soviet Union, a successive shift from Ad7a and Ad7a1-5 to Ad7f1 during 1976-1979 and 1986-1988 was reported (31). In South America, a shift from Ad7c to Ad7h occurred in 1986 (32), and Ad7h has subsequently caused serious respiratory illness in infants and young children in Chile

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and Argentina (33,34). In the early 1980s in China, a new genome type, Ad7d (27), replaced Ad7b as the predominant circulating virus. Recent reports suggest that Ad7d and Ad7h have spread beyond their formerly geographically restricted regions. Ad7d was identified in Japan in 1987 (35), and countrywide epidemics of Ad7 that began in 1995 in Korea (18; Hoan-Jong Lee, pers. comm.) and Japan (15, 36) were attributed to Ad7d and a closely related genomic variant, Ad7d2, respectively. Ad7d2 has emerged as the predominant strain circulating in Israel since 1992 (37). Ad7h was first reported outside South America in 1996, in Japan (36,38).

Beginning in the fall of 1998, an outbreak of Ad7 infection occurred at a pediatric chronic-care facility in Chicago and subsequently spread to a tertiary-care hospital, where staff from two clinic units were infected (17). This multi-site outbreak was associated with considerable illness and death among residents of the chronic-care facility. Isolates from this outbreak were identified by restriction enzyme analysis as Ad7d2. The appearance of this new genome type prompted us to study the temporal and geographic distribution of Ad7 genome types in the United States to better characterize the emergence and spread of this virus.

Materials and Methods

Ads

Of 297 Ad field isolates obtained from the Centers for Disease Control and Prevention (CDC) archives, state public health laboratories, university hospitals, and military training centers, 166 confirmed as Ad7 were selected for genome type analysis (Table 1). Of these, 116 were obtained from 1966 to 2000 from civilians in 25 states and eastern Ontario, Canada; 50 were obtained from February 1997 to May 1998 from military recruits attending training centers in five states (20). Isolates were selected to achieve broad geographic and temporal distribution. Because detailed demographic, epidemiologic, and clinical data from patients were limited, they were not included in this report. Most civilian isolates were obtained from individual cases or family clusters of Ad7 disease, ranging from mild upper respiratory illness to severe lower respiratory tract illness and death. Where civilian outbreaks of Ad7 illness were recognized, only one representative isolate was included in the 166 sample for analysis. Approximately 10% of Ad7 isolates from military recruits with respiratory illness were sampled from all five training sites and were selected to be evenly spaced over the designated time period. Reference strains Gomen and S-1058 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Reference isolates of Ad7d2 were obtained from a postmortem rectal swab from a 4-month-old baby in Israel in 1993, and Ad7h was obtained during a regional outbreak of respiratory illness in Chile in 1998. All Ad isolates were passaged at least once in A549 cells before restriction analysis. Type-specificity of all Ad7 field isolates was confirmed by neutralization or Ad7 type-specific polymerase chain reaction assay (PCR) (39).

Table 1. Human adenovirus 7 (Ad7) field isolates from the United States and Canada, 1966–2000

Location	Isolation year(s)	No. Ad7 isolates
Canada		
Eastern Ontario	1999, 2000	3
United States		
Alabama	1985, 1986	2
Arizona	1995, 1998	3
California[a]	1997	1
California	1981- 4, 1987, 1990, 1992, 1995, 1996	15
Colorado	1987	1
Florida	1986, 1996	2
Georgia	1996	1
Illinois[a]	1996 - 98	28
Illinois	1997	2
Iowa	2000	1
Kansas	1995, 1997	3
Louisiana	1996	2
Maine	1981	1
Maryland	1991, 1993 - 95	16
Massachusetts	1998, 1999	2
Michigan	1986	1
Mississippi	1986	1
Missouri[a]	1997	9
Missouri	1966, 1998, 1999	8
New York	1970, 1985, 1990, 1991, 1993, 1995-97, 1999, 2000	31
Ohio	1993-95, 1997, 1998	10
South Carolina[a]	1997, 1998	11
South Carolina	1998	1
South Dakota	1987	1
Tennessee	1997	2
Texas[a]	1998	1
Texas	1999, 2000	2
Virginia	1985	1
Washington	1996	1
Wisconsin	1996, 1998	3
North America (total)	1966-2000	166

[a]Ad7 isolates obtained from military recruit training centers.

DNA Restriction Analysis

Ad genomic DNA was extracted by a modification of the method of Deryckere and Burgert (40). Briefly, isolates were grown in 75-cm^2 confluent flasks of A549 cells until the 4+ stage of cytopathic effect was attained. The contents of the flask were centrifuged at low speed to remove cells, and the

supernatant was transferred to an ultra-centrifuge tube and centrifuged for 2 hours at 100,000 x *g*. The virus pellet was resuspended in 400 µL of Tris buffer (pH 7.4) with 1% sodium dodecyl sulfate and sequentially digested with DNAse free RNAse A (0.1 mg/mL) and proteinase K (0.5 mg/mL). The digest was extracted twice with equal volumes of phenol and chloroform/isoamylalcohol (24:1) and once with chloroform/isoamylalcohol alone. The purified DNA was then precipitated with absolute ethanol and washed once with 75% ethanol, and the pellet was resuspended in 100 µL of dH_2O. Enzyme digestions were carried out according to manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). DNA from all Ad7 isolates was digested with *Bam*HI and *Sma*I, and selected isolates were also digested with enzymes *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hpa*I, *Hin*dIII,*Sal*I, *Xba*I, and *Xho*I. Enzyme digests were electrophoresed at 100 volts for 5 hours on 0.8% agarose gels, and the DNA bands were visualized by ethidium bromide staining. Restriction fragment size(s) was interpolated from DNA molecular weight standards included in each run. Restriction patterns were compared with previously published profiles (24,37,41,42), and the identification of genome types followed the denomination system of Li et al. (24).

DNA Sequencing

The hypervariable region of the hexon protein gene corresponding to nucleotides 403 to 1356 (Gomen), which have been shown to encode the residues that define Ad serotype, was PCR amplified from selected Ad7 isolates as described (43) and sequenced by using the DyeDeoxy Terminator Cycle Sequencing Kit and ABI 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined for both PCR product strands. Sequence analysis was performed by using the Wisconsin Package ver. 10.0 (Genetics Computer Group, Madison, WI). Hexon gene sequence data for the reference Ad7d2 strain from Israel were submitted to GenBank (accession number AF321311).

Results

Ad7 Genome Types Identified

DNA restriction analysis of the 166 Ad7 field isolates identified 108 (65%) as Ad7b, 46 (28%) as Ad7d2, 4 (2%) as Ad7h, 3 (2%) as Ad7p, 3 (2%) as Ad7a, and 2 (1%) as Ad7a3. Restriction profiles of representative Ad7b, Ad7d2, and Ad7h isolates for selected endonucleases are shown in the Figure. All U.S. (and eastern Ontario, Canada) Ad7d2 isolates and an Ad7d2 reference strain from Israel (37) gave identical restriction patterns for *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Hpa*I, *Hin*dIII, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. Identical restriction profiles were also obtained with four U.S. Ad7h isolates and a 1998 isolate of Ad7h from Chile, which were similar to profiles described for Ad7h strains isolated in Argentina and Chile (formerly designated Ad3f) (41,42,44).

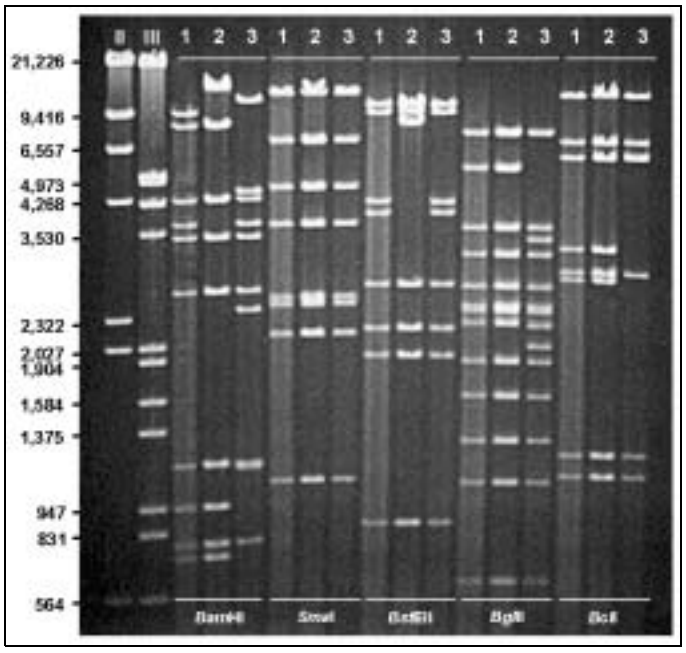

Figure. Restriction profiles of representative human adenovirus (Ad) genome types Ad7b (1), Ad7d2 (2), and Ad7h (3) after digestion with selected enzymes, *Bam*HI, Sma I, *Bst*EII, *Bgl*II, and *Bcl*I. DNA markers II (M *Hin*dIII) and III (M *Hin*dIII/*Eco*RI).

Ad7 Hexon Gene Sequencing

The hypervariable regions of the hexon gene (corresponding to nucleotides 403 to 1356 of the reference strain Gomen) of 24 temporally and geographically diverse Ad7 field isolates (including 11 Ad7b, 10 Ad7d2, and 3 Ad7h and laboratory strains S-1058, 55142 vaccine, and Gomen) were sequenced and compared with published Ad7 hexon sequences available from GenBank (Table 2). Nucleotide and deduced amino acid alignments of these sequences comprised two major genetic clusters as previously described (26,45): cluster 1, Ad7p (Gomen) and Ad7p1; and cluster 2, Ad7a Ad7b, Ad7c, Ad7d, Ad7d2, Ad7g, and Ad7h. Cluster 2 sequences were highly conserved, with over 98% nucleotide identity, and were generally uncorrelated with genome type. However, a unique Gln substitution for Leu (codon CTG > CAG) at amino acid position 443 of loop 2 of the predicted hexon protein was identified in all 10 Ad7d2 isolates from the United States and Israel; this substitution was also present in published hexon sequences of Ad7d isolates from China (45) and Japan (38).

Temporal Distribution of Ad7 Genome Types

The yearly distribution of the 166 Ad7 genome types is shown in Table 3. Ad7b was the only genome type identified from 1970 through 1992 and was the predominant genome type identified through 2000. Ad7d2 first appeared among 1993 isolates and accounted for approximately 28% of all Ad7 isolates obtained from 1993 to 2000. Four epidemiologically unrelated isolates of Ad7h were identified in 1998 and 2000.

Table 2. Human adenovirus 7 (Ad7) field isolates and laboratory strains used for hexon gene sequence comparisons

ID	Genome type	Location	Isolation year	Accession no.	Sequence source[a]
S-1058	7a	USA	1955	af053085	Inada & Mukoyama, direct submission; CDC
55142 vaccine	7a3	USA	1963	af065067	Crawford-Miksza et al. (26); CDC
BC30	7b	China	1958	u75951	Li & Wadell (45)
BC14	7b	China	1965	u77390	Li & Wadell (45)
KCH4	7b	England	1973	u77391	Li & Wadell (45)
v2026	7b	USA, MI	1986		CDC
v2124	7b	USA, SD	1987		CDC
2000017657	7b	USA, MD	1991		CDC
99026790	7b	USA, OH	1993		CDC
2000017667	7b	USA, MD	1994		CDC
2000026630	7b	USA, NY	1996		CDC
Kn T96-0620	7b	USA, CA	1996	af065068	Crawford-Miksza et al. (26)
99018141	7b	USA, MO	1997		CDC
2000016352	7b	USA, IL	1997		CDC
2000016376	7b	USA, SC	1997		CDC
2000016361	7b	USA, MO	1997		CDC
2000016376	7b	USA, SC	1997		CDC
37300	7c	Sweden	1964	u75952	Li & Wadell, (45)
BC3655	7d	China	1981	u77392	Li & Wadell, (45)
BC4492	7d	China	1984	u75953	Li & Wadell, (45)
BC4609	7d	China	1984	u77393	Li & Wadell (45)
BC8488	7d	China	1984	u77394	Li & Wadell (45)
383[b]	7d	Japan	1992	af053086	Hashido et al. (38)
Bal[b]	7d	Japan	1995	af053087	Hashido et al. (38)
2000017663	7d2	USA, MD	1993		CDC
2000026865	7d2	Israel	1993	af321311	CDC
2000017669	7d2	USA, MD	1994		CDC
2000026621	7d2	USA, NY	1995		CDC
99026817	7d2	USA, OH	1995		CDC
2000016333	7d2	USA, IL	1997		CDC
2000016364	7d2	USA, MO	1997		CDC
2000016375	7d2	USA, SC	1997		CDC
98034168	7d2	USA, IL	1998		CDC
2000017983	7d2	USA, WI	1998		CDC
BC25	7g	China	1985	u75954	Li & Wadell (45)
87-922	7h	Argentina	1987	u75956	Li & Wadell (45)
990179044	7h	Chile	1998		CDC
99018196	7h	USA, AZ	1998		CDC
2000016378	7h	USA, TX	1998		CDC
Gomen	7p	USA	1954	z48571	Li et al., direct submission; CDC
BC3423	7p1	China	1981	u75955	Li & Wadell (45)

[a]Sequencing and restriction analysis performed at Centers for Disease Control and Prevention (CDC) or obtained from previously published sources. Published sequences of Ad7 laboratory strains S-1058, Gomen, and 55142 vaccine confirmed at CDC.
[b]Ad7 strains 383 and Bal were originally reported as Ad7d with a "different restriction pattern by *Bst*EII" (38).

Table 3. Yearly distribution of 166 human adenovirus 7 (Ad7) genome types, United States and Canada, 1966–2000

Genome type	1966-1969	1970-1992	1993	1994	1995	1996	1997[a]	1998[a]	1999	2000	Total
7p	0	0	0	0	0	0	3	0	0	0	3
7a	3	0	0	0	0	0	0	0	0	0	3
7a3	2	0	0	0	0	0	0	0	0	0	2
7b	0	31	6	4	14	8	26	4	6	8	107
$7b_{var}$	0	0	0	0	0	0	0	0	1	0	1
7d2	0	0	2	1	3	4	32	2	0	2	46
7h	0	0	0	0	0	0	0	3	0	1	4
Total	5	31	8	5	17	12	61	9	7	11	166

[a]Data include 50 Ad7 isolates collected in 1997 (47 isolates) and 1998 (3 isolates) from military recruit training centers (Table 4).

Geographic Distribution of Ad7 Genome Types

Ad7b was identified among isolates from nearly all states (and eastern Ontario) sampled. Ad7d2 was first identified in isolates from Maryland and New York in 1993 and thereafter primarily from midwestern and northeastern states, including Wisconsin, Illinois, Kansas, Missouri, Louisiana, South Carolina, and Ohio, as well as eastern Ontario. Ad7h was only identified among isolates obtained from Texas and Arizona.

Ad7 Civilian Outbreaks, 1996–2000

During this study, we became aware of five separate outbreaks of Ad7 respiratory illness among civilians (Table 4). Four were institutional outbreaks that involved primarily infants and young children with underlying chronic disease that occurred in the fall or summer months of 1996, 1998, 1999, and 2000. A fifth communitywide outbreak of Ad7 in Tennessee, which occurred during March-July 1997, involved previously healthy children (16).Genome type analysis at CDC identified Ad7d2 in three of the four outbreaks where isolates were available. We attributed one outbreak to a novel *Sma*I restriction variant of Ad7b ($Ad7b_{var}$) that occurred in New York in 1999 (Jennifer Calder, manuscript in preparation).

Ad7 Gnome Types at Military Recruit Training Centers

In anticipation of increased Ad activity following termination of routine vaccination of new military recruits in 1996, the Naval Health Research Center (NHRC) in San Diego, CA, initiated surveillance for new cases of Ad-associated respiratory illness (20) [http://www.nhrc.navy.mil/geis/sites/nhrc.htm]. Clinical specimens collected at five designated training centers (San Diego, CA; San Antonio, TX; St. Robert, MO; Great Lakes, IL; and Columbia, SC) from recruits who reported respiratory illness were submitted to NHRC for identification of viral and bacterial pathogens. Of 50 Ad7 isolates, Ad7d2 was the most common genome type identified (58%), followed by Ad7b (34%), Ad7p (6%), and Ad7h (2%) (Table 5). Most Ad7d2 infections were reported from the Naval Recruit Training Center in Great Lakes, IL, where an outbreak of Ad7 (and Ad3) respiratory illness was documented during the fall of 1997 (21). Over 70% of the Ad7 isolates sampled from the Great Lakes Center from September 1997 to February 1998 were identified as Ad7d2. One of four Ad7h isolates identified in this study was obtained from a new recruit at Lackland Air Force Base, in San Antonio.

Table 4. Recognized civilian outbreaks of human adenovirus 7 (Ad7) respiratory disease, United States, 1996–2000

Location	Date	Setting	No. cases[a]	No. deaths	No. Ad7 isolates	No. restriction	Genome type
Houma, LA[b]	June 1996	Pediatric chronic-care facility	13	7	4	2	7d2
Memphis, TN[c]	Mar 1997	Community acquired	47	1	26	0	nd
Chicago, IL[d]	Nov 1998	Pediatric chronic-care facility	31	8	11	11	7d2
		and tertiary hospital	37	0	6	6	"
New York City, NY[e]	Oct 1999	Chronic-care facility formentally disabled persons	33	7	15	15	$7b_{var}$
Des Moines, IA[f]	Oct 2000	Pediatric chronic-care facility	20	4	9	9	7d2

[a]Suspected and confirmed cases of Ad7 respiratory disease.
[b]Robert Gohd, Children's Hospital, New Oreleans, LA (pers. comm.).
[c]Mitchell et al. (16).
[d]Gerber et al. (17).
[e]Jennifer Calder, The Mailman School of Public Health, Columbia University, New York, NY (manuscript in preparation).
[f]Michael Buley, Iowa Dept of Public Health, Des Moines, IA (pers. comm.).

Table 5. Genome types of 50 human adenovirus 7 (Ad7) isolates obtained from military recruit training centers, Feb 1997–May 1998[a]

Training center	No. Ad isolates	No. Ad typed	No. Ad7 (%)	No. restriction	Dates of isolation	Genome type			
						7b	7d2	7p	7h
Marine Corps Recruit Depot, San Diego, CA	129	128	10 (8)	1	April 1997			1	
Lackland Air Force Base, San Antonio, TX	1	1	1 (100)	1	April 1998				1
Fort Leonard Wood, St. Robert, MO	266	260	29 (11)	9	Feb 1997 to Nov 1997	4	5		
Naval Recruit Training Center, Great Lakes, IL	632	592	396 (67)	28	Sept 1997 to May 1998	8	20		
Fort Jackson, Columbia, SC	786	738	66 (9)	11	June 1997 to April 1998	5	4	2	
Total	1,814	1,719	502 (29)	50		17	29	3	1

[a]Isolation and serotyping of Ads conducted at the Naval Health Research Center, San Diego, CA.

Discussion

Our study represents the most comprehensive survey to date of Ad7 genome types circulating in the United States and provides a basis for future surveillance studies that can better delineate the disease impact of these viruses.

Before this study, the most comprehensive surveys of Ad7 genomic variants in the United States were conducted by Wadell et al. (27) and Adrian et al. (28) with field isolates of Ad7 collected from 1961 to 1985. These authors identified a diverse group of cocirculating Ad7 genome types (Ad7p, Ad7a, Ad7c, and others) that by the late 1960s to early 1970s were replaced by Ad7b, a change that preceded similar shifts to Ad7b seen in other parts of the world. Our data confirm this observation and show a continued dominance of the Ad7b genome type in the United States. Only one genome type from the earlier period, Ad7p, was still identified among currently circulating strains. We also documented the appearance of two new Ad7 genome types: Ad7d2, which was first identified in specimens collected in 1993 and subsequently detected over a wide geographic area in the eastern half of the United States and Canada; and Ad7h, which was first identified in specimens collected in 1998 in the Southwest.

Both epidemiologic and molecular evidence suggests that Ad7d2 entered the United States as part of its recent spread from evolutionarily related Ad7d strains formerly restricted to China. Ad7d2 shows the highest degree of genetic relatedness to Ad7d, differing by only one *Bst*EII restriction site in pairwise comigrating restriction fragment analysis with 12 different endonucleases (24,37); it possesses the unique amino acid substitution in the hexon protein also present in Ad7d isolates from China (45) and Japan (38). Ad7d was identified as early as 1980 in Beijing (24) and 2 years later in Changchin (46), and rapidly displaced Ad7b to become the major genome type circulating in China through 1990. Ad7d was identified in Japan during 1987 to 1992 (35) and in Korea in 1995 (18; Hoan-Jong Lee, pers. comm.), and Ad7d2 was the predominant genome type isolated during the 1995-1998 Ad7 epidemic in Japan (15,36). Ad7d2 was subsequently identified in Israel in 1992 (37) and in the United States in this study in 1993.

The emergence and apparent global spread of Ad7d2 are reminiscent of observations for another genome type of serotype 7, Ad7b. Originally described by Wadell and Varsanyi (25), Ad7b was associated with outbreaks of severe respiratory illness in Europe in the 1970s (12). Although first isolated in 1956 from a Paris orphanage outbreak (12,47), subsequent retrospective studies did not identify Ad7b in Europe again until 1969 (27). Before then, the earliest documented occurrence of Ad7b was in China in 1958 (24), where it was the predominant genome type circulating through the early 1980s (24,46). With the exception of Paris, the first appearance of Ad7b outside China was on the U.S. West Coast in 1962 (27). By 1970, Ad7b was the predominant genome type circulating throughout the United States (28) and eventually throughout many parts of the world.

The mechanism(s) underlying the apparent greater fitness of some Ad7 genome types, as reflected by their capacity to displace other circulating strains, remains speculative. Possible explanations include mutations or recombinations that yield strains with increased pathogenicity and therefore greater chance of causing recognized illness, or biological or antigenic changes that enhance transmission or infection compared with other Ad7 genome types. Although there is no conclusive evidence of differences in pathogenicity between Ad7 genome types, some types appear to be more frequently isolated from healthy carriers (e.g., Ad7p and Ad7a), while others are more often isolated from patients with more serious clinical outcomes (e.g., Ad7b, Ad7c, Ad7d, and Ad7h) (27,34). Some antigenic differences between Ad7 genome types have also been demonstrated; recent studies identified minor differences in neutralization titer between Ad7 prototype strain Gomen (Ad7p) and the vaccine strain 55142 (Ad7a) with rabbit hyperimmune antisera (26). In addition, a unique amino acid substitution in the hexon protein that distinguishes Ad7d/Ad7d2

strains from other genome types is predicted to impart substantial changes in the hydrophilicity of the protein and possibly associated antigenic changes (45).

Although Ad7 can be spread directly by the respiratory route, efficiency of transmission is typically lower than for some other respiratory viruses. Efficient spread usually requires crowding, such as that in closed communities like chronic-care facilities, military barracks, and day-care centers. Widespread community outbreaks of Ad7 can occur but appear to require low levels of herd immunity. For example, in Japan, >95% of persons <40 years of age lacked specific antibodies to Ad7 before the countrywide epidemic of Ad7 that began in 1995 (48,49). The most comprehensive recent seroprevalence data on Ad7 in the United States were obtained in 1992 from 364 military basic trainees attending new recruit training centers (50). Approximately 73% of screened trainees lacked specific antibodies to Ad7. In another study to evaluate the potential for use of Ad vectors in gene therapy for cystic fibrosis, 73.9% of 46 serum specimens collected from 1993 to 1995 from children (median age 4.7 years) were seronegative for Ad7 (51).

To achieve rapid spread, a novel genome type presumably requires an immunologically naive population, greater biological fitness than the indigenous circulating strains, and a means of introduction to the susceptible community. Azar et al. (37) noted that the appearance of Ad7d2 in Israel coincided with the arrival of large numbers of immigrants from the former Soviet Union and Ethiopia during the early 1990s. The global spread of Ad7b in the 1960s and 1970s may have been aided by the movement of unvaccinated U.S. and allied military personnel during the Vietnam War. In our study, the appearance of Ad7h in the U.S. Southwest in 1998 may be explained by the emigration of persons from Ad7h-endemic regions of South America, where communitywide outbreaks of respiratory illness due to Ad7h occurred as recently as 1998 in Chile (Rodrigo Fasce, pers. comm.). However, a more comprehensive survey of Ad7 isolates from Mexico and U.S. states on the Mexican Border would be necessary to substantiate this observation.

The five recognized civilian outbreaks of Ad7 respiratory illness that occurred during 1996-2000, three of which we attributed to genome type 7d2, might have been due to increased reporting as a result of our interest in this study or may represent a real increase in Ad7-associated disease, as occurred in Europe during the early 1970s and in Japan and Korea (12,15,18) beginning in 1995. One unsubstantiated possibility is that the discontinuation of vaccination of U.S. military recruits for Ad4 and Ad7 in 1996 and the subsequent increase in Ad-associated disease at military bases throughout the United States (20-22) provided a new focus for Ad7 dissemination to civilian populations. A possible example of this is the 1998 outbreak of Ad7d2 illness at a Chicago pediatric chronic-care facility described earlier (17). This outbreak occurred within a few miles of the Naval Training Center in Great Lakes, which had had an outbreak of Ad7d2 the preceding year (21). Most cases of Ad infection at military bases since 1996 have been attributed to Ad4 (20,22), but no comparable outbreaks of Ad4 disease among civilians have been reported. Unlike Ad7, which poses a risk to both civilian and military populations, Ad4 has only infrequently been associated with outbreaks of respiratory illness in civilian populations (2).

Although we identified individual cases of severe lower respiratory tract illness and deaths attributed to Ad7d2 and Ad7h in this study, the possibility that these two genome types may be associated with more severe disease is not yet clear. More extensive clinical and epidemiologic study is required to adequately address this question. The limited data from infected military recruits suggest no differences in clinical illness between those infected with Ad7d2 and Ad7b (data not shown). Reports of Ad7d2 infections in Israel (37) and Ad7d infections in China (46) also noted no clear differences in severity of disease. Cases of severe pneumonia and neurologic disease were reported from a recent regional epidemic of Ad7d2 in Japan (15), but there was no evidence that these severe cases were more common than those reported for outbreaks involving other Ad7 genome types. Ad7h, a genetically unique recombinant between Ad7 and Ad3 (42), has been linked to increased illness and death in infants in Chile and Argentina, where it is second only to *Human respiratory syncytial virus* as a cause of severe viral pneumonia in infants and young children (34,52). However, in this study, too few cases of Ad7h infection were identified to assess differences in disease severity.

In conclusion, our study documents the recent appearance in the United States of two new Ad7 genome types, Ad7d2 and Ad7h, and provides additional evidence of the global spread of these formerly geographically restricted viruses. The possibility that these genome types may be associated with more severe disease makes it prudent to monitor their spread and associated disease.

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A European Study on the Relationship between Antimicrobial Use and Antimicrobial Resistance

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In Europe, antimicrobial resistance has been monitored since 1998 by the European Antimicrobial Resistance Surveillance System (EARSS). We examined the relationship between penicillin nonsusceptibility of invasive isolates of *Streptococcus pneumoniae* and antibiotic sales. Information was collected on 1998-99 resistance data for invasive isolates of *S. pneumoniae* to penicillin, based on surveillance data from EARSS and on outpatient sales during 1997 for beta-lactam antibiotics and macrolides. Our results show that in Europe antimicrobial resistance of *S. pneumoniae* to penicillin is correlated with use of beta-lactam antibiotics and macrolides.

Antimicrobial resistance is a growing problem worldwide, requiring international approaches. The World Health Organization (WHO) and the European Commission have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control (1-3). In European countries, antimicrobial resistance has been monitored in selected bacteria from humans since 1998 through the European Antimicrobial Resistance Surveillance System (EARSS). Funded by the European Commission, EARSS is an international network of national surveillance systems intended to collect comparable and reliable resistance data. The purpose of EARSS is to document variations in antimicrobial resistance over time and place and to provide the basis for and assess the effectiveness of prevention programs and policy decisions.

One of the indicator organisms in EARSS is *Streptococcus pneumoniae*. It was included for three reasons: it is of major clinical importance for pneumonia, bacterial meningitis, and otitis media; many countries have reported that its resistance to penicillin is increasing; and *S. pneumoniae* is representative of organisms that are transmitted in the community.

A major risk factor for the development of resistance is thought to be inappropriate use of antimicrobial drugs. Most studies that have investigated the relationship of antimicrobial use and antimicrobial resistance have been undertaken in hospital, multicenter, or country settings (4-7). For infections with penicillin-nonsusceptible *S. pneumoniae* (PNSP), studies have demonstrated that at the individual level, previous use of beta-lactam antibiotics such as penicillin is an important risk factor (8-10). Studies on carriage of PNSP in children have shown that sulfamethoxazole-trimethoprim (co-trimoxazole) and macrolides such as erythromycin have also been associated with selection of PNSP (11,12). Translated to the population level, sales of beta-lactam antibiotics, co-trimoxazole, or macrolides in a given geographic region may be proportional to microbial resistance to penicillin. If on the European level a relationship between antimicrobial resistance and antimicrobial use could be found (as in the case of *S. pneumoniae* and resistance to penicillin), efforts to control antimicrobial use and misuse could be stimulated and monitored in Europe.

We used an ecologic study design to examine the correlation between use of relevant antibiotics in the outpatient setting and the proportion of PNSP among invasive isolates of *S. pneumoniae* in 11 European countries.

Methods

Antimicrobial Resistance Data

The estimated average coverage of the populations of countries participating in EARSS is 52% (range 10% to 90%) (13). Laboratories that participate in EARSS screen invasive *S. pneumoniae* isolates for oxacillin resistance (14). When an isolate is found to be nonsusceptible, the EARSS protocol requests confirmation as intermediate- or high-level resistance to penicillin by determination of MICs. Laboratories perform microbiologic testing and interpret results according to their own standards. National guidelines in Europe differ; isolates of *S. pneumoniae* are considered nonsusceptible to penicillin if the MIC is >0.06 (15-18) or >0.12 (19,20) mg/L. For this report, we use nonsusceptibility and intermediate resistance as synonyms; PNSP isolates are either intermediate or fully

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resistant to penicillin. Only the first invasive isolate per patient per quarter is reported.

To assess the comparability of susceptibility test results, a quality assurance exercise was performed in September 2000 among 482 laboratories from 23 countries participating in EARSS. The concordance (agreement of reported results with intended results) for the detection of penicillin resistance in the three *S. pneumoniae* control strains was 91% (21). Laboratories send standardized data to the national EARSS data manager, who checks data contents and ensures conformity with the EARSS data format. In collaboration with WHO, an export module from the laboratory-based software WHONET was developed for EARSS (22). Every quarter, data are forwarded to the central database at the National Institute of Public Health and the Environment (RIVM), Bilthoven, Netherlands, where the project is coordinated.

Antimicrobial Use Data

National outpatient sales data for antibiotics from 1997 were purchased from IMS Health Global Services, London, United Kingdom, for 13 of the 15 member states of the European Union. Corresponding data were obtained from the Danish Medicines Agency for Denmark and from the National Corporation of Swedish Pharmacies for Sweden (23). The IMS data were examined and adjusted according to the Anatomic Therapeutic Classification (ATC) system used by WHO (24). The amount in kilograms for an antimicrobial agent was converted to a number of defined daily doses (DDD). The DDD, which is based on the average daily dose used for the main indication of the drug, is appropriate for comparisons of drug use over time and in different geographic areas. For beta-lactam antibiotics, we combined ATC groups J01C (extended- and narrow-spectrum penicillins) and J01D (cephalosporins); macrolides were classified under code J01F. No data were available for the combination of trimethoprim and sulphonamide.

Nonadherence

We considered nonadherence of patients to the physician's prescription in individual countries as a possible confounder of antimicrobial resistance. Branthwaite et al. reported nonadherence levels from a population-based survey in seven countries (25). Data from four of the seven countries (Spain, Belgium, the United Kingdom, and Italy) were also captured in EARSS.

Statistical Analysis

We calculated the proportion of PNSP among all invasive *S. pneumoniae* isolates from each country reported during 1998-99. Because probabilities allow only values between 0 and 1, we modeled the natural logarithm of the odds of PNSP resistance (logodds).

Least-square linear regression analysis was used to assess correlation between antimicrobial use (of beta-lactam antibiotics and macrolides, expressed in DDD per 1,000 population per day) and the logodds of resistance. We correlated nonadherence levels with the logodds of resistance in the same way. We calculated the Spearman coefficient of determination (r-square) and its corresponding p value. For the calculation of the regression lines, we weighted the data points by the inverse of the variance of each data point. We used SAS software (SAS Institute Inc., Release 6.03., Cary, NC).

Results

Antimicrobial Resistance

During 1998-99, 337 laboratories from 11 European Union member states (Belgium, Finland, Germany, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden, and United Kingdom) and one nonmember state (Iceland) reported 4,872 invasive *S. pneumoniae* isolates to EARSS. The proportion of PNSP among isolates of invasive *S. pneumoniae* ranged from 1% to 34% (Table) (Figure 1). Southern European countries reported higher rates than northern European countries.

Antimicrobial Use

Data on outpatient sales of beta-lactam antibiotics and macrolides were available for 1997 from all 15 European Union member countries. Antimicrobial use varied widely between countries. Sales to outpatients ranged from 3.8 to 23.6 DDD per 1,000 inhabitants per day for beta-lactam antibiotics and from 0.97 to 5.98 DDD for macrolides. The three countries with the highest reported use were France, Spain, and Portugal for beta-lactam antibiotics and France, Spain, and Italy for macrolides; the three countries with the lowest use were the Netherlands, Germany, and Austria for beta-lactam antibiotics and Sweden, the Netherlands, and Finland for macrolides.

Correlation

For 11 countries, information was available for both antimicrobial resistance and antimicrobial use. Linear regression of the correlation of use of beta-lactam antibiotics and the logodds of resistance showed an r-square of 0.80 (p=0.0002) (Figure 2). The equation for the regression is

logodds of resistance =(-3.94)+(0.16xDDD).

For the use of macrolides, we calculated an r-square of 0.46. Figure 3 shows the graph for nonadherence to antibiotics and the logodds of resistance. The r-square is 0.8 (p=0.2).

Discussion

We present for the first time Europe-wide, country-specific, representative data on antimicrobial resistance collected by EARSS. Using an ecologic study design, we demonstrate through the correlation with data on antimicrobial use one aspect of the usefulness of surveillance for antimicrobial resistance. The results from 11 European countries show a linear relationship between use of beta-lactam antibiotics and macrolides and the proportion of PNSP among all invasive *S. pneumoniae* isolates.

Table. Number of submitting laboratories, number of isolates of *Streptococcus pneumoniae*, number (#) and percent (%R) of penicillin nonsusceptible *S. pneumoniae* isolates, logodds of resistance (ln(%R/[1-%R]), and outpatient sales of beta-lactam antibiotics and macrolides

Country	No. of laboratories	No. of *S. pneumoniae* isolates	Penicillin nonsusceptible *S. pneumoniae*			Outpatient sales of antibiotics in DDD[a]/1,000 inhabitants/day	
			No.	%R (95% CI)	ln (%R/[1-%R])	Beta-lactam antibiotics	Macrolides
Austria	-	-	-	-	-	6	3.7
Belgium	96	940	131	14 (12-16)	-1.82	14	4.1
Denmark	-	-	-	-	-	7	2
Finland	11	211	8	4 (2-8)	-3.18	8	1.9
France	-	-	-	-	-	24	6.0
Germany	15	222	4	2 (1-5)	-3.89	5	2.5
Iceland	2	54	1	2 (0-11)	-3.89	Not available	Not available
Ireland	12	157	30	19 (13-26)	-1.45	11	2.5
Italy	46	194	26	13 (9-19)	-1.87	15	5.1
Luxembourg	1	11	2	18 (3-52)	-1.52	14	4.7
Netherlands	20	760	8	1 (0-2)	-4.6	4	1.2
Portugal	12	134	25	19 (13-27)	-1.45	16	3.7
Spain	76	1,240	418	34 (31-36)	-0.66	21	5.9
Sweden	24	706	21	3 (2-5)	-3.48	8	1
United Kingdom	22	243	21	9 (6-13)	-2.31	9	3.2

[a]DDD = defined daily doses; CI = confidence interval.

EARSS data show that resistance for PNSP follows a north-south gradient. Southern European countries have higher proportions of PNSP than countries in northern Europe. A possible reason for this observation could be the difference in antimicrobial use, which also tends to be higher in southern European countries. If use of relevant antibiotics (beta-lactam antibiotics and macrolides) and the logodds of resistance are modeled through linear regression, a strong linear and statistically significant relationship is demonstrated.

Our findings agree with those of Austin et al., who modeled the relationship between antimicrobial use and endemic resistance, based on population genetic methods and epidemiologic observations (26). The correlation in Figure 2 is consistent with the model developed by Austin et al. on theoretical grounds.

We correlate antimicrobial sales data for 1997 with antimicrobial resistance data for 1998 and 1999. Others have observed that after a lag time of 1 or more years, changes in antimicrobial use may be followed by changes in antimicrobial resistance (27,28). Therefore, we believe that it is reasonable to correlate antimicrobial sales data in 1997 with antimicrobial resistance data from 1998-99.

We address several limitations in our study. First, because it is an ecologic study, we can make no inferences on the individual level. Second, resistance rates in some countries (Table) are calculated from a relatively limited number of isolates. However, based on communications with EARSS country representatives, our data are consistent with antimicrobial resistance levels derived from other sources (29). Third, an explanation for the differences in antimicrobial resistance could be sampling bias: clinicians in northern European countries may request blood cultures more frequently than their southern European colleagues, who may sample only in case of empirical treatment failure. Fourth, we have not addressed other, potentially important contributing factors for the development of antimicrobial resistance of organisms that are transmitted in the community, particularly nonadherence and over-the-counter sales of antimicrobial agents. Both these factors are difficult to measure. However, in 1993 nonadherence to prescribed antimicrobial agents was assessed in a survey in six

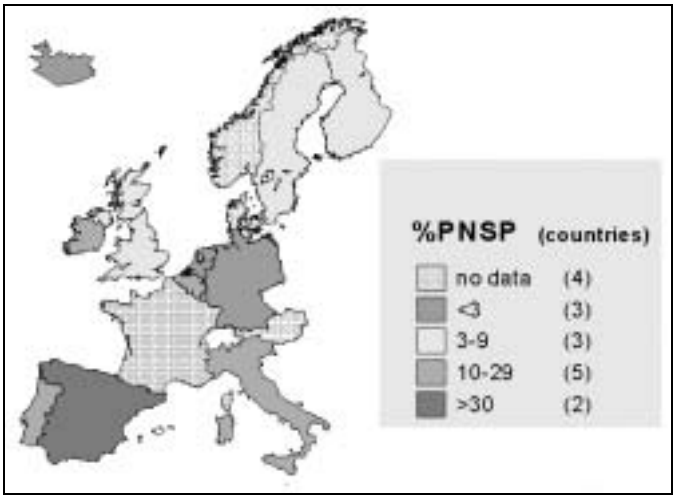

Figure 1. Proportions of invasive isolates of *Streptococcus pneumoniae* resistant to penicillin (PNSP) among 12 European countries, 1998–99.

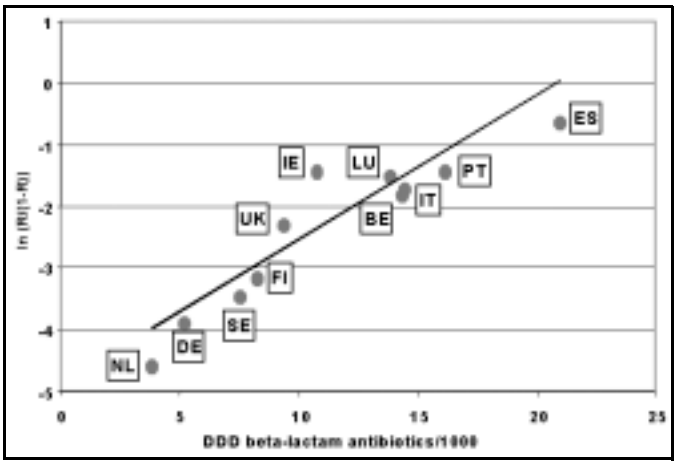

Figure 2. The logodds of resistance to penicillin among invasive isolates of *Streptoccus pneumoniae* (PNSP; ln(R/[1-R])) is regressed against outpatient sales of beta-lactam antibiotics in 11 European countries; antimicrobial resistance data are from 1998 to 1999 and antibiotic sales data are from 1997. DDD = defined daily dose; BE = Belgium; DE = Germany; FI = Finland; IE = Ireland; IT = Italy; LU = Luxembourg; NL = the Netherlands; PT = Portugal; ES = Spain; SE = Sweden; UK = United Kingdom.

European countries (25). Although the number of data points is limited, Figure 2 suggests a direct relationship between nonadherence rates and logodds of resistance. Thus, if nonadherence is also related to sales of antimicrobial agents, it could potentially confound the relationship between use and resistance. Data on the degree of over-the-counter use among European countries are not widely available; we know of one Spanish and one Greek study reporting an estimate of over-the-counter use (30,31). The influence of these and other parameters on the level of resistance should be quantified and understood. Finally, because children are the main reservoir of carriage of *S. pneumoniae*, an age-stratified analysis would be desirable, i.e., a correlation of resistance with antimicrobial use among children. However, this analysis would require more detailed use data, for example, of liquid formulations of antibiotics.

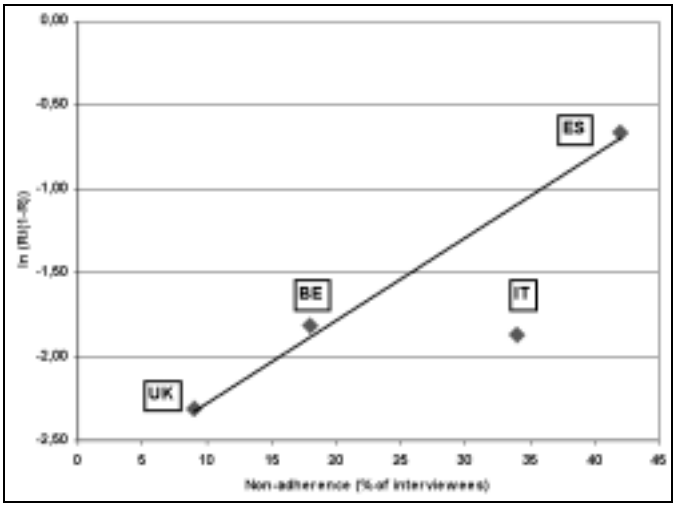

Figure 3. The logodds of resistance of invasive isolates of *Streptococcus pneumoniae* to penicillin (PNSP; ln(R/(1-R))) is regressed against nonadherence rates to antibiotic therapy in four European countries. Nonadherence rates are from 1993; PNSP data are from 1998-99. UK = United Kingdom; BE = Belgium; IT = Italy; ES = Spain.

At least two studies in northern Europe have demonstrated that PNSP rates can be halted or even reversed when physicians avoid the inappropriate prescription of antimicrobial agents (32,33). Our study is timely because it shows that even at the European level a correlation can be observed between antimicrobial resistance (of *S. pneumoniae* to penicillin) and antimicrobial use. In several European countries, national action plans for the appropriate use of antimicrobial agents are being planned or implemented; their effectiveness should be monitored through prospective and continuous surveillance of antimicrobial resistance and antimicrobial sales data (34-38).

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Eastern Equine Encephalomyelitis Virus Infection in a Horse from California

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A yearling quarter horse, which was raised in southern California, received routine vaccinations for prevention of infection by *Eastern equine encephalomyelitis virus* (EEEV). One week later, severe neurologic signs developed, and the horse was humanely destroyed. A vaccine-related encephalomyelitis was later suspected. A final diagnosis of EEEV infection was established on the basis of acute onset of the neurologic signs, histopathologic and serologic testing, and isolation and molecular characterization of EEEV from brain tissue**.** The vaccine was extensively tested for viral inactivation. Nucleotide sequences from the vaccine and the virus isolated in the affected horse were also compared. In California, arboviral encephalomyelitides are rarely reported, and EEEV infection has not previously been documented. This report describes the occurrence of EEEV infection in the horse and the investigation to determine the source of infection, which was not definitively identified.

Eastern equine encephalomyelitis virus (EEEV) is a mosquito-borne virus in the family *Togaviridae*, genus *Alphavirus*. EEEV, *Western equine encephalomyelitis virus* (WEEV), and *Venezuelan equine encephalomyelitis virus* (VEEV) are related but genetically distinct alphaviruses. EEEV and VEEV are lethal in up to 90% of recognized equine cases, whereas WEEV is least virulent in horses, which have a mortality rate of approximately 40% (1). EEEV may also cause fatal encephalitis in humans (mortality rate 50%-75%) (2). In the United States, enzootic EEEV occurs mainly from New England to Florida and along the Gulf Coast, with rare reports of foci as far inland as Michigan and South Dakota (3). In North America, sylvatic populations and the mosquito *Culiseta melanura* maintain the virus in hardwood, salt-water swamp habitats. Large populations of this mosquito allow amplification of the virus by transmission among wild birds (4). In wild birds indigenous to North America, the infection is usually innocuous, whereas in pheasants, cranes, and emus, the disease is often lethal. *C. melanura* feeds almost exclusively on passerine birds; however, spillover of EEEV from the enzootic vector into several other mosquito species (e.g., *Aedes* spp.), which feed on tangential hosts such as humans and equines, may result in large epizootics with high mortality rates (4-6). Our paper describes a sporadic case of EEEV infection in a horse outside the known geographic range of this virus and the ensuing investigation to determine the source of exposure.

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Materials and Methods

Case Report

In April 2000, a 14-month-old gelding quarter horse was seen at a veterinary referral hospital in southern California for sudden onset of quadraparesis and recumbency. The horse had no history of prior neurologic disease. He had been castrated approximately 90 days before the illness without complication. A multidose, multivalent vaccine containing formalin-inactivated EEEV and WEEV, influenza virus, and tetanus toxoid was administered to the affected horse and 27 stable mates 1 week before the onset of illness.

The horse appeared alert and healthy the night before onset of clinical signs. At 6:30 a.m. on April 21, he was found down in his stall and unresponsive to external stimuli. The referring veterinarian found a recumbent, comatose horse with spontaneous nystagmus and flailing, incoordinated movements. Initial therapy included intravenous corticosteroids, fluid therapy (including glucose to treat possible hyperkalemic periodic paralysis), and diazepam for intermittent seizures. The horse did not respond to therapy and was sent to the referral hospital.

On examination at the hospital, the horse was comatose with elevated heart and respiratory rates and a normal rectal temperature. A neurologic exam showed that pupillary light responses were absent bilaterally. Palpebral reflexes were present although weak. No organized motor movements occurred in response to stimuli. Initial emergency treatment consisted of intravenous fluids with dimethyl sulfoxide and

flunixin meglumine. Cervical and skull radiographs were performed and were within normal limits. An atlanto-occipital cerebrospinal fluid tap was also performed, and no abnormalities were seen on gross observation. At this point, diffuse cortical disease was evident. Trauma appeared very unlikely, and an infectious process or toxicosis seemed more probable. Because of the grave prognosis, the owners elected to euthanize the horse. The carcass was sent to the Animal Health and Food Safety Laboratory System, San Bernardino Branch, School of Veterinary Medicine, University of California, Davis, for necropsy.

Results

Pathology

Results of gross necropsy examination were unremarkable except for markedly hemorrhagic bladder mucosa. Histologic examination revealed lesions mainly confined to the cerebral cortex, thalamus, hypothalamus, and anterior portion of the spinal cord (C_1-C_4). Lesions in the brain were characterized by a multifocal to diffuse neutrophilic response with gradual progression to mononuclear cell infiltrates in some areas. Vascular damage and fibrin thrombi were evident (Figure 1). Some blood vessels had swollen endothelium surrounded by a thick layer of mononuclear cells. A mild degree of meningitis was present, with pleocellular response containing mainly mononuclear and neutrophilic infiltrates. The neuropil showed fine vacuolation, indicating edema. Some axons were markedly shrunken. The remaining portion of the spinal cord was unremarkable. The urinary bladder had diffuse submucosal hemorrhages. The lung showed flooding of the alveoli with eosinophilic fluid. The remaining tissues were unremarkable.

Virology

Portions of brain tissue were collected and sent for rabies testing at the local public health laboratory. Results were negative. Fresh, frozen brain tissues and serum were submitted to the National Veterinary Services Laboratories (NVSL). Tests for equine encephalomyelitides included virus isolation and serology for WEEV, EEEV, VEEV, *Equid herpesvirus 1* (EHV-1), and *West Nile virus* (WNV). For virus isolation, a 10% suspension of brain sample was prepared and injected into flasks of RK13, equine dermal, and Vero-MARU cells (Vero M). This cell line was obtained by NVSL at the 135th passage level from the Middle America Research Unit (MARU) as a multipurpose cell line for virus isolation in 1980 and has been maintained by the NVSL since that time. Additional brain suspension was injected intracerebrally into 16 suckling 4-day-old mice (from 2 litters). Cytopathic effects were observed in the RK13 and Vero M cells at 2 days after injection. Examination using electron microscopy of the RK13 cell culture fluids showed particles with morphologic features compatible with alphaviruses.

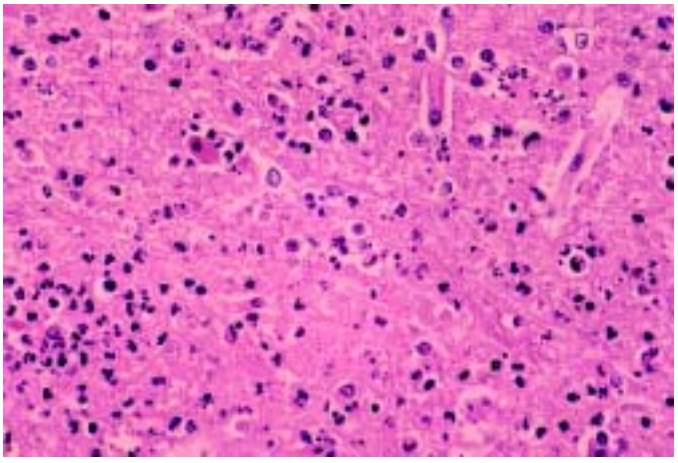

Figure 1. Photomicrograph of a section of the cerebral cortex from horse with *Eastern equine encephalomyelitis virus* infection. Note the dense neutrophilic response, vascular damage, and fibrin thrombi. Hematoxylin and eosin stain.

Virus preparations from both the cell culture supernatant and suckling mouse brains of mice that died were identified as EEEV by a complement fixation test with reference antisera. In that test, virus reacted strongly with EEEV antiserum and weakly or not at all with WEEV and VEEV antisera.

Serum collected from the yearling horse on April 21 before it died was tested for antibodies to EEEV, WEEV, and VEEV by hemagglutination inhibition (HI) and plaque reduction neutralization testing (PRNT). The serum had a HI titer of 20 against both EEEV and WEEV. HI antibodies to VEEV were not detected. In the PRNT, the serum neutralizing antibody titer versus EEEV was ≥100 but was undetectable against either WEEV or VEEV. The serum was also positive in an immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay for EEEV with a titer of ≥1000. Additional tests for antibodies to equine herpesvirus 1 and WNV were negative.

The Center for Vector-Borne Disease Research at the University of California, Davis (CVBDR), U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), and California Department of Health Services (CDHS) also received homogenized brain suspension material. Isolation of EEEV by mouse inoculation and cell culture supported NVSL's findings. The isolate was further characterized by USAMRIID by reverse transcriptase-polymerase chain reaction (RT-PCR) testing and sequencing as described (7,8), but with primers listed in Table 1. A 1,165-nucleotide portion of the viral genome including parts or all of the E2, 6K, and E1 genes was determined. Sequencing of the isolate showed an 18-nucleotide difference (98.5% homology) from the reference PE6 EEEV strain. Comparison with sequences that have been submitted to GenBank indicated that the virus is a North American antigenic variety in subtype 1 of the taxonomic scheme recently proposed by Brault et al. (9).

Field Investigation

Local, state, and federal agencies participated in a joint field and laboratory investigation to determine the source of infection. Four hypotheses were investigated to explain the occurrence of EEEV outside its usual range: 1) imported

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers used to sequence *Eastern equine encephalomyelitis virus* RNA

Title	Sense	Primer	Use	Region
9930	Forward	5´- GCGCTGCTTATTTTGCTGT -3´	RT-PCR	E1
11582	Reverse	5´- ATTATGCGCTGCCTGTAGTGTTTA -3´	RT-PCR	E1
4043	Forward	5´- GTGCGCGGCGACATAACAAAGAG- 3´	RT-PCR	NSP3
4976	Reverse	5´- CCGGGGGTACAGTGCCAGAGAA -3´	RT-PCR	NSP3
10470	Forward	5´- ATGCCAAACTCATCATAGGTCCACT -3´	Sequencing	E1
10938	Forward	5´- GTATAGCCACCGTTGCCTACAAATC -3´	Sequencing	E1
11345	Forward	5´- CAGGCAGTGTATAAGGCTGTCTTAC -3´	Sequencing	E1
T3	Forward	5´- AATTAACCCTCACTAAAGGGA -3´	Sequencing	NSP3

infection from an EEEV-endemic region, 2) autochthonous transmission by locally infected mosquitoes, 3) intentional inoculation of the horse with live EEEV by a person or by purposeful contamination of the vaccine and 4) incomplete inactivation of EEEV in a commercially inactivated viral vaccine.

Travel History

We found no evidence that this case was due to importation from an EEEV-endemic region. The horse was born in northern California and at 6 months of age was moved to southern California for training. The horse traveled as far east as Fort Worth, Texas, in July 1999 for showing purposes. The rest of the 1999 horse show season took place in southern California. The horse was last moved to a new stable (Farm A) in southern California during February 2000. He attended several shows in this area and as far east as Hurricane, Utah. A review of a list of participants at the horse shows recently attended showed no horses from EEEV-endemic areas and no reports of encephalomyelitis among other equine participants.

Surveillance for EEEV in Southern California

No evidence for autochthonous transmission of EEEV by local mosquito populations was found through surveillance in mosquitoes, sentinel chickens, wild birds, horses, and humans from the region. California has an extensive, long-established Arboviral Encephalitis Surveillance Program that is active from April through October each year (10,11). The program includes collection and testing of mosquito pools and sentinel chicken flocks for WEEV and *St. Louis encephalomyelitis virus* and surveillance for encephalomyelitis cases among equids, ratites (e.g., emus, ostriches), and humans. Following recognition of the equine EEEV case, CDHS began including EEEV screening in its routine testing program.

Coincidentally, a sentinel chicken flock was located on Farm A. Sera submitted from this flock in April were retrospectively tested for EEEV antibody by indirect immunoassay and found to be negative. The flock remained seronegative for EEEV from May through October. In addition, mosquitoes were collected at Farm A and within a 5-mile radius with carbon dioxide traps. A total of 74 mosquitoes, including the *Culex* spp. *tarsalis, quinquefasciatus, erythrothorax*, and *stigmatosoma*, and *Culiseta particeps*, were collected in 23 trap nights during May. Only 8 of 74 mosquito species were *Culex tarsalis*, a known vector species of WEEV and a potential vector species of EEEV in California (12). Surveys for resting adult mosquitoes in barns and other buildings yielded no mosquitoes. All mosquito pools were tested and found negative by virus isolation in tissue culture. Routine biweekly testing of sentinel chicken flocks and mosquito pools throughout California until the end of October showed no further evidence of EEEV activity in the state.

Despite enhanced surveillance, additional cases of EEV infection in local animal and human populations were not identified. Surveillance for encephalitis cases in horses and humans was heightened in southern California after the equine case was recognized. Veterinarians were alerted statewide through a newsletter published by the California Department of Food and Agriculture, and the local health department issued a press release. Following the publicity, a veterinarian reported three horses with acute neurologic disease during mid-May at another ranch, Farm B, approximately 50 km from Farm A. Necropsy and serologic testing of these cases performed at the California Animal Health and Food Safety Laboratory System, San Bernardino Branch, showed EHV-1 as the likely cause of the outbreak at Farm B; no evidence of EEEV infection was found. In addition, a brown-headed cowbird (*Molothrus ater*) die-off at another horse ranch, Farm C, approximately 80 km from Farm A, was investigated. No laboratory evidence of EEEV infection was found in three dead cowbirds collected from Farm C, although the causes of their deaths were not determined.

No other horses at Farm A had encephalitis. To further assess potential equine exposures at Farm A, a serosurvey of 10 randomly chosen stable mates of the affected horse were tested for EEEV antibodies. The sample ranged in age from weanlings to elderly horses; each had been vaccinated with the multivalent vaccine against WEEV, EEEV, influenza viruses, and tetanus (Vaccine A) from the same lot on the same day as the case. These horses showed positive neutralizing antibody titers by PRNT ranging from <20 to 320; none had IgM antibodies to EEEV by an ELISA-capture test. Previous vaccination histories were not available for these horses or the case,

but the findings in the stable mates were compatible with recent vaccination or the presence of maternal antibodies in younger horses without natural exposure.

Criminal Mischief

Although an intentional introduction of EEEV seemed highly unlikely, recent concerns about bioterrorism made this an important possibility to consider. We found no evidence of purposeful contamination of the vaccine or intentional inoculation of the horse. EEEV is not readily obtainable. Furthermore, no motive for such an act was found.

Vaccine Studies

An extensive evaluation of the final hypothesis, residual live EEEV in the vaccine, could not eliminate Vaccine A as the source of infection. The farm manager ordered the multivalent EEEV, WEEV, influenza viruses, and tetanus toxoid vaccine by mail from an out-of-state vendor and stored the vials at 6° C in a refrigerator at Farm A. The vaccine was a commercial, four-way, multidose product that was administered intramuscularly by farm personnel. The viruses in the vaccine were formalin-inactivated, adjuvant-type, and of tissue-culture origin.

Virus Isolation

Three unused vials and one partially used vial of Vaccine A were found in the refrigerator at Farm A. CDHS and CVBDR attempted virus isolation by mouse inoculation and cell culture by using the residual vaccine from Farm A. One-day-old mice were inoculated by either intraperitoneal or intracranial injections of the vaccine and were monitored for 18 days. Live EEEV was not isolated from any of the vials. Additionally, the Center for Veterinary Biologics Laboratory conducted safety tests on stored vaccine from the same lot as Vaccine A, which was available because of licensing procedures that require samples from each lot to be retained. Virus isolation attempts on these samples were also negative by cell culture and wet chick inoculations.

Molecular Comparison of Horse and Vaccine Strains

EEE viral RNA was extracted from the horse isolate and passaged once via BHK (baby hamster kidney) cell culture at CVBDR and directly from the residual vaccine and amplified and sequenced in two separate regions of the genome according to previously published protocols (7,8). The structural E1 region was amplified and compared with several other published EEEV E1 sequences in GenBank; 1,100-nucleotide sequences of the horse isolate and Vaccine A strain were compared with each other as well as with the 10 most closely matched published sequences in GenBank. The conclusions from this laboratory's study were very similar to the initial gene sequencing of the horse isolate by USAMRIID. Table 2 illustrates a comparison of each GenBank sequence to the sequence of the horse isolate. The phylogram is depicted in Figure 2.

Table 2. Comparisons of 1,100 nucleotide sequences of the horse virus isolate, Vaccine A virus strain, and selected GenBank isolates of the structural unit E1

GenBank accession no.	Strain/isolate	Mutations	% Match
Horse		0	100
Vaccine		5	99.5
AF159551	LA50	7	99.4
L37662	PE6 vaccine	11	98.9
U01552	Decuir	13	98.5
U01558	Tenbroeck	15	98.6
U01554	NJ/60	15	98.6
AF159556	FL96-14834	17	98.5
X63135	ssp. N. Am. Variant	18	98.4
AF159550	MA38-Mass	19	98.3
U01555	ME771332	19	98.2
U01034	82V2137	19	98.3

The NSP3 (nonstructural) region was also amplified and cloned to check for variability within Vaccine A and in the horse viral isolate, as well as to compare with published EEEV NSP3 sequences in GenBank. Four horse and five vaccine viral RNA clones were sequenced and analyzed. Of the 508 nucleotides in each fragment, only one nucleotide difference was evident among the cloned vaccine sequences, and only one was found among the four sequences from horse isolates. These differences could be a result of taq polymerase errors. The consensus sequences for both the vaccine and the horse EEEVl RNAs were compared with each other and with the only two EEEV GenBank sequences that came up in a BLAST search (Table 3, Figure 3).

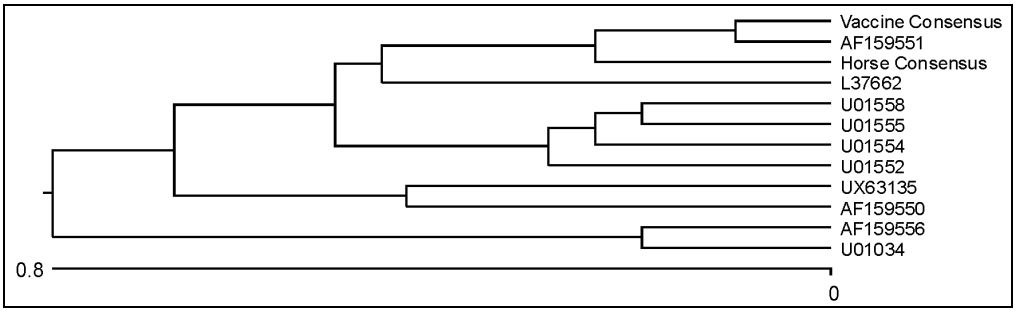

Figure 2. Phylogram based on nucleotide comparison from the E1 region of a horse infected with *Eastern equine encephalomyelitis virus*.

Table 3. Comparisons of 508 nucleotide sequences of the horse virus isolate, Vaccine A virus strain, and selected GenBank isolates of the nonstructural unit NSP3

GenBank accession no.	Strain/isolate	Mutations	% Match
Horse		0	100
Vaccine		0	100
X63135	ssp. N. Am. Variant	5	99.0
U01034	82V2137	5	99.0

Discussion

The identification of EEEV in a horse in California was unprecedented and clearly represented a potential human and animal health threat. In other areas of the country, equine epizootics have been recognized as precursors to human disease (13,14). The rapid recognition and reporting of the case permitted an extensive investigation into the source of exposure.

Several factors must be met to sustain epidemics, including virulent viruses, adequate vectors, neighboring intermediate hosts, and populations of susceptible horses and people (5,15). Such isolated cases as the one mentioned are sure to increase veterinary attention to the possibility of neurologic patients having EEEV infections, as well as elevating public awareness of the disease and methods of prophylaxis.

A diagnosis of EEEV infection was made on the basis of the rapid clinical onset of neurologic signs, compatible histopathologic and serologic findings, and isolation and molecular characterization of EEEV from brain tissue. Several neurologic conditions were considered in the differential diagnosis, including other viral encephalomyelitides (rabies, Aujesky disease, Borna disease, EHV-1 myeloencephalopathy, WEEV, and WNV encephalomyelitis), bacterial meningitis, listeriosis, leukoencephalomalacia, lead poisoning, equine protozoal myeloencephalitis, nigropallidal encephalomalacia, botulism, and verminous encephalitis.

California's Arboviral Encephalitis Surveillance Program is among the most comprehensive in the United States. The jurisdiction where the horse was stabled participated in the program, and a sentinel chicken flock was located adjacent to the farm. In this case, locally infected mosquitoes were apparently not the source of exposure. Furthermore, there was no evidence of spread from the infected horse to the local mosquito populations based on mosquito pool and sentinel chicken flock testing throughout the year. The likelihood of EEEV's having become established in California following this isolated equine case is remote but still important to monitor because of the public health implications. First, the primary vector of EEEV in North America, *C. melanura*, is not known to occur in California (16). In addition, our equine case was diagnosed in April, when mosquito populations are low in southern California; particularly the vector species known to feed on both birds and horses. Second, this case had a rapid clinical course, with euthanasia in <24 hours after onset of clinical signs. Since horses are known to have a short viremia (1 to 3 days' duration) it is unlikely that any mosquitoes acquired the infection from the horse during this short time period. However, if vector abundance were increased, this horse would have had the potential to amplify the virus (5). Incidental infections could have occurred among barn personnel and susceptible horses at Farm A and nearby locations by transmission from mosquitoes that acquired the infection from the case. Of even greater concern, competent vectors could then spread the disease further by feeding on susceptible wild bird populations, potentially establishing an enzootic cycle in southern California.

After we excluded disease by natural infection, bioterrorism, and importation, incomplete formalin inactivation of the EEEV in the vaccine had to be considered a likely possibility. Previous reports of residual virus in formalin-inactivated vaccines exist. Documented outbreaks due to *Poliovirus* (PV*), Foot-and-mouth disease virus,* and VEEV have been directly related to the use of formalin-inactivated vaccines (17-19). Attempts to isolate live EEEV from residual and stored vaccine were unsuccessful. However, this does not eliminate the possibility that the horse received live virus with its immunization. If inactivated viruses existed in the vaccine, they were likely present in undetectable levels during vaccine development and testing. Additionally, the live viruses were probably distributed sporadically throughout the vaccine lot, allowing for only an isolated recognized case. The situation could also be analogous to the 1955 "Cutter inactivated poliovirus incident," when children became infected with PV after vaccination and follow-up investigation disclosed that several lots of Salk PV vaccine contained live PV, despite being produced with formalin inactivation in full compliance with federal regulations (19). In the PV vaccine example, live virus was not uniformly distributed in that vaccine lot (20).

We further explored the hypothesis of residual live virus in the vaccine through molecular epidemiologic studies. Similar studies were used to examine the role of the VEEV vaccine in the 1967-1972 VEEV pandemic in Central America (21). Unfortunately, the North American variety of EEEV is the most genetically homologous of the alphaviruses and therefore the least conducive to molecular comparison of strains (9,22). In our study, the greatest nucleotide homology in the E1 region was among the horse virus isolate, Vaccine A virus, and the

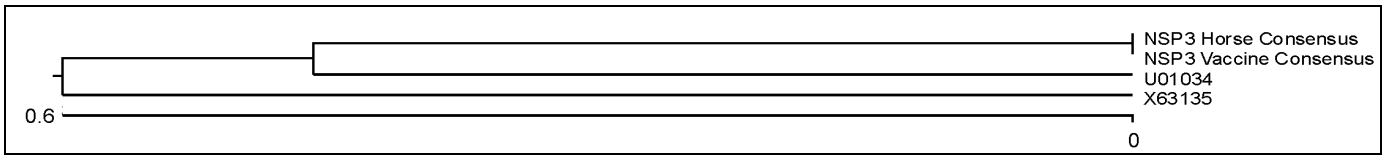

Figure 3. Phlyogram based on nucleotide comparison from the NPS3 (nonstructural) region of a horse infected with *Eastern equine encephalomyelitis virus*.

LA50 virus strain (Figure 2). Differences among sequences from Vaccine A EEEV and the horse viral isolate in the E1 region might represent mutations that occurred when virus passed through various hosts (horse brain/BHK cell culture/1) or genetic variants within the vaccine strain. However, we concluded on the basis of the limited number of clones analyzed that there were few to no other EEEV subclones in the horse viral isolate or vaccine virus. The NSP3 region proved to be more highly conserved and therefore less conclusive. Also, very few EEEV sequences that included the nonstructural regions have been published in GenBank, so comparison was limited. Regardless, the Vaccine A EEEV appears to be closely related to the horse viral isolate; thus, the possibility of live virus in the formalin-treated vaccine infecting the horse remains.

We are unaware of any reports of problems with this vaccine lot, despite notification of the manufacturer and other state veterinarians. If Vaccine A or portions of the lot contained live virus, many exposed horses may not have been susceptible because of previous immunization or presence of maternal antibodies. In addition, cases may have been unrecognized or unreported. If Vaccine A was the source of infection for this case or other cases, it was probably a rare event.

A definitive source of infection may never be revealed in this case. However, the case illustrates the need to maintain awareness that EEEV can occur outside its normal geographic boundaries; it also underscores the importance of prompt diagnosis, reporting, and surveillance for arboviral encephalomyelitides.

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Predicting the Risk of Lyme Disease: Habitat Suitability for *Ixodes scapularis* in the North Central United States

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The distribution and abundance of *Ixodes scapularis* were studied in Wisconsin, northern Illinois, and portions of the Upper Peninsula of Michigan by inspecting small mammals for ticks and by collecting questing ticks in state parks and natural areas. Environmental data were gathered at a local level (i.e., micro and meso levels), and a geographic information system (GIS) was used with several digitized coverages of environmental data to create a habitat profile for each site and a grid map for Wisconsin and Illinois. Results showed that the presence and abundance of *I. scapularis* varied, even when the host population was adequate. Tick presence was positively associated with deciduous, dry to mesic forests and alfisol-type soils of sandy or loam-sand textures overlying sedimentary rock. Tick absence was associated with grasslands, conifer forests, wet to wet/mesic forests, acidic soils of low fertility and a clay soil texture, and Precambrian bedrock. We performed a discriminant analysis to determine environmental differences between positive and negative tick sites and derived a regression equation to examine the probability of *I. scapularis* presence per grid. Both analyses indicated that soil order and land cover were the dominant contributors to tick presence. We then constructed a risk map indicating suitable habitats within areas where *I. scapularis* is already established. The risk map also shows areas of high probability the tick will become established if introduced. Thus, this risk analysis has both explanatory power and predictive capability.

Lyme disease, the most common vectorborne disease of humans in the United States, is caused by the spirochete *Borrelia burgdorferi* and transmitted by the blacklegged tick *Ixodes scapularis* (1). The distribution of Lyme disease in the Midwest has been determined largely by mapping the distribution of its vector, *I. scapularis*, which was first detected in northwestern Wisconsin in the late 1960s (2). Its range then expanded southward and eastward (3-6). Even though an isolated established population was discovered in northeastern Wisconsin in Marinette County (7), *I. scapularis* does not appear to have become established in several counties in northeastern Wisconsin. This area is heavily populated with white-tailed deer (*Odocoileus virginianus*) and white-footed mice (*Peromyscus leucopus*) (8), which serve as hosts for *I. scapularis* (1). Since host densities do not appear to be a limiting factor for the tick population (9), the physical environment, both at the macro and micro levels, may affect the tick's ability to survive in this habitat. Moreover, even if establishment is successful, environmental factors may limit tick population densities.

In northwestern Illinois, well-established *I. scapularis* populations were found along the Rock River in Ogle County and in Rock Island and Lee counties since the late 1980s (10-14). Through the early 1990s, Jo Daviess County was the only positive area along the Wisconsin border, and Putnam County was the only positive along the Illinois River. In southern Illinois, no blacklegged ticks were found among white-tailed deer in a survey conducted from 1980 to 1983 (15). Northern Illinois also maintains populations of white-tailed deer and white-footed mice (8), although a large proportion of land is used for agriculture (16).

The phenology of *I. scapularis* has been studied in Michigan (17), Wisconsin (18), and Illinois (19). In the Midwest, adults have both a longer activity period as well as higher peak densities in the spring than in the fall.

Studies of habitat preferences of *I. scapularis*, which have been conducted at various spatial scales (20-22), found environmental factors that are associated with vector and host distribution and densities. *I. scapularis* presence has been correlated with sandy soils (23, 24) and wooded vegetation (25-28). At the macro level, environmental risk factors for Lyme disease have been determined using satellite, climatological, and ecological data to characterize the habitat of the vector tick using geographic information systems (GIS), both in Europe (29-33) and the United States (22-24, 34-36).

The purpose of this study was to determine the distribution of *I. scapularis* in the upper Midwest based on data from Wisconsin, northern Illinois, and the Upper Peninsula of Michigan, and to explain the environmental factors that facilitate or inhibit the establishment of *I. scapularis*. Since host

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abundance is not a limiting factor for the maintenance of tick populations in this area, survival of *I. scapularis* may depend on a combination of several environmental risk factors, resulting in a patchy, discontinuous distribution of this vector. We propose a hierarchic interpretation, starting from the bedrock geology through glacial history and climate patterns, to explain the topography, soil, and vegetation patterns that may directly affect tick survival. By characterizing the habitat preferences of *I. scapularis* using digitized databases (some derived from satellite imagery) and field data integrated into a GIS, the distribution of Lyme disease and other diseases transmitted by the blacklegged tick can be predicted, and the risk of transmission to the human population can be assessed.

Methods

Site Selection

In Wisconsin, a statewide survey of parks and forests was conducted to determine the presence of *I. scapularis*. Sites were selected to represent each region in the state, with 28 of 59 states parks and forests selected. In Michigan, three sites were selected in Menominee County, where *I. scapularis* had previously been identified (7). In Illinois, paired positive and negative sites were sampled in Ogle and Rock Island counties, and additional sites were sampled along the Illinois River. Data are presented separately for each collection site.

Tick Collection

Tick collection was conducted at a total of 138 sites in July and September-October 1996, June 1997, and May-June 1998. The most comprehensive trips were made June 14-26, 1997, and May 27 through June 3, 1998, in the southern part of the study region. In several natural areas, more than one site was dragged, and results for each site were considered separately.

Questing *I. scapularis* ticks were collected in two ways: 1) by dragging a 1-m^2 white flannel cloth through vegetation for a total of at least two hours at each site (timed dragging), or 2) by dragging 1000 m on a grid (distance dragging). Timed dragging was conducted by teams of 4 or 5 persons, with each person dragging for 30 minutes. Distance dragging was also conducted by teams of 4 or 5 persons, which required an estimated 2 to 2.5 hours per grid. Thus, each site was dragged for a total of 2 or 2.5 hours per visit. All calculations of tick numbers are per 1 drag-hour.

Nymphs and adults were maintained alive in plastic vials with moistened cotton balls on ice for *B. burgdorferi* culture. Larvae were placed in vials containing 70% ethanol for later identification.

Vertebrate Collection

Small mammals were trapped overnight during July and October 1996, June 1997, and June 1998 at 13 selected sites in Wisconsin, and at all the Michigan and Illinois sites. Sherman live traps (H.B. Sherman Traps, Inc, Tallahassee, FL) were placed approximately 10 meters apart and baited with bread and peanut butter. Approximately 35 to 50 traps were placed per site, and 0 to 15 mice and 0 to 7 chipmunks were trapped at each site. White-footed mice and chipmunks were anesthetized with the inhalant anesthetic methoxyflourane (Shering-Plough, Inc., Madison, NJ), examined for ticks, and ear-tagged, and their sex and weight were recorded (LACAC animal use protocol # 99099). Ticks were removed and placed in vials containing 70% alcohol for identification.

Site Classification

For each site, the average number of each stage of the deer tick was calculated per hour of dragging. The number of ticks per dragging hour is based on an average of all drags. There was no situation where all or most ticks were found on one drag. The average number of larvae and nymphs was determined per small mammal captured. These data were not pooled with the dragging data because animals were not trapped at all sites.

A site was classified as negative (0) if *I. scapularis* was never found on vegetation or small mammal hosts. There was no case where ticks were found only on small mammals but not on drags. A site was rated 1 if only one stage of the tick was found, regardless of the quantity. A rating of 2 was given if all stages of the tick were found at low density (<10 larvae, <4 nymphs, <2 adults), and a rating of 3 indicated all stages were found at higher density.

We considered several types of classification, including calculating each stage separately and each collection trip separately. Although repeat visits increase the chance that a site will be classified as positive for the presence of ticks, there were no sites where more than one stage was found in only one visit. The finding of only one stage, however, may indicate accidental introduction without establishment. We selected a very conservative and coarse classification to account for the limitations of such an extensive field survey and to allow for differences in weather conditions, time of day, and other variables.

Soil Data

After removing the layer of leaf litter, soil samples were collected at each site from the uppermost 6 inches of topsoil. Data on predominant vegetation, leaf litter thickness, slope, and compass direction were also recorded at each site. Particle size analysis was performed on 10 gm samples of soil (37). The pH and the percentages of sand, silt, and clay were measured for each sample, and the soil texture class was determined from a combination of these percentages. The percentages and classes were compared with site positivity using Spearman rank correlation.

Forest Moisture Index

The classification of forest type was derived from the predominant trees at each site. The number of mature trees (>4 inches in diameter) was counted within a 50-m^2 grid at each site and identified according to species (38). The most

common species were used to classify the forests via a moisture index (38). The sites were divided into five categories: dry, dry/mesic, mesic, wet/mesic, and wet.

Georeferenced Databases

Data Sources

Geographic coordinates of sites were determined by using a Trimble Geoexplorer (Trimble Navigation, Ltd., Sunnyvale, CA) global positioning system (GPS) and exported by using the Trimble Pathfinder software into ARC/INFO and ArcView GIS (ESRI, Redlands, CA). The generated georeferenced database was overlaid on digitized state coverages of environmental data. Land cover and elevation data for Wisconsin were obtained from WISCLAND/GAP (University of Wisconsin and Wisconsin Department of Natural Resources, Madison, WI) at a scale of 1:40,000. WISCLAND/Upper Midwest GAP analysis created land cover classifications based on Landsat Thematic Mapper (TM) data and stratification of the satellite imagery with a hierarchic classification system into wetlands, urban areas, and upland areas. For Illinois, land cover, elevation, and quaternary geologic data were obtained from the Illinois GIS (Department of Natural Resources, 1996, Springfield, IL) at a scale of 1:500,000. Bedrock geology data were obtained from the Digital Geologic Map and Mineral Deposits of Minnesota, Wisconsin, and Michigan (U.S. Geological Survey, Reston, VA) at a scale of 1:1,000,000 for Wisconsin and Michigan, and from the Illinois GIS at a scale of 1:500,000. Soil data, including order, texture, drainage, and quaternary geology, were obtained from STATSGO (U.S. Department of Agriculture, Washington, DC) with a resolution of 2.5 km^2.

Climate data, gathered by the weather station closest to each site, were obtained from the National Oceanographic and Atmospheric Administration (National Climate Data Center, Asheville, NC). Variables included yearly and seasonal precipitation. Landsat TM satellite images were obtained for the entire study area from summer and fall of 1989 through 1993. For each site, average values of TM bands 3 (red), 4 (near-infrared), 5 (mid-infrared), and the normalized difference vegetational index (NDVI) were calculated for the surrounding 3x3 (0.01 km^2), 10x10 (0.1 km^2), and 30x30 (0.9 km^2) pixels. Indices of greenness, brightness, and wetness were obtained through the tasseled-cap transformation (39). Brightness is a measure of reflectance and is correlated to the texture and moisture content of soils, while greenness is a measure of the density of green vegetation present. Wetness is a measure of moisture in soils and vegetation. These remote sensing indices were treated as interval-level data and were associated with tick abundance at each site.

Environmental Variables

Land cover data were grouped into five ordinal categories: agriculture, grasslands, coniferous forest (in which $\geq$75% of trees maintain leaves all year), mixed forest (neither deciduous nor coniferous species make up >75% of land cover), and deciduous forest (at least 75% of trees shed foliage simultaneously in response to seasonal change).

Bedrock geology was classified as Precambrian, which consists of volcanic and metamorphic rocks, and sedimentary deposits from the Silurian, Ordovician, and Devonian eras (40). Quaternary geology information was obtained from the USDA Forest Service North Central Research Station (General Technical Report NC-178). Categories were classified as outwash plains and pitted outwash, lake plain, till plain, ground moraine, loess, and plateau.

Soil orders are defined by amount of organic matter present, pH, and the type of vegetation growing on the soil (40). In Wisconsin and northern Illinois, 8 of 12 soil orders are represented: mollisols (present under prairie), alfisols (deciduous forests), spodosols (coniferous forests), entisols and inceptisols (both of which are associated with poorly developed soils), histosols (peat and muck), and vertisols and paleosols (which represented <1% of the area). These orders were classified into ordinal categories based on increased fertility and decreased acidity: 0 = histosol and spodosol, 1 = entisol, 2 = inceptisol, 3 = mollisol, and 4 = alfisol.

Soil texture (40) was divided into seven groups in order of increasing particle size, ranging from clay (<2 mm) through silt (2 to 50 mm) to sand (0.05 to 2.0 mm). Drainage was divided accordingly into seven categories (STATSGO, Washington, DC), from very poorly drained to well drained. Excessively drained soils were ranked as 0 since they are too dry to support a biotic environment (40).

For each site, yearly and seasonal rainfall averages and average snowfall per year were obtained from the weather station (NOAA) nearest each site. Elevation ranged from 495 m in northern Wisconsin to 197 m in western Illinois. Precipitation, elevation, and remote sensing indices were treated as interval-level data.

Statistical Analysis

All analyses were performed by using SPSS software (SPSS, Chicago, IL). Soil texture classifications of samples from the sites were compared with those listed in STATSGO, the soils database (STATSGO, Washington, DC), and Spearman rank correlation was used to assess correlations between field data and data from the GIS. Univariate analysis was initially performed by using chi square contingency tables to determine significant associations between site positivity and environmental variables coded as previously described. Discriminant analysis was performed by using only the significant ($p<0.25$) environmental variables from the univariate analysis (41). A linear discriminant function was obtained from the combination of variables that best characterized the differences between the groups. A stepwise approach was used to enter variables one at a time until the discriminating power between tick abundance categories ceased to improve.

Analyses were performed by grouping the outcome variables into positive or negative sites and into the four abundance categories described previously.

As mentioned, since a site classified as category 1 (finding only one stage of the tick) could result from introduction into an unsuitable habitat, categories 0 and 1 were combined for additional analysis. Only 112 sites were used in the analysis, with no more than three sites included per natural area where multiple sites were sampled. The resulting classification functions were then used to predict tick abundance categories and assess how well the functions discriminated. Separate discriminant analyses were performed by using the seven indices obtained from the remote sensing data at three spatial scales and the precipitation data.

Logistic regression analysis was performed by using the primary environmental factors as independent variables and the positive and negative sites as outcome variables. Forest moisture index was excluded from the model because this variable was not available as digitized geographic coverage.

To develop a risk map for Lyme disease in the area studied, a grid was created encompassing the states of Wisconsin and Illinois with a resolution of 2.5 km^2 per cell. The grid was overlaid with the selected coverages by using ARC/INFO and ArcView GIS(ESRI, Redlands, CA), and data values corresponding to each layer were assigned to each cell. The Summarize Zones procedure from the ArcView Analysis Menu was used to calculate summary attributes for features by using a grid scheme that divided the entire study area into 2.5-km^2 cells. Each cell was assigned a value for each layer included in the logistic regression based on the most common category. The logistic equation was then used to generate the probability of the presence of *I. scapularis* within each 2.5-km^2 cell of the grid map. The map was generated with probabilities divided into quartiles and deciles.

Results

The locations of the 138 sites that were sampled in Wisconsin, Illinois, and Michigan are shown in Figure 1. Among the four categories, 56 sites were classified as negative, 24 were ranked as 1, 32 as 2, and 26 as 3. Most negative sites were in northeastern Wisconsin. In the southeastern part of Wisconsin, sites were negative except those situated in the Kettle Moraine State Forests (Sheboygan, Fond du Lac, Jefferson, Walworth, and Waukesha counties), which are located on the terminal glacial moraines. Negative sites in Illinois were at Blackhawk Nature Preserve (Rock Island County), located in a suburban area, and White Pines State Park (Ogle County), which has large stands of secondary growth pine forest. In Wisconsin, positive high-density sites were found in the southwestern driftless area and in the central sandy uplands, as well as in the well-recognized northwest part of the state (and across the state line into Minnesota).

In Michigan, where only a small area of the Upper Peninsula was sampled, all sites had very dense tick populations, except for a site that was classified as excessively drained

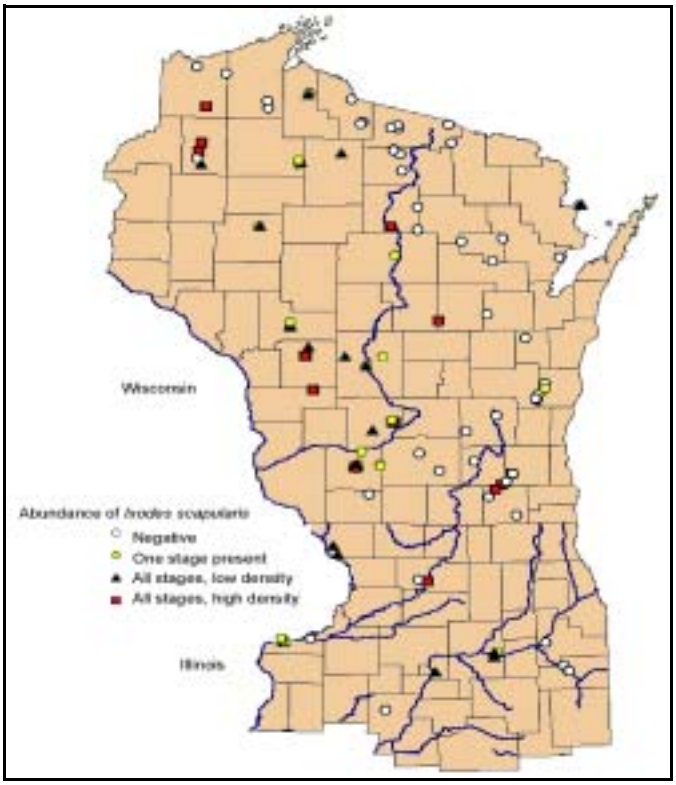

Figure 1. Geographic distribution of study sites ranked by abundance of *Ixodes scapularis* in Wisconsin, northern Illinois, and Menominee County in Michigan.

(>99% sand). The sites classified in the other two abundance categories (1 and 2) did not appear to cluster in any areas. In Illinois, the two parks that have been infested for at least a decade, Castle Rock State Park (Ogle County) and Loud Thunder Forest Preserve (Rock Island County), were classified as having dense tick populations, with lower populations in some sites along the Illinois River.

Particle size analysis, which is a function of the proportions of sand, silt, and clay, was performed at 82 sites (Figure 2). The positive sites were clustered in the sand/loamy sand texture classes. Individual percentages of sand, silt, and clay per sample were not correlated with tick abundance; however, texture class, which is a combination of these three percentages, correlated significantly ($r=0.42$, $p<0.05$) with greater tick densities found in soils with a greater proportion of sand. The soil texture class of samples determined from the soil analysis correlated significantly ($r=0.46$, $p<0.001$) with the soil texture class of each site as obtained from the STATSGO database.

The univariate analysis detected significant associations ($p<0.25$) between tick presence and land cover, soil order, bedrock geology, quaternary geology, soil texture, forest type, spring, summer, fall and winter precipitation, snowfall, and elevation (Figure 3). The results of the discriminant analysis are listed in Table 1. When negative and positive sites were contrasted, the variables forest type, soil order, land cover, soil texture and bedrock were significant. Tick presence was positively associated with deciduous (Figure 3a), dry/mesic and

Table 1. Significant environmental variables to determine favorable habitat for *Ixodes scapularis* using discriminant analysis

	Groups (sample size)						
	0 vs. 1,2,3 (47 vs. 65)		0,1 vs. 2,3 (63 vs. 69)		0 vs. 1 vs. 2 vs. 3 (47 vs. 16 vs. 24 vs. 25)		
Variable	Wilk's lambda	Disc F(x)	Wilk's lambda	Disc F(x)	Wilk's lambda	Disc F(x)1	Disc F(x)2
Forest type	0.784	0.552	0.789	0.789	0.754	0.665	-0.747
Soil order	0.618	0.521	0.699	0.542	0.569	0.633	0.774
Land cover	0.586	0.387					
Soil texture	0.564	0.381					
Bedrock	0.525	0.518					
Eigenvalue	0.904		0.431		0.681		0.045
% correctly classified	85.7		78.6		51.8		78.6
Canonical correlation coefficient	0.689		0.549		0.636		0.207

dry forests (Figure 3b), fertile soils such as alfisols (Figures 3c, 4), sand and loamy/sand soil texture (Figures 2, 3d), and sedimentary bedrock (Figure 3e). There was a negative association with grasslands and conifer forests (Figure 3a), wet and wet/mesic forests (Figure 3b), acidic soils such as spodosols (Figure 3c), clay soil texture (Figure 3d), and Precambrian bedrock (Figure 3e). Elevation was not an important discriminator in the model, nor was Quaternary geology (Figure 3f) important even though sites located on the plateaus and loess-covered areas were all positive. However, the distribution of the sites among the categories of Quaternary deposits was skewed because a large proportion of the state parks were located on terminal glacial moraines. The discriminate model was able to correctly classify 85.7% of the sites. The canonical correlation coefficient was 0.69, and the eigenvalue was close to 1 (0.91), indicative of a strong discriminant function. When the single stage category (1) was included with the negative group, only two variables, forest type and soil order, were significant. Most of the sites (78.6%) were still correctly classified; however, the eigenvalue decreased to 0.43. These same variables were significant when all the groups were considered separately; but the model only correctly classified 51.8% of the sites. Even though only 4/33 in the negative group were misclassified, there was very poor discrimination among the tick positive groups. No significant variables resulted from the discriminant analysis performed using the satellite data. Since all sites were located in forested areas, TM imagery may not have been able to discriminate well among suitable and unsuitable forested habitats. The precipitation variable was also not a significant discriminator between positive and negative sites in the model.

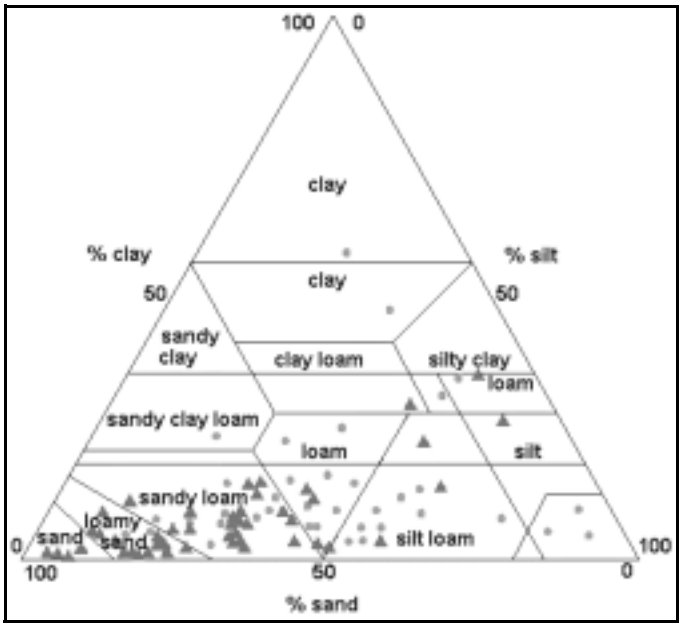

Figure 2. Soil particle size analysis of samples from positive and negative sites. Soil texture is expressed as the sum of percent sand, silt, and clay.

The results of the logistic regression analysis were in agreement with the discriminant analysis model in the positive versus negative group as seen in Table 2. The same variables were significant ($p<0.05$), and the model correctly classified 83.9% of the sites. The predictive risk map generated from the logistic regression model is shown in Figure 5. The higher probabilities indicate increased suitability of habitat for *I. scapularis.* In Wisconsin, the areas of moderate suitability (26%-40%) are located in the western half of the state. Patchy areas of higher probability (60%-100%) are found in the central and northern portion (Juneau, Adams, Waushara, and Marquette counties.) and along the border with Minnesota (Vernon and Crawford counties). In Illinois, the positive sites that were sampled corresponded to areas of increased suitability (60%-100%). Castle Rock State Park, where the highest tick densities are found, had a 90%-100% probability of suitable habitat. The areas bordering the Illinois River appear to be adequate habitat for *I. scapularis*, especially on the western side. Shawnee National Forest in the extreme southern portion of the state also appears to have a high probability (60%-80%), even though *I. scapularis* populations have not been detected (42).

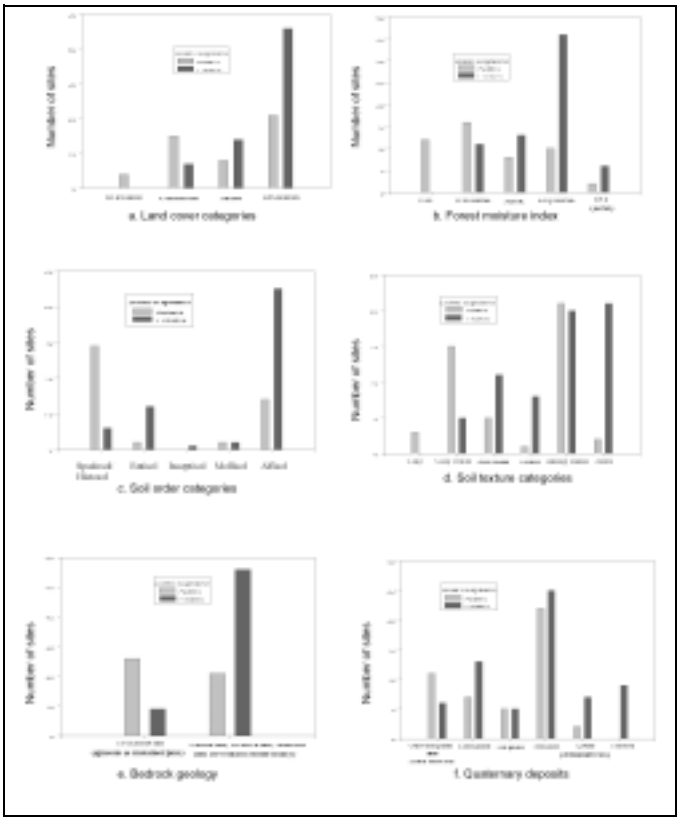

Figure 3. Categories of environmental variables and number of positive and negative sites.

Discussion

Ixodes scapularis may be introduced into new areas by several routes. Adult *I. scapularis* are carried into new areas primarily by deer (43), which are capable of ranging over wide areas, especially along riparian corridors. However, infected adult ticks have limited potential for spreading Lyme disease since transovarial transmission of *B. burgdorferi* is rare. Small mammals are efficient disease reservoirs, and juveniles tend to disperse during the spring and summer when tick larvae and nymphs are questing. However, the potential for long-range dispersal of Lyme disease by rodents is limited, since they occupy much smaller home ranges than deer (44). Birds have a high potential for introducing infected immature stages of *I. scapularis* into distant areas (45-47), especially during spring and fall migration.

To become successfully established in a new area, *I. scapularis* requires available hosts for feeding, which is not a limiting factor in our study area, and a suitable habitat for questing, molting, diapause, and oviposition. The vegetation, soil, topography, and climate are interrelated, and extremes of any one factor may adversely affect the tick's ability to survive.

The environmental characteristics vary throughout the two states, and certain combinations may determine whether introduced *I. scapularis* populations can become established. Tick abundance is an indicator of the suitability of environmental conditions for reproduction and survival. Finding only one stage of the tick may indicate either a poor microenvironment

Table 2. Significant environmental variables in the logistic regression model.

					95% Confidence interval	
Variable	Beta	Standard error	P value	Odds ratio	Lower	Upper
Land cover	0.85	0.40	0.03	2.36	1.08	5.15
Soil order	0.42	0.18	0.02	1.52	1.07	2.16
Bedrock	1.78	0.73	0.01	5.94	1.42	24.78
Soil texture	0.76	0.26	0.004	2.13	1.27	3.57
Constant	-9.06	1.95				

or a recent introduction. Finding all three stages at one site strongly suggests that a population has become established. A less than optimal habitat may account for low density in an established *I. scapularis* population, or it may indicate a recent introduction. Errors in classification may occur in an extensive field survey, as reported here, and a dynamic situation (i.e., the process of invasion of a new site) may mask the occurrence of some positive or potentially positive sites. By including a large number of sites and conducting repeat visits, we have tried to minimize such confounding effects.

Environmental factors such as bedrock geology, quaternary deposits, soils, vegetation, and climate influence each other directly and indirectly to create unique habitats. This is why we included risk factors that are not necessarily independent in a model that is most unbiased. The soil orders in the region (Figure 4) are influenced by the type of underlying bedrock and by quaternary deposits. The soils, in turn, influence the type of vegetation overlying them. Soil texture is the component of soil that influences the extent of drainage. The soil texture classes are independent of soil order and are usually a function of the degree of soil weathering and the parent material (bedrock or quaternary deposit). The tree composition of a forest is determined by a moisture gradient involving soil aeration, soil nutrient supply, and microclimatic features (38), and this gradient functions as a continuum. The forest types classified as dry and dry/mesic have oaks and jack pines as the dominant species that prefer well-drained, sandy soils. Oak forests also have a dense canopy layer that provides protection for the underlying vegetation. Wet and wet/mesic forests are composed of trees that have a high tolerance for very moist soils. The factors interacting at the microclimatic level within the topsoil and leaf litter appear to have an important influence on tick survival. Excessively moist conditions at the soil level were negatively associated with the establishment of *I. scapularis*. Soil texture, in addition to the topography, determines the extent of drainage, and the level of moisture of the ground layer, regardless of the amount of precipitation. However, given the effect of weather on tick abundance (19), associations between tick presence and amount of yearly precipitation or snowfall need to be analyzed further.

Our findings suggest that abiotic factors play a major role in determining whether populations of *I. scapularis* can

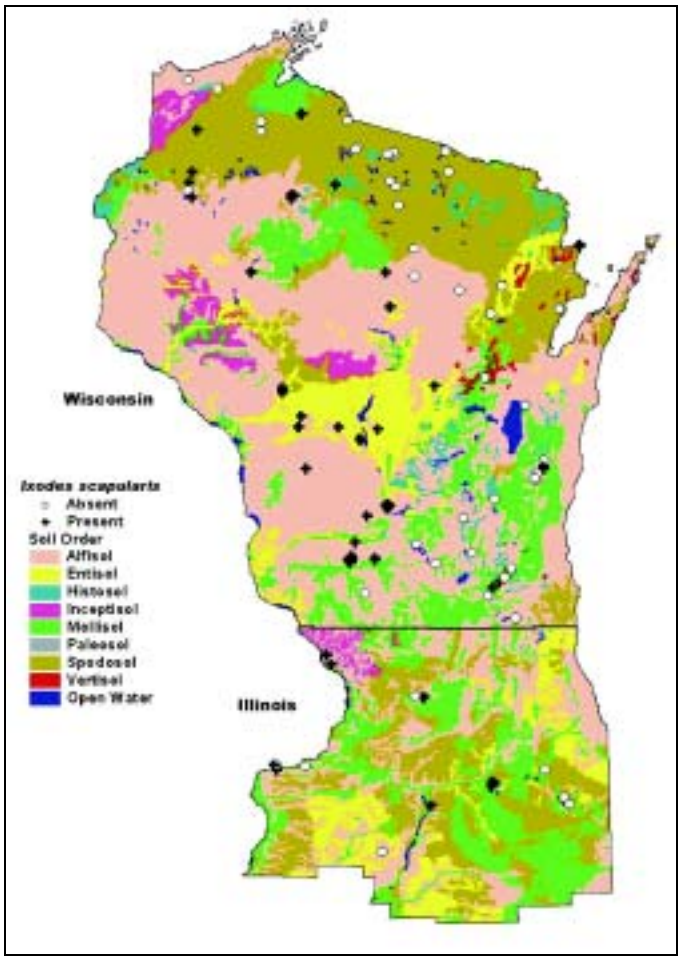

Figure 4. Map of soil orders in Wisconsin and northern Illinois, overlaid with tick study sites.

become established in an environment. Precambrian bedrock of volcanic origin results in the formation of acidic soils that are found mainly under coniferous forests, the forest type least likely to support tick populations. Soils containing increased acidity (spodosols) and a high proportion of clay that can retain excess moisture (48) were also more frequently present in negative sites. Excessive moisture in the soil may be deleterious to tick survival since they overwinter in the topsoil and leaf litter. It may also enhance the growth of organisms, such as fungi and entomophagous nematodes, which may have adverse effects on the tick population (49). Leaf litter is a necessary component for the survival of immature stages of *I. scapularis* (50). However, the type and quantity may determine the densities of ticks in a specific habitat. Tick densities were highest in forests dominated by oak, followed by maple, and lowest in coniferous forests that produce minimal amounts of leaf litter (38). Tick densities were also highest in areas with underlying sedimentary bedrock, which is associated with alfisol and mollisol soil orders and soil textures of increased particle size (38).

The statistically significant risk factors derived from the logistic regression analysis were in agreement with those obtained from the discriminant analysis, and allowed us to quantify and predict the environmental risk for the presence of *I. scapularis*. Several environmental factors must be evaluated simultaneously to assess the combination of factors required for successful establishment. Determining the environmental factors that limit survival can facilitate the development of measures for the control of the tick in the environment.

Using a GIS, we generated a risk map (Figure 5) to predict the presence of the tick vector, *I. scapularis*. The areas of suitable habitat for *I. scapularis* in Wisconsin corresponded to areas of increased incidence of human Lyme disease and known areas of tick endemicity. The extensive area of suitable habitat in the western portion of the state can explain the rapid expansion of the tick from the original northwestern focus to

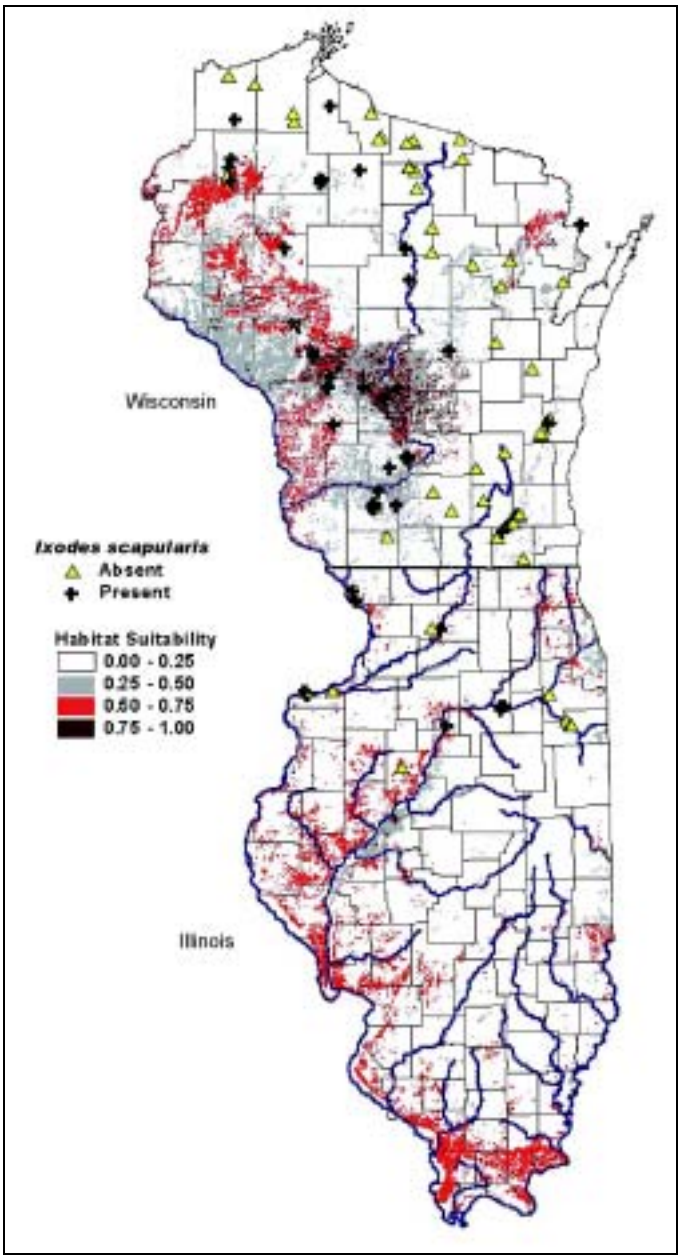

Figure 5: Predictive risk map of habitat suitability for *Ixodes scapularis* in Wisconsin and Illinois.

the southwestern portion of the state (2-5). While initial studies of tick distribution (3) and human granulocytic ehrlichiosis (51) point to the risk of tick-borne disease transmission in Northwest Wisconsin, our study points also to the sandy barrens of Central Wisconsin as most suitable habitats. Indeed, the highest numbers of ticks were collected in Council Ground State Park (Lincoln County), Fort McCoy (Monroe County), Hartman Creek State Park (Waupaca County), and Wildcat Mountain State Park (Vernon County), as well as in sites in the long-recognized Spooner area (Washburn County). Further, the highest prevalence of canine seropositivity to *B. burgdorferi* in northern Illinois and Wisconsin was found in dogs in the west-central counties of Wisconsin (52). Based on the risk map, most of the north-central and northeastern portions of Wisconsin have a <25% probability for tick presence. These are areas where our sampled sites were consistently negative for *I. scapularis*. In the eastern half of the state, the main areas of increased suitability were along the glacial terminal moraines, which is where the positive sites in the Kettle Moraine State Forest were located. There was also a higher probability in the northeastern corner of the state bordering Menominee County, Michigan, where positive sites were located.

In Illinois, areas of increased suitability corresponded to the same areas where the positive sites were located in Ogle, Rock Island, and Jo Daviess counties. The risk map indicated there is adequate habitat for *I. scapularis* populations to become established along the Illinois River, as well as the Mississippi River. However, in Illinois, tick populations may be limited to river corridors since extensive areas are used for agriculture. Where forested habitat is sparse, tick establishment may be restricted, even though geologic and soil factors are favorable. In southern Illinois, where climatic conditions may differ and other reservoir hosts may be present, the inclusion of additional parameters to the model may result in reduced risk probabilities. In contrast, the risk factor model and predictive map may be valid for other north-central areas that have similar environmental characteristics, particularly in parts of Minnesota, Michigan, northern Indiana, and Ohio. The model may be applied to other areas of the United States by using local geographic coverages.

In conclusion, this model can be used to help determine the risk of acquiring Lyme disease and other diseases transmitted by *I. scapularis* by predicting which locations may be currently infested with the tick. It can also be used to assess whether habitats that are currently nonendemic for *I. scapularis* would have the necessary combination of environmental factors to allow new populations of *I. scapularis* to become established. The model can thus be continuously refined based on findings from new areas. The risk of Lyme disease transmission could be predicted in areas capable of sustaining *I. scapularis* populations if ticks harboring *B. burgdorferi* are introduced by migrating deer or birds. The results obtained from these field studies can also form the basis for controlled experimental studies under field and laboratory conditions to further elucidate the preferred microenvironment of *I. scapularis*.

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Molecular Classification of Enteroviruses Not Identified by Neutralization Tests

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We isolated six viruses from patients diagnosed with aseptic meningitis or hand, foot, and mouth disease. The cytopathic effect of these viruses on cultured cells was like that of enteroviruses. However, viral neutralization tests against standard antisera were negative. Phylogenetic analysis with the complete VP4 nucleotide sequences of these 6 viruses and 29 serotypes of enteroviruses classified 3 of the viruses as serotype echovirus type 18 (EV18) and 3 as serotype human enterovirus 71 (HEV71). These results were confirmed by remicroneutralization tests with HEV-monospecific antisera or an additional phylogenetic analysis with the complete VP4 nucleotide sequences. Phylogenetic analysis with complete VP4 genes is more useful than neutralization tests with enterovirus serotype-specific antisera in identifying enterovirus serotypes.

The human enterovirus (HEV) genus of the family *Picornaviridae* includes the human pathogens that cause a wide spectrum of acute disease, including hand, foot, and mouth disease (1), aseptic meningitis (2,3), encephalitis (3-6), and neonatal sepsislike disease (7,8). Sixty-four serotypes of HEV have been recognized antigenically by neutralization tests with anti-HEV antibodies (9). HEVs have long been classified on the basis of serotype-specific antisera in virus neutralization tests (1,10), the only method available for serotyping HEVs. However, virus neutralization is both labor- and time-intensive, and antigenic variants in many serotypes of HEV can affect test results (1).

The HEV genome comprises a 5' nontranslated region (NTR), a long open reading frame that encodes a protein of approximately 2,100 amino acid residues, a short 3' NTR, and a polyadenylated tail. The polyprotein is co- and post-translationally cleaved to yield four structural proteins: VP4, VP2, VP3, and VP1 (1). Recently, attempts have been made to classify the HEV serotypes by using the partial nucleotide sequences of the HEV genomes (i.e., the 5' NTR [11-13], the VP4-VP2 junction [14-16], and VP1 [17-20]). Methods for molecular classification of HEVs should not only identify the serotypes rapidly but also detect antigenic variant strains or new serotypes. A new serotype of HEV has recently been identified by comparing the complete VP1 nucleotide sequences; its proposed name is HEV73 (19).

To investigate the HEV serotypes of six HEV-like viruses that were not neutralized by standard HEV typing sera, we determined the complete VP4 nucleotide sequences of these 6 viruses and 21 HEV antigenically defined serotypes, then performed phylogenetic analysis with another 8 HEV serotypes available from GenBank. The classifications of the untypeable viruses were confirmed by using HEV-monospecific antisera or an additional phylogenetic analysis with the VP4 sequences. The molecular classification of HEV with the complete VP4 sequences is useful for identifying the HEV serotypes.

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Methods

Virus Isolation and the Neutralization Test

The clinical specimens were injected into Vero, RD-18S, or MA104 cells to isolate viruses. All cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and maintained in MEM containing 1% to 2% FBS after being added to 48-well plates (Sumitomo Bakelite, Tokyo, Japan). The cells were incubated for 1 week, after which culture fluids were passaged and incubated for another week. Cultured cells showing cytopathic effects were regarded as virus isolation-positive and, together with the culture supernatant, were harvested and stored at -80°C before use. To serotype the viruses, microneutralization tests were performed with antiserum pools of Lim and Benyesh-Melnick (21) (Denka Seiken, Tokyo, Japan) or in-house monospecific immune sera against coxsackie virus A10 (CAV10), CAV16, and HEV71, respectively.

Viruses

Of the six viruses that could not be identified by the neutralization tests described above (Table 1), strains OC/0071, OC/0073, and OC/00272 were isolated from patients diagnosed with aseptic meningitis by using RD-18S cells. OC/00219, OC/00260, and OC/00261 were isolated from patients diagnosed with hand, foot, and mouth disease or aseptic meningitis by using Vero cells. No sera from these patients was available for analysis. Twenty-one serotypes were isolated and identified in our laboratory during 1995-2000 (Table 2); these strains were used in the experiments. For additional investigations of HEV71, we used eight HEV71 strains isolated and identified in our laboratory (Table 3).

Table 1. Unidentified enterovirus strains and patient information, Osaka, Japan, 2000

Strain	Patient age (years)	Specimen	Date of sampling	Clinical symptoms	Isolated cells
OC/0071	2	Stool	5/11/2000	AM[a]	RD-18S
OC/0073	2[b]	CSF	5/11/2000	AM	RD-18S
OC/00219	0	Throat swab	7/7/2000	HFMD	Vero
OC/00260	0	Throat swab	7/18/2000	HFMD, AM	Vero
OC/00261	0[c]	Stool	7/18/2000	HFMD, AM	Vero
OC/00272	6	Stool	7/18/2000	AM	RD-18S

[a]AM = aseptic meningitis; CSF = cerebrospinal fluid; HFMD = hand, foot, and mouth disease.
[b]Same patient as OC/0071.
[c]Same patient as OC/00260.

Table 2. Characteristics of 21 human enterovirus (HEV) serotypes antigenically defined, Osaka, Japan, 1995–2000

HEV serotype	Strain	Age (years)	Specimen	Date of sampling	Isolated cells
PV1	OC/00417	0	Throat swab	10/13/2000	Vero
PV2	OC/00138	0	Stool	6/10/2000	Vero
PV3	OC/99355	0	Nasal mucus	11/8/1999	Vero
EV3	OC/00467	7	Stool	11/13/2000	RD-18S
EV6	OC/99350	0	Stool	11/8/1999	RD-18S
EV7	OC/96221	7	Throat swab	7/22/1996	MA104
EV9	OC/00129	3	CSF[a]	6/8/2000	RD-18S
EV11	OC/98535	3	Stool	9/23/1998	RD-18S
EV16	OC/95378	1	Throat swab	9/11/1995	MA104
EV18	OC/99-Hanasaka	7	Stool	11/8/1999	RD-18S
EV25	OC/00263	0	Stool	7/17/2000	RD-18S
EV30	OC/97633	1	Stool	9/29/1997	RD-18S
CAV9	OC/96234	4	CSF	8/2/1996	RD-18S
CAV16	OC/00351	NA	Throat swab	8/31/2000	Vero
CBV1	OC/00364	0	CSF	9/6/2000	Vero
CBV2	OC/99284	0	Stool	9/11/1999	RD-18S
CBV3	OC/97620	6	CSF	9/19/1997	RD-18S
CBV4	OC/00362	1	Stool	9/8/2000	Vero
CBV5	OC/00223	0	Throat swab	7/7/2000	Vero
CBV6	OC/00325	0	CSF	8/8/2000	Vero
HEV71	OC/00168	2	Throat swab	6/21/2000	Vero

[a] CSF = Cerebrospinal fluid; NA = not available.

RNA Extraction and Reverse Transcription

Viral RNAs were extracted from the cell-culture supernatants by using ISOGEN-LS (Nippon Gene, Tokyo, Japan). cDNAs were synthesized with an Omniscript Reverse Transcriptase Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. The primers used for the synthesis were EVP-2 (5'-CCTCCGGCCCCTGAATGCGGCTAAT-3' relative to nt 444-468 in the genome of Poliovirus (PV) Sabin 1 strain) (22) and OL68-1 (5'-GGTAAYTTCCACCAC-CANCC-3' relative to nt 1178-1197 of Sabin 1), as described (23).

Polymerase Chain Reaction Amplification of cDNAs

Polymerase chain reaction (PCR) was performed by using 2 µL of each cDNA in a 50-µL reaction mixture containing 1.5 U of Taq DNA polymerase (Takara Shuzo, Shiga, Japan), 20 pmol of EVP-2 primer, and 20 pmol of OL68-1 primer. Each reaction was incubated in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) according to the following protocol: 5 minutes at 95°C, 40 cycles of 95°C for 30 seconds, 68°C for 30 seconds, 72°C for 1 minute, and then at 72°C for 5 minutes. After the appearance of approximately 750 bp-specific amplified fragments was confirmed by agarose gel electrophoresis, the amplicons were purified with a QIAquick PCR purification kit (QIAGEN).

DNA Sequence Analysis

Approximately 100 ng of purified amplicon was used in the reaction with the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems), and DNA sequencing was performed by using an ABI PRISM 310 DNA sequencer (Applied Biosystems). All DNA sequencings were performed on both strands using EVP-4 (5'-CTACTTTGGGT-GTCCGTGTT-3' relative to nt 541-560 in the genome of PV Sabin 1 strain) as the forward primer and OL68-1 as the reverse primer (23). Sequencer software (version 3.0; Hitachi Software, Tokyo, Japan) was used to determine the approximately 600-bp nucleotide sequence spanning 5' NTR to one third of VP2 (including all of VP4), translate nucleotide sequence to amino acid sequence, and decide the complete VP4 coding sequence of each virus.

Phylogenetic Analysis

A phylogenetic tree based on the complete VP4 nucleotide sequence was constructed by the neighbor-joining method (24) as implemented with the CLUSTAL X program (version 1.63b, December 1997; http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/). The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. The complete VP4 sequences of eight HEV serotypes not isolated in our laboratory were obtained from GenBank and included in the HEV analysis. Complete VP4 nucleotide sequences of another 18 HEV71 strains were obtained from GenBank and used in the phylogenetic analysis.

Remicroneutralization Tests

According to results of HEV phylogenetic analysis with the complete VP4 nucleotide sequences, remicroneutralization tests using monospecific antiserum against echovirus 18 (EV18; Denka Seiken), or HEV71 (anti-HEV71/BrCr and

Table 3. Characteristics of eight human enterovirus 71 strains, as antigenically defined, Osaka, Japan, 1996-2000

Strain	Age(years)	Specimen	Date of sampling	Clinical symptoms	Isolated cells
OC/9632	NA[a]	Stool	4/11/1996	HFMD	MA104
OC/99-Ikeda	6	Stool	9/11/1999	HFMD, AM	Vero
OC/0078	1	Throat swab	5/18/2000	HFMD, AM	Vero
OC/0080	NA	Stool	5/9/2000	Diarrhea	Vero
OC/0096	5	CSF	5/25/2000	Diarrhea, AM	Vero
OC/00114	0	CSF	5/31/2000	Fever	Vero
OC/00125	6	Throat swab	6/7/2000	Encephalitis	Vero
OC/00168	4	Throat swab	6/21/2000	Herpangina	Vero

[a] NA = Not available; HFMD = Hand, foot, and mouth disease; AM = aseptic meningitis; CSF = cerebrospinal fluid.

anti-HEV71/C7 sera; both supplied by the National Institute of Infectious Diseases, Japan) were performed to confirm the serotype of the untypeable strains from the first microneutralization assay.

Complete VP1 Nucleotide Compared with Deduced Amino Acid Sequences of HEV71 Strains

The complete VP1 nucleotide sequences of HEV71 strains OC/00168, 0C/00219, OC/00260, and OC/00261 were determined by the same procedure described above, except for the primers. The primers used for the analysis of VP1 nucleotide sequence were 71F2399 (5'-AGAAYTTYACCATGAAACTG-3' relative to nt 2380-2399 in the genome of HEV71 MS/7423/87 strain [25]; the nucleotide positions of the following are also relative to this strain: 71F2793 (5'-AGACATAACTG-GYTACGCCAC-3' nt 2774-2793) and 71F3042 (5'-CATGT-CACCYGCGAGCGCTT-3' nt 3023-3042) as the forward, 71R2712 (5'-CTACCAARCCTGCCCTACTG-3' nt 2693-2712), 71R3066 (5'-GGTACCCGTCGTAAAACCAC-3' nt 3047-3066) and 71R3376 (5'-AAGTTGCCCACGTAGAT-GGC-3' nt 3357-3376) as the reverse. The VP1 nucleotide sequence of HEV71 BrCr strain (25) was obtained from GenBank. Sequencer software (version 3.0; Hitachi Software) was used for determination and comparison of the complete VP1 nucleotide and deduced amino acid sequences of these HEV71 strains.

Results

Determination of Complete VP4 Nucleotide Sequences of HEVs

During May to July 2000, six viruses isolated in our laboratory (OC/0071, OC/0073, OC/00219, OC/00260, OC/00261, and OC/00272) could not be neutralized by standard pools of HEV typing sera and three antimonospecific sera (Table 1). However, the cytopathic effects of these viruses on RD-18S or Vero cells were all HEV-like (data not shown). To identify the serotypes of these untypeable HEV-like viruses by a method other than the neutralization assay, we determined the complete VP4 nucleotide sequences of all 6 strains and another 21 HEV serotypes identified in our laboratory over the past 6 years. The 3' end of the VP4 gene of each virus was determined from the deduced amino acid sequences as described (26,27). The complete VP4 nucleotide sequences of all HEV strains used in this study were 207 nt long, and the deduced amino acid sequences of all VP4 proteins were 69 amino acids long (data not shown).

Phylogenetic Analysis of HEVs

A phylogenetic tree was constructed based on the complete VP4 nucleotide sequences of the 6 HEV-like untypeable strains, the 21 HEV serotypes identified in our laboratory as prototype strains, and another 8 HEV serotypes available from the GenBank database (Figure 1). The 29 different HEV serotypes defined antigenically were clustered in four distinct lineages, as described (23). Three of the six untypeable strains (OC/0071, OC/0073, and OC/00272) were classified nearest to EV18. The VP4 nucleotide sequences of strains OC/0071 and OC/0073 were identical. The VP4 gene sequence of OC/00272 was the same as that of OC/99-Hanasaka, which was used as a prototype strain for EV18. The nucleotide sequences of these two clusters differed by 5 nt, but the deduced amino acid sequences were the same (data not shown). The other three untypeable strains (OC/00219, OC/00260, and OC/00261) were classified nearest to HEV71. The VP4 sequence of OC/00219 was the same as that of OC/00168, which was used as a prototype strain for HEV71. The VP4 nucleotide sequences of OC/00260 and OC/00261 were identical. The difference between these two clusters was 11 nt. The deduced amino acid sequences were the same (data not shown).

Remicroneutralization Tests

According to the results of the phylogenetic analysis based on the complete VP4 nucleotide sequences, remicroneutralization tests were performed. Microneutralization tests using the monospecific immune serum for EV18 were done against OC/0071, OC/0073, and OC/00272, and this serum neutralized these viruses. The same tests, using the two species of monospecific immune serum, anti-HEV71/BrCr and anti-HEV71/C7, were performed against OC/00219, OC/00260, and OC/00261, but neither serum neutralized the viruses (Table 4).

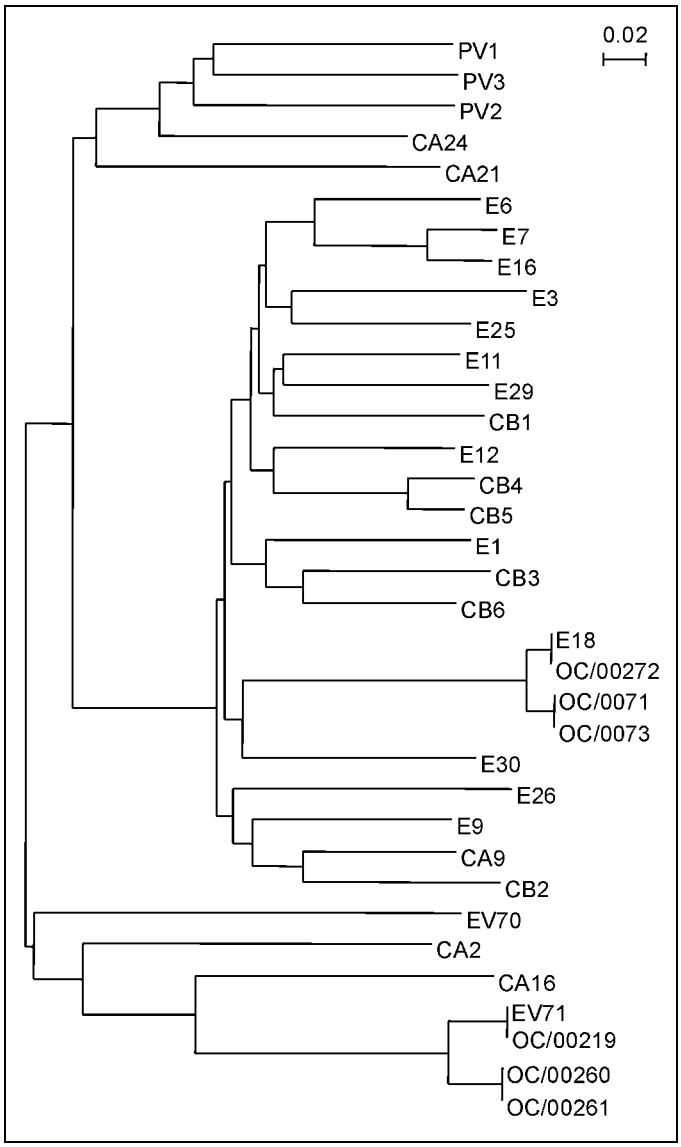

Figure 1. Phylogenetic analysis based on the human enterovirus (HEV) VP4 nucleotide sequences. The phylogenetic tree was constructed by the neighbor-joining method as implemented in CLUSTAL X program (version 1.63b). The marker denotes a measurement of the relative phylogenetic distance. The VP4 sequences of eight HEV serotypes described below are available from GenBank. The strain name and accession number are shown in parentheses: HEV1 (Bryson, AF250874), HEV12 (Travis, NC 001810), echovirus 26 (EV26, Coronel, AF117697), EV29 (JV-10, AF117698), coxsackie virus A2 (CAV2; Epsom/14448/99, AJ2296215), CAV21 (Coe, NC 001428), CAV24 (EH24/70, D90457), and HEV70 (J670/71, D00820).

Phylogenetic Analysis of HEV71 Strains

To establish whether OC/00219, OC/00260, and OC/00261 belong to HEV71, we used another phylogenetic analysis based on the complete VP4 nucleotide sequences of various HEV71 strains (Figure 2). In this analysis, we examined eight HEV71 strains isolated and identified in our laboratory from 1996 to 2000 (Table 3). All these HEV71 strains except for OC/9632 were identified by microneutralization tests with anti HEV71/BrCr serum (data not shown). Of the HEV71 strains available from GenBank, two were isolated in the United States in 1970 and 1987, respectively (25), four in Malaysia in 1997 (15), one in Singapore in 1998 (28), eight in Taiwan in 1998 (15,28), two in the United Kingdom in 1999, and one in China (year unknown). The HEV71 strains were clustered in three distinct genotypes, designated A, B, and C. The genotype nomenclature of HEV71 strains for phylogenetic analyses based on the VP1 (17,25,28) and VP4 (29) nucleotide sequences has been reported, and the results (Figure 2) were consistent with previous findings. Among the HEV71 strains that were identified in our laboratory, only OC/99-Ikeda was classified in genotype C. Seven of eight strains identified in our laboratory by neutralization tests were classified in genotype B; five of these had the same VP4 nucleotide sequence. OC/00219, OC/00260, and OC/00261 were also classified in this genotype. This result demonstrated that strains OC/00219, OC/00260, and OC/00261 were HEV71 serotypes.

Comparison of the Complete VP1 Nucleotide and Deduced Amino Acid Sequences of HEV71 Strains

Strains OC/00219, OC/00260, and OC/00261 were classified in HEV71 by the phylogenetic analysis, although these viruses were not neutralized by monospecific anti-HEV71 sera. Because the VP1 protein contains a number of important neutralization sites (1,30), we determined the complete VP1 nucleotide sequences and compared the deduced amino acid sequences of OC/00219, OC/00260, and OC/00261. OC/00168 used as a prototype strain for HEV71 was also analyzed because this strain was neutralized by anti-HEV71/BrCr serum; moreover, its VP4 gene was the same as that of OC/00219 (Figure 2). The complete VP1 nucleotide sequences of these strains were 891 nt long, and the deduced amino acid sequences were 297 amino acids long. The differences of VP1 nucleotide sequences were 4 to 42 nt (0.4% to 4.7%), and the difference of deduced amino acid sequences was one amino acid (0.3%) among these viruses. The differences of VP1 nucleotide sequences between OC/00168 and OC/00219 were 4 nt (0.4%), and the deduced VP1 amino acid sequences of these strains were the same. The VP1 nucleotide and deduced amino acid sequences of OC/00260 and OC/00261 were

Table 4. Results of re-microneutralization tests with human enterovirus (HEV) monospecific antiserum, Osaka, Japan, 2000

			HEV monospecific antiserum		
Strain	Isolated cells	Predicted HEV Serotype[a]	Anti-EV18	Anti-HEV71/BrCr	Anti-HEV71/C7
OC/0071	RD-18S	EV18[b]	+	ND	ND
OC/0073	RD-18S	EV18	+	ND	ND
OC/00219	Vero	HEV71	ND	--	--
OC/00260	Vero	HEV71	ND	--	--
OC/00261	Vero	HEV71	ND	--	--
OC/00272	RD-18S	EV18	+	ND	ND

[a]Serotypes were predicted from the phylogenetic analysis in Figure 1.
[b] EV18 = echovirus 18; ND = Test not done.

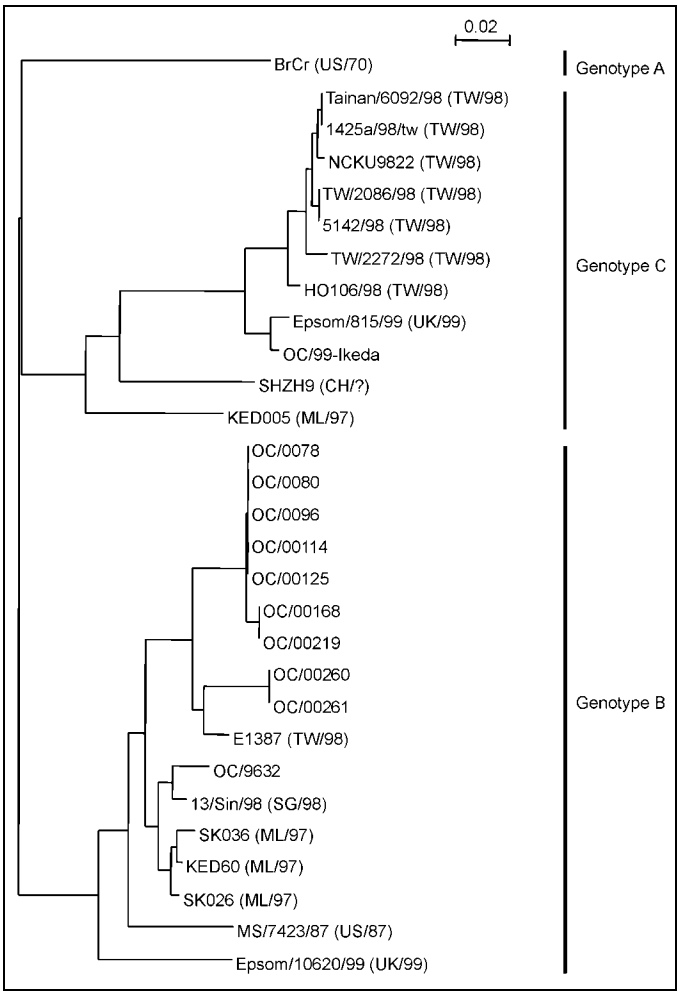

Figure 2. Phylogenetic analysis based on the human enterovirus 71 (HEV71) VP4 nucleotide sequences. The phylogenetic tree was constructed as described in the Figure 1 legend. The genotypes of the HEV71 cluster are denoted on the right. The VP4 sequences of 18 HEV71 strains available from GenBank are denoted by strain name, followed by the country and year isolated. Abbreviations for countries are as follows: US, United States; ML, Malaysia; CH, China; UK, United Kingdom; TW, Taiwan; and SG, Singapore. The accession numbers are as follows: BrCr; U22521, Tainan/6092/98; AF304459, 1425a/98/tw; AF176044, NCKU9822; AF136379, TW/2086/98; AF119796, 5142/98; AB037251, TW/2272/98; AF119795, HO106/98; AB037252, Epsom/815/99; AJ296213, SHZH9; AF302996, KED005; AB051334, E1387; AB051313, 13/Sin/98; AF251358, SK036; AB051333, KED60; AB051335, SK026; AB051332, MS/7423/87; U22522, Epsom/10620/99; AJ296214.

identical (data not shown). The deduced VP1 amino acid sequences of these four strains were compared with that of BrCr (Table 5). There were 18 amino acid (6%) differences between BrCr and other strains. The different amino acid positions of strains OC/00168, OC/00219, OC/00260, and OC/00261 against BrCr were the same. Any mutated residues distinguishable BrCr and OC/00168 from OC/00219, OC/00260, and OC/00261 were not recognized in the VP1 amino acid sequences.

Discussion

The serotype identification of HEVs has been performed by microneutralization tests using standard HEV antiserum pools (1,10). Since >60 serotypes of HEV are known to infect humans (1,19), the HEV serotype is almost impossible to identify by using monospecific antiserum from the first microneutralization test. Furthermore, the neutralization test is labor-intensive and time-consuming, requiring several weeks. As an alternative, identification based on nucleotide sequences has been used successfully in several laboratories (15,19,20, 23,29,31-35). To investigate the serotypes of the six untypeable HEV-like viruses that were not neutralized by the standard HEV antisera, we used phylogenetic analyses based on the complete VP4 nucleotide sequences of HEVs and were able to determine the serotype of each virus in the light of these results. OC/0071, OC/0073, and OC/00272 were thought to be EV18 strains by the phylogenetic analysis (Figure 1) and were neutralized by the monospecific anti-EV18 serum. These results indicate that the phylogenetic analysis based on the VP4 nucleotide sequence is consistent with the result of the microneutralization tests using the serotype-specific sera. OC/00219, OC/00260, and OC/00261 were thought to be HEV71 strains by the same analysis (Figure 1), but these viruses were not neutralized by the two monospecific anti-HEV71 sera. The phylogenetic analysis based on the HEV71 VP4 sequences confirmed that these viruses were HEV71 strains belonging to genotype B (Figure 2). We considered that OC/00219, OC/00260, and OC/00261 were all HEV71 strains not neutralized

Table 5. Differences in deduced VP1 amino acid sequences (aa 1-297) of human enterovirus 71 strains BrCr, OC/00168, OC/00219, OC/00260, and OC/00261

Amino acid position	Strain				
	BrCr[a]	OC/00168	OC/00219	OC/00260	OC/00261
18	Lys	Arg	Arg	Arg	Arg
22	Pro	Gln	Gln	Gln	Gln
30	Pro	Gln	Gln	Gln	Gln
31	Asp	Asn	Asn	Asn	Asn
43	Lys	Glu	Glu	Glu	Glu
58	Ala	Thr	Thr	Thr	Thr
98	Lys	Glu	Glu	Glu	Glu
145	Arg	Glu	Glu	Glu	Glu
164	Asp	Glu	Glu	Glu	Glu
167	Asp	Glu	Glu	Glu	Glu
172	Pro	Gln	Gln	Gln	Gln
183	Ser	Leu	Leu	Leu	Leu
184	Ser	Thr	Thr	Thr	Thr
244	Glu	Lys	Lys	Lys	Lys
246	Ser	Pro	Pro	Pro	Pro
249	Ile	Val	Val	Val	Val
275	Ser	Ala	Ala	Ala	Ala
282	Asp	Asn	Asn	Ser	Ser

[a]VP1 nucleotide sequence was obtained from GenBank (accession no. U22521) and translated into the deduced amino acid sequence.

by anti- HEV71/BrCr and anti-HEV71/C7 sera, both available as standard monospecific anti HEV71 serum in Japan. These results also indicate that phylogenetic analysis with the VP4 sequences of HEVs can identify the serotypes in the same way as neutralization tests with HEV serotype-specific antisera. We are now preparing antiimmune sera against OC/00219, OC/00260, and OC/00261, respectively, to confirm antigenically that these are the prime strains of HEV71 neutralized by anti-HEV71/BrCr serum.

Oberste et al. have shown that HEV VP1 nucleotide sequences correlate with antigenically defined serotypes and have demonstrated the utility of VP1 sequences as a molecular surrogate for antigenic type (19,35). They have also shown that the VP1 sequences have a better correlation with HEV serotypes than the 5' NTR or the VP4-VP2 junction (36). The phylogenetic analysis based on the VP4 sequences we have described also correlates well with HEV serotypings by anti-immune sera. We used 21 HEV serotypes antigenically defined in our laboratory and another 8 strains available from GenBank as prototype strains in this analysis. We do not know whether 29 serotypes are sufficient for the phylogenetic analysis of HEV, as there are >60 serotypes. The good result of HEV phylogenetic classification based on the VP4 sequences might depend on the prototype numbers (29 of 64 serotypes) that we used. Ishiko et al., who performed HEV phylogenetic analyses based on VP4 sequences (23), used 45 HEV serotypes as prototype strains and obtained a phylogenetic tree similar to ours (Figure 1) except for a difference in the prototype strain numbers. Another phylogenetic analysis based on the VP4 sequences in this article was performed against the HEV71 strains (Figure 2). For this analysis, the HEV71 strains were clustered in three distinct genotypes, and the nomenclature was almost the same as for the HEV71 analyses based on the VP1 nucleotide sequences (17,25). Recently, Chu et al. also reported the appropriateness of the phylogenetic analysis with the VP4 sequences for the molecular epidemiology of HEV71 outbreak in Taiwan in 1998 (29). These results suggest that the phylogenetic analysis based on the VP4 nucleotide sequences is also useful as a molecular surrogate for antigenic HEV serotyping. The analysis was more convenient based on the VP4 sequences than the VP1 sequences, since the complete VP4 sequence is 207 nt and the complete VP1 sequences are 834 to 951 nt (35), although the 3' third of the VP1 sequence of 365 nt was used (32).

The VP4 nucleotide sequences of OC/99-Hanasaka and OC/00272 were identical, but the results of neutralization assays were different. OC/99-Hanasaka was easily neutralized by HEV pooled sera against EV18, but OC/00272 was not. The same results were observed for strains OC/00168 and OC/00219 HEV71, i.e., the results of their neutralization tests differed in spite of the VP4 sequence identity. These results indicate that the VP4 nucleotide sequences are highly conserved even though the neutralizable epitopes are antigenic variants. We compared the VP4 nucleotide and deduced amino acid differences of HEV71 strains, BrCr (25), E1387 (15), OC/9632, OC/99-Ikeda, OC/0078, OC/00219, and OC/00260. HEV71 genotypes indicated 1 to 37 nt (0.5% to 17.9%) differences. However, we found no amino acid differences (100% identity) (Table 6). Complete homology of the HEV71 VP4-deduced amino acid sequences has also been described (20,29), and Singh et al. demonstrated amino acid substitutions in the VP2 and VP3 regions, with the greatest variation in VP1 (20). These results indicate that VP4 is the most stable protein; accordingly, VP4 genes will be suitable for the molecular identification of HEV serotypes in the future.

VP4 is not exposed on the outer surface of the capsid, and no neutralizable epitopes appear to exist in VP4. On the other hand, VP1, VP2, and VP3 are outer capsid proteins and contain neutralizable epitopes (37,38). A number of important neutralization epitopes may exist on VP1 (1,30,39). To confirm the important neutralization sites on VP1, we compared the deduced VP1 amino acid sequences of HEV71 strains OC/00168, OC/00219, OC/00260, and OC/00261. OC/00168 was neutralized by anti-HEV71/BrCr serum, while OC/00219, OC/00260, and OC/00261 were not. Comparison of the deduced VP1 amino acid sequences showed that no mutated residues on the VP1 region corresponded to the result of the neutralization tests. This result indicates that either the important neutralization epiotopes for anti-HEV71/BrCr serum do not exist on the VP1 protein, or the epitopes are specifically masked in the cases of OC/00219, OC/00260, and OC/00261. Further analysis against the VP2 and VP3 regions of these strains should allow interpretation of these findings.

Table 6. Number of nucleotide and deduced amino acid differences between the VP4 genes of human enterovirus 71 strains,[a]

	BrCr	E1387	OC/9632	OC/0078	OC/00219	OC/00260	OC/99-Ikeda
BrCr		33	37	36	37	32	34
E1387	0		10	7	8	7	33
OC/9632	0	0		13	14	15	37
OC/0078	0	0	0		1	10	37
OC/00219	0	0	0	0		11	36
OC/00260	0	0	0	0	0		34
OC/99-Ikeda	0	0	0	0	0	0	

[a]Nucleotide numbers are given above the diagonal and amino acid numbers below it.

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Listeria monocytogenes Infection in Israel and Review of Cases Worldwide

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Listeria monocytogenes, an uncommon foodborne pathogen, is increasingly recognized as a cause of life-threatening disease. A marked increase in reported cases of listeriosis during 1998 motivated a retrospective nationwide survey of the infection in Israel. From 1995 to 1999, 161 cases were identified; 70 (43%) were perinatal infections, with a fetal mortality rate of 45%. Most (74%) of the 91 nonperinatal infections involved immunocompromised patients with malignancies, chronic liver disease, chronic renal failure, or diabetes mellitus. The common clinical syndromes in these patients were primary bacteremia (47%) and meningitis (28%). The crude case-fatality rate in this group was 38%, with a higher death rate in immunocompromised patients.

Listeria monocytogenes (Lm) is a ubiquitous pathogen in the environment, capable of causing human and animal infection. Although uncommon in humans, it occurs in sporadic and epidemic forms throughout the world (1-3); a recent multistate outbreak was reported in the United States (4). Most and perhaps all forms of listeriosis in humans result from foodborne transmission (5). In its most severe form, listeriosis is an invasive disease that affects immunocompromised patients and has the highest case-fatality rate of foodborne illnesses (6-10). In immunocompetent persons, it can also cause severe disease (attributed by some investigators to ingestion of high infective doses), as well as outbreaks of benign febrile gastroenteritis (11). Another form of human disease is perinatal infection, which is associated with a high rate of fetal loss (including full-term stillbirths) and serious neonatal disease (12).

Lm infection has been a reportable disease in Israel since 1993. A preliminary report from the Ministry of Health (MOH) claimed a fivefold increase in incidence from 1996 to 1998, but the information was incomplete (13). Our study was undertaken to delineate trends and better characterize the epidemiologic and clinical features of this emerging infection in Israel and to compare these findings with those reported in recent publications worldwide.

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Material and Methods

The Israeli Survey

Of the 24 general (acute-care) hospitals in Israel, 11 are large, with 500-1,200 beds, 8 have 300-499 beds, and 5 have <300 beds. Information on Lm infections was collected by contacting infectious disease specialists in each of the 19 larger hospitals. The specialists were asked to identify retrospectively all patients with listeriosis (as defined below) from the period 1995-1999 in their hospitals and to complete a questionnaire on each. Questionnaires were completed from 17 of the hospitals (11 large, 6 intermediate), and complementary information was retrieved from the MOH passive and active surveillance files on 4 additional hospitals (1 intermediate and 3 small). These 21 hospitals represented approximately 95% of the total acute-care beds in Israel during the study period.

One hundred sixty-one patients with Lm infection were identified. Clinical information was available for all patients except five (3%: two with positive blood cultures and one with a positive vaginal culture who were not hospitalized, and two with positive blood cultures whose hospital charts could not be retrieved).

Lm isolates were identified by standard methods in the microbiology laboratory in each medical center, then sent to the Reference Laboratory for Listeria in Jerusalem for confirmation.

Listeriosis was defined as the growth of Lm (as confirmed at the reference laboratory) from any body site. An infection in a pregnant woman and her fetus or neonate was considered a single perinatal event.

[1]Dr. Lang died November 18, 2001.

Worldwide Review

We conducted a MEDLINE search for studies describing nonselective, population-based surveys of Lm infections in the English language literature of the last decade (1990-2000). All case series describing at least 15 nonperinatal, nongastroenteritis infections were included in the review of nonperinatal listeriosis. All case series describing at least 15 perinatal cases were included in the review of perinatal listeriosis.

Results

The Israeli Survey (1995-1999)

The 161 cases identified during the 5-year study period included 91 (57%) nonperinatal and 70 (43%) perinatal infections. The average annual incidence during the study period was 0.6/100,000 population. The marked increase in 1998 (Figure 1) was exclusively in perinatal cases; the reason for the increase remains unclear. There were no clusters in place during any of these years. Infection occurred throughout the year, but more often during summer and fall, with 70% of cases occurring from May to October (Figure 2).

Nonperinatal Cases

The mean age of the 87 nonperinatal cases with available clinical information was 67 years (range 4-91), 66 (76%) were ≥60 years of age (Figure 3); 56 (64%) were male. Sixty-four patients (74%) had severe immunocompromising conditions (Table 1). Of 45 patients (52%) with malignant disease, most had received chemotherapy, steroid therapy, or both during the month before the Lm infection. Other immunocompromising conditions were chronic renal failure (11 patients, 4 of whom were on hemodialysis), chronic liver disease (10 patients, mostly with cirrhosis), and diabetes mellitus (13 patients). Some of these patients had additional immunocompromising conditions (Table 1). Twenty-three patients (26%) were not immunocompromised. Most (19 [83%] of 23) were ≥63 years of age; some had concomitant conditions not considered to be immunocompromising, including three patients with valvular heart disease, predisposing them to endocarditis. Only four immunocompetent patients were <60 years of age, including a 4-year-old girl and a 38-year-old man with primary bacteremia, a 22-year-old woman with typical pyelonephritis and Lm cultured from blood only, and a 51-year-old man who had gastroenteritis and positive blood cultures (stool was not cultured for Lm).

Clinical syndromes in the 87 nonperinatal cases were primary bacteremia in 41 (47%), meningitis in 24 (28%), bacteremia with a focal infection in 18 (21%), and focal infection without bacteremia in 4 (5%) (Table 2). Six patients with meningitis had Lm isolated from both blood and cerebrospinal fluid (CSF). Two patients with primary bacteremia had symptoms suggestive of meningitis (confusion, aggressiveness), but they died shortly after admission without having a lumbar puncture performed.

The case-fatality rate in the nonperinatal group was 38% (33 of 87). Twelve of the 33 deaths occurred within 48 hours of admission or disease onset. We observed a lower mortality rate (6 [19%] of 31) among persons who received a penicillin (mostly ampicillin) as empiric therapy, compared with those who received a penicillin only after culture results were reported (9 [30%] of 30), but this difference was not statistically significant (p=0.25). The difference in death rates in immunocompromised (28 [44%] of 64) compared with immu-

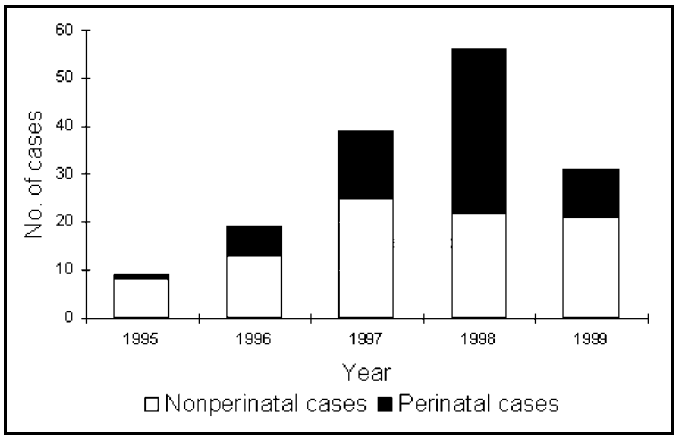

Figure 1. Number of cases of perinatal and nonperinatal *Listeria monocytogenes* infection, Israel, 1995–1999.

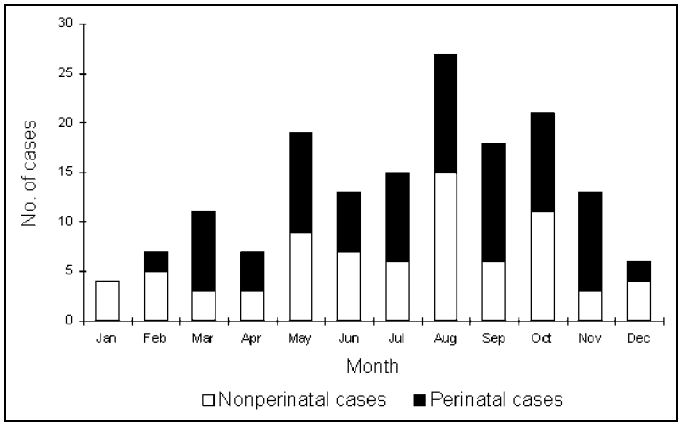

Figure 2. Seasonal occurrence of *Listeria monocytogenes* infection, Israel, 1995–1999.

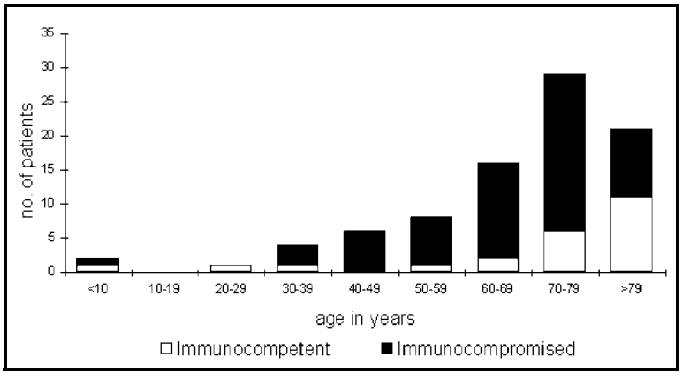

Figure 3. Age distribution of 87 nonperinatal cases of *Listeria monocytogenes* infection by immune-status group, Israel, 1995–1999.

Table 1. Immunocompromising conditions in 64 cases of non-perinatal *Listeria monocytogenes* infection, Israel, 1995–1999

		Additional underlying conditions				
Main underlying illness	No. of cases	Steroids/ chemotherapy	Chronic renal failure	Chronic liver disease	Diabetes mellitus	Others
Hematologic malignancy	23	19	3	2	7	8[a]
Solid malignancy	22	9				
Chronic renal failure	8[b]	1			2	1[c]
Chronic liver disease	8	1			1	2[c]
Diabetes mellitus	3	1				

[a]Splenectomy (2 cases) neutropenia (5) vasculitis (1).
[b]Hemodialysis (4 cases).
[c]Severe congestive heart failure (3 cases).

nocompetent patients (5 [22%] of 23) had borderline statistical significance (p=0.05). There was no correlation between death and age for the whole group; however, all five immunocompetent patients who died were >80 years of age.

Perinatal Cases

Clinical information was available on 69 pregnant women (mean age 28 years; range 21-40 years) and their offspring. Twenty-seven pregnancies (gestational age 9-26 weeks) resulted in intrauterine fetal death and miscarriage, one full-term infant was stillborn, and three others (born at 26, 29, and 39 weeks) died within 24-48 hours of birth (Table 3), for a mortality rate among offspring of 45%. Seventeen (55%) of the 31 infected mothers whose offspring died were bacteremic. For the other 14 mothers, Lm was isolated from other sites, including placenta, amniotic fluid, and fetal tissue (Table 3).

The other 38 mothers gave birth to live infants, 16 of whom had no evidence of Lm infection. Eleven of the 16 uninfected infants were delivered when the mothers had active Lm amnionitis (gestational ages 25 to 40 weeks), and 5 were delivered several weeks after the maternal infection, which occurred at weeks 19, 21, 35, 36, and 37. Twenty-two infants had evidence of Lm infection after birth, 18 within 48 hours of delivery and 4 on days 4-8. Only two (11%) of the 18 infants with early infection had meningitis, compared with all 4 with later onset of infection.

All infected mothers had mild illness and recovered uneventfully; none had meningitis. One mother had an underlying immunocompromising condition (systemic lupus erythematosus).

Table 2. Clinical syndromes in 87 cases of nonperinatal *Listeria monocytogenes* infection, Israel, 1995–99

Clinical syndrome	Immuno-compromised	Immuno-competent	Total
Bacteremia without focus	34 (53%)	7 (30%)	41 (47%)
Meningitis	17[a] (27%)	7 (30%)	24 (28%)
Bacteremia with focus	9[b] (14%)	9[c] (39%)	18 (21%)
A focus without bacteremia	4[d] (6%)	0	4 (5%)
Total	64 (100%)	23 (100%)	87 (100%)

[a] With bacteremia (6 cases), with pneumonia (1 case).
[b] Endocarditis (3 cases), peritonitis (2), pneumonia (4, one with shunt infection).
[c] Endocarditis (3 cases), gastroenteritis (3), pyelonephritis (2), anal abscess (1).
[d] Peritonitis (3 cases), pleuritis (1).

Worldwide Review (1990-2000)

Nine case-series of nonperinatal listeriosis and five case-series of perinatal infection matched the inclusion criteria. These reports and our study provided 1,250 cases of nonperinatal and 494 cases of perinatal listeriosis for analysis (Tables 4 and 5). Nonperinatal infection constituted, on average, 65% (1,025 of 1,583) of cases among studies that supplied this information (Table 4). In total, 1,250 cases of nonperinatal infections were reviewed; information about mortality was provided for 1,129 patients. The patients' ages ranged from <1 year to >90 years, but most were >60 years of

Table 3. Types of infection, sources of cultures, and outcome in 69 cases of perinatal *Listeria monocytogenes* infection, Israel, 1995–99

		Mothers' cultures			Infants' cultures		
Type of mother-infant infection	No. of cases	Blood only	Blood and tissue	Tissue only	Blood only	Blood and tissue	Tissue only
Uninfected mother and infected infant	13 (19%)				4	7 (3)	2 (2)
Infected mother and infected infant	9 (13%)	2	1	6	3	2	4 (1)
Infected mother and uninfected infant	16 (23%)	9	3	4			
Fetal/neonatal death (amnionitis)	31[a] (45%)	4	13	14	1	1 (1)	
Total	69 (100%)		54			24	

[a]Includes 27 intrauterine deaths with abortions, 1 stillbirth, and 3 early neonatal deaths. Numbers in parenthesis are cases of meningitis. Tissue denotes any material that is not blood, such as cerebrospinal fluid, placenta, and amniotic fluid.

age. The mean ages in the different series ranged from 50 to 67 years; 60% were male. Annual incidence rate varied widely (i.e., 0.1-1.1 per 10^5), not only between countries but also between consecutive years in the same setting. Most authors also described seasonal variation, with a peak incidence in summer possibly related to seasonal consumption of specific food products (8) or to more frequent breakdowns in food handling in higher temperatures.

Most (74%) of the persons affected in the reported cases (Table 4) were immunocompromised. Malignancy, chemotherapy, steroid therapy, organ transplantation, alcoholism, liver disease, renal insufficiency, and diabetes mellitus were most commonly reported, with few cases of acquired immunodeficiency syndrome.

With regard to clinical syndromes, the most common (47%) site of infection was the central nervous system (CNS) (Table 4), frequently associated with bacteremia. An additional 48% of patients were bacteremic without CNS involvement.

Perinatal infection constituted 34% (470 of 1,378) of cases among studies that supplied this information (Table 5). In total, 494 cases of perinatal infections were reviewed. Infection during the first two trimesters of pregnancy was almost invariably fatal to the fetus. One hundred six (21%) of the 494 pregnancies reviewed here resulted in intrauterine death.

Two hundred seventy-eight (56%) live-born infants had neonatal listeriosis and survived. Most of this neonatal infection was of early onset (209 cases), but the definition of early onset varied (from ≤5 to ≤7 days), and information concerning day of onset was incomplete in some series (Table 5). Almost all the late-onset infections (69 cases) were of the CNS. An additional 61 infants (12%) with neonatal listeriosis died from the infection in the postnatal period, for an overall intrauterine and postnatal mortality rate of 34% (167 of 494). In 49 (10%) of the affected pregnancies, the infant was born alive and without evidence of listeriosis.

Table 4. Characteristics of nonperinatal listeriosis from 10 recently reported series

	First author, year (ref)										
Characteristic	McLauchlin, 1990 (8)	Gellin, 1991 (6)	Cherubin, 1991 (14)	Skogberg, 1992 (15)	Nolla-Salas, 1993 (16)	Jones, 1994 (17)	Paul, 1994 (18)	Bula, 1995 (10)	Goulet, 1996 (9)	Siegman-Igra, 2001 (present study)	Total or average
Country and scope	England, national	USA, six areas	USA, four centers	Finland, Helsinki	Spain, Barcelona	England, Bristol	Australia, Sydney	Switzerland, western part	France, national	Israel, national	Worldwide
Study period	1967-1985	1986	1982-1999	1971-1989	1990	1983-1992	1983-1992	1983-1997	1992	1995-1999	1967-1999
Total no. of cases	722	246	119	74	31	29	84	122	225[a]	156	1,808
Nonperinatal cases (% of total)	474 (66%)	179 (73%)	54 (45%)	58 (78%)	29 (94%)	16 (55%)	71 (85%)	57 (47%)	225 (NA)	87 (56%)	1,025/1583 (65%) 1,025+225=1,250
Mean age (range) (years)	59 (1-97)	NA (<1-95)	NA	50 (29-66)	58 (17-89)	60 (1-95)	39% (>60)	66 (31-96)	65 (1-101)	67 (4-91)	50-67
Male gender	265 (58%)	101 (56%)	NA	NA	24 (77%)	9 (56%)	NA	33 (58%)	135 (62%)	56 (64%)	623 (60%)
Peak season	Autumn and spring	Late spring to fall	May-Aug	NA	39% in July-Sept	76% in July-Dec	NA	NA	NA	70% in May-Oct	Summer and fall
Annual incidence[b]	NA	0.7	NA	0.09 - 0.65	1.1	0.35	0.3	NA	NA	0.6	0.1-1.1
Immunocompromised	261/337 (77%)	NA	53/ 54(98%)	47/58 (81%)	24/29 (83%)	13/16 (81%)	59/71 (83%)	25/57 (42%)	159/225 (71%)	64/87 (74%)	705/934 (74%)
CNS infection	268/474 (57%)	55/179 (31%)	19/54 (35%)	29/58 (50%)	9/31 (29%)	6/16 (37%)	29/71 (41%)	45/57 (79%)	110/224 (49%)	24/87 (28%)	594/1,251 (47%)
Bacteremia ± focus[c]	183/474 (39%)	119/179 (66%)	35/54 (65%)	24/58 (41%)	20/31 (65%)	10/16 (73%)	40/71 (56%)	12/57 (21%)	97/224 (43%)	59/87 (68%)	599/1,251 (48%)
Focal disease only[d]	9/474 (5%)	5/179 (3%)	-	5/58 (8%)	2/31 (6%)	-	2/71 (3%)	-	17/224 (8%)	4/87 (5%)	44/1,124 (4%)
Mortality	164/371 (44%)	63/ 179(35%)	17/ 54(31%)	15/ 58(26%)	16/31 (52%)	6/16 (37%)	27/71 (38%)	18/57 (32%)	54/225 (24%)	33/87 (38%)	413/1,149 (36%)

[a]Includes nonperinatal cases only.
[b]Estimated annual incidence per 10^5 population.
[c]Bacteremia with or without a non-CNS focus of infection (e.g., pneumonia, endocarditis, urinary tract infection, prostatitis, peritonitis, gastroenteritis, rectal abscess, osteomyelitis, and cellulitis).
[d]For example, peritonitis, pleuritis, arthritis, pericarditis, cholecystitis, appendicitis, and cellulitis.
NA= not available; CNS= central nervous system

Table 5. Characteristics of perinatal listeriosis from six recently reported series

Characteristic	First author, year (ref)						Total or average
	McLauchlin, 1990 (12)	Gellin, 1991 (6)	Cherubin, 1991 (14)	Craig, 1996 (19)	Nolla-Salas, 1998 (20)	Siegman-Igra, 2001 (present study)	
Country and region	England, national	USA, six areas	USA, four centers	Australia, Melbourne	Spain, Barcelona	Israel, national	Worldwide
Study period	1967-1985	1986	1982-1999	1983-1994	1990-1996	1995-1999	1967-1999
Total no. of cases	722	246	119	24[a]	135	156	1,400
Perinatal infection (% of total)	248 (34%)	67 (27%)	65 (55%)	24 (NA)	21 (16%)	69 (44%)	470/1,378 (34%) 470+24=494
Estimated incidence per 10^4 births	NA	0.8-2.4	NA	2	0-4.1	1.4	0.6-4.1
Average maternal age (range) (years)	NA	26 (17-35)	NA	NA (18-39)	30 (26-34)	28 (21-40)	NA (26-30)
Early neonatal infection and survival	114[b] (46%)	31 (46%)	20 (31%)	14[c] (58%)	11 (52%)	19 (28%)	209/494 (42%)
Late neonatal infection and survival	36[d] (15%)	8[e] (12%)	21(32%)		1[d] (5%)	3[d] (4%)	69/494 (14%)
Infected mother and uninfected infant	9 (4%)	13 (19%)	2 (3%)	4 (17%)	5 (23%)	16 (23%)	49/494 (10%)
Intrauterine death	42 (17%)	14 (21%)	15 (23%)	4 (17%)	3 (14%)	28 (41%)	106/494 (21%)
Postnatal death	47 (19%)	1 (1%)	7 (11%)	2 (8%)	1 (5%)	3 (4%)	61/494 (12%)
Gestational age at abortion (weeks)	12-28	11-30	NA	18-29	10-27	9-26	9-29
Immunocompromised mothers	5			1	1	1	8[f]

[a]Includes only perinatal cases.
[b]Including 29 with unknown time of onset.
[c]No differentiation between early and late neonatal infection.
[d]>5 days.
[e]>7 days.
[f]2 diabetes mellitus, 2 renal transplant, 2 systemic lupus erythematosus, 1 Crohn disease and steroids, 1 HIV infection.
NA= not available

Discussion

Ingestion of Lm is a very common occurrence (1,2) since it has been isolated from many food products in Israel (unpub. data, MOH) as well as in many countries worldwide. Development of invasive disease secondary to Lm ingestion is determined primarily by the integrity of the immune system of the host (predominantly cell-mediated immune defects) and possibly also by inoculum size (11). The organism crosses the mucosal barrier of the intestine and invades the bloodstream. It may disseminate to any organ, but it has a clear predilection for the placenta and CNS, thereby determining the main clinical syndromes.

The case-fatality rate in the collected data on perinatal infection was 36% (413 of 1,149 patients for whom this information was available). This high mortality reflects both the severity of Lm infection and the seriousness of the underlying conditions. Higher mortality rates were correlated with older age, presence of CNS infection, and immunodeficiency (5,6,8,15,21). One study reported that deaths in immunocompetent patients occurred exclusively in the elderly (9), a finding that correlates well with our observations.

An unexpected observation in our study was the occurrence of hospital-acquired listeriosis in adults. The presumed hospital acquisition occurred on day 3-67 of hospital stay in 59 (16%) of 369 cases with relevant information, as reported in four studies, including ours (9,16,18). All patients acquiring listeriosis in the hospital (except one) were immunocompromised. No clustering of cases in time or place occurred, and no case had an obvious source for nosocomial acquisition of Lm. Because the incubation period of listeriosis is long (11-70 days) and fecal carriage not uncommon (5%-10%) (1,2), colonization could have been acquired before hospitalization and infection developed in the hospital, possibly even triggered by increased immunosuppression. Another possible explanation is consumption of contaminated food brought in from sources outside the hospital, but this could not be documented. We found only one description of a hospital outbreak of Lm among adults (three cases secondary to an index one), but the method of transmission was not established (22). Hospital transmission among neonates in nurseries was thought to occur more frequently (24%) (12) and was described by several investigators (18,23,24).

Among perinatal infections, we report the highest case-fatality rate (45%). This observation could be related to the frequency of taking cultures from aborted tissues. The diagnosis of Lm can easily be missed if cultures are not routinely

taken from aborted fetal tissues or if blood (and other) cultures are not obtained from febrile pregnant women. The great variability in incidence rates and other epidemiologic features between studies and among medical centers within studies suggests that many cases escaped diagnosis.

Concerning the mothers, all authors described a mild febrile "influenzalike" illness, without maternal deaths. Only one of the 494 mothers had meningoencephalitis with Lm isolated from the cerebrospinal fluid, but underlying condition or maternal and fetal outcomes were not reported (12). Eight mothers (<2%) were immunocompromised (Table 5), but no comparable data are available on the prevalence of these conditions among pregnant women in general.

In conclusion, listeriosis is an emerging zoonosis that constitutes a life-threatening disease for human fetuses and neonates, the elderly, and patients with certain predisposing conditions. Documented cases may not represent the true incidence in the community, especially with regard to perinatal infection. Fetal and maternal cultures should be obtained in every case of spontaneous abortion or stillbirth, to ensure proper diagnosis. Empiric ampicillin therapy should be included in the treatment of neonatal meningitis, sepsis, or meningitis in the elderly and immunocompromised patients and in febrile pregnant women without a source of infection.

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Epidemiologic Features of Four Successive Annual Outbreaks of Bubonic Plague in Mahajanga, Madagascar

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From 1995 to 1998, outbreaks of bubonic plague occurred annually in the coastal city of Mahajanga, Madagascar. A total of 1,702 clinically suspected cases of bubonic plague were reported, including 515 laboratory confirmed by *Yersinia pestis* isolation (297), enzyme-linked immunosorbent assay, or both. Incidence was higher in males and young persons. Most buboes were inguinal, but children had a higher frequency of cervical or axillary buboes. Among laboratory-confirmed hospitalized patients, the case-fatality rate was 7.9%, although all *Y. pestis* isolates were sensitive to streptomycin, the recommended antibiotic. In this tropical city, plague outbreaks occur during the dry and cool season. Most cases are concentrated in the same crowded and insanitary districts, a result of close contact among humans, rats, and shrews. Plague remains an important public health problem in Madagascar, and the potential is substantial for spread to other coastal cities and abroad.

Plague is enzootic in the central highlands of Madagascar, where approximately 200 to 400 bacteriologically confirmed or presumptive cases are reported each year to the World Health Organization; 1,500 to 2,500 clinically suspected cases are reported by the national surveillance system (1,2). In this island, the main reservoir of *Yersinia pestis* is the black rat (*Rattus rattus*) and the main vector the rat flea (*Xenopsylla cheopis)* (3). From the arrival of plague in Madagascar in 1898 until the 1920s, plague occurred in several harbors around the island. It disappeared progressively from the coastal areas as soon as it reached the central highlands where, above an altitude of about 800 m, it found a suitable environment. A period of quiescence in the coastal areas lasted more than 60 years. Then, in August 1991, a sudden outbreak lasting 7 months occurred in the harbor of Mahajanga (4). During the next 3 years, when neither an epizootic nor a human case was reported, the outbreak was considered an isolated epidemiologic incident. However, in 1995 a new epidemic occurred (5,6), which was followed by three others, in 1996, 1997, and 1998. These outbreaks accounted for approximately 30% of the reported cases of plague in Madagascar during the period. We describe the main epidemiologic features of these four urban outbreaks in this exceptional resurgent coastal plague focus.

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Population and Methods

In Madagascar, health workers must report all clinically suspected cases of plague to the national surveillance system. For each patient, a biological sample (bubo aspirate, sputum, or postmortem liver or lung puncture, whenever appropriate) has to be collected and sent by mail to the National Reference Laboratory at the Institut Pasteur de Madagascar in the capital, Antananarivo. The delivery time is frequently 2-3 weeks after the specimen was collected, and the only reliable method to allow bacteriologic diagnosis remains bubo aspiration (as well as sputum and postmortem liver or lung puncture, whenever appropriate) and transportation on a swab in Cary-Blair medium. Blood samples for culture were not adopted in Madagascar since the likelihood of isolating *Y. pestis* is approximately twice as high in buboes as in blood. In August and September 1997, a temporary bacteriology laboratory was established in Mahajanga; thus, all the biological samples collected during these 2 months were processed on site.

The confirmatory diagnosis was based on bacteriologic methods. A case of plague was considered to be confirmed as soon as a strain of *Y. pestis* was isolated by culture or mouse inoculation. A patient was considered to have a presumptive plague case when *Y. pestis* could not be isolated but gram-negative bacillus, with morphologic patterns of *Y. pesti*, could be detected on smear. Microscopy lacks both sensitivity and specificity, and the isolation of *Y. pestis* requires at least 6 days. Prior treatment of patients with antibiotics impedes the culture and may lead to false-negative results. Physicians were asked to collect an acute-phase serum sample before treatment and a convalescent-phase serum sample at least 7 days after the onset of disease. Whenever available, the sera and bubo aspirates were tested for F1-antigen by immunocapture enzyme-linked immunosorbent assay (ELISA) (7,8) and for anti-F1 antibodies by the classical indirect ELISA method (9).

In this study, the bacteriologically confirmed or presumptive patients, the ELISA-positive patients (F1 antigen or antibodies), or both were defined as having laboratory-confirmed cases. Clinical and epidemiologic data were collected on standard forms, after the patients or their families were interviewed. Reporting to the national surveillance system was assumed to be thorough, as confirmed by a seroepidemiologic survey in 1999 (10). In Madagascar all *Y. pestis* isolates are screened for their in-vitro resistance to streptomycin,

gentamicin, chloramphenicol, tetracycline, sulfamethoxazole-trimethoprim, and ampicillin.

The geographic distribution of plague patients was visualized by using a simplified map of Mahajanga. For this purpose, the city was divided into four areas. We defined the boundaries of these areas by aggregating districts that were comparable for population density, sanitation level, and housing type.

Results

In 1995, the first identified case occurred in March, followed by several sporadic cases in May and July. The outbreak proper started by mid-August. From 1995 to 1998, 1,702 clinically suspected bubonic plague cases were reported; 335 were considered confirmed (297) or presumptive (38) cases after bacteriologic testing. None of the *Y. pestis* isolates was recovered from sputum. When either F1 antigen capture or anti-F1 ELISA assays were used, 180 more cases were laboratory confirmed. In all, 515 persons were considered to have contracted plague from January 1, 1995, to December 31, 1998.

For each of the 4 years we studied, a biological result was available for 88.5%, 98.7%, 97.2%, and 99.5% of the suspected patients, respectively. When bacteriologic methods were used, the annual confirmation rates were 22.2%, 14.8%, 30.1%, and 30.3%, respectively. The proportions represented by bacteriologically confirmed cases among the total number of laboratory-confirmed patients were 72.3%, 83.6%, 98.7%, and 88.1%, respectively. Detailed laboratory results are summarized in the Table.

Two of the 297 *Y. pestis* isolates from Mahajanga patients were resistant to one of the tested antibiotics, one to chloramphenicol in 1996 and one to ampicillin in 1998.

The proportion of males (56.1%) was significantly higher among cases than in the general population (p=0.006). The age and sex-distribution of patients with laboratory-confirmed cases remained unchanged during the 4 years (Figure 1). The median age of patients was 15 years, and 75% of patients were <25 years old. Although the highest incidence of the disease was observed in 5-to 19-year-old persons, 59 cases occurred in children <5 years old; 2 were <1 year old.

Among laboratory-confirmed cases, a significantly higher frequency of cervical and axillary buboes occurred in children; by contrast, inguinal buboes represented about 80% of cases in persons ≥20 years of age ($p<10^{-7}$). The distribution of bubo location according to age is shown in Figure 2. Body temperatures were available for 454 of persons with laboratory-confirmed cases: the median temperature was 39.5°C (25th and 75th percentiles were 38.2°C and 40°C). Diarrhea (7.1%), prostration (4.5%), and coma (1%) were the other most frequently reported symptoms.

A total of 507 laboratory-confirmed patients were admitted to hospital; 40 (7.9%) of them died. The case-fatality rate was not significantly different by year (7.1%, 9.3%, 6.7%, and 10.3% in 1995, 1996, 1997, and 1998, respectively). Nor was this rate related to age, sex, bubo location, or delay between onset of disease (as reported by the patients) and initiation of treatment. Lethality was also not correlated with drug susceptibility of *Y. pestis* isolates, since they were all sensitive to streptomycin, the drug recommended by the national program. Only the body temperature at admission to the hospital was significantly higher in the group of deceased patients than in recovered (39.6°C vs. 39.1°C, p=0.01).

Table. Results of bacteriology testing for *Yersinia pestis*, Mahajanga, Madagascar, by year

	1995	1996	1997	1998	Total
Total suspected cases	558	399	539	206	1,702
Laboratory-confirmed[a] cases	117	97	214	87	515
Bacteriology					
Number tested	342	330	501	195	1,368
Number confirmed	55	41	149	52	297
Number presumptive	21	8	2	7	38
Number positive[b]	76	49	151	59	335
Percent positive	22.2	14.8	30.1	30.3	24.5
F1 antigen ELISA					
Number tested	433	335	413	189	1,370
Number positive	38	25	131	35	229
Percent positive	8.8	7.5	31.7	18.5	16.7
Anti-F1 antibodies ELISA					
Number tested	365	344	396	191	1,296
Number positive	68	59	137	48	312
Percent positive	18.6	17.2	34.6	25.1	24.1

[a]Confirmed + presumptive and/or enzyme-linked immunosorbent-assay (ELISA) positive.
[b]Confirmed + presumptive cases

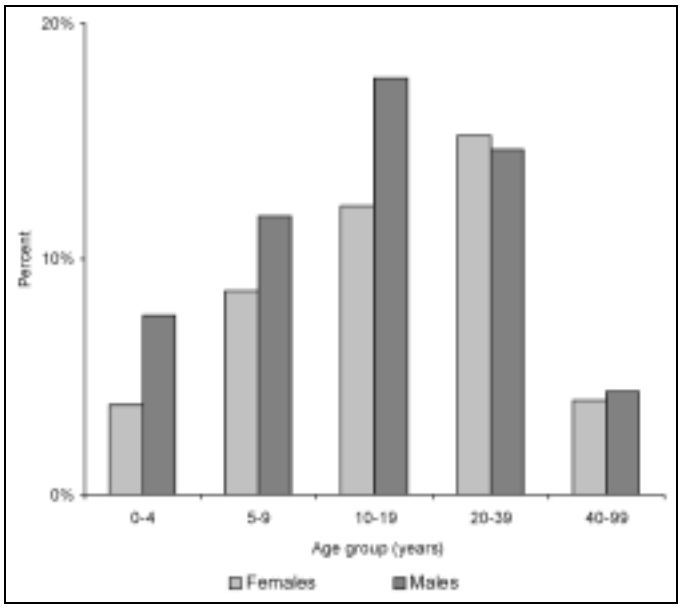

Figure 1. Age and sex-distribution of laboratory-confirmed bubonic plague cases, Mahajanga, Madagascar

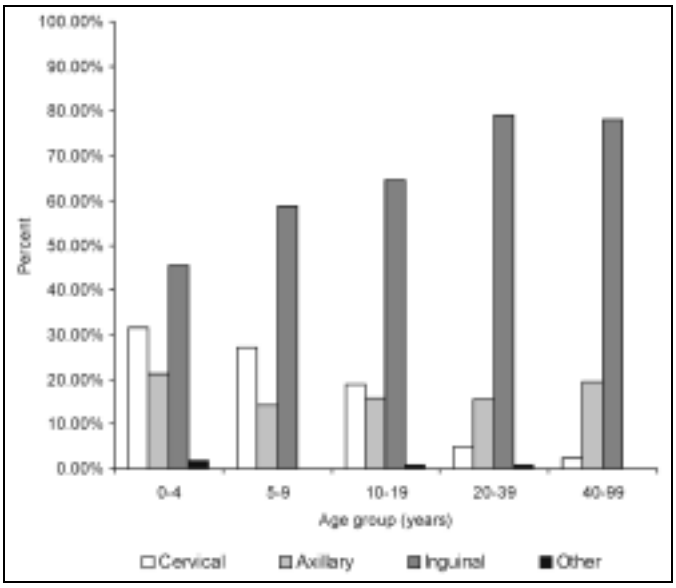

Figure 2. Distribution of bubo location according to age in laboratory-confirmed bubonic plague cases, Mahajanga, Madagascar.

Most (76%) patients were reported during August through October during the dry season, a peak that occurs every year. The temperature patterns in Mahajanga and the monthly distribution of laboratory-confirmed cases are related. The outbreaks used to occur in July, when the temperature is the lowest of the year, and ceased in November, when the temperature rebounds (Figure 3).

Among the 357 laboratory-confirmed plague patients for whom data were available about rat deaths within the 15 days preceding onset of disease, 203 (56.9%) had found dead rats indoors or in the vicinity of their homes; 154 (43.1%) had not noticed dead rats in their surroundings. Of the 203 who had, 117 (57.6%) had found the dead rats inside their homes. The confirmation rate was higher among persons who reported rat deaths in their surroundings than among persons not reporting such deaths (57.7% versus 24.6%, $p<10^{-7}$).

The geographic distribution of plague laboratory-confirmed cases according to districts is shown in Figure 4. The residence of patients could be clearly identified on this map for 473 (91.8%) of the laboratory-confirmed cases. The incidence of plague differed sharply according to districts: most patients (82.9%) lived in Area 1, which pools the most unhealthy and densely populated districts of the town. The southwestern part of the city (Area 2), including the harbor and the old colonial town, had few cases and did not show any trend towards increasing incidence. Areas 3 and 4, greener and less populous suburbs of Mahajanga, showed an intermediate incidence. The increase in cases in 1997, especially in Area 3, was no longer occurring in 1998.

Discussion

World plague foci are mostly restricted to temperate climate highlands such as regions in East Africa, central Asia, and the American Southwest; outbreaks in coastal areas have become rare. Thus, the situation in the harbor of Mahajanga, where plague has found favorable conditions and seems to have established itself, is noteworthy. During 4 successive years, 97 to 214 laboratory-confirmed plague cases were reported annually, raising questions about epidemiologic determinants of this disease's having taken roots in this tropical city. Before its sudden reemergence in 1991 after more than 60 years of quiescence, plague in Madagascar was supposedly restricted to areas above 800 m because of climatic constraints that influence the proliferation of fleas and *Y. pestis* (11). In Mahajanga, as had already been observed in 1907 (12), the plague season starts in July or August, during the dry season, when the air temperature is the lowest. This is in contrast with the central highlands, where most of the cases occur between October and February, during the warmer rainy season (2). Indeed, during the plague season for both the coastal and plateau regions, the minimum temperature is about the same, between 17° and 22°C. Recent studies have shown that the plague season in the central highlands is clearly linked to the abundance both of fleas and the black rat, *R. rattus*, the main reservoir and virtually the only small mammal found in houses (95% of captures in traps) (13). In Mahajanga, ongoing studies have shown that the Asiatic shrew, *Suncus murinus*, accounts for up to 75 % of the trapped animals and is a regular carrier of the rat flea, *X. cheopis* (Duplantier et al., unpub. data). Moreover, the seroprevalence among shrews trapped during the postepidemic period was 43% in 1995. *Y. pestis* strains were also isolated from five shrews in 1996 and 1997 (Chanteau et al., unpub. data). All these findings strongly suggest the determinant role of shrews as a previously

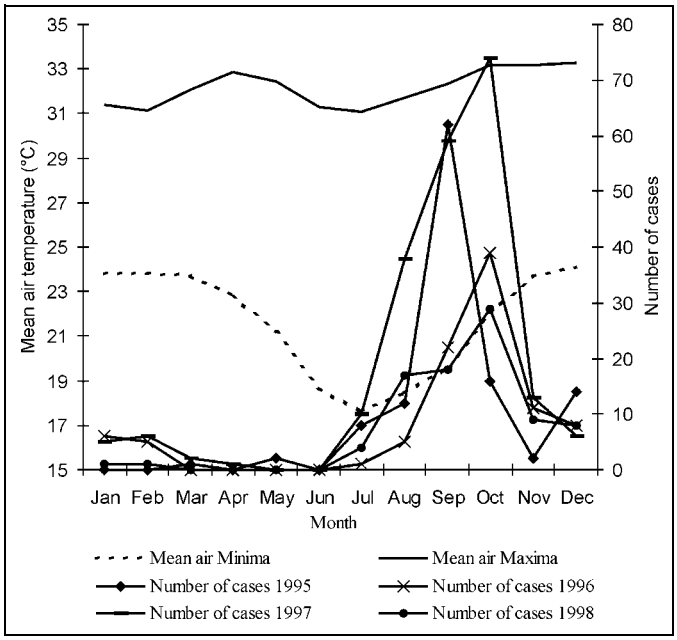

Figure 3. Mean air temperatures and month-distribution of laboratory-confirmed cases of bubonic plague, Mahajanga, Madagascar, since 1995.

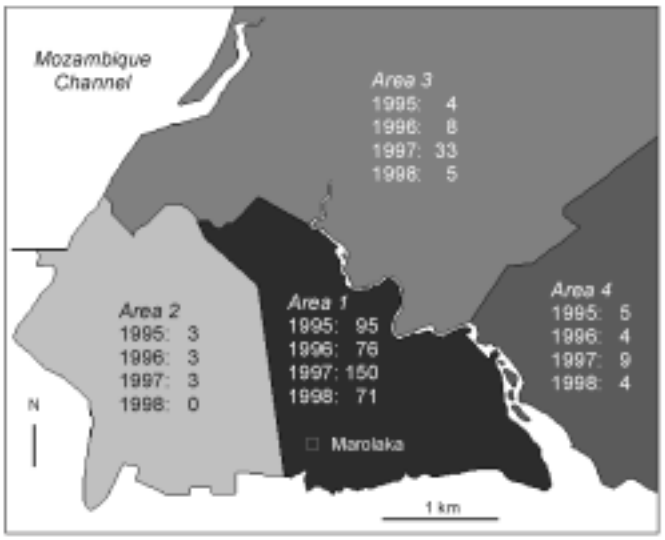

Figure 4. Incidence of laboratory-confirmed bubonic plague cases according to the patients' place of residence, in Mahajanga, Madagascar.

unrecognized host of *Y. pestis* in the epidemiologic cycle of plague in Mahajanga. This new parameter complicates and revises the classical rodent-flea-human triad. In Southeast Asia, the role of *S. murinus* in plague is established (14,15).

The epidemic wave of plague that started in August 1991 and lasted until February 1992 was confined to the neighborhood surrounding the main market in Area 1; this region, and more precisely a place named Marolaka, was considered to be the epicenter of the outbreak (4). The source of the initial contamination was probably inland, due to the trading of agricultural products from the northern plague foci to the marketplace, as suggested by results of the *Y. pestis* genotyping by pulsed-field gel electrophoresis (Buchrieser et al., manuscript in preparation). Although we think that an inland reintroduction of the infection is unlikely to occur for 4 consecutive years under the same pattern, we cannot exclude this hypothesis until molecular analysis of the isolates is available. The epidemic ring did not extend to the other areas of the town, and this quiescence lasted 3 years, during which information about hosts and fleas was scarce. The starting point for the second epidemic wave described here was in exactly the same zone of the marketplace; however, this time, the plague front extended to the other three areas. Over the 4 years, the incidence was higher in Area 1 than in the others, although in 1997 Area 3 was clearly affected. The plague front did not extend further than 10 to 15 km from town.

A geomedical survey (Rakotoarisoa S, unpub. data) concluded that three different types of structures were present in the entire city of Mahajanga**.** Area 2 is almost comparable with a European city with its wide, paved streets, sewer network, store buildings with apartments in the upper floors, and low population density); Areas 3 and 4 are semirural suburbs with low population density. In contrast, Area 1, which was the epicenter for the two waves of the outbreak, is densely populated with very poor people. This area also includes the two largest markets, which generate the town's largest amount of rubbish. Therefore, while no physical barrier separates Area 1 from Area 2, the lower incidence in the latter could be related to the slimmer chance of contact between humans and reservoirs of plague, in short, to better housing conditions.

Clinically, despite some published claims that the clinical diagnosis of bubonic plague is straightforward**,** field data show a bacteriologic confirmation rate no greater than 30% for the best years. This rate improved in 1997 and 1998, compared with 1995 and 1996, suggesting that physicians are becoming more skilled at making this diagnosis. The increased confirmation rate was also due to their increased skill in collecting bubo pus and the progressive shortening of the delay before the samples were analyzed in a laboratory. Except during the 2 months of August and September 1997, bubo samples arrived at the central plague laboratory in Antananarivo as long as 2 or 3 weeks after being collected, which led to false-negative results because they were contaminated or the plague bacillus had died. The use of ELISA methods to detect either F1 antigen in acute-phase bubo samples or antibodies in convalescent-phase serum contributed to the confirmation of 35% of the total laboratory-confirmed cases. However, bacteriology and ELISAs are used as retrospective tools to confirm plague. Only a rapid diagnostic test such as the F1 dipstick assay is a valuable tool for health workers (8).

The higher incidence of bubonic plague in males than females and in young persons rather than in adults is a constant epidemiologic feature in Madagascar, whether in Mahajanga or the highlands (1-3). In published studies, gender differences in incidence rates differ by country (16). In India, females were more frequently infected; in the city of Hai-nan, China, males and females were equally affected; and in Manchuria, the situation was similar to that in Madagascar (16)**.** Despite its being well accepted that incidence is linked to extrinsic more than intrinsic factors, we could not find any link to occupational behavior.

The effects of human age on the relationship between rat fleas and people and of gender on this disease deserve further study. The observation of a relationship between the age and the bubo location is common in Madagascar, although it has not been described elsewhere. As it is widely accepted that the location of the bubo is dependent on the place where the injection of *Y. pestis* occurred, we infer that infective flea bites more often involve the upper extremities in children than in adults. In the urban plague focus of Mahajanga, as in the central highlands, transmission is believed to occur mostly inside houses. Although we do not have any indications that children have specific risks, such as handling dead rodents in play, children do spend more time close to the floor (e.g., games, sleeping) than adults and therefore are closer to fleas.

From the start, the absence of pneumonic plague has been remarkable. The natural course of bubonic plague can lead to secondary pneumonic plague, which can give rise to highly

contagious cases of primary pneumonic plague in contacts, as seen every year in the highlands of Madagascar (17). Yet not a single contact case has been reported, even though several bubonic patients died before being diagnosed and treated and thus contacts remained unidentified who could have benefited from chemoprophylaxis. This observation fits with early studies describing peumonic plague only in temperate places. As far back as 1929, Thiroux pointed out that pneumonic plague outbreaks had never been observed in Madagascar in areas where the absolute minimum temperatures did not remain regularly under 16°C for several consecutive days (18). As early as 1907, in the absence of effective treatment and chemoprophylaxis, only four cases of pneumonic plague had been observed among 72 plague cases during the first epidemic in Mahajanga. The absence of lung infection apparently is not related to a particular strain of the plague bacillus since the *Y. pestis* strain that currently circulates in Mahajanga was likely introduced from the highlands in 1991, as demonstrated by pulsed-field gel electrophoresis genotyping (Buchrieser et al, manuscript in preparation).

The case-fatality rate is somewhat higher than reported in published studies, and it does not show any trend towards decreasing. However, we considered only laboratory-confirmed cases, and technical conditions in Mahajanga hospital are poor. We believe that a major proportion of treatment failures could be avoided if patients did not wait 2 days or more before coming to hospital. Moreover, dates of onset seem to be questionable for some serious cases; apparently families are often reluctant to imply that they have been negligent in managing the patient at home, or they have resorted to traditional healing before referring the patient to a hospital. Up to now, antibiotics used to treat the disease (streptomycin is the recommended treatment in Madagascar) have been totally effective against *Y. pestis* strains from Mahajanga. The discovery of one chloramphenicol-resistant isolate and one ampicillin-resistant isolate requires that the country maintain an efficient bacteriology surveillance system. The appearance and spread of multiresistant *Y. pestis* strains, such as the two isolated in 1995 in the highlands, are cause for concern (19,20).

Plague is still a threat in Madagascar and is no longer restricted to areas in the highlands over 800 m. A bubonic plague outbreak has been reported recently at 500 m altitude (21,22). Such epidemics as we describe may occur in any other coastal city where the shrew *S. murinus* and the flea *X. cheopis* are present. Because trade between the highlands and the ports has intensified, an active program for surveillance and monitoring of plague borders must be maintained. Improved ways to rat-proof structures should also be encouraged.

Dr. Boisier is a physician and specialist in epidemiology, working for the French Army Health Service. His research interests include the epidemiology and control of tropical diseases.

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Rickettsia felis in *Ctenocephalides* spp. Fleas, Brazil

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In June 2000, suspected cases of Brazilian spotted fever (BSF) occurred in Coronel Fabriciano Municipality, Minas Gerais State, Brazil. Pooled fleas collected near two fatal cases contained rickettsial DNA. The nucleotide sequence alignment of the 391-bp segment of the 17-kDa protein gene showed that the products were identical to each other and to the *R. felis* 17-kDa gene, confirming circulation of *R. felis* in Brazil.

The pathogenic rickettsiae are a group of intracellular bacteria responsible for various human diseases. *Rickettsia rickettsii* and *R. typhi* and the diseases they cause—Brazilian spotted fever (BSF), transmitted by the *Amblyomma cajennense* tick, and murine typhus, transmitted by the Oriental rat flea—have been recognized in Brazil since the 1920s (1-3). Molecular methods, including detection by DNA amplification by polymerase chain reaction (PCR) and DNA sequence analysis, are useful in characterizing rickettsial agents in arthropods. This approach has allowed the identification of new species, such as *R. felis* in opossums, fleas (4,5), and blood and skin from ill humans from the United States, Mexico, France, and Brazil (6-9). We report the identification of *R. felis* in *Ctenocephalides* fleas collected during the investigation of an outbreak of spotted fever group rickettsiosis in Brazil.

Material and Methods

In June 2000, fleas and ticks were collected in a periurban area of the city of Coronel Fabriciano, Steel Valley, Minas Gerais State, Brazil (Figure 1). This survey was performed during an outbreak of suspected BSF in which two children died. They were brothers who lived in the same house. The first child who became ill was 12 years old; during the course of his disease he had fever, nausea, vomiting, diarrhea, abdominal pain, headache, myalgia, and edema. Later, renal failure and stupor occurred. The second patient had fever, rash, nausea, vomiting, diarrhea, abdominal pain, headache, myalgia, jaundice, and renal failure. Both patients reported a tick bite a day before the onset of disease. One death was later confirmed as a case of spotted fever group rickettsiosis by immunohistochemical technique in tissues collected at autopsy. PCR was performed on brain, stomach, liver, spleen, and kidney tissues collected at autopsy, preserved in formalin, and sent to the University of Texas Medical Branch at Galveston. Because the DNA was not preserved, the death could not be attributed specifically to *R. rickettsii*, *R. felis*, or other species of *Rickettsia*.

The ticks were collected from three dogs and five horses near the house where the deaths occurred and were stored in 70% ethanol at room temperature. Ticks were separated into 15 pools with three specimens per pool, undifferentiated by life stage or sex. Fleas were also removed from 10 dogs in the home of the child whose death was confirmed as being due to BSF (Galvão et al., unpub. data) and from 3 cats near this residence; fleas were stored at –70°C. The fleas were separated into six pools with five specimens per pool. The ticks and fleas were identified as *A. cajennense* and *Ctenocephalides* spp., respectively.

PCR amplifications were done as previously described (9) with the DNA extracted from pools of ticks and fleas (Figure 2). Each PCR product was cycle sequenced with the primers described above and fluorescein-labeled dideoxynucleotide bases in the Applied Biosystems model (11) DNA sequencing system (ABI, Foster City, CA). Sequences were edited and assembled by using Chromas software (http://www.technelysium.com.au/chromas.html).

To arrive at the most accurate sequence for each PCR product, both forward and reverse sequences were determined. Where differences in nucleotide bases were observed, a predominant base was assigned if most of the sequences contained it. If one base did not predominate, the original chromatographs were consulted to resolve ambiguities.

Figure 1. Map of Brazil and Minas Gerais State, showing Coronel Fabriciano municipality.

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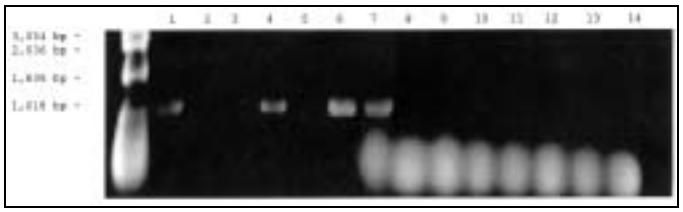

Figure 2. Detection of the *Rickettsia* specific 17-kDa gene by polymerase chain reaction amplification in DNA extracted from ticks and fleas. The vectors were first placed in 1.5-mL microcentrifuge tubes containing 200 µL of 10 mM phosphate-buffered saline, pH 7.4, and were crushed with a micropestle. The suspensions were lysed in 0.5% sodium dodecyl sulfate and incubated with 100 µg/mL proteinase K at 37°C for 1 hour in the case of fleas or overnight in the case of ticks. The lysed suspensions were extracted twice with an equal volume of phenol-chloroform, followed by a single chloroform extraction. The extracted DNA was amplified with primer 1 (5′-GCTCTTGCAACTTCTATGTT-3′) and primer 2 (5′-CATTGTTCGTCAGGTTGGCA-3′) as described by Webb et al. (10) for amplification of a 434-bp fragment from the rickettsial 17-kDa protein gene. PCR was performed at 30 cycles for 1 minute at 94°C, 5 minutes at 48°C, and 2 minutes at 72°C. The PCR products were then separated by electrophoresis in 1% agarose gel and stained with ethidium bromide. Lanes 1-3: DNA from cat fleas, Lanes 4-6: DNA from dog fleas, Lane 7: 17- kDa gene *Rickettsia felis* DNA (Positive Control), Lanes 8-14: DNA from ticks.

Sequences were compared by using the BLAST software program with 17-kDa sequence from other *Rickettsia* species obtained from the GenBank database. These sequences were aligned for maximal homology by using the Multialign software program (12).

Results

Of the 15 samples of pooled ticks and 6 samples of pooled fleas examined, 3 samples of pooled fleas had the 434-bp product expected for a *Rickettsia* (Figure 2). Nucleotide sequence analysis of the aligned 391-bp segment of 17 kDa confirmed that the three PCR products were identical to each other and to the 17-kDa protein gene of *R. felis* in the database.

Discussion

Recent research on rickettsial diseases in Latin America has included tropical Mexico, Andean Peru, and northern Argentina. The investigation in Minas Gerais State, Brazil, added another ecologic zone and geographic region of Latin America to those in which novel rickettsioses and ehrlichioses have been detected and identified.

BSF is the best-recognized rickettsial disease in Brazil; few reports have been published about human cases of other rickettsioses such as murine typhus and Q fever (13). BSF is known to occur in the states of Minas Gerais, São Paulo, Rio de Janeiro, Bahia, and Espírito Santo. Minas Gerais State has a surveillance program for BSF, and since 1990 interest has grown in the study of this disease in areas where residents seeking employment are increasingly exposed to tick-infested habitats. From 1990 to 1994, the incidence of BSF was 0.35 per 100,000 inhabitants, with a higher incidence in the latter half of the year (13). The age range most affected was 5 to 14 years (13), and the case-fatality ratio was 19% during 1993 to 1995 (14).

Our results show that, in addition to *R. rickettsii* and *R. typhi*, *R. felis* is also found in Brazil, as indicated by positive serology in human cases (8). Our data are the first indication by PCR of the presence of *R. felis* in fleas from Brazil. The *Ctenocephalides* spp. flea is proposed as a possible vector of this new rickettsial disease in Brazil.

Because of the complicated differential diagnosis of febrile exanthems, which includes dengue fever and other viral, rickettsial, and bacterial diseases, more attention should be paid to diagnostic laboratory investigation of rickettsial diseases. *R. felis* has been identified as the etiologic agent of a new rickettsiosis wherever it has been investigated: United States (Texas), Mexico, Brazil, and France (8). In Latin America, two reports have been published of human rickettsioses caused by *R. felis* in Mexico and Brazil (7,8). In both these reports, neurologic involvement was described, suggesting a severe clinical course associated with *R. felis* (15).

Although our investigation does not provide evidence for widespread flea infection by *R. felis* in Brazil, we demonstrate for the first time the presence of infection by this bacteria in Brazilian *Ctenocephalides* fleas. The descriptions of *R. felis*-positive human cases (8) in the same area where *R. felis* was identified in *Ctenocephalides* fleas indicate the possibility of this flea's being the vector of human *R. felis* rickettsiosis in Brazil.

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Severe *Ehrlichia chaffeensis* Infection in a Lung Transplant Recipient: A Review of Ehrlichiosis in the Immunocompromised Patient

Nasia Safdar,* Robert B. Love,* and Dennis G. Maki*

We describe a case of human ehrlichiosis in a lung transplant recipient and review published reports on ehrlichiosis in immunocompromised patients. Despite early therapy with doxycycline, our patient had unusually severe illness with features of thrombotic thrombocytopenic purpura. Of 23 reported cases of ehrlichiosis in immunocompromised patients, organ failure occurred in all patients and 6 (25%) died.

Since the discovery in 1987 of *Ehrlichia* as a cause of tick-borne disease in humans (1), ehrlichiosis has been recognized as an increasingly important cause of acute febrile illness (2,3). The two main pathogenic species are *Ehrlichia chaffeensis*, which causes human monocytic ehrlichiosis (HME), and the as-yet-unnamed agent of human granulocytic ehrlichiosis (HGE) (4). A third species, *E. ewingii*, which has been recently described, causes clinical disease indistinguishable from infection caused by *E. chaffeensis* or the agent of human granulocytic ehrlichiosis (5).

Delineation of the epidemiology of human ehrlichiosis has greatly enhanced our understanding of this emerging infection. However, information on the manifestations of ehrlichiosis in immunocompromised patients is limited. We report a case of severe monocytic ehrlichiosis in a lung transplant recipient who had pancytopenia, acute renal failure, and encephalopathy. Despite early diagnosis and treatment with doxycycline, his illness progressed and took on features of thrombotic thrombocytopenic purpura (TTP). A review of reported cases of *Ehrlichia* infection in immunocompromised patients shows that the infection is far more severe in this population and is often fatal.

Case Report

A 38-year-old man with cystic fibrosis had undergone bilateral lung transplantation in 1998 and had been well. In September 2000, he visited a physician with a 3-day history of fever as high as 38.3°C, myalgias, and headache. A resident of Columbia, Missouri, the patient had spent much time outdoors but did not recall tick infestation or recent tick bite. His medications included cyclosporine, mycophenolate, prednisone, diltiazem, trimethoprim-sulfamethoxazole, and valacyclovir.

On physical examination, the patient appeared acutely ill with temperature 38.3° C, blood pressure 140/64, heart rate 110 per minute, and respiratory rate 20 per minute. He was lethargic but could follow commands, and his neurologic exam was unremarkable. Fine bibasilar crackles were present bilaterally, but heart sounds were normal. Examination of the abdomen was negative. Synovitis was not evident, and no cutaneous lesions were found.

The leukoctye count was 3.7×10^9 per L with 68% neutrophils, hemoglobin was 64 g/L, and platelet count was 23,000/L. Serum creatinine was 4.6 mg/dL, aspartate aminotransferase 420 U/L, alanine aminotransferase 96 U/L, and bilirubin 3.2 mg/dL. International normalized prothrombin time ratio (INR) was 1.4. Examination of a peripheral blood smear showed schistocytes and other microangiopathic changes.

Multiple blood cultures were negative. Cytomegalovirus DNA was not detected in peripheral blood. Noncontrast computed tomography of the brain was normal. Chest radiograph showed bilateral infiltrates.

The patient was treated initially with intravenous piperacillin-tazobactam and vancomycin. Cyclosporine and trimethoprim-sulfamethoxazole were discontinued. The next day, his mental status continued to deteriorate. Lumbar puncture was deferred because of thrombocytopenia. Antibiotic therapy was changed to intravenous meropenem. Four days after admission, the bone marrow was examined because of worsening pancytopenia; intracytoplasmic morulae were seen in monocytoid cells, characteristic of monocytic ehrlichiosis (Figure). Leukocytes in a peripheral blood smear also contained morulae. Intravenous doxycycline was begun for treatment of presumed *Ehrlichia* infection. Whole-blood polymerase chain reaction (PCR) (Viromed, Minneapolis, MN) in the first week of illness was subsequently reported positive for *E. chaffeensis*

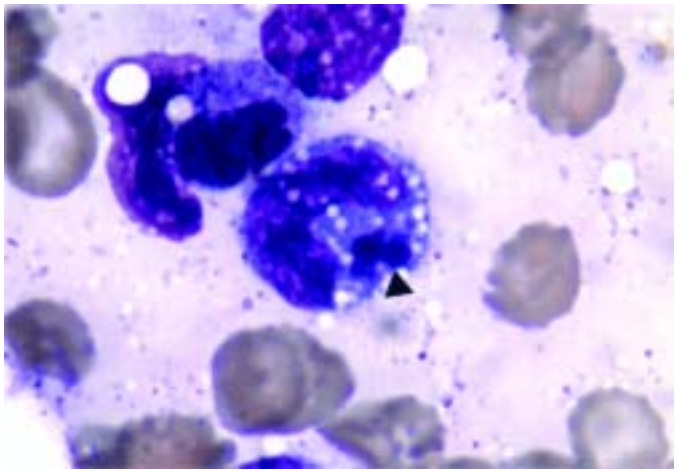

Figure. Bone marrow examination (Wright's stain x1000). Intraleukocytic morulae of *Ehrlichia* can be seen (arrow) within monocytoid cells.

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Table. Reports of immunocompromised patients with *Ehrlichia* infection

Immunocompromised state	Age	Form of ehrlichiosis	Clinical features	Outcome	Reference
Rheumatoid arthritis, on methotrexate	49	*Ehrlichia ewingii*	Fever, headache	Survived	5
Emphysema, on prednisone	65	*E. ewingii*	Fever, headache	Survived	5
Renal transplant	11	*E. ewingii*	Lyphadenopathy, fever	Survived	5
Liver transplant	47	HME[a]	Multiorgan failure	Survived	9
Leukemia	6	HME	Hepatitis, pancytopenia, rash, renal failure	Survived	10
Asplenia	71	HGE	Fever, neurologic dysfunction	Survived	11
Asplenia	30	HGE	Rash, fever	Survived	11
HIV infection (CD4 45/mm^3)	33	HME	Cardiomyopathy, heart failure	Survived	12
Renal transplant	35	HME	Rash, pancytopenia, renal failure	Survived	13
HIV infection (CD4 NS)	38	HME	Multiorgan failure	Died	14
Sickle beta-thalassemia	NS	HME	Respiratory failure	Survived	15
Renal transplant	NS	HME	NS	Survived	15
HIV infection (CD4 164/mm^3)	52	HME	Hepatitis, thrombocytopenia	Died	16
Renal transplant	67	HGE	Pancytopenia, renal failure, hepatitis	Survived	17
HIV infection CD4 (18/mm^3)	36	HME	Hepatitis, renal failure	Died	18
Liver transplant	51	HME	Pancytopenia, shock	Survived	19
Asplenia, chronic lymphocytic leukemia, on steroids	80	HGE	Multiorgan failure	Died	7
HIV infection (CD4 199/mm^3)	37	HME	Fever, pancytopenia, toxic-shock-like illness	Survived	20
Splenectomy	46	HME	Pancreatitis, shock, encephalitis	Survived	20
HIV infection (CD4 64/mm^3)	41	HME	Pancytopenia, pulmonary hemorrhage	Died	21
Asplenia	67	*E. canis*	Renal failure respiratory failure, encephalitis	Died	8
Crohn disease requiring prednisone	57	*E. canis*	Pancytopenia, hepatitis	Survived	22
Bilateral lung transplant	38	HME	Pancytopenia, renal failure, TTP-like illness	Survived	Current case

[a]HME = human monocytic ehrlichiosis; HGE = human granulocytic ehrlichiosis; NS = not specified

DNA. Serology by immunofluorescence antibody testing for both *E. equi* and *E. chaffeensis* performed 2 weeks after onset of illness was negative, with titers <1:40.

Despite treatment with doxycycline, the patient's confusion, thrombocytopenia, and microangiopathic anemia did not improve, and on the fifth hospital day he was transferred to the University of Wisconsin Hospital and Clinics. Physical examination showed blood pressure 144/94 mmHg, heart rate 77/minute, temperature 36.5°C, and respiratory rate 24/minute. Multiple ecchymoses were present on the torso and extremities. Neurologic examination was nonfocal. There were coarse bibasilar crackles in the lungs bilaterally. Examination of the heart and abdomen was unremarkable.

Leukocytes were 2.9 x 10^9/L, hemoglobin 86 g/L, and platelets 30,000/L. Serum creatinine was 6.2 mg/dL (548 mol/L), total bilirubin 2.0 mg/dL, aspartate aminotransferase 105 U/L, and alanine aminotransferase 55 U/L. INR was 1.1, and the activated partial thromboplastin time was 26 seconds. A peripheral blood smear showed numerous fragmented red blood cells. Chest radiograph showed persistence of bilateral infiltrates.

The patient's fever resolved 2 days after doxycycline was started; however, oliguric renal failure necessitated hemodialysis. Hematologic studies showed progressive microangiopathic anemia and thrombocytopenia with a normal INR, suggestive of TTP, presumably secondary to *Ehrlichia* infection. Daily plasmaphereses were begun and continued for 8 weeks. Gradually the hematologic abnormalities and renal function improved, and the patient's mental status returned to normal. Doxycycline was given for 2 weeks.

The patient ultimately made a full recovery with no apparent sequelae. Cyclosporine was not resumed, and he was maintained on sirolimus and prednisone to prevent transplant rejection. No rejection occurred, despite a reduction in immunosuppressive therapy during the treatment of *Ehrlichia* infection.

Review of Published Reports

Ehrlichiosis is a zoonotic illness caused by *Ehrlichia* species, which are pleomorphic, intracellular, rickettsia-like organisms (2-4). The clinical spectrum of ehrlichisis varies from a mild, influenzalike illness to a fulminant sepsis syndrome, but in most patients is self-limiting and not fatal. Death rates of documented ehrlichial infection in large, unselected series have been 1% to 8% (3,6-8). This low rate contrasts sharply with the high death rate of ehrliochiosis in immunocompromised patients (Table).

Cellular immunity represents the most important host defense against rickettsial infection (23). Acute-phase sera of patients with HGE contain elevated levels of interferon gamma, which is associated with the clearance of *Ehrlichia* from peripheral blood (24). In a mouse model of ehrlichiosis, immunocompromised mice have persistent infection, and most eventually die (25). Impairment of cellular immunity, whether from immunosuppresssive therapies or underlying disease, retards recovery, leading to more severe disease and higher death rates.

The population of immunocompromised patients is large and growing; many have asplenia or solid organ or bone marrow transplants (26). An analysis of the published reports of ehrlichial infection shows that the disease in immunocompromised patients is far more severe and prolonged and more likely to be fatal (Table) (5,7-22). Virtually all these patients had signs of organ dysfunction, including pancytopenia (40%), renal failure (24%), respiratory distress (14%), shock (28%), and neurologic dysfunction (18%). Six (25%) of 23 patients died; 4 of the 6 deaths were in HIV-infected patients. Two patients died within 24 hours after coming to medical attention, despite initiation of appropriate antimicrobial therapy; in the third, the diagnosis was not considered until late in the hospital course; and in the fourth, the diagnosis was made postmortem. Two deaths occurred in asplenic patients; in both, *Ehrlichia* infection was not suspected until 1 week after onset of illness.

In a recent series of ehrlichial infection in 21 HIV-infected patients, 6 of which are included in our review, Paddock et al. reported a high frequency (71%) of moderate to severe disease in HIV-infected patients, particularly with *E. chaffeensis* (27). Low CD4 counts were associated with a poor outcome.

Discussion

To our knowledge, this is the first reported case of acute ehrlichiosis in a lung transplant recipient. Our patient had laboratory features typical of *Ehrlichia* infection (thrombocytopenia, leukopenia, and transaminase elevation). However, he also had microangiopathic anemia, renal failure, and neurologic dysfunction characteristic of TTP. Ehrlichiosis with features of TTP has been described in two reports (28,29), one case each of HME and HGE. Both cases were in immunocompetent persons: one was treated with doxycycline and plasmapheresis; in the other, the diagnosis was made postmortem.

Our patient's multiorgan failure and hematologic aberrations persisted, despite doxycycline therapy, until he underwent plasmapheresis. He was receiving cyclosporine, which is a well-known cause of a rare hemolytic uremic syndrome-TTP-like condition that does not respond to plasmapheresis and nearly always proves fatal (30). That our patient's TTP-like illness coincided with *Ehrlichia* infection and responded to doxycycline and plasmapheresis makes it most likely that it was a consequence of acute ehrlichiosis, not cyclosporine.

Neurologic manifestations, ranging from confusion to frank meningitis, have been reported in up to 20% of patients with erhlichiosis (31). Our patient had obtundation and delirium that persisted after doxycycline therapy was initiated and his fever had resolved. The presence of headache and confusion in conjunction with pancytopenia and transaminase elevation should raise suspicion of *Ehrlichia* infection, especially if the patient has had potential tick exposures.

The diagnosis of ehrlichiosis is often delayed because of its nonspecific clinical and laboratory manifestations. In the immunocompromised person, the search for opportunistic infections may further preclude consideration of *Ehrlichia* infection. The empiric antimicrobial regimens used in immunocompromised patients for suspected cryptogenic bacterial and fungal sepsis rarely include a drug or drugs effective against *Ehrlichia*. PCR to detect *Ehrlichia* DNA is invaluable for the diagnosis and has >90% sensitivity and even better specificity (32). This technique is particularly useful in the immunocompromised host in whom rapid diagnosis is of utmost importance. Peripheral blood and bone marrow examinations show intracellular morulae in HME in only 1% to 5% of cases and cannot be relied on diagnostically, unless positive. Serologic testing does not allow rapid diagnosis and may be negative in the immunocompromised patient (21), as was the case with our patient.

The diagnosis of ehrlichiosis should be considered in any patient with fever, transaminase elevations, and new-onset thrombocytopenia or leukopenia who has had potential tick exposures in an endemic area. In the immunocompromised host, clinical manifestations are more severe and can include neurologic deterioration, multiorgan failure, and even a TTP-like illness. Response to appropriate therapy with doxycycline may be delayed. A high index of suspicion, the use of PCR for confirmatory diagnosis and early empiric therapy can be life-saving.

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Enzootic *Angiostrongylus cantonensis* in Rats and Snails after an Outbreak of Human Eosinophilic Meningitis, Jamaica

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After an outbreak in 2000 of eosinophilic meningitis in tourists to Jamaica, we looked for *Angiostrongylus cantonensis* in rats and snails on the island. Overall, 22% (24/109) of rats harbored adult worms, and 8% (4/48) of snails harbored *A. cantonensis* larvae. This report is the first of enzootic *A. cantonensis* infection in Jamaica, providing evidence that this parasite is likely to cause human cases of eosinophilic meningitis.

Angiostrongylus cantonensis is the most common infectious cause of eosinophilic meningitis worldwide (1). Although human infections with *A. cantonensis* are traditionally associated with Southeast Asia and the Pacific Basin, sporadic cases have been reported in several countries outside this region (1,2). In the Caribbean, eosinophilic meningitis has not been commonly reported, although *A. cantonensis* has been found in rats from Cuba, Puerto Rico, and the Dominican Republic (3-5).

A case of eosinophilic meningitis was described in 1994 in an adult Jamaican who had never traveled outside the country (6). In the absence of confirmatory histology or serology, the question of the endemicity of *A. cantonensis* in Jamaica at that time was raised (6). In May 2000, 12 persons in a group of 23 U.S. tourists who visited Jamaica for a week met the clinical definition for eosinophilic meningitis within 6-30 days (median 11) of returning home (7). Nine persons required hospitalization; there were no deaths. There was serologic evidence of exposure to *A. cantonensis* in eight persons who had eaten salad at the same restaurant, a common exposure that might account for all cases (7).

Since *A. cantonensis* has not been documented in Jamaica and many restaurants in Jamaica's tourist areas serve imported vegetables, the source of contamination of the vegetables was not necessarily on the island. We investigated whether *A. cantonensis* occurs naturally in the wild rat and snail populations of Jamaica.

The Ministry of Health collected 109 rats through the rat control program run by the Public Health Department. Rats were collected in eight sites across the island (Table) and sent to the Parasitology Research Laboratories at the University of the West Indies, where the cardiopulmonary system was dissected to determine infection status. In addition, staff from University of the West Indies and the Centers for Disease Control and Prevention (CDC) collected snails from four sites (Table) and examined them for infection.

Adult worms were recovered from the cardiopulmonary systems of 24 rats (20/78 *Rattus norvegicus*; 4/31 *R. rattus*) (Table). These worms had features characteristic of *Angiostrongylus*, including size (males measured 14-15 mm in length; females 24-26 mm in length), body shape, and prominent dark intestine (Figure 1A). The long copulatory spicules in the male worms, which measured approximately 1.2 mm (Figure 1B), are diagnostic for *A. cantonensis*, as the spicules of other species in the genus are generally <0.5 mm long (8).

Overall, 22% of the rats were infected with *A. cantonensis*. Infection rates did not differ significantly between *R. rattus* and *R. norvegicus* (chi square 2.10; p=0.148). The mean number of worms recovered per infected rat was 17±3.5 (range 3-27).

Table. Recovery of *Angiostrongylus cantonensis* from rats and snails, Jamaica, 2000

Location	Host	No. infected/no. examined (%)
Rats		
Freeport	*Rattus norvegicus*	6/10 (60)
	R.rattus	0/0
Mandeville	*R. norvegicus*	10/17 (59)
	R. rattus	0/0
Black River	*R. norvegicus*	2/11 (18)
	R.rattus	1/4 (25)
Kingston	*R. norvegicus*	1/12 (8)
	R.rattus	1/11 (9)
Lucea	*R. norvegicus*	1/15 (7)
	R.rattus	0/0
Montego Bay	*R. norvegicus*	0/0
	R.rattus	1/12 (8)
Port Antonio	*R. norvegicus*	0/8
	R. rattus	0/3
Lime Hall	*R. norvegicus*	0/1
	R.rattus	1/1 (100)
Snails		
Mandeville	*Thelidomus asper*	4/10 (40)
Brown's Town	*Orthalicus jamaicensis*	0/27
	Dentellaria sloaneana	0/2
Yallahs	*Orthalicus jamaicensis*	0/6
Scott's Pass	*Orthalicus jamaicensis*	0/3

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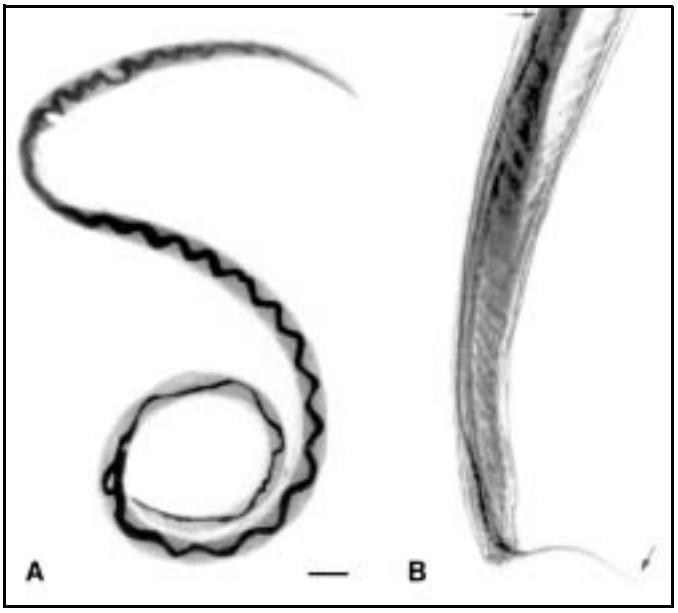

Figure 1. Adult *Angiostrongylus cantonensis* recovered from rat lungs. A. Adult female worm with characteristic barber-pole appearance (anterior end of worm is to the top). Scale bar = 1.5 mm. B. Tail of adult male, showing copulatory bursa and long spicules (arrows). Scale bar = 85 µm.

Land snails (Figure 2) were collected by hand from small farms and residential gardens and sent to the Division of Parasitic Diseases laboratory, CDC, Atlanta. A portion of the muscular head-foot region was excised from each surviving snail, cut into smaller fragments, and placed in separate dishes containing digestion fluid (0.01% pepsin in 0.7% v/v aqueous HCl [9]). Dishes were examined for nematode larvae at 4-5 hours and 24 hours after digestion.

Four of 10 *Thelidomus asper* collected in Mandeville were found positive for *A. cantonensis* larvae, but neither *Orthalicus jamaicensis* (n=36) nor *Dentellaria sloaneana* (n=2) were infected (Table). Living larvae digested from *Thelidomus* were easily recognized and recovered because they retained motility in the digestion fluid. Larvae were examined microscopically, and the morphologic features compared with those in published reports (10) and reference *A. cantonensis* larvae to confirm identification. Two species of lungworm (metastrongyles) larvae were recovered. Most larvae were *Angiostrongylus cantonensis* (375 to 420 [mean 402] µm in length after fixation in hot alcohol), but a small number of *Aelurostrongylus abstrusus* (400 to 440 [mean 427] µm in length after fixation in hot alcohol), a lungworm of cats, were also observed. Typical of lungworm larvae, the two species were similar in size and the presence of characteristic sclerotized rhabdions at the anterior end of the larvae. The larvae were easily distinguished, however, by the shape of the tip of the tail; *A. cantonensis* had a constriction near the end of the tail and ended in a fine point, while *A. abstrusus* terminated in a knob (10,11).

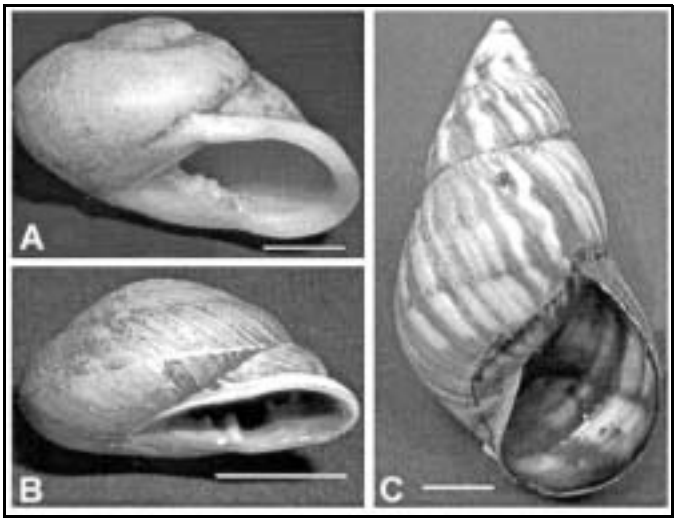

Figure 2. Three species of land snails collected in Jamaica and examined for *Angiostrongylus* larvae. A. *Thelidomus asper.* B. *Orthalicus jamaicensis*. C. *Dentellaria sloaneana*. Scale bar = 1 cm.

This is the first report of enzootic *A. cantonensis* infection in Jamaican rats and snails; our data show that the range of the parasite extends to another Caribbean country outside Cuba, the Dominican Republic, and Puerto Rico (3-5). The occurrence of the parasite at high rates in rats and in specific groups of snails, earlier findings of eosinophilic meningitis in a resident, and the recent outbreak of *A. cantonensis*-associated eosinophilic meningitis in visitors to the island suggest that autochthonous transmission to humans is probable in Jamaica. These studies are being extended to determine the full distribution of the parasite and the species of snails involved in its transmission. Furthermore, serologic tests need to be developed to confirm infections in persons in the Caribbean.

Public health officials, clinical parasitologists, and travel medicine practitioners should consider *A. cantonensis* as a causative agent of eosinophilic meningitis in Jamaican residents and travelers to the island.

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Recent Increase in Meningitis Caused by *Neisseria meningitidis* Serogroups A and W135, Yaoundé, Cameroon

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From 1991 to 1998, *Neisseria meningitidis* serogroups A, B, and C represented 2%-10% of strains isolated from cases of bacterial meningitis in Yaoundé. During 1999 to 2000, the percentage of meningococci reached 17%, a proportion never reported since recordkeeping began in 1984. The increase of serogroup A meningococci and the emergence of W135 strains highlight the need for increased surveillance for better diagnosis and prevention.

Neisseria meningitidis serogroup A causes major epidemics of meningitis in Africa, essentially within the African meningitis belt (1)**.** Epidemics of cerebrospinal meningitis in this belt are often enormous (1). During the first 9 months of 1996 in the World Health Organization (WHO) African Region, 146,166 cases were reported to WHO; 15,783 were fatal. During that year, 42,129 cases occurred in Burkina Faso, 7,244 in Mali, 16,050 in Niger, and 75,069 in Nigeria. These four countries reported 95% of the cases in Africa in 1996, for an overall case-fatality rate of 10.6% (2).

The recommended control practices in Africa involve vaccination with the meningococcal bivalent polysaccharide A/C vaccine in response to epidemics. Efficient public health practice necessitates that epidemics be detected early, stocks of vaccines be set up in target regions, and field vaccination with the bivalent vaccine be rapid, since the quadrivalent ACYW135 vaccine has limited worldwide supply and is more expensive.

The presence of *N. meningitidis* serogroup W135 has been confirmed in Africa for some time. In Burkina Faso in 1980, 1.3% of the meningococcal strains isolated from rhinopharyngeal carriers belonged to serogroup W135. In 1981 and 1982, monitoring of the serogroups responsible for meningococcal meningitis at Dakar (Senegal) and Niamey (Niger) showed that 4% and 3% of strains, respectively, belonged to serogroup W135 (3). In 1984 and 1985**,** 7% of *N. meningitidis* strains isolated from meningitis cases in Gambia belonged to serogroup W135 (4). In 1993 and 1994, two strains of *N. meningitidis* W135 were isolated from patients in Mali; both belonged to the ET-37 complex (5). More recently, in 1994, six strains of serogroup W135 isolated from clinical cases in Gambia were studied; they also belonged to the ET-37 complex. DNA macrorestriction analysis of these strains identified four different profiles in pulsed-field gel electrophoresis (PFGE), indicating that the strains involved were closely related but different (6). W135 strains are often isolated after intensive campaigns of vaccination against meningococci of serogroups A and C (3,4,6).

In spring 2000, an epidemic of *N. meningitidis* W135 infection broke out among Hajj pilgrims (for whom vaccination against meningococci of serogroups A and C is mandatory) and their close contacts. In all, 241 cases were reported in Saudi Arabia and 90 in 13 other countries (7), including the United States (4 cases) (8), the United Kingdom (33 cases), and France (19 cases). All these strains showed markers of the ET-37 complex; had an antigenic formula W135:2a:P1-5,2; a sequence type ST-11; and the same profile on PFGE (9), confirming the clonal origin of the epidemic. Four W135 strains isolated in U.S. patients epidemiologically linked to Hajj pilgrims were further studied. The sequence of the *porA* gene showed that these four strains had variable regions VR1 and VR2 identical to those of the prototype P1.5,2 strain (8).

Apparently, the W135 strains isolated in Africa until 1995 did not cause large epidemics, even if isolated in the countries in the African meningitis belt in which epidemics due to serogroups A meningococci are frequent (e.g., Niger, Mali, Senegal, and Gambia). In Niger in 1981, only one W135 strain of 231 meningococci was isolated from a meningitis case (3). Similarly, W135 accounted for 7 of 42 strains in 1982 in Niger, and 3 of 76 strains in Senegal in 1981 to 1982 (3), 3 of 41 in Guinea in 1984 to 1985 (4), and 2 of 75 strains isolated in 1991 to 1994 in six countries in the African meningitis belt (5). However, available information shows that the case-fatality rate due to W135 strains was relatively high in Africa before 1995, as in Europe during the recent Hajj 2000 epidemic: 6 (35%) of 17 cases in Africa before 1995 (in Senegal, Gambia, and Niger) and 10 (18%) of 56 cases in Europe in 2000 (in the United Kingdom, France, and the Netherlands).

The Study

We report here a sudden increase in the number of meningococcal strains isolated from cerebrospinal fluid (CSF) sent to the Medical Biology Laboratory of the Pasteur Centre of Cameroon (CPC) at Yaoundé in the 1-year period 1999 to 2000 (note that in Cameroon the administrative year begins on July 1). Yaoundé, the capital of Cameroon, is a city of approximately 1,500,000 inhabitants. Located in the forest zone at an altitude of 750 m, about 400 km south of the southern limit of

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the African meningitis belt, it has a humid, tropical climate. The CPC laboratory receives samples from patients admitted to the principal hospitals of Yaounde and, in 1999 to 2000, 91.5% of CSF samples sent to the laboratory were from children <15 years old; 81% were from children <5 years.

From 1984 to 1990, bacteria were isolated from 767 (5.8%) of 13,134 CSF samples; 42 (5.5%) of these were *N. meningitidis.* This proportion was significantly lower than during the 1991 to 2000 period (8.7%; $p<0.05$). We do not know if there were changes in the population of patients using the CPC services that might account for the slow increase in cases of meningococcal disease. Slow improvement of laboratory practices and medical competence might account for slowly increasing proportions of *N. meningitidis* over a 20-year period. However, no major changes in laboratory techniques occurred in 1999 at CPC that could account for the sudden increase observed in 1999 and 2000. Moreover, during that 20-year period, most CSF samples came from the same pediatric wards of the neighboring Central Hospital of Yaoundé and other major children's hospitals.

The table shows the changes in isolation rates of *N. meningitidis* from clinical cases at Yaoundé during 1991-92 to 2000-01. The number of meningococcal strains isolated has remained small for the last 10 years, as would be expected in a zone located at a considerable distance south of the African meningitis belt and one in which pneumococci and *Haemophilus influenzae* are the two most frequent bacterial agents of meningitis. Most meningococcal strains isolated were serogroup A, the most frequent group in Africa. The proportion of meningococci identified in cases of bacterial meningitis varied significantly in this period ($p<0.01$).

In the 2-year period 1998 to 2000, two events occurred. The first was an increase in the number of isolates of *N. meningitidis*; such isolates accounted for >19% of strains isolated from cases of bacterial meningitis in 1999 to 2000, and 21% in 2000 to 2001—two to three times more than normal. We checked records back to 1984 and found that in none of the years in this period was such a large number of meningococci isolated at Yaoundé. The second noteworthy event was the appearance of serogroup W135 strains, which accounted for 9 (19%) of 48 meningococcal strains isolated in 1998 to 2000 versus 0 of 46 in 1991 to 1997 ($p<0.01$).

One W135 case occurred in January 1999 in a 12-year-old boy from Yaoundé who had no known contact with a Hajj pilgrim and no recent history of travel. On the four W135 patients from 1999 to 2000, one was male and three were female. Ages were 2, 3, 29, and 37 years. Onsets of disease were in July 1999 and in May and June 2000, i.e., after the usual meningococcal peak in the dry season, and none had known direct or indirect contact with each other or with a Hajj pilgrim. In 2000 to 2001, four cases occurred, all in males (aged 9, 15, 23, and 40 years); onsets of diseases occurred in January 2001, then in March, May, and June. One of them was in a 23-year-old student, who had been studying in Dakar (Senegal) for 2 years; he became ill while observing holy days in Cameroon. He could have been in indirect contact with Hajj pilgrims, since he was Muslim and Senegal is largely Muslim. Vaccination status was obtained for five of these nine patients: two were vaccinated against meningococcal meningitis, including the student.

The five strains of *N. meningitidis* W135 isolated in 1999 to 2000 were serotyped, subtyped, and studied by molecular biology techniques. All belonged to the ET-37 complex and had the antigenic formula W135:2a:P1.2,5. Two strains were subjected to multilocus sequence typing; both were of sequence type ST-11, typical of isolates of the ET-37 complex (10). These five strains were indistinguishable by multilocus DNA fingerprinting and showed markers of E-37 complex (11). Finally, *Spe*I restriction profiles were determined by PFGE: four of the strains were indistinguishable, and the final strain

Table. Isolation of *Neisseria meningitidis* from meningitis cases at Yaoundé, Cameroon, 1991–2001

Year	No. of CSF samples	No. (%) of cases of bacterial meningitis	No. (%) of cases of meningococcal meningitis	No. of strains of each serogroup
1991-1992	1,246	131 (10.5)	8 (6.1)	6 A; 2 C
1992-1993	1,049	105 (10)	11 (10.5)	11 A
1993-1994	961	88 (9.2)	9 (10.2)	8 A; 1 B
1994-1995	722	69 (9.6)	6 (8.7)	2 A; 4B
1995-1996	998	70 (7)	4 (5.7)	1A; 1B; 1C; 1NT
1996-1997	1,255	97 (7.7)	2 (2.1)	2C
1997-1998	1,282	92 (7.2)	6 (6.5)	4A; 1C; 1NT
1998-1999	1,505	116 (7.7)	8 (6.9)	6A; 1B; 1W135
1999-2000	1,812	120 (6.6)	23 (19.2)	17A; 2B; 4W135
2000-2001	1,612	81 (5)	17 (21)	13A; 4W135
Total	12,442	969 (7.8)	94 (9.7)	68A; 9B; 6C;9W135; 2NT

NT: not serogrouped; CSF: cerebrospinal fluid.

differed by one band only. All these clones differed slightly (by two bands for four isolates and by three bands for one isolate) from the clone isolated from the Hajj pilgrims in 2000.

Conclusions

These results show an increase of serogroup A meningococci in Yaoundé and demonstrate the presence and circulation of at least one indigenous clone of *N. meningitidis* W135 of the ET-37 complex in Central Africa. The clone is very similar to, but differs slightly from, the clone responsible for a meningitis outbreak among Hajj pilgrims in 2000 (8,9). Since none of the patients with W135 meningococci had direct contact with Hajj pilgrims and Cameroonian W135 strains are slightly different by PFGE from the W135 clone isolated in Europe and the United States in 2000, these strains from Cameroon seem to predate the 2000 Hajj-associated outbreak. A larger study of the W135 strains isolated in Africa, Europe, and Asia, from patients with no direct link to the pilgrimage to Mecca (indigenous strains) would make it possible to identify the geographic origin of the strain responsible for the Mecca epidemic in 2000. Such studies would also make it possible to elucidate the role of A and C vaccination in the selection of W135 clones belonging to the ET-37 complex.

We cannot explain with certainty why serogroup A meningococci has increased in Yaoundé, but the finding stresses the importance of continuous surveillance. The circulation of W135 strains in Central Africa raises questions about their epidemic potential and highlights the microbiologic surveillance of meningococcal meningitis. Thus, anti-W135 serogrouping antibodies are necessary for all National Reference Laboratory services. Antigen-detection kits for the diagnosis of meningitis should also contain anti-W135 antibodies. Moreover, the problem of the availability of a quadrivalent vaccine, including the W135 antigen, should be resolved. Strengthening the capacities for epidemiologic and microbiologic surveillance of meningitis in Africa is a prerequisite for prevention and control of meningococcal epidemics.

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HIV Prevalence in a Gold Mining Camp in the Amazon Region, Guyana

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The prevalence of HIV infection among men in a gold mining camp in the Amazon region of Guyana was 6.5%. This high percentage of HIV infection provides a reservoir for the virus in this region, warranting immediate public health intervention to curb its spread. As malaria is endemic in the Amazon Basin (>30,000 cases/year), the impact of coinfection may be substantial.

In Guyana and other South American countries containing large tracts of Amazon jungle, few studies have investigated the prevalence of HIV infection in isolated communities (1). Geographic isolation would lead to low infection rates because of lack of exposure to the disease. In addition, prevalence data on HIV can be negligible even in urban areas. For example, the only reported HIV studies in the Guyanese population have focused on HIV prevalence in commercial sex workers in the capital city of Georgetown (2). No studies have reported the prevalence of HIV in Guyanese men or in the interior Amazon region of the country. We evaluated a group of men living and working in the Amazon region of Guyana to determine the prevalence of HIV infection.

This study was conducted after we obtained Institutional Review Board approvals as well as permission from the Guyanese Ministry of Health and the director of a local Guyanese gold mining camp. Informed consent was obtained from each participant. Typically, men live in gold mining camps for periods of 6 to 8 weeks, working 12-hour days, 7 days per week. At the end of a 6- to 8-week shift, the men rotate out of camp to their homes on the coast for 2 weeks of rest. Mining gold in the Amazon region requires considerable manual labor and long hours working in a hot, humid jungle environment. Salaries paid to gold miners, however, are much better than those of typical jobs in the city. Thus, jobs in the mining industry are attractive, and many men leave their families and work as miners in the jungle for a few years to provide a better standard of living for their families.

The mining camp in this study was approximately 400 km inland from Georgetown, the capital of Guyana, in the heart of the Amazon region of the country. It was typical of many of the mining camps in the jungle (Figure). Men sleep in rows of 20 to 40 hammocks strung underneath a large tarp-like covering. The tarp coverings are not enclosed, but the men usually sleep under mosquito netting, as malaria infection is a constant problem. Pit latrines are available in the camp as are rainwater shower stalls. Water is obtained from a nearby stream, and a generator provides light in the camp in the evenings. The facility is fenced in and heavily guarded by armed patrols, as gold is stored in the camp from daily operations. The camp is a living facility only as all mining occurs outside the camp itself.

We enrolled almost the entire workforce of the mining camp (n = 216) for participation in this study. Only four declined the free HIV test and were excluded from the analysis. All 216 subjects were Guyanese men (age range 18-35 years). Pre- and post-HIV counseling was completed, and informed consent was obtained from all participants. Seven milliliters of venous blood was obtained from each participant after precounseling was completed. Onsite screening for HIV was completed, and serum was stored on ice and transported for confirmatory testing in a laboratory. Onsite HIV testing was by the Determine (Dainabot, Tokyo, Japan) rapid immunochromatographic test for the qualitative detection of HIV-1/2; in previous field work this test yielded 100% sensitivity and specificity (3). The test required 50 µL of serum, with results available for visual interpretation within 15 to 60 minutes. However, most of the positive samples produced a clearly visible red line within 10 minutes. HIV testing by enzyme-linked immunosorbent assay (ELISA) with Western blot confirmation (Abbott, Abbott Park, IL) was completed on all sera on our return from the jungle. Participants were not given results until confirmatory testing was completed. All participants were notified that results were available by a letter from the collaborating local physician, and all were offered personal counseling when they visited him for their results.

Fourteen (6.5%) of the 216 men were found to have HIV infection by results of both the onsite rapid strip test and

Figure. Typical living quarters for miners in the jungle, Amazon Basin, Guyana.

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subsequent ELISA and Western blot tests. Results obtained with the rapid test performed onsite had 100% agreement with those of the laboratory tests performed after our return.

Results of this small HIV screening study, indicating that 6.5% of men living in this remote camp were HIV positive, suggest enormous potential for further transmission of HIV in Guyana, in both jungle and urban environments. Migration of city dwellers into the Amazon jungle region may increase the risk of transmission of HIV to indigenous people. Conversely, gold miners can become infected with HIV during contact with commercial sex workers in small villages near the mining areas. HIV can then be further transmitted to the miner's spouse and unborn children on his return to the city. Whether the miners contracted the infection while living in the jungle or whether they entered the region already infected is unclear; however, the high percentage of HIV infection in this population provides a reservoir for the disease in this region and warrants immediate public health intervention to curb its spread.

Intervention is warranted to increase public awareness of HIV in underserved remote jungle and urban regions in Guyana. Rapid HIV screening tests, which can be completed without equipment or ancillary supplies, may provide an important tool for rapid screening and providing immediate feedback to patients. Initial counseling on risk-reducing behavior can be initiated onsite to provide an immediate intervention strategy to prevent the spread of the disease while follow-up testing with a confirmatory HIV test is completed.

Given the high numbers of malaria cases in the Guyana Amazon region, combined with this new evidence of potentially escalating HIV rates, studies are warranted to measure the impact of HIV/malaria coinfection. Reports showing an average of >30,000 cases of malaria per year over the past decade clearly designate this region as having a high rate of endemic malaria (4). Since T-cell and B-cell function, thought to provide a defense against malaria, are both adversely affected at the early stages of HIV infection and continue to deteriorate, this may contribute to higher rates of malaria mortality or more severe malaria symptoms, as the infected person's impaired immune system is less effective against the invading parasites. Conversely, malaria could exacerbate HIV infection (5), since the already compromised immune system may be overwhelmed by the multiple infection. Thus, HIV/malaria coinfection may contribute to increased rates of illness and death in the Amazon region. Recent studies on HIV/AIDS and malaria in Africa suggest that coinfection with these two diseases has become a concern in Africa (6,7). This problem also merits attention in the Americas so that further research, planning, and interventions will be focused in this region.

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Hajj-Related *Neisseria Meningitidis* Serogroup W135 in Mauritius

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Meningococcal disease is rare in Mauritius; only one case was reported from 1992 to 1999. However, since June 2000, four cases have occurred. Epidemiologic information and typing results indicate that these recent cases probably followed the introduction of *Neisseria meningitidis* W135 in Mauritius by pilgrims returning from the Hajj in 2000 and 2001.

Mauritius is a small tropical island in the Indian Ocean (population 1.2 million). The country is classified as a middle-income country by the World Bank; its primary commercial links are with Europe, the Indian subcontinent, and southern Africa.

Bacteriologic investigations for government health institutions in Mauritius are conducted in only one laboratory. These centralized results indicate that meningococcal disease is extremely rare in Mauritius. From 1992 to 1999, the only recorded infection occurred in a patient 3 days after he returned from the United Kingdom. However, since June 2000, four cases of meningococcal disease have occurred in Mauritians who have no history of travel outside the island.

Case Reports

Case 1

In June 2000, a 49-year-old Muslim woman was admitted to the hospital with a purpuric rash. She was initially thought to have a hematologic disorder before meningococcal septicemia was suspected. Despite treatment with intravenous penicillin and cefotaxime, she died the following day. *Neisseria meningitidis* was isolated from her blood cultures. A latex agglutination test with polyvalent reagent was positive for groups ACYW135 (Wellcogen bacterial antigen kit, Murex, Dartford, UK), but negative results were obtained with monovalent sera for group A, B, and C antigens (Slidex, Biomérieux, Marcy l'Etoile, France). The isolate was subsequently typed by the World Health Organization (WHO) Collaborating Centre for Meningococcal Infections in Marseilles, where it was confirmed as *N. meningitidis* serogroup W135, type 2a, subtype 1.2,5. It belonged to ST-11, the same sequence type as isolates obtained from English and French pilgrims returning from the Hajj that same year (1). The patient had no history of travel or close contact with a returning pilgrim, according to relatives.

Case 2

In July 2000, a 5-year-old Muslim child was admitted to the hospital with fever, vomiting, and ecchymoses. Initially she was thought to have a bleeding disorder. She had already received intravenous amoxicillin when meningococcal disease was suspected and specimens for microbiologic investigation taken. Her cerebrospinal fluid (CSF) was turbid with a leukocyte count of 11,500 per µL. CSF was positive for *N. meningitidis* antigens, groups ACYW135 with polyvalent serum, and negative for *Haemophilus influenzae* type b, pneumococcus, and meningococcus groups A, B, and C. Cultures of CSF and blood were negative. Cefotaxime was added to her treatment, and she made a good recovery.

The child's father, who returned from the Hajj 3 months earlier, had received the meningococcus A+C bivalent vaccine before travel. He had not been clinically ill during the pilgrimage or after his return.

Case 3

In November 2000, a 27-year-old man was admitted to the hospital with ecchymoses and signs and symptoms of meningitis. He had received antibiotics before investigations were performed. His CSF was turbid, with 26,000 leukocytes and 10,000 red cells per µL. Results of antigen testing on blood and CSF specimens were similar to those of the child in case 2, and cultures were negative. He was treated with cefotaxime and fully recovered. The patient was not Muslim and had not had close contact with a returning pilgrim. However, he and the patient in case 1 lived in the same village (population 10,000) as 22 pilgrims from the 2000 Hajj.

Case 4

In April 2001, a 4-month-old Muslim infant was hospitalized with fever and ecchymoses. Her CSF was turbid with 900 leukocytes per µL, and *N. meningitidis* was confirmed by culture. She was treated with cefotaxime and made a full recovery. Her father had returned from the Hajj 2 weeks before her onset of symptoms, but apart from a cough and cold, he had been clinically well. However, meningococcus was isolated from his oropharynx, as well as from the throat of the patient's 2-year-old brother. Isolates from the patient and her father and brother were positive by agglutination with meningococcus ACYW135 polyvalent reagent and negative with monovalent sera for meningococcus A, B, and C.

The pilgrim's vaccination certificate confirmed that he had received a quadrivalent meningococcal vaccine. All three isolates were sent to the WHO Collaborating Centre, which confirmed meningococcus serogroup W135, type 2a, subtype 1.2,5. They were indistinguishable by pulsed-field gel electrophoresis from isolates obtained from French pilgrims in 2000.

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Close contacts of all four patients with meningococcal disease received antimicrobial prophylaxis within 24 hours of diagnosis: a single 500-mg dose of ciprofloxacin was given to adults, and 2 days of rifampicin (10 mg/kg twice a day) was given to children. Close contacts of the infant (case 4) were also given the quadrivalent meningococcal vaccine. The only meningococcal carriage studies were conducted on some close contacts of this infant. No case secondary to those reported above is known to have occurred.

Two of these patients had meningococcal disease that was shown by typing to have been caused by the same W135 clone isolated from pilgrims returning from the Hajj in 2000 and 2001. Case 2 was culture negative but epidemiologically linked with a returning pilgrim and was probably caused by the same clone. Case 3 was also culture negative, but antigen tests indicated that infection was caused by meningococcus group W135 or group Y.

Conclusions

Cases of W135 meningococcal disease in returning pilgrims and their contacts have been reported in several countries following the Hajj of 2000 (1) and 2001 (2). In many European countries, further cases later occurred in persons with no history of close contact with a returning pilgrim (3).

About 16% of the Mauritian population are Muslims, and approximately 1,800 and 2,200 pilgrims traveled from Mauritius to Saudi Arabia for the Hajj in 2000 and 2001, respectively. In 2000, Mauritian pilgrims received the bivalent A+C meningococcal polysaccharide vaccine, but five Mauritians reportedly died in Saudi Arabia of meningitis of unspecified type. Cases of meningococcal disease in Mauritius itself occurred 3 months after the pilgrims returned, a period that coincided with the annual peak in upper respiratory tract infections (4). Viral upper respiratory tract infections are known to increase the risk of meningococcal disease (5). After the infections were reported in 2000, the Mauritian government decided to import a quadrivalent meningococcal polysaccharide vaccine (Mencevax ACWY, SmithKline Beecham, Genval, Belgium) for pilgrims attending the Hajj in 2001, and almost all of them received that vaccine. Although no case of meningitis was subsequently reported among these pilgrims, case 4 suggests that some have become carriers of meningococcus W135.

Evidence indicates that the quadrivalent meningococcal polysaccharide vaccine may not prevent asymptomatic nasopharyngeal infection with *N. meningitidis* serogroup W135 (6). Prophylactic administration of antibiotics to returning pilgrims may be indicated to reduce the risk for transmission to close contacts. A recent study in the United States showed that 0.8% of 727 returning pilgrims in 2001 were carriers of W135 meningococcus, compared with none on departure (7). All these pilgrims are presumed to have received the quadrivalent meningococcal vaccine. In view of the low carriage rate, administration of chemoprophylaxis to all returning pilgrims was not recommended (7). However, these findings may not be applicable to pilgrims from developing countries. During the Hajj, pilgrims from poorer countries often live in more overcrowded accommodations than those from more affluent regions, which may increase the risk of droplet transmission and result in higher rates of asymptomatic carriage.

Because several countries do not have scheduled flights to Saudi Arabia, many pilgrims travel on chartered airplanes, which would facilitate the administration of prophylaxis at the airport to returning pilgrims. The effectiveness of single-dose oral ciprofloxacin (8) simplifies the task. The disadvantages of ciprofloxacin prophylaxis must, however, be considered, including the small risk for anaphylaxis-like reaction (9). The growing problem of antibiotic-resistant organisms cannot be ignored, as fluoroquinolone resistance in the related species *N. gonorrhoeae* has already emerged in many places (10). Children and pregnant women, who likely represent only a small proportion of pilgrims, could be given intramuscular ceftriaxone, but there is also a risk of anaphylaxis besides the disadvantages of parenteral therapy. The decision to administer chemoprophylaxis to all returning pilgrims should therefore depend on whether transmission of W135 meningococcus during future pilgrimages continues. Information regarding continuing transmission would be useful before pilgrims return to their homes.

During Hajj 2001, many pilgrims, especially from developing countries, were unlikely to have received the quadrivalent vaccine. The Ministry of Health of Saudi Arabia has recently specified that, beginning in 2002, pilgrims must have been vaccinated with a quadrivalent vaccine (2). This requirement may reduce future transmission. However, if cases continue to occur, many countries should consider prophylaxis for returning pilgrims. Clearly, surveillance for meningococcal disease in general and serogroup W135 in particular will remain important in the next few years.

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Contagion on the Internet

To the Editor: Computer viruses are designed to be pests, proliferating in uncontrolled ways and causing severe damage to electronic data. These malignant programs, which amplify between files and computers, are strikingly similar in virulence, modes of spread, and evolutionary pathways over time to the microbes that cause infectious diseases. Both biological viruses and these virtual viruses are transmitted from host to host. Computer viruses are a human invention; however, their development follows a well-recognized biological route. Relatively harmless ancestors gradually or step-by-step evolve into "pathogens;" the host develops adaptive defense mechanisms, which in turn select for new virus "variants;" eventually, equilibrium is reached between infection and host defenses. Comparing "virtual microbes" with their biological counterparts can help us control both.

The term "computer virus" is loosely used to describe computer "malware," an umbrella term that includes the following categories. 1) Viruses. A computer virus is a program that implants a version of itself in any program it can modify. The modified program, once run, attempts to modify other programs directly associated with it. Computer viruses spread by sharing data on infected disks or diskettes. Unlike their biological counterparts (which are fast and very infectious), computer viruses spread slowly and infrequently between computers. 2) Worms. A worm is a self-contained program that replicates itself and sends copies to any connected computer, with little or no user interaction. Unlike biological worms (which spread slowly), computer worms spread rapidly and without much user interaction between computers of a network, including the Internet. (In view of the contagiousness of biological worms and viruses, the terms should have been reversed.) 3) Trojan horses. A Trojan horse is a program concealing harmful code that usually makes a computer or network available to unauthorized users in an appealing or unsuspicious package. A virus, worm, or Trojan horse can be latent (then also called a logic bomb) and become active only after a certain period.

Each class of computer malware has hundreds of variants, and many variants have several slightly modified versions, paralleling microbial diversity. Worms, such as the infamous "ILOVEYOU" worm in 2000, may employ a universal message of gratification to entice users. Their wide dissemination parallels the spread of socially transmitted diseases (e.g., influenza) that have the potential to infect everyone susceptible. In contrast, computer viruses (spread by sharing data on infected diskettes) parallel sexually transmitted diseases, whose spread is related to specific behavioral practices. Viruses or worms that are spread undetected but are activated at a later date (as was the case with the Michelangelo virus, discovered in 1991 and still around) resemble latent microbes, such as HIV. Denial-of-service attacks, which block access to a server by an onslaught of messages, are the equivalent of toxins, since neither can reproduce in their host and are only harmful above a critical concentration. Spam (unwanted but harmless e-mail), the curse of computer users with slow modems through expensive telephone connections, resembles bacterial commensals that can injure the host only under specific conditions.

Biological viruses can mutate rapidly, create novel pathogenic and transmission routes, and develop antigenic variation to evade host immunity. In the computer world, worms exhibit similar behavior. Once a worm has been transmitted successfully, variants quickly emerge. These variants cause damage in similar ways but evade detection and impediments installed to provide "immunity" to the original "strain." Therefore, knowledge of biological infections can be used to predict and anticipate highly virulent computer infections.

Although the computer user has some recourse against computer viruses, the costs may be high. As with biological viruses, good hygienic practice is helpful. Just as they should wash hands frequently, avoid exposure to people with colds, or use condoms to protect against infectious diseases, computer users should mistrust (and thus not open) files received through unexpected channels or with unknown extensions or subject lines, request confirmation from the sender before opening attachments, and regularly back up hard disks to reduce the risk of losing data. The consequences of such actions in terms of time, disk space, and efficiency illustrate a biological truth: immunity has cost. Effective antiviral barriers are impediments to communication. Moreover, virus protection programs are only as good as the last virus recognized, providing only partial protection at best. Computer users have not always taken inconvenient precautions, even in view of serious consequences. ILOVEYOU was a worm that used the same mechanism of spread as Melissa, which had been released a year earlier. Yet, ILOVEYOU turned out to be even more destructive than Melissa.

Biological immune disorders in which host defenses turn against the host and actually cause damage are known as autoimmune diseases. Computer autoimmune disorders parallel their biological counterparts. Recently, a warning (defense mechanism used by computer users) turned out to be a not-so-harmless hoax. The hoax warning stated that certain files were infected by a computer virus. Heeding the warning, unsuspecting computer users removed the affected utility files from their computers' operating systems. The harm mediated by this "host defense" was relatively small in this particular case, resembling the discomfort of allergies, in which immune responses to benign

agents cause limited damage. However, more malignant forms of "auto-mutilating" hoaxes are likely to emerge that could be as devastating to computers as some autoimmune diseases are to humans.

The electronic monoculture that improves communication also increases the risk for contagion. Predominant use of a single operating system has improved communication and sharing of electronic data but has also facilitated ready amplification of virulent programs. As with biological infection, transmission of computer infection depends on susceptibility of the population. Virus producers saw an opportunity in the popular preference worldwide for PCs with Microsoft Windows operating systems. The enormous popularity of these systems, along with their long-recognized inadequate protection against misuse, made computer users susceptible. Virtual viruses able to infect multiple operating systems are rare (as are biological viruses with broad host specificity), and even when infected, computers that run on different operating systems (e.g., Mac, Unix) or other-than-Outlook e-mail programs usually are dead-end hosts for PC viruses.

Pathogens do not reinvent the wheel. Virulence genes are constantly "stolen" and reused. Thus, new combinations of virulence genes can result in new pathogenic strategies, and such combinations frequently accumulate in pathogenicity islands. Reuse and combination of effective (and infective) strategies are also common in computer malware. A recent example demonstrates the value of just the right amount of virulence. A highly dangerous worm called Nimda (Admin in reverse) was released exactly 1 week after the September 11, 2001, terrorist attack in the United States. Nimda combined the most powerful strategies of Code Red and SirCam and spread more rapidly than any previous worm. Clicking on the subject line of an infected e-mail (to delete it, for instance) itself activated the worm. However, because of the immensity of the threat, the Internet community responded extremely rapidly. Within hours after its release, alerts to system administrators on how to block the worm had effectively slowed its spread. Early surveillance and barrier development averted disaster. As in contained epidemics of hemorrhagic fevers, the immense threat of high contagion and lethality prompts effective measures to rapidly recognize outbreaks and prevent pandemics.

The types of measures to be used against computer contagion can be learned from biology. Immune effectors of plants and animals protect against a broad range of pathogens; however, in nature this system evolved over millions of years. Engineering protective computer systems with similar efficacy within a few years is a great challenge. Current protection programs mainly resemble innate immunity, but programs that learn from exposure (thus resembling adaptive immunity) are under development. Vaccination with relatively harmless microbes primes the immune system. Biological hosts also naturally carry protective microflora that compete with pathogens. Could we produce "virtual vaccines" that are beneficial to the computers carrying them (e.g., by blocking preferred sites of entrance for viruses or repairing viral damage automatically) and let these "good" microbes circulate on the Internet just as malignant viruses do? Crude versions of such vaccines have already been developed. Using "good" microbes would have its costs: occupation of Internet capacity and consequent slowdown of data transmission and presence of malicious worms disguised as beneficial ones to elude detection.

Knowledge of infectious diseases may help control computer contagion. Conversely, study of computer malware may help curb infectious disease emergence. Internet contagion illustrates how pathogens emerge and spread in our increasingly small world. The speed of virtual pathogen evolution makes it possible to follow the process of mutation and selection in real-time. With countless interlinked computers, the risk for virtual contagion is so great that urgent steps are needed to avoid catastrophe. How many pandemics will it take before we accept the risks and costs of computer immunity? Similarly, to protect against emerging pathogens, we must use all tools available, including virtual pandemics. A task force to collect data on the epidemiology of virtual infections as a model for infectious diseases might be an important first step.

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Emergence and Rapid Spread of Tetracycline-Resistant *Vibrio cholerae* Strains, Madagascar

To the Editor: The Indian Ocean was free of cholera for decades, until January 1998, when an outbreak was detected in Comoros Islands (1). On March 23, 1999, the Malagasy Epidemiological Surveillance System reported the first case of cholera in Mahajanga, a harbor on the northwest coast (2). In May 1999, the Malagasy sanitary authorities set up sanitary barricades at the borders of the two provinces—Mahajanga and Antananarivo—affected by the epidemic. Oral doxycycline was systematically given to all the travelers crossing the barricades. In addition, doctors in hospitals and dispensaries in these two provinces gave doxycycline to patients

with acute diarrhea. Despite these measures, cholera had reached all six provinces of the island 10 months later. In June 1999, a specific cholera surveillance system was established in every Malagasy province with close collaboration between the Malagasy Ministry of Health and the Institut Pasteur de Madagascar.

The first strain isolated in Mahajanga was *Vibrio cholerae* serogroup O1, serotype Ogawa, biotype El Tor. Its antibiotype showed resistance to trimethoprim-sulfamethoxazole, sulfonamides, trimethoprim, chloramphenicol, streptomycin, and vibriostatic agent O129 (a molecule naturally active against *V. cholerae* and used for identification). Susceptibility was conserved for tetracycline, ampicillin, cephalotin, and pefloxacin (2). This strain showed a rRNA gene restriction pattern similar to those of African and Comorian strains isolated since 1994 and 1998, respectively (2,3).

From July 1999 to March 2001, we monitored the tetracycline resistance of *V. cholerae* isolated from the stool samples sent to the Institut Pasteur de Madagascar in Antananarivo, using the standard disk-diffusion method (4). Stool samples were collected in sterile containers, on Whatman paper, or on rectal swabs. Isolation of *V. cholerae* was carried out immediately after reception. Every *V. cholerae* strain identified belonged to serogroup O1, biotype El Tor. All the tetracycline-resistant *V. cholerae* isolated and 60 randomly selected tetracycline-susceptible strains were tested for sensitivity to the following drugs: ampicillin, cephalotin, doxycycline, sulfonamide, trimethoprim, trimethoprim-sulfamethoxazole, chloramphenicol, streptomycin, spectinomycin, neomycin, kanamycin, nalidixic acid, pefloxacin, erythromycin, rifampicin, and nitrofurantoin, as well as to vibriostatic agent 0129.

During the study period, we isolated 351 (46.1%) *V. cholerae* strains from 761 stool samples analyzed. The provinces of Antananarivo, Mahajanga, and Toliary accounted for 85.9% of the stool samples sent to our laboratory. From these provinces, we isolated 288 strains; by contrast, from the three other provinces (Antsiranana, Fianarantsoa, and Toamasina, located on the east coast), 63 strains were isolated. Rates of isolation, tested by a chi-square test, did not differ significantly between the six provinces ($p=0.32$).

Fifty five (15.7%) of the 351 strains isolated were found to be tetracycline resistant (cross-resistance with doxycycline) but had the same resistance pattern as the index strain isolated in Mahajanga for the other antibiotics tested. During the first rainy season following the epidemic (November 1999 to March 2000), a unique tetracycline-resistant strain

was isolated (in February 2000), in the capital Antananarivo; it was also resistant to ampicillin, nalidixic acid, and nitrofurantoin. During the dry season (from April to October 2000), five (13.2%) of 38 *V. cholerae* new tetracycline-resistant strains were found. However, during the last rainy season (November 2000 to March 2001), 49 (69 %) of 71 strains isolated were tetracycline resistant. They were mainly from the city and suburbs of Antananarivo (95.3%, 41/43 strains). The eight other resistant strains came from the provinces of Antananarivo, Toliary, and Fianarantsoa.

As observed in Tanzania (5), the extensive prophylactic use of tetracycline may have triggered the rapid emergence and spread of tetracycline-resistant strains in Madagascar. The high rate of resistance in Antananarivo, where the major Malagasy hospitals are located, could be due to easier access to drugs in the capital than in the other provinces.

Of the 60 randomly selected tetracycline-susceptible strains, 56 had the original antibiotype; four became susceptible to vibriostatic agent O129 and to all the antibiotics tested, except trimethoprim. Four (3.5%) of the 115 strains tested (55 tetracycline-resistant and 60 tetracycline-susceptible strains) on a large panel of antibiotics were susceptible to trimethoprim-sulfamethoxazole. As usually observed in other African cholera-endemic countries (6), only a small proportion of the strains were susceptible to trimethoprim-sulfamethoxazole, one of the most frequently dispensed drugs.

Faced with this first emergence of cholera in Madagascar and its rapid spread, medical authorities reacted immediately by using doxycycline as chemoprophylaxis (contrary to World Health Organization recommendations [7]), probably because of its easy availability**.**

Our study demonstrates that 2 years after the epidemic began, neither trimethoprim-sulfamethoxazole nor tetracycline, the two first-line drugs used in Madagascar, can be recommended any longer for treating severe cases of cholera. This may represent a critical public health problem in the country, especially as most of the population cannot afford more effective but expensive antibiotics.

Therefore, Malagasy medical authorities should a) abandon any systematic chemoprophylaxis, b) advise only oral rehydration therapy for mild-to-moderate cases, and c) reserve antibiotic therapy for severe illness (7). These measures against the cholera epidemic should be accompanied by general reinforcement of microbiologic surveillance to monitor antibiotic resistance so that the island can respond effectively to any future bacterial epidemics.

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Guidelines for Letters. Letters discussing a recent Emerging Infectious Diseases article (400-500 words, 5-10 references) should be received within 4 weeks of the article's publication. Letters reporting preliminary data (500-1,000 words, 10 references) should not duplicate other material published or submitted for publication, should not be divided into sections, and should avoid figures or tables. All letters have the same authorship, financial disclosure, and acknowledgment requirements as full articles and should include a word count. For more guidance on manuscript preparation, see Emerging Infectious Diseases Instructions to Authors. Send letters to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA, or e-mail: eideditor@cdc.gov .

Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

Fifth Annual Conference on New and Re-Emerging Infectious Diseases

University of Illinois,
Urbana-Champaign, IL
April 18-19, 2002

The Fifth Annual Conference on New and Re-Emerging Infectious Diseases will be held April 18-19, 2002. On April 18, 2002 the conference will be held at 4:00 p.m. at the University of Illinois at Urbana-Champaign, Bevier Hall Auditorium.

The keynote speakers are Dr. Beatrice H. Hahn, Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL (SIV reservoirs and human zoonotic risk) and Dr. Dr. Matthew K. Waldor, Howard Hughes Medical Institute, Division of Geographic Medicine/Infectious Diseases, New England Medical Center and Tufts University School of Medicine, Boston, MA, (Cholera toxin). On April 19, 2002, morning and afternoon sessions will be held at Hawthorne Suites Hotel, Champaign from 8:15 am to 6:00 p.m. Invited speakers and topics include: Dr. Edward M. Eitzen, Commander, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland (Biological weapons); Dr. John Donelson, Department of Biochemistry, College of Medicine, University of Iowa (The genome of African trypanosomes); Dr. Daniel Goldberg, Howard Hughes Medical Institute, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, (Genome-wide analysis of malaria parasites); Dr. Barry N. Kreiswirth, Director, Public Health Research Institute Tuberculosis Center, New York, N.Y. (Global dissemination of the multidrug resistant Mycobacterium tuberculosis).

Those interested in presenting a poster must submit an Abstract Form available at our web site: http://www.cvm.uiuc.edu/idc/. Submission deadline to abstracts is April 5, 2002.

Keystone Symposia on Gene-Based Vaccines:

Mechanisms, Delivery Systems and Efficacy

Beaver Run Resort
Breckenridge, Colorado
April 10–15, 2002

Gene-based vaccines (both plasmid- and vector-based) are considered a promising approach for making prophylactic and therapeutic vaccines against infectious diseases and cancer. However, a number of issues need to be resolved, including increasing the potency of the vaccines by understanding the mechanisms of DNA uptake and antigen presentation and determining how to harness the unexpected immunostimulatory effects of DNA itself. Participants will include scientists from a variety of disciplines (microbiology, virology, cell biology, vaccinology, gene therapy, oncology, infectious diseases, and polymer chemistry) to address the fundamental cellular mechanisms involved.

For more information, call 800-253-0685 or 970-262-1230 or visit www.keystonesymposia.org

Ninth International Conference on Lyme Borreliosis and Other Tick-Borne Diseases

Hyatt Grand Central
New York, New York, USA
August 18-22, 2002

Participants include health-care practitioners, public health officials, and allied health professionals who are actively involved and/or interested in research or management of Lyme borreliosis and other tick-borne diseases. Major program topics include Lyme borreliosis: state of the art and future research directions; diversity of Borrelia —clinical, pathogenetic, and diagnostic implications and impact on vaccine development; genetics of *Borrelia burgdorferi;* laboratory diagnosis; and strategies for prevention of Lyme borreliosis and other tick-borne diseases.

The deadline for abstracts is May 10, 2002. For further information, contact: Heather Drew, Imedex, 70 Technology Drive, Alpharetta, GA 30005; tel: + 1 770-751-7552; fax: +1 770-751-7334, e-mail: h.drew @imedex.com or online at www.imedex.com/infectiousdisease.htm

About the Cover

Flying Fox

Vincent van Gogh, 1885
(oil on canvas, 41 cm x 79 cm)

Courtesy of Van Gogh Museum, Amsterdam
(Vincent van Gogh Foundation)

According to the Van Gogh Museum in Amsterdam, the origins of Flying Fox are not well documented. Van Gogh probably saw a flying fox in a museum or private collection in Brabant, Antwerp, or Paris. The dark brown background colors in the painting are similar to those in other works of his Nuenen period. The brighter colors and rough brushstrokes in the wings are more avant garde and suggest techniques used in his later paintings.

Flying foxes like the one that captured van Gogh's imagination are very large fruit-eating bats (order Chiroptera, suborder Megachiroptera). These mammals are found in tropical and subtropical regions between Africa and the South Pacific, including the Philippines, where there are 70 species of bats. Flying foxes can weigh as much as 1.5 kg and have a wingspan of up to 1.8 m. Occasionally, they are hunted and used as a food source (1).

Worldwide, bats are a major predator of night-flying insects and farm pests. Throughout the tropics, they are vital to the survival of the rain forest through their seed dispersal and pollination activities. Studies of bats have contributed to medical advances, including the development of navigational aids for the blind.

Paul Arguin

Centers for Disease Control
and Prevention, Atlanta, Georgia

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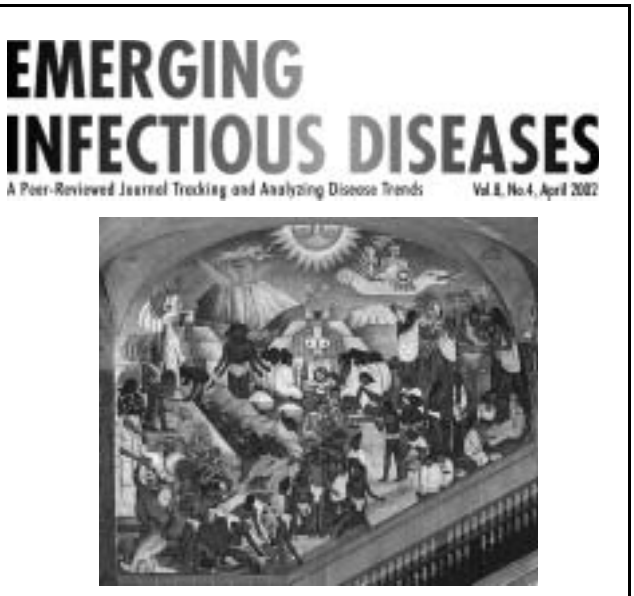

In the next issue

Feline Host Range of Canine Parvovirus (CPV): Recent Emergence of New Antigenic Types of CPV in Cats

Megadrought and Megadeath in 16th Century Mexico

Experimental Infection of Horses with West Nile Virus and Evaluation of Their Potential to Infect Mosquitoes and Serve as Amplifying Hosts

Outcomes of Persons with Treated Human Granulocytic Ehrlichiosis

Nine Years of Survey of European Bat Lyssavirus Infection in Spanish Bat Populations

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

Editorial Policy and Call for Articles

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

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

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

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

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). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Arial. Mac files should be sent in MS Word or RTF formats.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in 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 in full. List the first six authors followed by "et al."

Tables and figures. Create tables within MS Word's table tool. Do not format as columns or tabs; do not submit tables in Excel. For figures, use color as needed. Send digital files (see above) or camera-ready slides, photographs, or laser prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or 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: 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. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles 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. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Another Dimension: Thoughtful essays on philosophical issues related to science and human health.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.