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On the Cover

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(detail on front cover)
(China circa 16th century)
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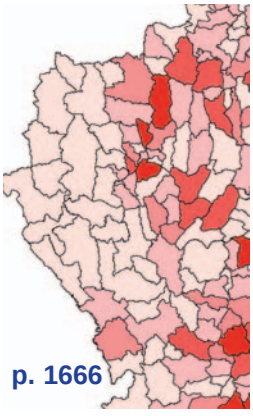
About the Cover p. 1810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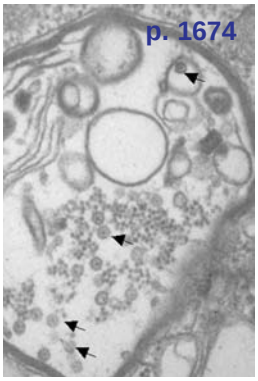
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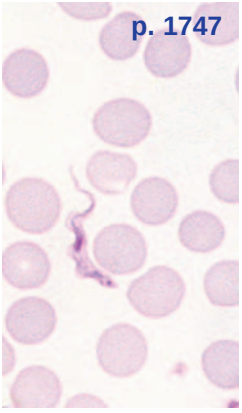
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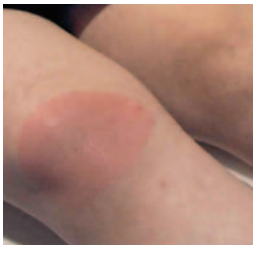
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Emergence of Toscana Virus in Europe

Rémi N. Charrel,* Pierre Gallian,*† José-María Navarro-Marí,‡ Loredana Nicoletti,§ Anna Papa,¶ Mária Paz Sánchez-Seco,# Antonio Tenorio,# and Xavier de Lamballerie*

Toscana virus (TOSV) is an arthropodborne virus first identified in 1971 from the sandfly *Phlebotomus perniciosus* in central Italy. Many case reports in travelers and clinical research and epidemiologic studies conducted around the Mediterranean region have shown that TOSV has a tropism for the central nervous system (CNS) and is a major cause of meningitis and encephalitis in countries in which it circulates. In central Italy, TOSV is the most frequent cause of meningitis from May to October, far exceeding enteroviruses. In other northern Mediterranean countries, TOSV is among the 3 most prevalent viruses associated with meningitis during the warm seasons. Therefore, TOSV must be considered an emerging pathogen. Here, we review the epidemiology of TOSV in Europe and determine questions that should be addressed in future studies. Despite increasing evidence of its major role in medicine as an emerging cause of CNS infections, TOSV remains an unstudied pathogen, and few physicians are aware of its potential to cause CNS infections.

Toscana virus (TOSV) was originally isolated in 1971 from the sandfly *Phlebotomus perniciosus* collected in Monte Argentario (Grosseto province, central Italy) (1,2). Thus far, most clinical and epidemiologic studies have been conducted in Italy, although studies from other Mediterranean countries have been published recently. From these, TOSV appears to be 1 of the 3 major viral pathogens involved in aseptic meningitis acquired during the summer in these countries. A bibliographic search using "Toscana virus" as keyword in the PubMed database retrieved 54 research and review articles. Less than 50% of them report imported or autochthonous human cases acquired in Italy, Spain, Portugal, France, and Cyprus. Even though evidence that TOSV plays a major role in human disease is increasing, it remains poorly studied, and physicians have little awareness of its potential to cause CNS infections.

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Virus Properties and Classification

According the 8th report of the International Committee on Taxonomy of Viruses, TOSV is a serotype of Sandfly fever Naples virus within the genus *Phlebovirus* in the family *Bunyaviridae*. TOSV is an arthropodborne virus. The lack of biochemical and genetic data for most phleboviruses dictates that the species are defined by serologic relationships and are distinguishable by 4-fold differences in 2-way neutralization tests. Phleboviruses contain a negative-sense, single-stranded RNA genome that consists of 3 segments, designated large, medium, and small, which encode the RNA-dependent RNA polymerase, the envelope glycoproteins, and the nucleoprotein, respectively.

Epidemiology of Phleboviruses and Toscana Virus

Phlebotomus (sandfly) fever viruses have been isolated from sandflies in southern Europe, Africa, central Asia, and the Americas, and evidence exists for the presence of different viruses in the same sandfly population. Sandfly fever Naples (but not the TOSV serotype) and Sicilian viruses have the widest geographic distribution, in parallel to their vector's (*Phlebotomus papatasi*) distribution. Until recent years, the known distribution of TOSV was limited to Italy and Portugal (3,4). In Italy, the virus was isolated from the vectors *P. perniciosus* and *Phlebotomus perfiliewi* and from humans, whereas the presence of the virus in Portugal was suspected on the basis of a strain isolated from the cerebrospinal fluid (CSF) of a Swedish patient who was returning to his home country from Portugal. More recently, the geographic distribution of the virus has been extended to France, Spain, Slovenia, Greece, Cyprus, and Turkey, according to results from viral isolation and serologic surveys (5–9).

Geographic Distribution of Toscana Virus

Italy

Preliminary clues pointing to the role of TOSV in CNS infections in Italy were provided by reports of imported cases diagnosed in the United States (10) and Germany (11). A large study carried out from 1977 to 1988 showed that the virus was the cause of meningitis in 2 regions of Italy, Tuscany and Marche, with a seasonal peak in August, which corresponded to the peak of sandfly activity (3). Since then, the virus has been isolated in other regions of central and southern Italy. More recently, research into TOSV as an etiologic agent of neurologic diseases has been carried out in Emilia-Romagna and Piedmont (12). Striking evidence that TOSV was the most prominent viral etiologic agent in summertime meningitis was reported in the late 1990s (13); in one of the most comprehensive studies, TOSV represented 81% of the viruses detected in CSF from patients who sought treatment for meningitis and other CNS infections (14). TOSV sequences were detected in 85 of 104 CSF specimens that provided positive results for viral sequence; However, 173 CSF specimens were negative by polymerase chain reaction (PCR); therefore, TOSV sequences were detected in 30% of the patients admitted for meningitis and in 40% of the patients admitted from June to November. A study of children living in rural or suburban areas of Siena (central Italy) showed that 40% of meningitis or encephalitis cases could be linked to TOSV infection (15). A 7-year study performed in Siena showed that 52% of aseptic meningitis cases in adults were associated with TOSV (seroconversion, presence of immunoglobulin M [IgM], PCR detection) (16). All studies agree regarding the monthly distribution of human cases of TOSV infections: the highest risk of acquiring TOSV is in August, then July and September, and finally June and October. Populations living in rural areas and with high levels of outdoor activity are at the greatest risk of TOSV infection. An occupational risk study conducted on forestry workers in Siena, Florence, and Arezzo showed that 77.2% of them had positive IgG for TOSV, compared with an urban population who exhibited a 22% prevalence for IgG. In contrast, 6% of forestry workers of the Piedmont area showed TOSV IgG (17). The first report of TOSV infection in Umbria was published in 2003 in the form of a retrospective study of 93 aseptic meningitis and meningoencephalitis cases. Of interest is the observed 16% of the healthy control population who were IgG positive (12). TOSV infections in Emilia-Romagna were documented for the first time in 2002 (18).

France

The first case of TOSV infection acquired in France was reported in a German traveler who was returning from southern France (19). During surveillance for West Nile virus in southern France, serum specimens from patients with suspected cases (meningitis) were tested for TOSV, and several contained specific IgM. Two cases of meningitis caused by TOSV were diagnosed by seroconversion and by viral isolation (6). Two cases (1 meningitis and 1 febrile illness) were recently reported (5). Together, data confirm that TOSV circulates in southeastern France and causes disease in humans.

Spain

The first case of TOSV infection reported from Spain occurred in a Swedish tourist after a visit to Catalonia and was documented by plaque reduction neutralization test (PRNT) (20). In the last 3 years, Spanish researchers and physicians have reported many cases and conducted large epidemiologic studies that established TOSV as 1 of the 3 leading causes of meningitis in Spain (Figure 1) (8,21,22,23). A large study conducted in different regions of Spain showed the presence of IgG antibodies to TOSV (26.2%), sandfly fever Naples virus (2,2%), and sandfly fever Sicilian virus (11.9%) in 1,181 adults and 87 children (21). In 2003, the EVITAR (Enfermedades Viricas Transmitidas por Artropodos y Roederes) network for the study of arthropod- and rodentborne viral diseases was created and sponsored by the Spanish Ministry of Health. Within this context, a study on seroprevalence in Granada showed a 24.9% seroprevalence rate. A significant increase was observed with age (9.4% in persons <15 years vs. 60.4% in persons >65 years). In addition, several cases of TOSV have been documented in the south, central, and Mediterranean areas. These data suggest that the situation in Spain is similar to that observed in France, with lower prevalence of CNS infections than that observed in central Italy.

Cyprus

Several studies were conducted in Swedish United Nations soldiers based in Cyprus in 1985. Blood samples were obtained from a 362-soldier battalion just before and immediately after their 6-month tour of duty. Of 298 serum pairs available, seroconversion to TOSV was observed in 1 patient who did not show any clinical manifestations (9). Seroprevalence studies showed that 20% of the healthy population had TOSV IgG (23).

Greece

Phleboviruses are found in Greece (24). Recent studies of populations living on the Ionian Islands and western mainland of Greece showed a seroprevalence of 60% and 35% respectively, by enzyme-linked immunosorbent assay (ELISA). However, so far, no studies have reported meningitis or encephalitis cases caused by TOSV in Greece.

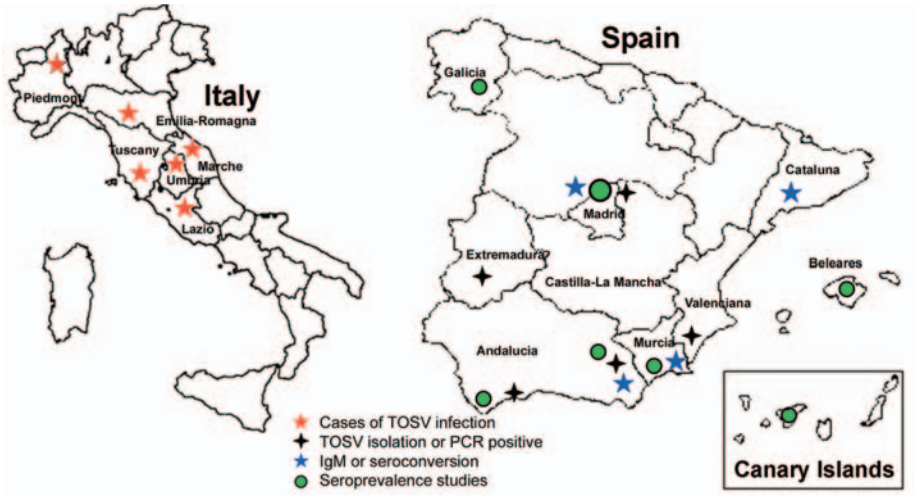

Figure 1. Provinces of Italy and Spain in which clinical cases of Toscana virus (TOSV) infection have been documented, and seroprevalence studies were conducted. PCR, polymerase chain reaction; IgM, immunoglobulin M.

Portugal

To date, Sweden has had 1 imported case in a man who had a severe headache and fever without neck stiffness after returning from Portugal. Viral isolation was successful and identification was performed by plaque neutralization (4). In addition, 1 German patient returning from vacation in Portugal had meningitis; diagnosis was established by ELISA and confirmed by immunoblot assay (25).

Germany

In a seroepidemiologic survey of 859 healthcare workers and medical students, anti-TOSV IgG was detected in 1.0% of samples by immunofluorescent assay (IFA), and in 0.7% by enzyme immunoassay (EIA). In 2,034 German patients, who were hospitalized for various diseases, 1.6% were positive for anti-TOSV IgG by IFA, and 0.8% by EIA. Anti-TOSV IgG was detected in 43 samples of commercial immunoglobulins at titers of 10–1,000 by EIA. Although the seroprevalence of antibodies to TOSV is low in Germany, TOSV infection should be considered in patients returning from virus-endemic areas who have fever and headaches or symptoms of meningitis (26).

Cycle in Nature

Vectors

TOSV was isolated from *P. perniciosus* and *P. perfiliewi* but never from *P. papatasi*. TOSV has also been isolated from the brains of the bat *Pipistrellus kuhli*, which was trapped in areas where *P. perniciosus* and *P. perfiliewi* are found (1,2). Transovarial transmission has been demonstrated in the laboratory and by viral isolation from male *Phlebotomus* spp. Venereal transmission from infected males to uninfected females has also been demonstrated. *P. perniciosus* is distributed throughout the Mediterranean region as 2 races. The typical *P. perniciosus* race occurs in Italy as well as in Malta, Tunisia, and Morocco. The Iberian race replaces it in southern Spain (with the pni mtDNA sublineage) (27).

Reservoir

The reservoir of TOSV is most likely the vector. Neither mammals nor birds have been recognized as a potential reservoir, although few studies have been carried out on mammals and almost none on birds. Whether humans can play a role in the virus cycle by infecting naïve sandflies is not known.

Although a number of phleboviruses have been isolated from the blood of sick persons and from wild animals, the role of vertebrates in the maintenance of the transmission cycle of these viruses remains unclear. Transient and low-level viremia is present after phlebovirus infection in humans and in susceptible laboratory animals (28–30). Moreover, sandflies must ingest a large quantity of virus to become infected (31). Verani et al. (1) examined different species of wild vertebrates (wild mouse, bank vole, stone marten, coypus, porcupine, bat, fox, and hedgehog) through serologic testing and viral isolation.

Disease in Humans

Clinical Forms

Seroprevalence studies suggest that a proportion of infections by TOSV are asymptomatic or paucisymptomatic. Additional studies will be necessary to evaluate the ratio of symptomatic versus asymptomatic or paucisymptomatic infections.

In some cases, TOSV infection causes a self-limiting febrile illness without CNS manifestations; these patients are not usually hospitalized, and their cases are not usually investigated further. This fact may account for the probable underestimation of TOSV infection rates.

After an incubation period ranging from a few days to 2 weeks, disease onset is intense (70%) with headache

(100%, 18 h–5 days), fever (76%–97%), nausea and vomiting (67%–88%), and myalgias (18%). Physical examination may show neck rigidity (53%–95%), Kernig signs (87%), poor levels of consciousness (12%), tremors (2.6%), paresis (1.7%), and nystagmus (5.2%) (L. Nicoletti, pers. comm.). In most cases reported so far, CSF contained >5–10 cells with normoglycorachia and normoproteinorachia. Blood samples may show leukocytosis (29%) or leukopenia (6%). The mean duration of the disease is 7 days, and the outcome is usually favorable.

Although TOSV infection in most cases consists of a mild disease with a favorable outcome, a small number of severe cases have been reported in the literature. Two young brothers and a sister living in Umbria experienced TOSV infection in the form of severe meningoencephalitis with stiff neck, deep coma, maculopapular rash, diffuse lymphadenopathy, hepatosplenomegaly, renal involvement, skin rash with lamellar desquamation, a tendency to bleed, and diffuse intravascular coagulopathy. CNS manifestations occurred after 3 weeks of fever. Convalescence was marked by hydrocephalus that required a ventriculoatrial shunt. Diagnosis was established by serologic means and by PCR sequencing (32). Two cases of encephalitis without meningitis were recently diagnosed by serologic testing and by detecting TOSV sequences in CSF (33). One case of meningitis, complicated by abducens nerve palsy, was reported (34). To date, no published data exist that suggest that TOSV causes any other manifestations. However, a substantial proportion of infection likely results in asymptomatic or paucisymptomatic cases (5,35).

Laboratory Diagnosis

Serologic Testing

Seroconversion and the detection of IgG, IgM, or both, can be achieved in cells infected with TOSV. However, cross-reactivity exists between members of the genus *Phlebovirus* and specifically between TOSV and other serotypes of sandfly fever Naples virus.

ELISAs have been developed with either crude antigens or purified virus obtained from infected cells. The advantage of ELISA resides in its capacity to rapidly test a large number of specimens; however, cross-reactions most likely will be observed. Recently, an ELISA test based on a recombinant nucleoprotein gene was developed and is now available commercially from an Italian company. Recent seroprevalence studies were based on this test (8,21).

PRNT is the test of choice when the virus species must be confirmed. Therefore, seroprevalence data must be carefully interpreted since in most cases, analyses were performed with ELISA or IFA that cannot discriminate between sandfly fever Naples virus, sandfly fever Sicilian virus, and TOSV.

Virus Isolation

Viruses can be isolated from clinical samples by using CSF but not serum. CSF specimens that yield virus through cell culture are collected in the first 2–4 days of the disease.

TOSV replicates in a variety of animals. Intracranial, intraperitoneal, and subcutaneous routes lead to death in newborn mice, and intracranial and intraperitoneal routes lead to death in weanling mice. This effect is seen with viruses from only a few families, including flaviviruses, which are also implicated in viral encephalitis. In guinea pigs and rabbits, intracranial injection results in paralysis and death, whereas intraperitoneal injection is not fatal and results in antibody synthesis.

TOSV replicates in Vero, BHK-21, CV-1, and SW13 cells with cytopathic effect and not in C6/36 cells. However, cell culture appears to have a low sensitivity for detecting TOSV since only 14% of the PCR-positive CSF specimens added to Vero cells led to viral isolation.

Molecular Techniques

In some cases, the relatively low level of virus in blood and CSF samples hampers attempts to isolate the virus. In such cases, molecular techniques based on PCR are more sensitive than IgM detection or viral isolation. Three different methods for molecular diagnosis of TOSV have been developed (Table). To date, all studies aimed at the molecular detection of TOSV sequences in the CSF of patients with meningitis or other CNS manifestations have used classic PCR detection through single-round or nested protocols. Tests of an RT-PCR assay alone, without a further nested PCR step, showed that this method did not appear to be valid for detecting TOSV, since no sample was positive after the first reaction. Two systems (14,36) use specific primers in the S segment, and the other is based on degenerate oligonucleotides targeting of the L segment (37). The most widely used has been successful for TOSV diagnosis in Italy (13) and France (R. Charrel et al. unpub. data). In 2003, a new method for detecting TOSV of Italian or Spanish origin was produced by using degenerate primers. The description of 2 genotypes of TOSV demonstrates a need for caution when designing molecular methods for diagnosis to avoid false-negative results. Recently, real-time PCR systems, including a fluorescent dye–labeled probe, have dramatically reduced the risk of contamination. The sensitivity of real time RT-PCR is close to that obtained by nested PCR protocols, and the results are obtained within 3 hours. However, to develop real-time PCR assays that detect all variants of TOSV circulating in Mediterranean countries and causing diseases in humans, a considerable amount of work must be done to determine the sequences of strains reflecting viral heterogeneity observed in different countries. The recent report

Table. Primers described in the literature for TOSV virus RT-PCR and nested PCR detection*

TOSV strain	Primer	Gene	Assay	Reference
TV1	5′-CCAGAGGCCATGATGAAGAAGAT-3′	N	RT-PCR	14
TV2	5′-CCACTCCTATGAGCAGCTTCT-3′	N	RT-PCR	14
TV3	5′-AACCTGATTTCAGTCTACCAGTT-3′	N	Nested	14
TV4	5′-TTGTTCTCAGAGATGGATTTATG-3′	N	Nested	14
TosN123	5′-GAGTTTGCTTACCAAGGGTTTG-3′	N	RT-PCR	37
TosN829	5′-AATCCTAATTCCCCTAACCCCC-3′	N	RT-PCR	37
TosN234	5′-AACCTTGTCAGGGGNAACAAGCC-3′	N	Nested	37
TosN794	5′-GCCAACCTTGGCGCGATACTTC-3′	N	Nested	37
NPhlebo1+	5′-ATGGARGGITTTGTIWSICIICC-3′	L	RT-PCR	37
Nphlebo1–	5′-AARTTRCTIGWIGCYTTIARIGTIGC-3′	L	RT-PCR	37
Nphlebo2+	5′-WTICCIAAICCIYMSAARATG-3′	L	Nested	37
Nphlebo2–	5′-TCYTCYTTRTTYTTRARRTARCC-3′	L	Nested	37
ATos2–	5′-RTGRAGCTGGAAKGGIGWIG-3′	L	Nested†	37
T1	5′-CTATCAACATGTCAGACGAG-3′	N	RT-PCR	36
T2	5′-CGTGTCCTGTCAGAATCCCT-3′	N	RT-PCR	36
T3	5′-CATTGTTCAGTTGGTCAA-3′	N	Nested	36
T4	5′-CGTGTCCTGTCAGAATCCCT-3′	N	Nested	36

*TOSV, Toscana virus; RT-PCR, reverse transcription–polymerase chain reaction.
†Primer used in combination with Nphlebo2+ for a nested reaction specific for TOSV.

of a Spanish genotype, genetically divergent from the strains circulating in Italy, which is not detected by PCR systems previously reported in Italy, underlines the requirement for a large program of strain isolation and full-length genome sequencing to achieve this goal.

Genetic Diversity of TOSV Strains

Strains Isolated

The prototype TOSV strain, ISS Phl.3, isolated from *P. perniciosus* in 1971 has been completely sequenced. A total of 84 virus strains were obtained from 16,374 male and female sandflies (*P. perniciosus* and *P. perfiliewi*) collected in 2 localities of the Tuscany region of Italy between 1980 and 1985. Thirty-seven (44%) were identified as TOSV and 47 (56%) as a new member of the phlebotomus fever serogroup, Arbia virus. The overall virus isolation rate from sandflies was 0.5%. Viral isolation rates for both viruses were similar in different years and in the 2 localities, suggesting that the 2 virus types were active in the sandfly population simultaneously (maximum activity in July) (3). Seventeen strains of TOSV have been isolated in Spain from patient specimens (22). Several strains have been isolated in southeastern France from patients with clinical cases and remain to be characterized.

Genetic Diversity

A number of strains from Italy have been partially sequenced, and only minor differences in the nucleoprotein were found among strains isolated in the early 1980s from both species of sandflies, from the bat, and from humans, with $\leq$1 amino acid substitution (L. Nicoletti, pers. comm.). Similar results were described in a study on some variants in the N gene of strains isolated from humans from 1995 to 1998; only 1 variant showed a single amino acid substitution of an 80-amino-acid region (38). Changes in the amino acid sequence that make this protein less efficient in its interaction with the viral nucleic acid may kill the virus.

A different situation has been described in Spain for partial sequences in the large segment encoding the polymerase activity. A phylogenetic analysis performed from L segment sequences obtained from 11 clinical isolates from Granada and compared with the homologous sequence of an Italian reference strain showed that Spanish sequences were closely related to one another and distantly related to the Italian strain (37). This finding suggests the presence of at least 2 geographically distinct populations of TOSV.

Phylogeny and Evolution

To date, sequence data are too scarce to perform significant phylogenetic analyses. We must therefore set up a large program of complete genome sequencing of the strains collected in different regions and simultaneously to encourage the development of viral isolation programs in all countries surrounding the Mediterranean where vectors are circulating to better understand the genetic diversity, phylogenetic relationships, and mechanisms driving the evolution of TOSV (Figure 2).

Future Concerns

Nature of the Vector in Different Regions

The virus has been isolated from *P. perfiliewi* and *P. perniciosus*, the most abundant sandfly species present in Italy. However, other vector species, found in different

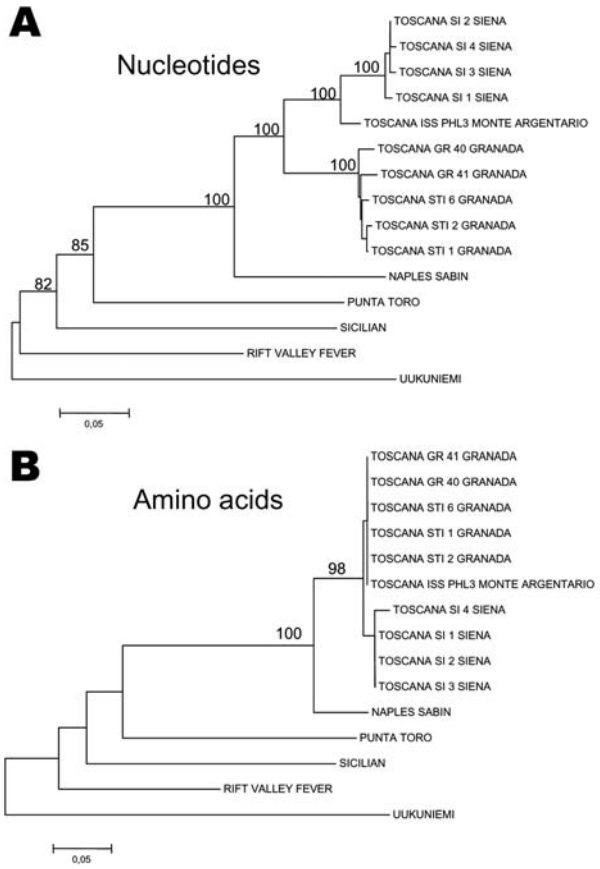

Figure 2. Phylogenetic trees reconstructed from nucleotide (A) and amino acid (B) sequences corresponding to a 236-nucleotide fragment of the N gene. Alignments were obtained with ClustalX 1.8 and p-distance matrices were obtained. Neighbor-joining by using 100 pseudoreplications for the bootstrap tests were carried out after excluding gaps from the alignments. Bootstrap values <75% are not shown. The numbers attached to branches are bootstrap values. A value of 0.05 substitutions per site is equivalent to 5% changes.

geographic areas, could transmit the virus. Serologic results indicate that the virus is present in many areas of the Mediterranean basin. Entomologic studies must be conducted to better understand the distribution and identification of potential vectors of TOSV.

TOSV and Blood Donation

The recent introduction of West Nile virus into North America has stimulated a renewed interest among health authorities regarding arthropodborne viruses, specifically concerning human blood products. Until 2002, the risk of transmitting West Nile virus to a naïve patient from a blood donation was considered negligible, given the supposed short time (≈6 days) and low viremia titers. However, ≈30 cases of viral transmission were documented in 2002 and 2003 in the United States and Canada, as well as cases of West Nile virus infections after organ transplantation from a viremic donor. Moreover, 540 positive blood donation samples were detected by using PCR, which underlines the necessity of this kind of test in an epidemiologic situation similar to that seen in the United States. Recent data on TOSV circulation in Mediterranean countries during the summer raise concerns about potential implications for blood donations.

Genotypes and Their Distribution

Limited studies have been conducted on the genetic variability of TOSV. The work of Sanchez-Seco on the L segment demonstrated the presence of 2 geographically distinct populations of the virus (37). However, the study was performed on strains isolated from patients with acute neurologic disease. On the basis of seroprevalence in a healthy population, Magurano and Nicoletti hypothesized that among the different strains of TOSV that may circulate in the same area and infect humans, only a few cause severe disease, whereas most strains induce antibody response with minor or no symptoms of illness (39). The role of different strains in the symptoms and influence on the severity of TOSV infection requires clarification.

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Dr Charrel is a virologist who works in a hospital diagnostic laboratory and with a university research group. His research interests are arthropodborne and rodentborne viruses that cause disease in humans, with a special interest in emerging and reemerging viruses such as arenaviruses, flaviviruses, and phleboviruses.

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Highly Pathogenic Avian Influenza H5N1, Thailand, 2004

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In January 2004, highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype was first confirmed in poultry and humans in Thailand. Control measures, e.g., culling poultry flocks, restricting poultry movement, and improving hygiene, were implemented. Poultry populations in 1,417 villages in 60 of 76 provinces were affected in 2004. A total of 83% of infected flocks confirmed by laboratories were backyard chickens (56%) or ducks (27%). Outbreaks were concentrated in the Central, the southern part of the Northern, and Eastern Regions of Thailand, which are wetlands, water reservoirs, and dense poultry areas. More than 62 million birds were either killed by HPAI viruses or culled. H5N1 virus from poultry caused 17 human cases and 12 deaths in Thailand; a number of domestic cats, captive tigers, and leopards also died of the H5N1 virus. In 2005, the epidemic is ongoing in Thailand.

Highly pathogenic avian influenza (HPAI) is a devastating disease in poultry; it is associated with a high death rate and disrupts poultry production and trade (1,2). HPAI viruses may be transmitted from birds to humans (3,4), and they are a potential source of future human influenza pandemics (5). HPAI outbreaks were relatively rare until 1990 but occurred in many countries in the last decade (1). In Asia, since the HPAI H5N1 epidemic in Hong Kong in 1997, HPAI viruses have been isolated continuously through routine surveillance in Hong Kong (6,7), South Korea (8), and China (9–11). In Thailand, no evidence of HPAI infection was recorded before 2004 (12). In 2003 and 2004, HPAI H5N1 outbreaks were reported in several Asian countries (South Korea, Vietnam, Japan, Thailand, Cambodia, Hong Kong, Laos, Indonesia, China, and Malaysia) (1,13), and these outbreaks were not easily halted (11,14). Furthermore, H5N1 viruses crossed from birds to humans and caused 116 laboratory-confirmed cases in Vietnam, Thailand, Cambodia, and Indonesia with 60 deaths (as of September 29, 2005) (11,15). We describe epidemiologic features of the HPAI H5N1 epidemic in Thailand in 2004, with focus on introduction of the virus, distribution of disease in Thai poultry, control measures, and consequences.

Poultry in Thailand and HPAI Virus Introduction

Before 2004, Thailand was among the world's major poultry exporters and produced ≈1 billion chickens per year (16); >400,000 persons were employed in the poultry industry (17). Aside from commercial hybrid broilers and layers, backyard poultry are raised for food in most villages (18) (Table 1). The poultry population is concentrated in the Central and Eastern Regions of Thailand (Figure 1). Table 2 categorizes Thai poultry production into 4 sectors on the basis of farm management, biosecurity, and market orientation (14).

In late 2003, poultry farms in the Central and Northern Regions of Thailand experienced large-scale die-offs (19–22). Beginning in mid-December 2003, H5N1 outbreaks were reported in South Korea, Vietnam, and Japan. Meanwhile, Vietnam confirmed the first human death from H5N1 (13). In December 2003, a nationwide surveillance program was initiated to detect human cases in Thailand (22). Subsequently, the surveillance program was strenuously implemented for poultry in mid-January 2004. Cloacal swabs were collected from poultry flocks throughout Thailand, and all samples were tested for avian influenza by virus isolation (2) at national and regional

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Table 1. Poultry population categorized by geographic region in Thailand in 2003

Poultry population	North	Central	East	South	Northeast	Total	RR (95% CI)*
Backyard chickens							
Birds	18,067,529	9,312,042	3,880,535	6,280,375	25,551,093	63,091,574	
Flocks	543,793	143,829	81,804	241,886	1,125,352	2,136,664	
Infected flocks	491	296	107	31	94	1,019	
Incidence, %	0.0903	0.2058	0.1308	0.0128	0.0084	0.0477	1.0
Layers							
Birds	2,288,485	7,682,667	8,304,081	2,113,035	3,924,255	24,312,523	
Flocks	4,209	6,396	3,941	7,666	14,264	36,476	
Infected flocks	29	42	14	1	6	92	
Incidence, %	0.6890	0.6567	0.3552	0.0130	0.0421	0.2522	5.3 (4.4–6.4)
Broilers							
Birds	12,442,797	70,414,281	53,681,571	6,565,161	22,210,976	165,314,786	
Flocks	4,588	6,242	6,507	6,166	22,274	45,777	
Infected flocks	44	54	8	3	2	111	
Incidence, %	0.9590	0.8651	0.1229	0.0487	0.0090	0.2425	5.1 (4.3–6.1)
Ducks							
Birds	2,567,666	8,026,701	6,110,934	1,777,466	5,317,325	23,800,092	
Flocks	58,606	33,607	17,917	95,216	478,483	683,829	
Infected flocks	85	355	29	9	13	491	
Incidence, %	0.1450	1.0563	0.1619	0.0095	0.0027	0.0718	1.5 (1.3–1.7)
Quails							
Birds	199,357	2,920,216	189,342	302,291	81,597	3,692,803	
Flocks	147	324	114	1,797	211	2,593	
Infected flocks	12	26	–	1	1	40	
Incidence, %	8.1633	8.0247	0	0.0556	0.4739	1.5426	32.4 (26.5–39.5)
Geese							
Birds	8,098	154,723	101,465	9,980	34,401	308,667	
Flocks	1,650	1,870	923	2,596	7,646	14,685	
Infected flocks	4	8	3	–	1	16	
Incidence, %	0.2424	0.4278	0.3250	0	0.0131	0.1090	2.3 (1.4–3.7)
Other							
Infected flocks	10	14	6	–	10	40	
Total							
Birds	35,573,932	98,510,630	72,267,928	17,048,308	57,119,647	280,520,445	
Flocks†	612,993	192,268	111,206	355,327	1,648,230	2,920,024	
Infected flocks‡	665	781	161	45	117	1,769	
Incidence, %	0.1085	0.4062	0.1448	0.0127	0.0071	0.0606	
RR (95% CI)	1.0	3.7 (3.4–4.1)	1.3 (1.1–1.6)	0.1 (0.01–0.15)	0.05 (0.06–0.08)		

*Relationship between cumulative incidence and relative risk (RR) of influenza H5N1 epidemic. Cumulative incidence of infected flocks of backyard chickens and the Central Region were assigned an RR of 1.0.
†Included only the number of flocks of backyard chickens, layers, broilers, ducks, quails, and geese. Some flocks are mixed types of poultry on site.
‡Included only the number of infected flocks of backyard chickens, layers, broilers, ducks, quails, and geese.

laboratories of the Thai Department of Livestock Development (DLD).

On January 23, 2004, the Thai national reference laboratory (National Institute of Animal Health [NIAH]) officially confirmed the presence of an H5 HPAI virus in a layer chicken farm in Suphanburi Province (13). The route by which this virus was introduced could not be traced. The virus was characterized as the H5N1 subtype (13), a member of the 2000 avian influenza lineage; most of its genetic sequences were closely related to influenza A/Duck/China/E319.2/03 (23); it belonged to genotype Z (11). That same day, the Thai Ministry of Public Health (MOPH) announced 2 laboratory-confirmed cases of H5N1 virus in children from Suphanburi and Kanchanaburi Provinces; the children eventually died (24,25).

Spread of the Epidemic

The onset of H5N1 human cases (22) showed that the H5N1 virus was already introduced into Thailand by the end of 2003 (Figure 2), before the first identification of the virus. In addition, the 149 reported outbreaks in poultry in 144 villages in 32 of the 76 provinces during the first week of the epidemic indicated that the virus had been widespread throughout the country. The epidemics in Thailand took place in 2 distinct periods, January–May 2004 (termed P1 or the first wave) and July–December 2004 (termed P2 or the second wave) (Figure 2). The epidemic

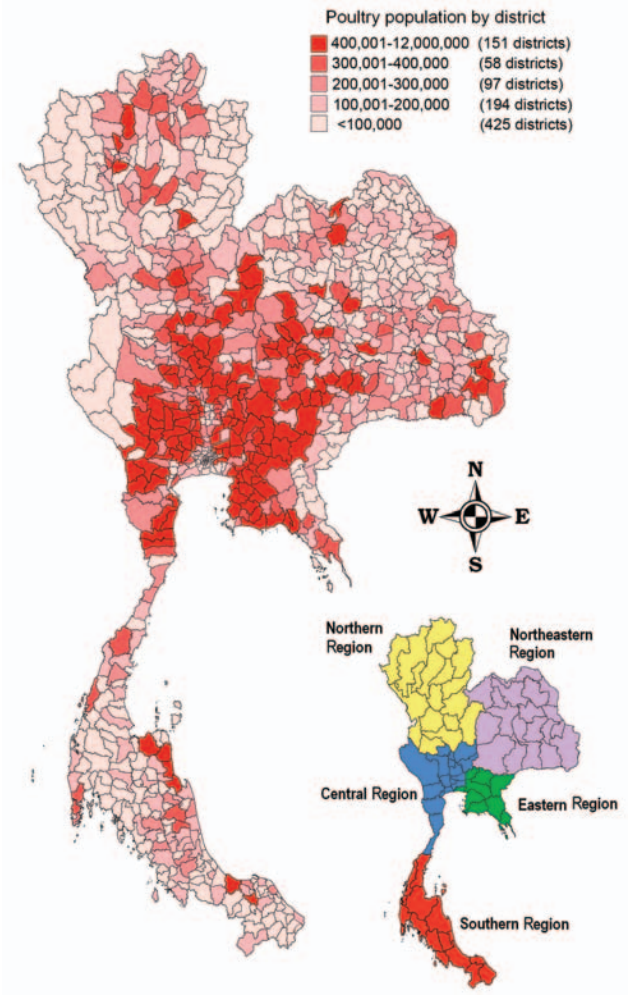

Figure 1. Distribution of poultry population in Thailand in 2003.

is ongoing in Thailand in 2005, but the current analysis includes only outbreaks from January to December 2004.

From January to May 2004, HPAI infections were detected in 188 villages in 42 of 76 provinces throughout Thailand (Table 3). The outbreaks occurred in all parts of Thailand but particularly in the Central, the southern part of the Northern, and the Eastern Regions. The last outbreak of the first wave was reported on May 24, 2004, from a layer farm in Chiangmai Province (13).

On July 3, 2004, the recurrence of HPAI was confirmed in layer farms in Ayudthaya and Pathumthani Provinces, north of Bangkok. These viruses were characterized as the H5N1 subtype, with genetic sequences similar to the H5N1 isolated in January 2004 (26). During P2, HPAI infections were detected in 1,243 villages in 51 provinces (Table 3), which were concentrated in the same 3 regions (Figure 3). From July 3 onward, ≈1–5 cases per day were detected in the first weeks of the epidemic. It reached a peak of 61 cases per day in mid-October 2004 (Figure 2B).

The geographic distribution of the second wave differs markedly from that of the first wave, and the number of confirmed outbreaks was 8 times higher. Most HPAI outbreaks were found in the Central and Northern Regions where chicken and duck flocks are relatively more abundant. In the Northern Region, 99% of infected flocks were detected in the southern part. Figure 3 shows that HPAI was sporadic in the Southern, the northern part of the Northern, and the Northeastern Regions, which have a lower number and density of poultry populations. Figure 2 shows a dramatic increase in HPAI-positive flocks in January and October 2004, which coincided with the nationwide surveillance programs implemented at that time. Also, the number of infected flocks, particularly of backyard chickens and ducks, increased markedly in these months (Figure 4).

Type of Poultry Affected

Table 1 shows the various types of poultry in HPAI-positive flocks in 2004. Eighty-three percent of infected flocks were backyard chickens (56%) or ducks (27%); the rest were broilers (6%), layers (5%), quails (2%), and other birds (3%). From field studies in early 2004, ducks were determined to be silent carriers of HPAI virus (10,27). Accordingly, the proportion of infected ducks diagnosed

Table 2. Poultry production system in Thailand*

Poultry production	Biosecurity	Market orientation	Example
Sector 1	High	Commercial	Industrial integrated system: all components of the production chain (e.g., hatchery, feedmill, poultry farm, slaughterhouse, processing plant, transportation) owned by company with strictly implemented procedures for biosecurity
Sector 2	Moderate to high	Commercial	Semivertical integrated system (or contract farming system): poultry houses owned by the farmer but chicks, feed, and veterinary service supplied by private company. Birds kept indoors with basic physical barriers and hygiene to prevent contact with other animals
Sector 3	Low	Commercial, local, or live-bird market	Layer farm with caged birds in open sheds or free-roaming birds that spend time outside the shed
Sector 4	None	Local	Village or backyard poultry: birds freely roam the village around people and other animals, including cockfighting

*Source: Food and Agriculture Organization (14).

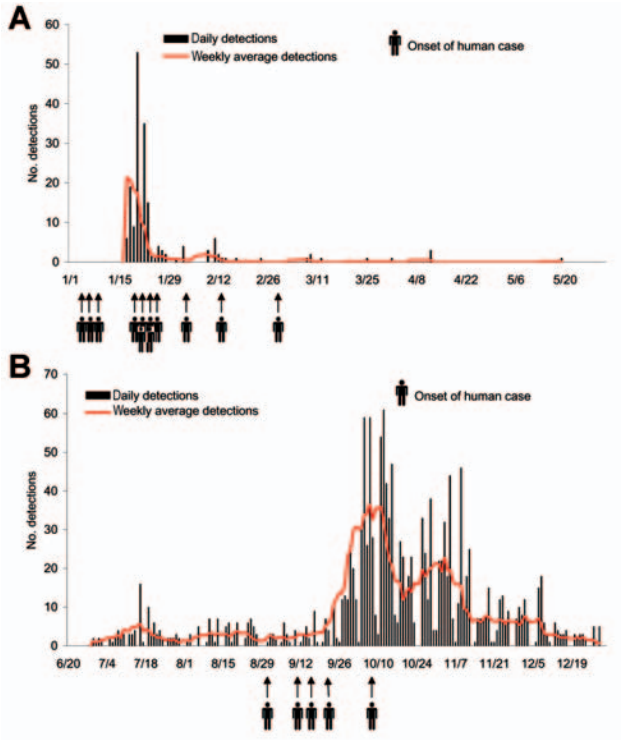

Figure 2. Epidemic curve of the confirmed highly pathogenic avian influenza H5N1 outbreaks in poultry in Thailand by date of notification. A) January–May 2004. B) July–December 2004.

during P2 markedly increased when compared to the number diagnosed during the early epidemic (P1) because more samples from ducks were submitted to laboratories.

Figure 4 shows epidemic curves by species; consistent dissemination of infection was confined to backyard chickens and ducks. Figure 5 illustrates the percentage of HPAI-infected poultry by region. More than 50% of infected flocks were of the backyard type in all regions except the Central Region (≈40% of infected flocks were backyard), which suggests that backyard chickens played a crucial role in the epidemic. However, during P2, 46% of infected flocks in the Central Region were ducks, which shows that they also contributed substantially to the epidemic. Free-grazing ducks are common in the Central Region (Table 1), with its abundance of wetlands and rice paddies. In 102 flocks (6.05%), HPAI was detected in >1 species (mixed farms).

Table 1 also shows cumulative incidence and relative risk (RR) of HPAI outbreaks. RRs of a flock's becoming inflected were 3.7 and 1.3 times higher, respectively, in the Central and Eastern Regions compared to the Northern Region. Moreover, risks for HPAI infection were 5.3, 5.1, 1.5, 32.4, and 2.3 times higher, respectively, in layers, broilers, ducks, quails, and geese compared to backyard chickens.

Spread to Other Species

In the early epidemic, domestic cats, captive tigers, and leopards also died from H5N1 viruses, which indicates that avian influenza can cross species barriers (13,20,25). In October 2004, the infection of H5N1 viruses was confirmed in captive tigers at Sriracha tiger zoo in Chonburi Province, eastern Thailand (28). A total of 147 of 441 tigers kept in the zoo died or were euthanized to prevent possible spread to other zoo animals. Fresh chicken carcasses used to feed the zoo animals, contaminated with HPAI viruses, were considered to be the most plausible source of the infection (28).

Control Measures

Basic Control Measures

Several measures were taken after the first isolation of HPAI virus in January 2004. Initially, all poultry, their products, feed, bedding, waste, and manure from infected flocks were destroyed immediately by the veterinary authorities. Culling infected birds in each flock was generally completed 1–2 days after the virus was confirmed by virus isolation (confirmatory diagnosis took ≈2–8 days after submission of samples). Meanwhile, a restriction on moving poultry and their products within a 5-km radius around the infected flocks was enforced by DLD inspectors in collaboration with local police, and control checkpoints were temporarily established in these areas. Moreover, infected premises and equipment were cleaned and disinfected.

In July 2004, DLD implemented a series of control measures to enable quick action. Specifically, if the poultry death rate in any facility was >10% within a single day, all birds, their products, and other potentially contaminated materials had to be destroyed without delay. Cloacal swabs of affected flocks were then collected for laboratory

Table 3. Number of detections of highly pathogenic avian influenza H5N1 outbreaks in each administrative division during epidemic in Thailand, 2004 (n = 1,685 flocks, record with missing data excluded)

Administrative division	No. detections (Jan–May)	No. detections (Jul–Dec)	Total (Jan–Dec)*
Province (N = 76)	42	51	60
District (N = 926)	89	264	305
Subdistrict (N = 7,409)	146	781	903
Village (N = 71,864)	188	1,243	1,417

*Some HPAI outbreaks during P2 (Jul–Dec) occurred repeatedly in the same administrative division as during P1 (Jan–May).

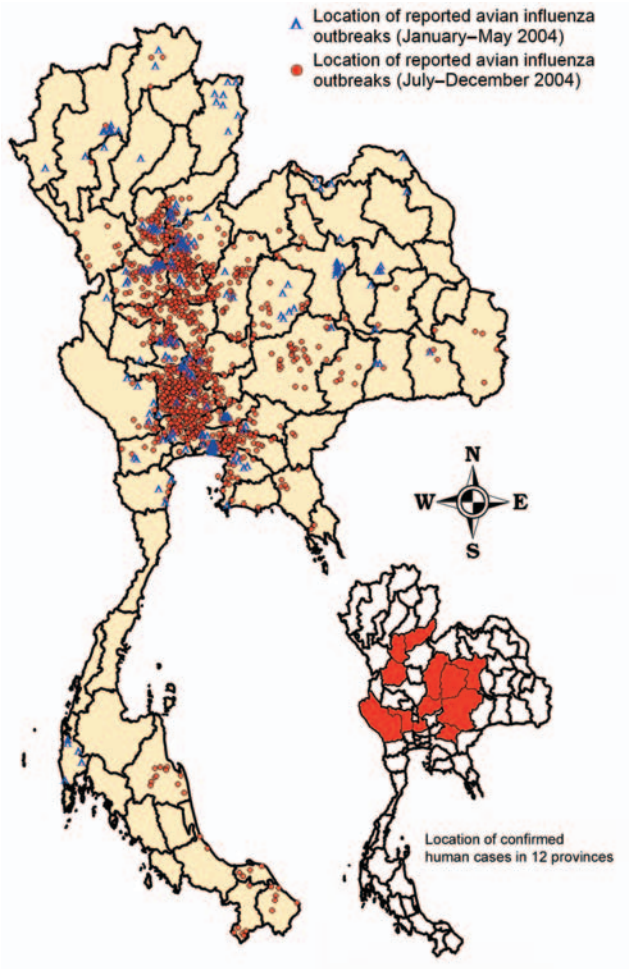

Figure 3. Distribution of reported highly pathogenic avian influenza H5N1 outbreaks in villages in Thailand, January–May 2004 (188 villages of 193 flocks) and July–December 2004 (1,243 villages of 1,492 flocks).

confirmation. Subsequently, neighboring flocks were destroyed immediately or quarantined until H5N1 laboratory confirmation. Upon a confirmative laboratory result, quarantined flocks were culled. Furthermore, movement of poultry and their products was restricted within a 1- to 5-km radius around the infected area.

Preemptive Culling

In January 2004, contiguous flocks were preemptively culled as quickly as possible within a 5-km radius of a confirmed outbreak. After July 2004, preemptive culling was implemented only within a village, within an area of 1 km around an outbreak, or on suspected farms. This new strategy was adopted because the density of poultry flocks decreased after the massive culling during P1. Negative public perception of massive culling was another reason that this strategy was revised.

Surveillance and Diagnosis

In mid-January 2004, DLD launched a nationwide surveillance program to detect possible HPAI infections in poultry. Cloacal swabs were randomly collected from 4 flocks in each village (5 birds per flock). Swab samples were placed in tubes that contained virus transfer medium; usually 5 swabs were pooled per tube. During P1, >100,000 tubes of swab samples were tested for avian influenza virus. During P2, ≈130,000 tubes of swab samples and 72,000 serum samples were collected for diagnosis.

Swab samples as well as sick or dead bird specimens were submitted to NIAH or regional laboratories. All samples were processed for virus isolation in embryonated chicken eggs (≈1–2 days) (2); 2 serial passages in embryonated chicken eggs were performed before a specimen was regarded as negative (≈8 days). In January 2004, the first avian influenza isolate was sent to the University of Hong Kong to identify the virus and serotype hemagglutinin (HA) and neuraminidase (NA) antigens. Thereafter, NIAH itself established the necessary facilities to identify and serotype virus. Furthermore, real-time reverse transcription–polymerase chain reaction analyses for avian influenza were used to detect the virus at all laboratories to reduce the time of diagnosis. Hemagglutination inhibition (HI) test was used to detect antibodies to avian influenza virus in serum samples (2).

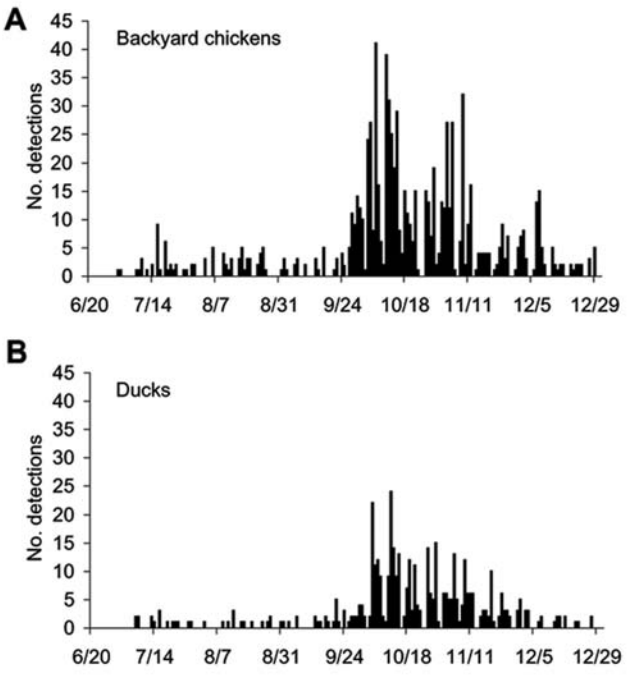

Figure 4. Infected flocks by day of detection and type of poultry, July–December 2004. A) Backyard chickens. B) Ducks. A full version of this figure, including data for January–May 2004 and for broilers and layers, is available online from http://www.cdc.gov/ncidod/EID/vol11no11/05-0608-G4.htm

A nationwide comprehensive surveillance program (known as "x-ray survey") was implemented October 1–31, 2004. The goal of this survey was to detect HPAI infection in any village. In close collaboration among the Ministry of Agriculture and Cooperatives, MOPH, and provincial governors, volunteer public health MOPH workers and DLD livestock workers searched for and reported sick and dead poultry in villages. Through the surveillance program, farmers were also persuaded to report sick or dead poultry in their flocks to authorities.

In 2005, x-ray surveys were implemented continuously every 6 months. Moreover, commercial poultry flocks spend ≈8 days waiting for the results of cloacal swab or blood tests; only if birds are free of the virus will their owners be allowed to move them to slaughterhouses or new areas.

Other Supportive Measures

A public awareness campaign was started to educate the public on avian influenza and to bolster consumers' confidence that poultry was safe. In addition, the so-called "Big Cleaning Week" was promoted from March 1 to 7, 2004, to encourage relevant parties to be aware of HPAI and to disinfect their facilities, e.g., poultry houses, farm equipment and vehicles, slaughterhouses, and retail markets. Soaps, detergents, alkalis, acids, aldehydes, chlorine, and quaternary ammonium compounds were used as disinfectants. Poultry exhibition and cockfighting were prohibited (since early 2004). A violation of this regulation is subject to fine. Additionally, the practice of allowing ducks to freely graze was discontinued. Because of traditional farming styles, however, these practices are unlikely to change in a short period of time. After an affected flock was culled, a wait of ≥60 days in broiler farms and ≥90 days in layer farms and backyard chickens was imposed before a new flock could be established. Farmers must also improve sanitary measures in their farms to meet DLD's requirements.

Consequences of Epidemic

In early 2004, lack of information and communication with regard to HPAI caused the public to lose confidence in poultry products. The decrease in domestic consumption and bans on Thai poultry products by importing countries damaged the poultry industry. In addition, H5N1 virus from poultry caused 17 human cases with 12 deaths in 12 provinces (Figures 2 and 3) (15,22).

The Thai government used a stamping-out policy to control HPAI outbreaks and compensated farmers for their losses. According to the Animal Epidemic Act, farmers are entitled to compensation of 75% of the value of animals that are destroyed. However, 100% compensation was provided during P1 because the epidemic was widespread and devastating to Thai farmers; compensation was reduced to 75% during P2. Compensation per bird was (in US dollars) $0.38–$65, depending on the type of poultry ($0.38 for quail; $1.13 for broiler; $2 for meat duck; $2.25 for backyard chicken; $3.5 for layer chicken, layer duck, or goose; $7.25 for turkey; and $65 for ostrich).

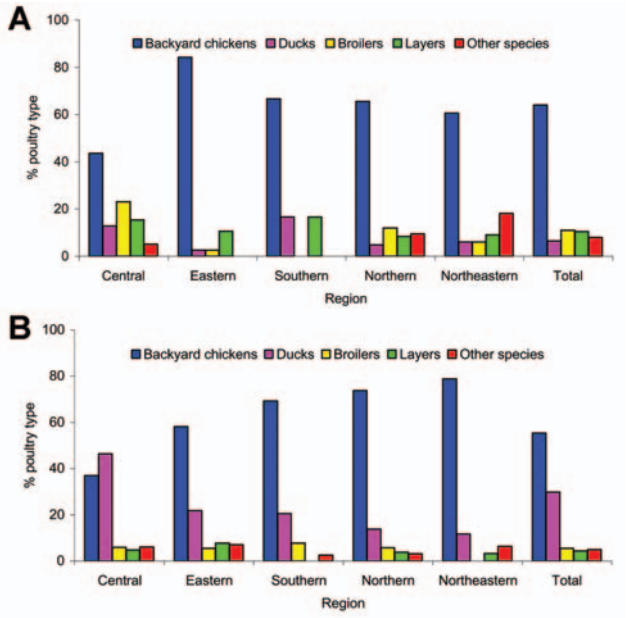

Figure 5. Percentage of main poultry types in infected flocks by region during the 2004 HPAI H5N1 epidemic in Thailand. A) January–May 2004. B) July–December 2004.

Approximately 62 million birds were either killed by H5N1 viruses or culled for disease control and animal welfare reasons. The government allocated a budget of ≈5.3 billion Thai baht (US $132.5 million) for direct compensation to affected farmers (29). All costs of implemented basic measures were covered by the government. As of March 2004, the HPAI epidemic had an estimated effect on the national gross domestic product of 0.39%. These losses amount to 25.24 billion Thai baht (US $631 million) (30).

Conclusions

Detection and Early Distribution of HPAI Virus

Epidemiologic data from the early epidemic indicate that the period between the introduction of the virus into Thailand and its conclusive identification was too long. The route of virus introduction could not be traced. Also the delay between primary infection, first diagnosis, and finding the initial case allowed widespread dissemination of the virus and contributed to the large scale of the epidemic (31,32). Early warning, early detection, and early response are essential to prevent and control HPAI. In view of potential public health implications of HPAI, notifying

and collaborating with public health authorities is equally important.

Geographic Regions, Affected Species, and Incidence

The epidemic differed by region. The Central and Northern Regions contained 82% of the total outbreaks (Table 1). Infections were prominent in backyard chicken flocks in the southern part of the Northern Region and in free-grazing ducks in the area adjacent to the Central Region. Backyard chickens and free-grazing ducks played essential roles as H5N1 hosts (Figure 4); 83% of confirmed flocks were backyard chickens or ducks. Because of improved surveillance during P2, disease detections increased markedly (Figure 2). The difficulty of clinically detecting HPAI in ducks (27,33) and free-ranging backyard chickens and ducks also made controlling the disease difficult.

The 2004 cumulative incidence and RR also show a higher number of detections in the Central, Northern, and Eastern Regions relative to other parts of Thailand (Table 1). This finding reflects that the high density of poultry, the local geography (e.g., wetlands, water reservoirs, and rice paddies), and farming practice in these regions might be risk factors for outbreaks. Other studies showed a strong association between free-grazing duck populations and the practice of free-grazing farming with spread of the virus in the Central Region (M. Gilbert et al., pers. comm.). In our exploratory analysis, the RR for HPAI infection in Thai poultry production could not be clearly elucidated. The RR for HPAI infection was high in broilers, layers, quails, geese, and ducks compared to backyard chickens (Table 1). The number of detections in these types of poultry increased substantially in backyard chickens and ducks when national surveillance was implemented in January and October 2004. This observation suggests that when larger-scale farmers observed suspected cases in layer and broiler farms, they immediately reported them to local authorities, encouraged by the compensation that they received. In contrast, small farmers most likely did not report their few dead poultry. Consequently, the number of outbreaks in small farmers may have been underestimated. Additionally, size of flock may be a confounding factor in the higher risk for infection in broiler, layer, and quail flocks (34,35).

Course of the Epidemic

The epidemic curve during P1 shows a steep rise in the first week; detections decreased sharply after control measures were taken (Figure 2A). In the early epidemic, samples of culled flocks were not tested during massive culling. Undoubtedly, the quantity of infected flocks was underestimated, thus obscuring the effectiveness of control measures to stem the outbreaks. High numbers of HPAI detections coincided with low temperatures in Thailand from October to February, when wild birds from central and northern Asia migrate into Thailand (7). Therefore, seasonal conditions and bird migration might have contributed to the introduction of HPAI virus. Furthermore, the lower temperature supports survival of the virus in the environment and facilitates transmission (33). In addition, several festivals, which are associated with raising, selling, and transporting poultry, occurred around the end of the year. Illegal transportation and cockfighting may have worsened the HPAI situation.

Effectiveness of Control Measures

Because of differences and changes to control measures and surveillance programs during P1 and P2, HPAI outbreak data are difficult to compare. The start of the outbreak was an emergency period, during which epidemiologic data could not be effectively or completely collected. However, our results indicate that although several measures were implemented in 2004, the epidemic could not be controlled. HPAI outbreaks can be controlled rapidly with highly restrictive measures by totally depopulating all poultry in the entire areas in some countries (6,32,36). However, given that HPAI was widespread in all parts of Thailand, total depopulation was not a practical option. But a combination of depopulation with improved early detection and response practiced during P2, combined with the culling rigor practiced during P1, may be a realistic option.

The Thai epidemic shows that the virus continues to circulate in the country. The immediate challenge is, therefore, to control avian influenza in free-ranging animals in rural areas, particularly in backyard chickens and free-grazing ducks. However, control of outbreaks in these types of poultry is difficult because of traditional farming practices. Control could be achieved by improving biosecurity of poultry farms and changing farming practices (6,36,37). Meanwhile, educating farmers and staff on early detection and the basic concepts of biosecurity may be the most critical way to eliminate avian influenza virus (32).

Since January 2004, a stamping-out policy has been used to control avian influenza outbreaks in Thailand; vaccination has been not allowed. According to the Office International des Épizooties Terrestrial Code 2005, 2 broad vaccination strategies exist, inactivated whole avian influenza viruses and hemagglutinin expression–based vaccines. Thus, vaccination may be worthwhile to consider as an additional control measure (36). Vaccination significantly reduces excretion of viruses (38,39), which may reduce viral load in the environment and decrease the risk for human exposure. However, HPAI infection could become endemic if vaccination is not managed appropriately (40).

Early detection of all cases was essential to rapidly implement control measures. Meanwhile, comprehensive veterinary surveillance and long-term control measures are required (11). The success of HPAI elimination, therefore, depends on a collaboration of all stakeholders, including farmers, industries, veterinarians, public health authorities, academic institutions, media, and the government (36,37).

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Coltiviruses and Seadornaviruses in North America, Europe, and Asia

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Coltiviruses are tickborne viruses of the genus *Coltivirus*. The type species, Colorado tick fever virus (from North America), has been isolated from patients with flulike syndromes, meningitis, encephalitis, and other severe complications. Another coltivirus, Eyach virus, has been isolated from ticks in France and Germany and incriminated in febrile illnesses and neurologic syndromes. Seadornaviruses are endemic in Southeast Asia, particularly Indonesia and China. The prototype virus of the genus, Banna virus (BAV), has been isolated from many mosquito species, humans with encephalitis, pigs, and cattle. Two other seadornaviruses, Kadipiro and Liao Ning, were isolated only from mosquitoes. The epidemiology of seadornaviruses remains poorly documented. Evidence suggests that BAV is responsible for encephalitis in humans. Infection with BAV may be underreported because it circulates in regions with a high incidence of Japanese encephalitis and could be misdiagnosed as this disease.

Vertebrate viruses belonging to the family *Reoviridae* and having 12-segmented dsRNA genomes at one time were classified in the genus *Coltivirus* (Colorado tick virus [CTFV]). At the time the genus was created, it included tickborne and mosquitoborne viruses. Presently, the genus *Coltivirus* contains only CTFV, California hare coltivirus (CTFV-Ca), which is considered a serotype of CTFV, Eyach virus (EYAV), which is distinct from CTFV, and Salmon River virus (SRV), which may be a serotype of both CTFV and CTFV-Ca. Alternatively, analysis of sequence data and antigenic properties of the mosquitoborne viruses led to their reassignment to a new genus designated *Seadornavirus* (Southeast Asian dodeca RNA virus) (1,2).

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Coltiviruses

Historical Aspects and Epidemiology

Tickborne vertebrate viruses include CTFV isolated from humans and ticks, EYAV isolated from ticks, CTFV-Ca isolated from a hare (*Lepus californicus*, black-tailed jackrabbit) in northern California, and SRV isolated from a person in Idaho. Many mosquitoborne viruses were also considered tentative species in this genus, including Banna virus (BAV) from humans. The tickborne viruses in this genus have many distinctive features and sequence data have led to a reevaluation of their taxonomic status.

Coltiviruses have been isolated from ticks of the family *Ixodidae* and from rodents and humans. CTFV is endemic in northwestern North America, where it causes CTF, a human disease initially confused with a mild form of Rocky Mountain spotted fever, which is caused by *Rickettsia rickettsii*. The causative agent was isolated by Florio and others (3) in 1946 from human serum by injection into adult hamsters. The virus was adapted to egg and mice, and suckling mice became the routine isolation system for CTFV (3–5).

CTF is found in the Rocky Mountain region of the United States and in Canada. The virus distribution closely matches that of its vector *Dermacentor andersoni*. CTFV-Ca, identified as strain S6-14-03, was isolated from the blood of the white hare, *L. californicus*, in California (outside the range of *D. andersoni*) (6). Antibodies to CTFV antigen have been detected in sera of humans in South Korea (C. Calisher, pers. comm.). SRV was isolated from a patient with moderately severe CTF-like illness in Idaho.

EYAV was isolated in Europe in 1976 from *Ixodes ricinus* ticks (EYAV-Gr) and in 1981 from *I. ricinus* (EYAV-Fr578) and *I. ventalloi* (EYAV-Fr577) (7). Its antigenic relationship to CTFV was established by a complement-fixation assay (7). Genome sequence analysis showed that

CTFV and EYAV are closely related (8) and confirmed the antigenic observations.

Serologic surveys in France identified antibodies to EYAV in 1.35% of animals, including the European rabbit (*Oryctolagus cunniculus*), mice, mountain goats, domestic goats, sheep, and deer (9). The presence of EYAV was suspected in Europe because anti-EYAV antibodies were detected in patients with neurologic disorders. However, the natural cycle of the virus is still unclear, and whether it circulates continuously in Europe is not known, although the rabbit *O. cunniculus* is suspected of being main host (6,9). In 2003, the virus was reisolated from *I. ricinus* ticks in Germany (10).

Vectors, Host Range, and Transmission

Ticks are the principal vectors of coltiviruses. CTFV is transmitted by the wood tick *D. andersoni*, but other ticks such as *D. occidentalis*, *D. albopictus*, *D. arumapertus*, *Haemaphysalis leporispalustris*, *Otobius lagophilus*, *Ixodes sculptus*, and *I. spinipalpis* are also infected with the virus. EYAV has been isolated from *I. ricinus* and *I. ventalloi* (7). Ticks become infected through blood meals from an infected vertebrate host. CTFV is transmitted transstadially, but not transovarially. Infected larvae and nymphs can hibernate, and the nymphal and adult ticks become persistently infected. In certain rodents viremia can persist for >5 months. These ticks and rodents may provide hosts by which the virus could overwinter. The prevalence of viremia in rodents in a virus-endemic area ranges from 3.5% to 25%, and the prevalence in ticks ranges from 10% to 25% (7). CTFV has a wide host range that includes ground squirrels, chipmunks, wild mice, wood rats, wild rabbits and hares, porcupines, marmots, deer, elk, sheep, and coyotes.

Person-to-person transmission of CTFV can occur by blood transfusions (11). This virus is included on the list of agents screened before bone marrow transplantation in the United States (www.guideline.gov/summary/summary.aspx?doc_id=2573&nbr=1799&string=pertussis). Prolonged viremia observed in humans and rodents is due to the intraerythrocytic location of virions, which protects them from immune clearance (12–14).

Properties, Genome, and Replication

Coltivirus particles are ≈80 nm in diameter and have a core ≈50 nm in diameter. Electron microscopic studies (8,15) have shown particles with a relatively smooth surface capsomeric structure and icosahedral symmetry (Figure 1A). Most viral particles are nonenveloped, but a few acquire an envelope structure during passage through the endoplasmic reticulum (8,15). The buoyant density of CTFV in CsCl is 1.36–1.38 g/cm^3. The virus is stable between pH 7.0 and 8.0 but loses infectivity at pH 3.0.

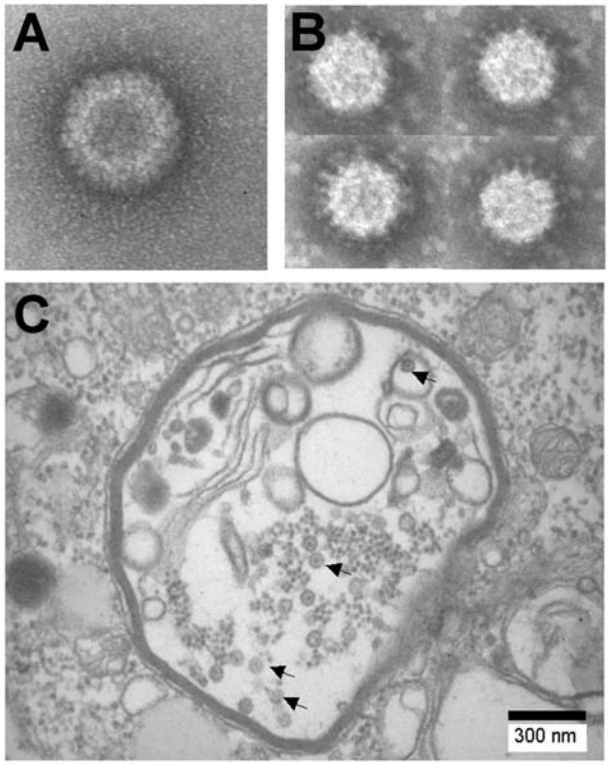

Figure 1. Negative contrast electron micrographs of A) Colorado tick fever virus and B) Banna virus (BAV). C) Thin section of BAV-infected C6/36 cells showing viral particles (arrows) in vacuolelike structures.

CTFV can be stored at 4°C for 2–3 months in 50% fetal calf serum, 0.2 mmol/L Tris-HC1, pH 7.8, or for years at –80°C. Upon heating to 55°C, CTFV loses its infectivity. The virus is stable when treated with nonionic detergents (such as Tween 20) or with organic solvents (such as Freon 113 or its ozone-friendly substitute Vertrel XF), but viral infectivity is abolished by treatment with sodium deoxycholate or sodium dodecyl sulfate (16,17).

The genome consists of 12 dsRNA segments designated Seg-1 to Seg-12 in order of reduced molecular weight as observed during agarose and polyacrylamide gel electrophoresis. The genome contains ≈29,000 bp and segment size ranges from 675 bp to 4,350 bp (18,19). The genomic dsRNA of CTFV has an electropherotype (Figure 2A) similar to that of CTFV-Ca. CTFV produces a cytopathic effect (CPE) in mammalian cells, including human carcinoma cells, monkey kidney cells (buffalo green monkey [BGM] and Vero), hamster kidney cells (BHK-21), and mouse fibroblasts (L-929). Cells infected with CTFV develop granular matrices that contain virus-like particles in the cytoplasm. These structures are similar to viral inclusion bodies produced during orbivirus infections (15). In addition, bundles of filaments (tubules)

characterized by cross-striations and kinky threads are found in the cytoplasm and, in some cases, in the nucleus of infected cells (8,15,17). These tubules may also be comparable to those found in orbivirus-infected cells. More than 90% of virus particles remain associated with debris after cell disruption.

Sequence analysis of coltivirus genomes has shown that segment 6 of viral protein 6 (VP6) of CTFV is homologous to segment 7 (VP7) of EYAV (Figure 3). The amino acid sequence (residues 370–490) of VP7 of EYAV showed 50% similarity to the sarcolemmal-associated protein of the European rabbit *O. cunniculus*, which may be the major host of EYAV. By comparison, VP6 of CTFV showed no similarity with this rabbit protein (8), which may be the result of insertion of a sequence encoding a lagomorph protein into segment 7 of EYAV. Reading through a stop codon, which is common in retroviruses and alphaviruses, was reported in coltiviruses, particularly in segment 9, where long and short proteins are produced from a single open reading frame (20).

Virus Relationships

Antigenic variation between CTFV strains is low, especially between strains from humans (21). Distinct CTFV serotypes are difficult to define (22), and immunity to reinfection has been observed (23). CTFV from North America and EYAV from Europe show little cross-reactivity in neutralization assays. CTFV-Ca cross-reacted with CTFV, but not with EYAV, and may be a serotype of CTFV. Two species of coltiviruses have been identified: CTFV, with 2 serotypes represented by CTFV-F1 and CTFV-Ca, and EYAV. Overall identities between nucleotide sequences from segments 9, 10, 11, and 12 of CTFV strains range from 90% to 100%: 97%–100% for segment 9, 96%–99% for segment 10, 90%–94% for segment 11, and 94%–96% for segment 12. The degree of identity between nucleotide sequences of segments 1–12 of CTFV and EYAV isolates ranges from 55% to 88% (19).

The genome of CTFV contains 29,174 nucleotides, and that of EYAV contains 29,210 nucleotides (18). All 12 segments of CTFV and EYAV have conserved sequences that are located at their termini. The motifs 5′-G/CACAUU-UG-3′ and 5′-UGCAGUG/C-3′ are found in the 5′ and 3′ noncoding regions of CTFV, respectively, and the motifs 5′-GACAA/UUU-3′ and 5′-UGC/UAGUC-3′ are found in these noncoding regions in EYAV. The 5′ and 3′ terminal trinucleotides of all segments in both viruses are inverted complements (8).

Genome characterization has helped shed light on the origin of EYAV. Genetic findings support the hypothesis that EYAV was derived from a CTFV-like ancestor virus that was introduced into Europe through Asia when lagomorph ancestors migrated from North America 5–50 million years ago (8). The antigenic and genetic relationships between CTFV and EYAV are further corroborated by their identical morphologic features, as analyzed by electron microscopy (8).

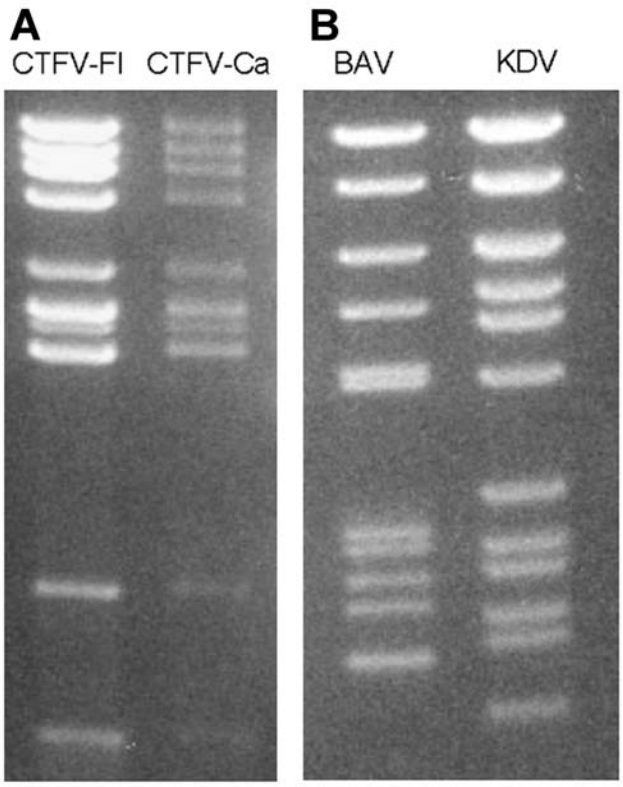

Figure 2. Electropherotypes of coltiviruses and seadornaviruses on 1% agarose gels. A) Colorado tick fever virus (CTFV-Fl) and California hare coltivirus (CTFV-Ca). B) Banna virus (BAV) and Kadipiro virus (KDV).

Clinical Features

Infection of humans with CTFV is characterized by abrupt onset of fever, chills, headache, retroorbital pain, photophobia, myalgia, abdominal pain, and generalized malaise. Diphasic or triphasic febrile patterns have been observed, usually lasting for 5 to 10 days. Severe forms of the disease that involve infection of the central nervous system (CNS) or hemorrhagic fever, pericarditis, myocarditis, and orchitis have been rarely observed, mainly in children. Severity is sufficient to result in hospitalization of ≈20% of patients. Offspring of mice experimentally infected with CTFV showed teratogenic effects (24). Mother-to-infant transmission has been reported in pregnant women.

The incidence of complications in different reports of infection with CTFV has been reported as ≤7% (23–26). CTFV causes leukopenia (65% of infected humans), with

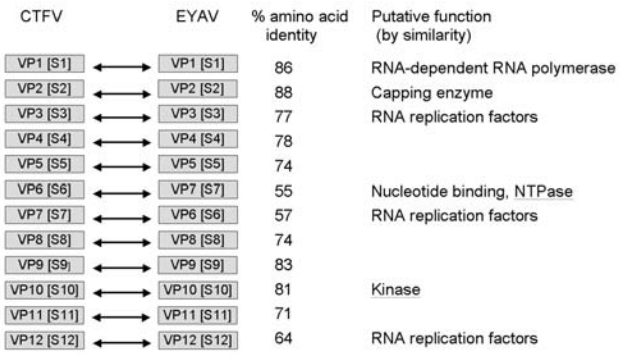

Figure 3. Comparison of nucleotide and amino acid sequences of genome segments of viral proteins (VP) and dsRNA segments (S) of Colorado tick fever virus (CTFV) and Eyach virus (EYAV). NTP, nucleoside triphosphatase.

mean leukocyte counts of 900/μL to 3,900/μL, and thrombocytopenia, with platelet counts of 20,000/μL to 95,000/μL (27). Patients with neurologic disorders (meningitis, meningoencephalitis, encephalitis) show lymphocyte infiltration of cerebrospinal fluid (CSF), and the virus has been isolated from CSF.

CTFV infections have been confused with other tickborne diseases such as Rocky Mountain spotted fever (a rickettsial disease), tularemia, relapsing fever, and Lyme disease. However, rash and leukocytosis distinguish Rocky Mountain spotted fever from CTF. Signs in the CNS confuse CTF with other causes of viral meningitis and encephalitis, including St. Louis encephalitis virus, Western equine encephalitis virus, and enteroviruses.

CTFV can be isolated from blood because it is present in circulating erythrocytes for as long as 4 months (28), and it infects the hematopoietic progenitor cells and remains sheltered in erythrocytes after maturation. Intracerebral injection of blood into suckling mice is considered the most sensitive isolation system. Reverse transcription–polymerase chain reaction (RT-PCR) was developed for diagnosis of CTFV infection, and this method detected human and tick virus isolates ≥3 days postinfection in experimentally infected mice (29). As little as 1 genome could be detected by using a PCR assay (29).

Serologic diagnostic methods based on CTFV-infected cell cultures have also been developed (29). These include 1) a complement-fixation test that is relatively insensitive because in 25% of patients complement-fixing antibodies are not detected and in 75% of patients these antibodies appear late after infection; 2) a seroneutralization assay for detection neutralizing antibodies that appear 14–21 days after onset of disease; 3) an immunofluorescence assay that uses CTFV-infected BHK-21 or Vero cells and is an easy and rapid test for detecting anti-CTFV antibodies; and 4) an enzyme-linked immunosorbent assay (ELISA) for immunoglobulin M (IgM) and IgG that appear concurrently or a few days after neutralizing antibodies (peak 30–40 days after infection), However, IgM titers decrease sharply after day 45. An ELISA based on recombinant VP7 and a Western blot based on synthetic VP12 showed good sensitivity in detecting antibodies to CTFV (29,30).

A complement fixation assay has been developed for detection of EYAV (6). It detected anti-EYAV antibodies in 158 Czechoslovakian patients with encephalitis in whom tickborne encephalitis had been diagnosed. This population was also tested for tickborne encephalitis viruses (Kemerovo, Lipovnik, or Tribec) and antibodies to EYAV. Seventeen serum specimens (11%) had only anti-EYAV antibodies. The same test identified anti-EYAV antibodies in 8 (17%) of 47 patients with polyradiculoneuritis and meningopolyneuritis (31). Recently, an ELISA based on recombinant VP6 of EYAV was developed. This test selectively identified anti-EYAV antibodies (32). An RT-PCR assay based on the sequence of genome segment 12 has also been developed for specific detection of EYAV (29).

Seadornaviruses

Historical Aspects and Epidemiology

BAV is the type species of the genus *Seadornavirus* (19) that includes Kadipiro virus (KDV) and Liao Ning virus (LNV). Vectors for this genus include *Anopheles*, *Culex*, and *Aedes* mosquitoes. These viruses are endemic in Southeast Asia, particularly Indonesia and China (2). BAV was first isolated in 1987 from CSF (2 isolates) and sera (25 isolates) of patients with encephalitis in southern China (Yunnan Province). Numerous isolates were also obtained from other patients with encephalitis (33,34). A virus described as an isolate of BAV was identified in western China (Xinjiang Province) from patients with fever and flulike manifestations (35). Virus isolates from pigs and cattle with genomes having the same electropherotype as BAV were also reported (34). The isolation from mosquitoes of 12 segmented dsRNA viruses antigenically related to BAV has been reported in various provinces of China, including Beijing, Gansu, Hainan, Henan, and Shanshi, and in central Java, Indonesia. BAV is now classified as a biosafety level 3 arboviral agent (www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s74.htm).

Vectors, Host Range, and Transmission

Seadornaviruses have been isolated from *Culex vishnui*, *Cx. fuscocephalus*, *Anopheles vagus*, *An. aconitus*, *An. subpictus*, and *Aedes dorsalis*. BAV, KDV, and LNV are found in tropical and subtropical regions where other mosquitoborne viral diseases, especially JE and dengue, are endemic. Several cases of encephalitis in China have been

diagnosed as Japanese encephalitis (JE) without any detection of JEV or specific anti-JEV antibodies. Recently, 89 paired serum samples from these patients were tested by ELISA for anti-BAV IgG antibodies. At least a 4-fold (up to 16-fold) increase in IgG antibodies was observed in 7 cases. An additional 1,141 serum specimens of patients from a large number of health institutes in China, who supposedly had JE or viral encephalitis, were tested for anti-BAV IgM antibodies; 130 samples were positive (34).

Seadornaviruses were shown to replicate in adult laboratory mice and were detected in infected mouse blood at 3 days postinfection until day 5 postinfection (36). To date, BAV has been isolated only from humans, and KDV and LNV have been isolated only from mosquitoes.

Properties, Genome, and Replication

Seadornavirus has 7 structural proteins, 5 of which are present in the core (37). The viruses are icosahedral with a diameter of 60–70 nm, and the core has a diameter of ≈50 nm. The surface of virus has spikes (Figure 1B) that are similar to those of rotaviruses. The viruses are stable at pH 7.0, and acidity decreases their infectivity (which is lost at pH 3.0). Purified virus can be stored at 4°C or for long periods at –80°C. Viral infectivity is decreased considerably upon heating to 55°C. Organic solvents such as Freon 113 or Vertrel XF can be used for purification of viral particles from cell lysates and do not affect infectivity.

The seadornavirus genome consists of 12 segments of dsRNA known as Seg-1 to Seg-12 in order of decreasing molecular mass observed by gel electrophoresis (Figure 2B). The genome of BAV or KDV is ≈21,000 bp, and the segment length ranges between 862 bp and 3,747 bp (2). During replication, viruses are found in the cell cytoplasm within vacuolelike structures (Figure 1C) that are believed to be involved in morphogenesis (37).

Seadornavirus isolates replicate in various mosquitoes cell lines such as C6/36 and AA23 (both from *Ae. albopictus*), A20 (*Ae. aegypti*), and Aw-albus (*Ae. W. albus*). More than 40% of the virus particles are released into the culture medium before cell death and massive CPE (fusiform cells). Infected cells are not lysed, and the virus leaves cells by budding, thus acquiring a temporary envelope (37). Late infection results in cell lysis from cell death. Intracellular radiolabeling of viral polypeptides has shown termination of host cell protein synthesis (37).

In addition to its capacity to replicate in a large number of mosquito cell lines, LNV is the only seadornavirus that replicates in a variety of transformed or primary mammalian cell lines such as Hep-2 (human carcinoma cells), BGM, Vero, BHK-21, L-929, and MRC-5 (human lung fibroblasts). Infection with LNV results in a massive lytic effect and also kills adult mice.

BAV	KDV	% amino acid identity	Putative function of BAV proteins (location)
VP1 [S1]	VP1 [S1]	42	RNA-dependent RNA polymerase (core)
VP2 [S2]	VP2 [S2]	28	T=2 protein, nucleotide binding (core)
VP3 [S3]	VP3 [S3]	39	Capping enzyme (core)
VP4 [S4]	VP4 [S4]	33	(outer coat)
VP5 [S5]	VP6 [S6]	30	(non-structural)
VP6 [S6]	VP5 [S5]	33	NTPase (non-structural)
VP7 [S7]	VP7 [S7]	30	Protein Kinase (non-structural)
VP8 [S8]	VP9 [S9]	25	(core)
VP9 [S9]	VP11 [S11]	26	Cell attachment (outer coat)
VP10 [S10]	VP10 [S10]	24	(core)
VP11 [S11]	VP12 [S12]	26	(non-structural)
VP12 [S12]	VP8 [S8]	27	dsRNA-binding (non-structural)

Figure 4. Comparison of nucleotide and amino acid sequences of genome segments of viral proteins (VP) and dsRNA segments (S) of Banna virus (BAV) and Kadipiro virus (KDV). NTP, nucleoside triphosphatase.

Relationships among Seadornaviruses and other *Reoviridae*

Antigenic relationships between seadornaviruses were investigated by using mouse immune sera. BAV from southern China and Indonesia and KDV from Indonesia are classified as distinct species (1) and show no cross-reactivity in neutralization tests. These viruses are also antigenically distinct from LNV. Antigenic variations were observed in many isolates from China that showed cross-reactivity with BAV.

Amino acid sequence analysis identified BAV, KDV, and LNV as 3 distinct species (2). Identities between homologous proteins ranged from 24% to 42%, with the highest value found in the polymerase gene. Analysis of different BAV isolates has shown the existence of 2 genotypes identified as genotype A (represented by isolates BAV-Ch [China] and BAV-In6423 [Indonesia]) and genotype B (represented by isolates BAV-In6969 and BAV-In7043 [Indonesia]). This grouping is based on sequences of segments 7 and 9: amino acid sequences of segment 7 show an identity of 72% between the 2 genotypes, while those of segments 9 show an identity of 41%. All other proteins among BAV isolates have identities ranging from 83% to 100% (1,19). Based on a seroneutralization assay, these 2 genotypes were found to represent 2 serotypes of BAV (38).

Sequence comparison of the structural proteins of BAV (VP1, VP2, VP3, VP4, VP8, VP9, and VP10) (Figure 4) with those of other members of the *Reoviridae* have shown that VP9 and VP10 of BAV have similarities to VP8 and VP5 subunits of the outer coat protein VP4 of rotavirus A (37). This finding was further confirmed when the crystal structure of BAV VP9 was determined and showed structural similarities to rotavirus VP8 (38). In addition, VP3 of BAV, which is the guanylyltransferase of the virus (39),

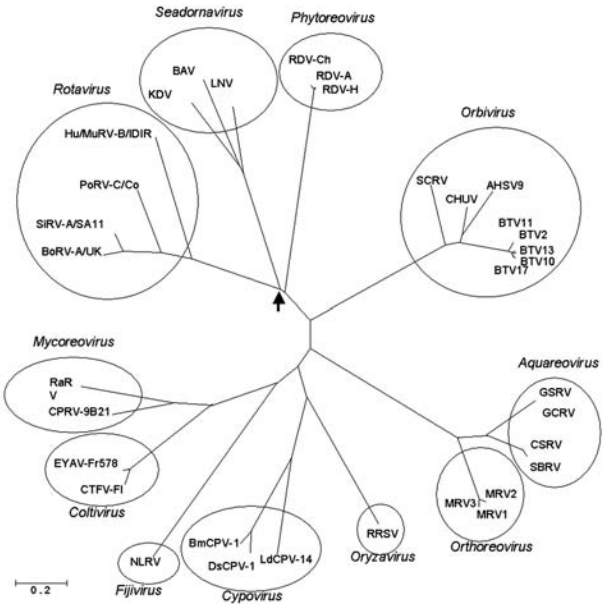

Figure 5. Phylogenetic comparison of the viral polymerase protein sequences of viruses of the family *Reoviridae*, including seadornaviruses and coltiviruses. Accession numbers and further details of the sequences and viruses are available in the report by Attoui et al. (8). Analysis shows coltiviruses and seadornaviruses as 2 distinct phylogenetic groups and that seadornaviruses share a common phylogenetic origin with rotaviruses (arrow). KDV, Kadipiro virus; BAV, Banna virus; LNV, Liao Ning virus; RDV, Rice dwarf virus; RDV-Ch, RDV Chinese isolate; SCRV, St. Croix River virus; AHSV, African horse sickness virus; CHUV, Chuzan virus; BTV, Blue tongue virus; GSRV, Golden shiner reovirus; GCRV, Grass carp reovirus; CSRV, Chum salmon reovirus; SBRV, Striped bass reovirus; MRV, Mammalian orthoreovirus; RRSV, Rice ragged stunt virus; BmCPV, *Bombyx mori* cytoplasmic polyhedrosis virus; DsCPV, *Dendrolimus spectabilis* cytoplasmic polyhedrosis virus; LdCPV, *Lymantria dispar* cytoplasmic polyhedrosis virus; NLRV, *Nilaparvata luguens* reovirus; CTFV, Colorado tick fever virus; EYAV, Eyach virus; CPRV, *Cryphonectria parasitica* reovirus; RaRv, Rotavirus A; BoRV, Bovine rotavirus; SiRV, Simian rotavirus; PoRV, Porcine rotavirus; Hu/MuRV, Human/Murine rotavirus.

exhibited significant amino acid identity with the VP3 of rotavirus, which is also a guanylyltransferase. These data suggest an evolutionary relationship between rotaviruses and seadornaviruses (Figure 5).

Clinical Features

The only seadornavirus isolated from humans and associated with human disease is BAV. Persons infected with BAV exhibited flulike symptoms, myalgia, arthralgia, fever, and encephalitis (33). A serologic diagnostic assay was developed (40) based on VP9, the outer coat protein responsible for cell attachment and neutralization. Patients infected with BAV have shown a 4-fold increase in anti-BAV antibody titers in paired serum specimens tested by ELISA, showing an immune response to the virus infection (34). Molecular diagnostic assays have also been designed for detecting BAV and KDV (36). RT-PCR assays were validated in infected murine model, in which the genome could be detected as early as 3 days postinfection. PCR assays identify differences between genotypes A and B of BAV based on the length of the amplicon obtained by specific primers in segment 9. A PCR assay was recently developed for LNV based on sequence of segment 12 that allows detection of the genome in infected mouse blood (H. Attoui, unpub. data).

Treatment and Immunity

No specific treatment exists for infections caused by any members of the family *Reoviridae*. In infections with CTFV or BAV, symptomatic treatment includes acetaminophen for relief of fever and pain. Patients infected with CTFV show long-lasting immunity. An experimental vaccine was developed in the 1960s and produced long-lasting immunity, but production was stopped in the 1970s. Patients infected with BAV show a strong immunologic response (35). Mice experimentally infected with BAV develop viremia. Clearance of the virus from the circulation occurs concomitantly with the appearance of anti-BAV antibodies (36,40).

Dr Attoui is a virologist at the university research unit in Marseille, France. His research interest is human arboviruses of the family *Reoviridae* (coltiviruses, seadornaviruses, and orbiviruses).

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Cervical Human Papillomavirus Screening among Older Women

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Rates of acquisition and clearance of cervical human papillomavirus (HPV) during a 3-year period in women 51 years of age were compared with rates in younger women to provide data on cervical screening for women >50 years of age. Paired, cytologically negative, archived cervical smears taken 3 years apart from 710 women in Nottingham, United Kingdom, were retrieved and tested for HPV infection with polymerase chain reaction (PCR) with GP5+/6+ primers. Seventy-one (21.3%) of 333 women 51 years of age who were HPV negative at baseline were positive 3 years later. This percentage was higher than the corresponding acquisition rates among women 21 (15.2%), 31 (14.1%), and 41 (13.3%) years of age, although these differences were not significant. This retrospective study shows that HPV-negative women >50 years of age can acquire HPV and, therefore, require cervical screening.

Prospective studies of young women (≤25 years of age) have reported high rates of cervical human papillomavirus (HPV) acquisition; the average duration of these infections is <1 year (1–4). Little evidence shows that the converse is true among older women, considering the decline in HPV prevalence that has been found with age (5,6). In studies from Brazil and Colombia, an incidence of high-risk (carcinogenic) HPV of ≈5% per year was observed among women ≥35 years of age (7,8); recent data from Costa Rica showed that the rate of high-risk HPV acquisition during a 5-year interval decreased slightly with increasing age (9). All these studies were conducted in areas where the risk of cervical cancer is considered to be high. In a small study from Canada, 7.7% of women 45–49 years of age who were negative for high-risk HPV at baseline tested positive 1 year later, similar to the rate in women 20–25 years of age (10). From studies of women in adulthood, estimates of the percentage of HPV infections (high and low risk) that persist >12 months range from 25% to 50% (10–13); HPV persistence after 5 years increased with age in the study from Costa Rica (9).

Data on acquisition and clearance of HPV in women 50–64 years of age in the United Kingdom and other Western nations are needed. The presence of HPV is a requisite to develop invasive cancer of the cervix (14). Therefore, women within this age range with cytologically negative specimens, tested with a sensitive testing method such as the polymerase chain reaction (PCR), should have a virtually zero lifetime risk of developing cervical cancer, unless they acquire a new HPV infection. A mathematic modeling study based on the UK screening population estimated that not providing screening for HPV-negative women at age 50 would save as much as 25% of resources for smear tests and 18% for colposcopies; however, the savings would be accompanied by an increase in the incidence of invasive cancers of ≈2/100,000 women annually (15). These estimates would be more accurate if the risk of acquiring high-risk HPV infection at these ages was known.

In this study, archived cervical smears were used to compare rates of HPV acquisition during a 3-year period (1 screening interval in the United Kingdom) between women of different ages. This approach also allowed us to estimate rates of HPV persistence and clearance during the same 3-year interval for women whose baseline smears were HPV positive.

Methods

Anonymous data were provided by the Nottingham Cervical Screening Laboratory, which holds computerized details of all cytology smears of women who have lived in Nottingham since 1987. This laboratory also stores all specimens from women for whom routine cervical screen-

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ings were conducted by general practitioners in this area. All specimens used for this study were cytologically normal and had been stored for ≥10 years (in accordance with UK legal requirements) but were scheduled for routine destruction.

Women eligible for this study were 21, 31, 41, or 51 years of age when a normal baseline smear was taken in 1988. We intended to select a sample of 400 women 51 years of age and 100 women from each of the other age groups to participate in the study. The main entry criterion was a normal cervical smear taken in 1988, followed by a technically adequate subsequent smear (of any cytologic grade) taken from October 1990 to December 1991. The sample for women 51 years of age was supplemented by including women whose baseline smear was obtained in 1989 and who had a subsequent smear taken before July 1992; all eligible women of this age were selected. Women 21, 31, or 41 years of age were selected randomly from the list of participants who met the main entry criteria. For all participants, results from subsequent cytology examinations conducted through December 2002 were provided by the Nottingham Cervical Screening Laboratory, including any cervical biopsy results from which histologic confirmation of cervical intraepithelial neoplasia (CIN, grades 1–3) or cervical cancer was obtained.

When cytology slides were retrieved for HPV testing, all identifiers (name, date of birth, and cytology number) were replaced with a unique study number. Only investigators based at the cytology laboratory (P.V., J.J.) had access to clinical data. These investigators did not have access to HPV testing results for specific patients. Another investigator (M.G.) was responsible for linking HPV test results from the same patient with subsequent cytology results and had access to data on screening histories but not information through which patients could be identified. The study was approved by the Nottingham City Hospital ethics committee.

Cytology slides were immersed in xylene (40 mL) for 2–3 days to remove coverslips. Cells were then scraped with a sterile scalpel blade into an Eppendorf tube containing ethanol (1 mL) and centrifuged to remove any trace of xylene. DNA was extracted by using Qiagen (Crawley, West Sussex, United Kingdom) DNA extraction kits (16).

HPV DNA was amplified by using real time PCR with Mx4000 (Stratagene, La Jolla, CA, USA) (17). The GP5+ and GP6+ consensus primer pairs in the L1 region of the HPV genome identified infection with any genital HPV type (18). The PCR master mix Quantitect (Qiagen) (19) contained optimized amounts of SYBR green dye (Molecular Probes, Inc., Eugene, OR, USA) to which primers (5 pmoL/tube) and DNA template (5 μL) were added. Positive controls containing tubes of HPV 16 DNA diluted at various strengths (0.01–10 pg/tube) and negative controls containing the PCR master mix but no DNA template were included in all assays. Forty amplification cycles were performed. Samples that were HPV positive with the GP5+/6+ primer sequence were tested with type-specific HPV16 and HPV18 primers located in the E7 667–686 and 753–774 regions of the genome, respectively (Sequences: HPV16 forward primer, 5′-GAT GAA ATA GAT GGT CCAGC-3′; HPV 16 reverse primer, 5′-GCT TTG TAC GCA CAA CCG AAG C-3′; HPV18 forward primer, 5′-TGA AAT TCC GGT TGA ACC TTC-3′; and HPV 18 reverse primer, 5′GGT CGT CTG CTG AGC TTT CT-3′). A total of 471 cervical DNA samples collected for related studies (although not from women in this study) were tested for the β-globin gene to assess the integrity of the DNA extraction process; 456 (96.8%) tested β-globin positive.

HPV acquisition was calculated as the percentage of women who were HPV negative at baseline but HPV positive on follow-up (and the converse applied for HPV clearance). Clearance of baseline infections was assumed if the equivalent test at follow-up was negative. Nonsymmetric confidence intervals were calculated to measure acquisition and clearance, as these are more appropriate when observed percentages are low (20). Rates of HPV acquisition in women of different ages were compared with the χ^2 test; Fisher exact test was used as a consequence of small numbers for HPV clearance. Rates of HPV prevalence (at baseline and follow-up), acquisition, and clearance across the 4 ages were compared by using the χ^2 test for trend.

Results

Cervical smears were retrieved for HPV testing for 710 women (104 were 21 years of age, 105 were 31 years of age, 105 were 41 years of age, and 396 were 51 years of age). Of the study sample (N = 710), 11 were subsequently excluded because of a cervical abnormality before their baseline smear. Follow-up smears could not be retrieved for 27 women; the smear was cytologically abnormal in 20 cases, and the follow-up smear could not be retrieved in the 7 remaining cases. Sixteen women were excluded because an HPV result could not be ascertained at either time because of insufficient DNA. The final sample was 656 women. The mean length of time between the baseline and follow-up smear was 3.08 years (range 1.98–3.84 years). For 76% of the sample (n = 499), the time gap was 2.75–3.25 years. The average time gap between the smears did not vary substantially between age groups.

Rates of HPV infection in this sample are shown in Table 1. In 1988, rates of HPV infection declined with age; the lowest rate was seen in women 51 years of age (10.5%). Three years later, the trend was reversed with the highest rate of HPV in women who were 51 years of age

Table 1. Human papillomavirus (HPV) positivity rates in baseline and follow-up samples

Age (baseline)	Baseline, 1988; no. HPV positive (%)	Follow-up, 1991; no. HPV positive (%)
21 (n = 86)	20 (23.3)	13 (15.1)
31 (n = 98)	13 (13.3)	15 (15.3)
41 (n = 100)	17 (17.0)	14 (14.0)
51 (n = 372)	39 (10.5)	81 (21.8)
	χ^2 (trend): p = 0.004	χ^2 (trend): p = 0.059

at baseline. A test to determine HPV infection trend with age was significant at baseline and borderline significant at follow-up (Table 1).

Age-specific rates of HPV acquisition and clearance (any genital type amplified by the GP5+/6+ primer pair) are shown in Table 2. The HPV acquisition rate was highest for women who were 51 years of age at baseline, although this rate was not significantly higher than the corresponding rate for any of the other ages. A test for trend in the acquisition rates across the 4 ages reached borderline significance (χ^2 trend = 3.18, p = 0.07). Clearance of HPV infection was lowest for women 51 years of age, although again, no substantial differences were observed between this rate and those for the other 3 ages, and the trend in clearance rates across the 4 ages was not significant (χ^2 trend = 0.72, p = 0.40). When analyses for overall HPV acquisition and clearance were conducted again and included the 20 women with abnormal cytology at follow-up, assuming that these were HPV positive, the results for both HPV acquisition and clearance remained similar.

Type-specific HPV acquisition and clearance rates are shown in Table 3. Twenty (11 in women 51 years of age) new HPV16 infections were found in this sample; 24 (14 in women 51 years of age) new HPV18 infections were found. The low number of new HPV16 and HPV18 infections meant that the confidence intervals for the estimated rates of acquisition of these types were wide. Linear trends to assess rates of acquisition across age were not significant for either HPV16 (χ^2 trend 0.08, p = 0.77) or HPV18 (χ^2 trend 0.02, p = 0.89). Three women (21, 31, and 51 years of age, respectively) acquired both HPV16 and HPV18 infections at follow-up, after testing negative for both at baseline. Of the 27 HPV16 or HPV18 infections that were found at baseline over all 4 ages, only 3 were still present at the time of follow-up. This finding indicated overall clearance rates were close to 90% for both HPV16 and HPV18 (Table 3).

Abnormal findings, including symptoms of mild severity in 10 women and moderate severity in 6 women, developed in 38 women in this study during a follow-up period of 11 years; severe dyskaryosis developed in 5 women. CIN or cancer was histologically confirmed in 8 women; CIN grade 1 was diagnosed in 4 women, CIN 3 was diagnosed in 3, and invasive squamous carcinoma was diagnosed in 1. Cytologic and histologic results stratified by HPV status in 1988 and 1991 are summarized in Table 4. Rates of cervical abnormality during follow-up were slightly higher for women who were HPV positive at baseline, although HPV status at either time was not significantly associated with subsequent abnormal cytologic results (Table 5). Two of the 5 women with severe dyskaryosis, however, had acquired HPV at the time of the second smear (Table 4). Both of these women were 51 years of age at baseline. Squamous carcinoma was diagnosed in 1 of these women in 1998 (7 years after HPV was detected). This woman was negative for HPV types 16 and 18. The other woman also had the cytologic diagnosis in 1998; however, no biopsy result was recorded on this occasion. This woman was positive for both HPV16 and HPV18 at baseline.

Discussion

Our study found a high 3-year rate of HPV acquisition rate in women 51 years of age at baseline. Although differences in HPV acquisition with age were not statistically significant, this rate was at least as high as that observed among younger women. This rate is equivalent to the 1-year acquisition rate for high-risk HPV in a Canadian study of a sample of 39 women 45–49 years of age (10). Our estimated incidence among 51-year-old women was slightly higher than the rate of high-risk HPV (6.1/100) found in 50- to 54-year-old women in a study from Colombia (8), a country with a high rate of cervical cancer.

Table 2. Numbers of women infected with human papillomavirus (HPV) at baseline and follow-up and HPV acquisition and clearance rates by age

Age (baseline)	HPV status – (1988)		HPV status + (1988)		% HPV acquisition in 3 y (95% CI)*	% HPV clearance in 3 y (95% CI)
	– (1991)	+ (1991)	– (1991)	+ (1991)		
21	56	10	17	3	15.2† (8.4–25.7)	85.0‡ (64.0–94.8)
31	73	12	10	3	14.1† (8.3–23.1)	76.9‡ (49.7–91.8)
41	72	11	14	3	13.3† (7.6–22.0)	82.4‡ (59.0–85.4)
51	262	71	29	10	21.3 (17.3–26.0)	74.4 (58.9–85.4)
Total	463	104	70	19	18.3 (15.4–21.7)	78.7 (69.0–85.9)

*CI, confidence interval.
†Not significantly different from acquisition rate in women 51 years of age when using χ^2 test (p>0.05)
‡Not significantly different from clearance rate in women 51 years of age when using Fisher exact test (p>0.05)

Table 3. Numbers of women infected with human papillomavirus (HPV) 16 and 18 at baseline and follow-up and HPV16 and HPV18 acquisition and clearance rates by age

Age (y, baseline)	HPV status – (1988)		HPV status + (1988)		% HPV acquisition in 3 y (95% CI)	% HPV clearance in 3 y (95% CI) *
	– (1991)	+ (1991)	– (1991)	+ (1991)		
HPV16						
21	79	3	3	1	3.7 (1.3–10.2)	–
31	94	3	1	0	3.1 (1.1–8.7)	–
41	94	3	3	0	3.1 (1.1–8.7)	–
51	360	11	1	0	3.0 (1.7–5.2)	–
Total	627	20	8	1	3.1 (2.0–4.7)	88.9 (56.5–98.0)
HPV18						
21	77	2	6	1	2.5 (0.7–8.8)	–
31	89	5	3	1	5.3 (2.3–11.9)	–
41	95	3	2	0	3.1 (1.0–8.6)	–
51	352	14	5	0	3.8 (2.3–6.3)	–
Total	613	24	16	2	3.8 (2.5–5.5)	88.9 (67.1–96.9)

*Clearance rates of HPV 16/18 were not calculated for individual ages because of insufficient numbers; CI, confidence interval.

The combined acquisition rate for HPV types 16 and 18 in this study was ≈7%. Only type-specific PCR was conducted for these types, a limitation of this study. Further research is, therefore, required to estimate the combined acquisition rate for all high-risk HPV among older women (and, conversely, to determine whether a substantial proportion of incident infections are with low-risk HPV).

A selection criterion for this study was a cytologically normal cervical smear taken during 1988. In the United Kingdom, changes to the cervical screening program had just been implemented at that time, and coverage rates of the UK screening population were ≈50% (21). Whether this change could have caused selection bias remains speculative; women who received cervical screening may have been at higher risk than all eligible women. Another criterion was that all women in the study have cytologically normal results at both phases of the study. As stated earlier, only a small number of women (n = 20) had abnormal cytologic findings at follow-up, and when analyses that indicated these women were HPV positive were conducted again, no noticeable impact was seen on results.

The high rate of HPV acquisition in 51-year-old women may be due to either a higher-than-anticipated rate of new partners among older women or partner infidelity. However, the former supposition is not supported by data from 2 large-scale national surveys of sexual behavior among women in the United Kingdom conducted in 1990 and 2000 (22,23). The second of these surveys found that only 10.9% of women 35–44 years of age reported a new sex partner in the year preceding the survey, compared with 39.2% of women 16–24 years of age (23). Therefore, a 3-year HPV acquisition rate of 21% for women 51 years of age would be higher than expected, based solely on the rate of new partner acquisition among a group of women 35–44 years of age. The 1990 version of this survey, however, reported that 5.4% of men in the 45- to 59-year age group reported ≥2 sex partners during the previous year (22). While these may not have all been new sex partners, and the rate just among married (or cohabiting) men may be lower (separate data not available), this finding indicates that a small part of the total HPV acquisition rate could be a consequence of partner infidelity.

Also, the high rate of HPV acquisition in 51-year-old women may be due to the reemergence of latent HPV infections caused by hormonal changes resulting from menopause or alterations to the cervix caused by hormone replacement therapy. A latent infection can be defined as one where HPV genomes are established in the basal cells but differentiation of the host cells does not take place (24). If such infections were below detection with increasingly sensitive PCR technology until the point of reactivation, then women could possibly become HPV positive in the absence of sexual activity. This possibility could also explain the finding of a second age-related peak in HPV

Table 4. Association between human papillomavirus (HPV) infection in 1988 and 1991 and subsequent cytologic and histologic outcomes

Grade*	HPV status – (1988)		HPV status + (1988)	
	– (1991)	+ (1991)	– (1991)	+ (1991)
Normal	440	97	62	19
Borderline	11	2	4	0
Mild	6	2	2	0
Moderate	3	1	2	0
Severe	3	2	0	0
Any abnormal cytologic results	23	7	8	0

*Worst grade of dyskaryosis diagnosed during 11 years of cytologic follow-up.

Table 5. Human papillomavirus (HPV) status and cytologic outcome summarizing results presented in Table 4

HPV status	Cytologic outcome		Total	p*
	Normal, n (%)	Abnormal, n (%)		
1988				
HPV –	537 (94.7)	30 (5.3)	567	0.22
HPV +	81 (91.0)	8 (9.0)	89	
1991				
HPV –	502 (94.2)	31 (5.8)	533	1.0
HPV +	116 (94.3)	7 (5.7)	123	

*Comparing rates of subsequent abnormality between HPV + and – women by using Fisher exact test.

prevalence in some South American populations after 55 years of age (6,25,26). However, less evidence for this second peak in HPV prevalence is seen in the United Kingdom and other countries where the risk of cervical cancer is lower.

Additionally, false-negative results occurring during the first phase of testing would cause HPV infections present at baseline and follow-up (i.e., persistent infections) to be misclassified as incident infections, therefore overestimating rates of HPV acquisition. This study relied on archived cervical smears taken a decade earlier to detect HPV infection. While studies have validated the use of this type of specimen by showing agreement between results from archived and fresh specimens from the same women (27), concerns exist that the quality of extracted DNA may be poorer for older specimens because of changes in the methods of sampling and fixation (28).

A less understood but still plausible reason for these results also relates to the use of archived cervical smears; the possibility of cross-contamination of samples at the time of collection could have accounted for false-positive results at the second phase of testing. This contamination could have occurred either where cervical smears were taken or at the screening laboratory during fixation and staining. In a study from Sweden, this risk of cross-contamination was largely excluded when smears with registration numbers adjacent to the HPV-positive study smears were also tested for HPV (29). Results from this study are difficult to generalize to the present setting, where the risk of such cross-contamination could only be assessed if the quality of procedures conducted in doctors' offices and screening laboratories between 1988 and 1991 could be examined.

This report also contains details of HPV infection 3-year clearance rates for women who tested positive in the first phase of the study. As baseline rates of HPV infection in this sample were 10%–25%, depending on age (Table 1), few smears were included for comparison in the analysis of HPV clearance, which resulted in insufficient statistical power for clearance rates between women of different ages to be compared. Among women 51 years of age, a 74% HPV 3-year clearance rate was observed. The potential for false-negative results at the second phase of testing means that these HPV clearance rates are likely overestimated.

In the United Kingdom, screening intervals were recently lengthened to 5 years for women 50–64 years of age, after an analysis of UK screening data showed that the protective effect after a negative cervical smear persisted longer for older women (30). Whether cervical screening could be discontinued after 50 years of age for low-risk women has also been debated (31–33). Whether HPV testing could identify low-risk women would depend on the natural history of the virus in older women and the personal and social implications of introducing a test for a sexually transmitted disease into a national screening program (34). Our findings do not support discontinuing cervical screening for HPV-negative women at age 50 because of the high risk of HPV acquisition observed in women 51 years of age during a 3-year interval. By conducting a prospective study in which follow-up is ensured over a period of time at 4- to 6-month intervals, many of the limitations of this study could be circumvented. The probability of viral persistence and progression of incident HPV infections among older women in the United Kingdom and other western countries also should be recognized, as only persistent HPV infections lead to cervical cancer (35). Outcome data from this study are limited; further data are required from larger prospective studies to determine more precisely the positive and negative predictive values (for high-grade CIN and cancer) of incident HPV infections that develop in middle age. These issues require consideration before the true impact of discontinuing cervical screening programs worldwide for HPV-negative women ≥50 years of age can be accurately ascertained.

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Cryptococcus gattii in AIDS Patients, Southern California

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Cryptococcus isolates from AIDS patients in southern California were characterized by molecular analyses. Pheromone *MFα*1 and *MFa*1 gene fragments were polymerase chain reaction–amplified with fluorescently labeled primers and analyzed by capillary electrophoresis (CE) on DNA analyzer. CE–fragment-length analyses (CE-FLAs) and CE–single-strand conformation polymorphisms (CE-SSCPs) were used to determine *Cryptococcus gattii* (*Cg*), *C. neoformans* (*Cn*) varieties *neoformans* (*CnVN*) and *grubii* (*CnVG*), mating types, and hybrids. Corroborative tests carried out in parallel included growth on specialized media and serotyping with a commercial kit. All 276 clinical strains tested as haploid *MATα* by CE-FLA. CE-SSCP analyses of *MFα1* showed 219 (79.3%) *CnVG*, 23 (8.3%) *CnVN*, and 34 (12.3%) *Cg* isolates. CE-FLA and CE-SSCP are promising tools for high-throughput screening of *Cryptococcus* isolates. The high prevalence of *Cg* was noteworthy, in view of its sporadic reports from AIDS patients in North America and its recent emergence as a primary pathogen on Vancouver Island, Canada.

The encapsulated basidiomycete *Cryptococcus neoformans* was recently divided into 2 species, *C. neoformans* (*Cn*) and *C. gattii* (*Cg*) (1). *Cn* consists of 2 varieties, *grubii* (*CnVG*) and *neoformans* (*CnVN*), which are opportunistic pathogens and predominantly infect immunocompromised persons (2,3). *CnVG* is the major causative agent of cryptococcosis worldwide, except in central Europe, where *CnVN* infection is most prominent. In contrast, *Cg* is a primary pathogen, which predominantly infects immunocompetent persons (4). *Cg* was previously thought to be restricted to tropical and subtropical climates with a special ecologic niche on *Eucalyptus* trees (5,6). However, the recent outbreak of *Cg* infection in healthy humans and animals in the temperate climate of Vancouver Island, British Columbia, Canada, and its isolation from several species of trees other than *Eucalyptus* have raised the strong possibility that this fungus might have broader geographic distribution (7–9).

The mechanisms underlying pathogenic and environmental differences between *Cn* and *Cg* are not known. Within *Cn* species, *CnVN* infections are more likely to display skin involvement and to afflict older patients, whereas *CnVG* infections are reported to cause a higher mortality rate (3,10). In contrast, infections caused by *Cg* result in a lower mortality rate but are frequently complicated by neurologic sequelae and require surgery and prolonged therapy (4,11). Our recent studies with the Cu,Zn SOD (*SOD1*) and MnSOD (*SOD2*) knockout mutants of *Cg* indicated that these antioxidants are crucial for *Cg* pathogenesis (12,13). In contrast, the antioxidant function of *SOD1* in *CnVG* is less crucial for pathogenesis (14). These observations are the first molecular evidence of a likely divergence in the pathogenic mechanisms used by *Cn* and *Cg*.

Both *Cn* and *Cg* have a single locus, 2-allele mating system comprising *MATα* and *MATa* strains**.** The *MATα* strains of *Cn* and *Cg* predominate in nature and in clinical settings, and this predominance over *MATa* strains is linked to high virulence and reproduction by haploid fruiting (3,15). Generally, *Cryptococcus* strains are haploid, but hybrid strains have also been characterized from both clinical and environmental sources (16–20). Thus, characterizing clinical *Cryptococcus* isolates to the individual species or varieties and according to mating and hybrid types could be useful for managing cryptococcosis cases and for further understanding the epidemiology of this disease.

Several laboratory typing methods have been used in epidemiologic studies of cryptococcosis, including serotyping, electrophoretic karyotyping, use of mitochondrial DNA probes, use of genomic DNA probes, determination of allelic variations at the *URA5* locus, multilocus enzyme typing, measurement of creatinine utilization, polymerase chain reaction (PCR), fingerprinting and

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amplified fragment-length polymorphism (reviewed in [2]). Previously, we described a PCR–restriction fragment length polymorphism (PCR-RFLP) typing scheme for *Cn* and *Cg* pheromone genes, which could be used for characterizing mating types, hybrids, and variety (21). In the present study, we developed a capillary electrophoresis–fragment length analysis (CE-FLA) test, and a CE–single stranded conformation polymorphism (CE-SSCP) test by using the pheromone genes *MFα1* and *MF**a**1*. These tests were used in parallel with more traditional specialized culture medium and a commercial serotyping kit to characterize *Cryptococcus* isolates from AIDS patients in southern California.

Materials and Methods

Cryptococcus Isolates

Two hundred seventy-six *Cryptococcus* isolates originating from patients with HIV/AIDS were obtained from the Infectious Diseases Laboratory, Los Angeles County Hospital, Los Angeles, California. The isolates were suspended in sterile skim milk and stored at –20°C. The isolates were transferred frozen to the Mycology Laboratory of the Wadsworth Center in Albany, New York, USA, where they were streaked on Niger seed agar plates (3) to check for purity and reconfirmation of their identity; a typical colony was picked for further analysis. The subcultures were placed in long-term storage in sterile 15% glycerol at –70°C. These isolates were further characterized in our laboratory by testing their growth on canavanine-glycine-bromothymol blue (CGB) agar for differentiation of *Cryptococcus* species (22) and serotyping with Crypto Check Kit (Iatron Laboratories Inc., Tokyo, Japan). Several investigators gave strains to put together a panel of reference isolates that were either currently being used in molecular pathogenesis studies, represented type strains, or were otherwise unique. The details of these 16 reference isolates are listed in Table 1. Six additional A/D hybrid strains, characterized in our earlier study, were also used (18).

Multiplex PCR for Pheromone Genes

A previous report from this laboratory described the use of specific primers for amplification of *MFα1* and *MF**a**1* gene fragments, which could be separated as 100-bp and 117-bp fragments on a specialized agarose gel (21). The primer sets V290/V291, which was earlier designed to amplify *MF**a**1* gene from *CnVN*, did not amplify similar genes from *MAT**a*** strains of either *CnVG* or *Cg*. Multiple alignment of *MF**a**1* indicated that this gene is highly polymorphic among *CnVG*, *CnVN*, and *Cg* (Figure 1). Therefore, 2 new sets of primers were designed to obtain *MF**a**1* amplicons from *CnVG* and *Cg*. These primers are listed in Table 2. A multiplex PCR for simultaneous amplification of *MFα1* and *MF**a**1* in a 50-μL reaction volume was performed with 5 μL of 10× PCR buffer with 15 mmol/L $MgCl_2$, 2.5 μL of each of 8 primers (10 μmol/L stock), 3.0 μL dNTP mix (10 μmol/L each), and 2.0 U Taq DNA polymerase (Perkin Elmer, Foster City, CA, USA). The template DNA was 5.0 μL of either a boiled cell suspension or 50 ng genomic DNA. Initial denaturation was conducted at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57.5°C for 1 min, amplification at 72°C for 1 min, and final extension at 72°C for 7 min, in a GeneAmp PCR System 9600 (Perkin Elmer). In preliminary experiments, PCR products (10-μL aliquots) were resolved by electrophoresis on 3.5% MetaPhor agarose (FMC Bio-Products, Rockland, ME,

Table 1. *Cryptococcus neoformans* (*Cn*) and *Cryptococcus gattii* (*Cg*) strains used in this study for standardization of reagents*

Strain identity	Variety/species	Mating type	Source
H99 (NYSD 1649)	*CnVG*	α	New York State Herbarium, Albany, NY
KN99α	*CnVG*	α	J. Heitman, Duke University, Durham, NC
KN99**a**	*CnVG*	**a**	J. Heitman, Duke University, Durham, NC
IUM96–2828	*CnVG*	**a**	B.L. Wickes, University of Texas Health Sciences Center, San Antonio, TX
NIH12 (ATCC 28959)	*CnVN*	α	ATCC, Manassas, VA
JEC21	*CnVN*	α	J.C. Edman, University of California San Francisco (UCSF), San Francisco, CA
JEC20	*CnVN*	**a**	J.C. Edman, UCSF, San Francisco, CA
NIH430 (ATCC 28958)	*CnVN*	**a**	ATCC, Manassas, VA
NIH433 (ATCC 34875)	*CnVN*	**a**	ATCC, Manassas, VA
NIH444 (ATCC 32609)	*Cg*	α	ATCC, Manassas, VA
NIH191 (ATCC 32608)	*Cg*	**a**	ATCC, Manassas, VA
NIH198	*Cg*	**a**	K.J. Kwon-Chung, National Institutes of Health, Bethesda, MD
WM0135	*Cg*	**a**	W. Meyer, University of Sydney, Sydney, Australia
WM-138	*Cg*	**a**	W. Meyer, University of Sydney, Sydney, Australia
UM2	Hybrid (A/D)	α/**a**	F. Dromer, Institute Pasteur, Paris, France
UM8	Hybrid (A/D)	α/**a**	F. Dromer, Instotite Pasteur, Paris, France

*ATCC, American Type Culture Collection; *VG*, var. *grubiii*; *VN*, var. *neoformans.*

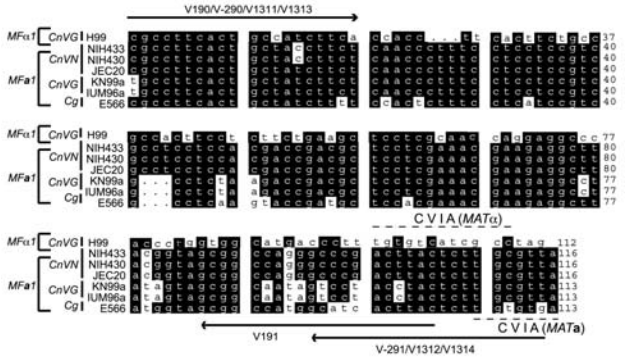

Figure 1. Primers for pheromone polymerase chain reaction (PCR). Nucleotide sequence alignment for *MFα1* and *MF**a**1* genes is shown with characteristic Cys-Val-Ile-Ala (CVIA) motifs. Both sense and antisense primers were designed from within the open reading frames of pheromone genes, to ensure high specificity of the multiplex PCR. The *MFα1* sequence from *Cryptococcus neoformans* var. *grubii* (*CnVG*) (AF542529) and the *MF**a**1* sequences from *CnVG* (AY129299), *Cryptococcus neoformans* var. *neoformans* (*CnVN*) (AF542530), and *Cryptococcus gattii* (*Cg*) (AY710429) were used for multiple alignments with GCG (Wisconsin package version 10.0). A common primer pair, V190/V191, was designed to get *MFα1* PCR amplicons from *CnVG*, *CnVN*, and *Cg* (*MFα1* sequence from *CnVG* was used as a reference), while unique primer pairs V290/V291, V1311/V1312, and V1313/V1314 were designed to get *MF**a**1* PCR amplicons from *CnVN*, *CnVG*, and *Cg*, respectively. All the 3′-PCR primers contained a sequence from CVIA motif, which provided specificity to PCRs for pheromone genes.

USA) gels in Tris-borate-EDTA (TBE) buffer, and were detected by ethidium bromide staining. The PCR experiments were repeated twice, and identical results were obtained.

Gene Scan Analysis

The *MFα1* sense primer (V190) was labeled with FAM (6-carboxyfluorescein) at the 5′ end, the antisense primer (V191) was labeled with tetrachloro-fluorescein (TET) at the 3′ end, and *MF**a**1* sense primers (V290, V1311, V1313) were labeled with 6-carboxy-2′, 4′, 4′, 5′, 7′, 7′-hexachlorofluorescein (HEX) at the 5′ end. The fluorescent dye–labeled primers were custom ordered (Operon Technologies, Inc., Alameda, CA, USA). FLA and SSCP of the *MFα1* and *MF**a**1* PCR amplicons were determined by CE with an ABI PRISM 310 Genetic Analyzer, and the electronic images were analyzed by using GeneScan analysis software (Applied Biosystems Inc., Foster City, CA, USA). The sample preparation for CE consisted of 1 μL *MFα1* and *MF**a**1* PCR amplicons, 12 μL highly deionized formamide, and 0.5 μL GeneScan-500 (TAMRA) size standard (Applied Biosystems). The sample mixture was denatured for 5 min at 95°C and was then rapidly cooled on ice before loading on the instrument. For CE-FLA, the samples were analyzed under denaturing conditions (POP-4 polymer [Applied Biosystems] in buffer supplied by manufacturer) at 60°C, and for CE-SSCP, the samples were analyzed under nondenaturing conditions (3% GeneScan polymer in 1× TBE buffer with 10% glycerol) at 30°C. A capillary (47 cm × 50 μm inside diameter) was installed, and POP-4 or 3% polymer was filled according to manufacturer's instructions. The electrophoresis conditions for CE-FLA were 5-s injection time, 15-kV injection voltage, 15-kV electrophoresis voltage, 150-s syringe pump time, 120-s preinjection electrophoresis, and 20-min collection time for each sample, and the run was performed at 60°C. The electrophoresis conditions for CE-SSCP were 5-s injection time, 15-kV injection voltage, 13-kV electrophoresis voltage, 30-s syringe pump time with no preinjection time, and 20-min collection time for each sample, and the run was performed at 30°C. CE-FLA and CE-SSCP standardization experiments were carried out on ≥4 independent occasions, and unknown sample analyses were repeated at least once.

Results

Multiplex PCR

The 4 sets of primers (*MFα1*/*MF**a**1*) produced reproducible results for control *CnVG*, *CnVN*, *Cg* haploids (Figure 2A), and A/D hybrid strains (Figure 2B). These results validated the robustness of the primers, which had been designed from well within the open reading frames of 2 pheromone genes, to prevent amplification of any nontarget DNA. The latter objective also informed the deci-

Table 2. Primers used in this study*

Primer name	Sequence	Target	Source/reference
V190–5′	5′-CTTCACTGCCATCTTCACCA-3′	*MFα1–Cg*, *CnVN*, and *CnVG*	(21)
V191–3	5′-GACACAAAGGGTCATGCCA-3′		
V290–5′′	5′-CGCCTTCACTGCTACCTTCT-3′	*MF**a**1–CnVN*	(21)
V291–3	5′-AACGCAAGAGTAAGTCGGGC-3′		
V1311–5′	5′-TGCCTTCACTGCTATCTTCT-3′	*MF**a**1–CnVG*	This study
V1312–3′	5′-AACGCAAGAGTAGGTAGGAC-3′		
V1313–5′	5′-CGCCTTCACTGCTATCTTTTC-3′	*MFa1–Cg*	This study
V1314–3′	5′-CACACAAGAGTAAGTGATGC-3′		

**Cn, Cryptococcus neoformans; Cg, Cryptococcus gattii*; *VN*, var. *neoformans; VG*, var. *grubii*.

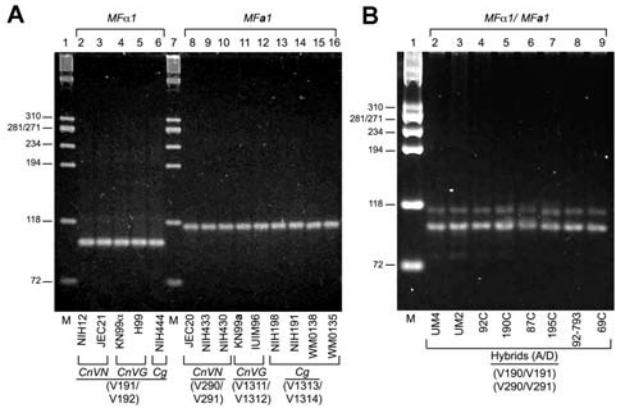

Figure 2. Multiplex polymerase chain reaction (PCR) for pheromone fragment analysis. A) Multiplex PCR with 4 sets of primers comprising *MFα1* (V190/V101) and *MF**a**1* (V290/V291, V1311/V1312, V1313/V1314) genes were carried out as described in Materials and Methods. Approximately 100-bp *MFα1* and 117-bp *MF**a**1* PCR amlicons were detected on 3.5% MetaPhor agarose in Tris-borate-EDTA buffer for *MATα* and *MAT**a*** strains comprising *Cryptococcus neoformans* var. *grubii* (*CnVG*), *Cryptococcus neoformans* var. *neoformans (CnVN*), and *Cryptococcus gattii* (*Cg*) Lanes 1 and 7, molecular mass marker. B) Multiplex PCR depicting *MFα1* and *MF**a**1* PCR amplicons from the 8 known hybrid (A/D) isolates. Lane 1, molecular mass marker.

sion to anchor the 3′ ends of all PCR primers within the characteristic Cys-Val-Ile-Ala (CVIA) motifs; this eliminated any possible amplification of other pheromone genes since this is the only sequence shared among fungal pheromones (18). Even though multiple copies of *MFα* and *MF**a*** genes have been reported in *C. gattii* and *C. neoformans* by Southern hybridization and whole genome-sequencing, PCR primers only amplify single amplicons because these genes have identical nucleotide sequences (18,23,24). Although this multiplex method was well suited for identifying mating types and hybrids, further delineation of species and varieties would require restriction digestion with several unique enzymes as we stated previously (21). Therefore, we decided to use CE-FLA and CE-SSCP to further characterize pheromone gene amplicons. These techniques have been successfully used to delineate fragment length as well gene mutations for characterizing various fungal and bacterial isolates (25–27). The SSCP analysis displays migration of the amplified DNA fragment as a function of that fragment's structural conformation. Given that the tertiary structure of a fragment is sensitive to single nucleotide substitutions, this method was shown to be suitable for detecting single nucleotide changes when 100-bp to 300-bp DNA fragments were analyzed (28). Since amplified pheromone fragments yield ≈100- to 120-bp products, they were an ideal substrate for this method of mutant detection.

CE-FLA

The 16 reference strains of known *Cryptococcus* species, varieties, mating types, and hybrids were used to establish a robust CE-FLA protocol with denaturing POP-4 polymer at 60°C. The electrophoretic runs with POP-4 polymer produced a 112-bp DNA fragment for *MF**a**1* and 97-bp fragment for *MFα1*, which were easily distinguished with the GeneScan software by the characteristic peak sizes (Figure 3). CE-FLA allowed *Cryptococcus* mating types and hybrids to be identified, but not *CnVG*, *CnVN*, and *Cg*.

CE-SSCP

CE-SSCP test under nondenaturing conditions with 3% GeneScan polymer at 30°C allowed characteristic peak patterns to be detected in *MFα1* and *MF**a**1* genes because of the individual differences within the nucleotide sequences. Distinct patterns obtained for *CnVG*, *CnVN*, and *Cg* by using *MFα1* gene are shown in Figure 4. The sense strand (labeled blue) yielded 1 characteristic peak pattern, while the antisense strand (labeled green) yielded 2–3 characteristic peak patterns. We subsequently decided to label only the sense strand to reduce the cost of the PCR primers as well the complexity of the peak patterns observed with antisense strand. For determining an unknown sample, the instrument analyses needed to yield

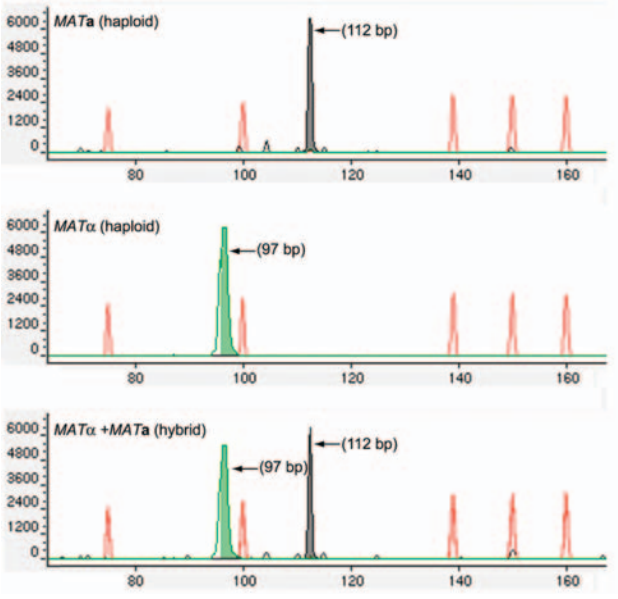

Figure 3. Capillary electrophoresis fragment-length analyses (CE-FLA) for the identification of mating types and hybrids. The ABI PRISM 310 Genetic Analyzer and GeneScan analysis software were used for the fragment length analysis of the pheromone genes. Sense strands of *MFα1* and *MF**a**1* were labeled with fluorescent probes TET (green) and HEX (black), respectively, and polymerase chain reaction amplicons were analyzed with POP-4 polymer under denaturing conditions at 60°C. Green peak, *MFα1*; black peak, *MF**a**1*. These peaks were aligned by using an internal size standard, GeneScan-500 TAMRA (red peaks).

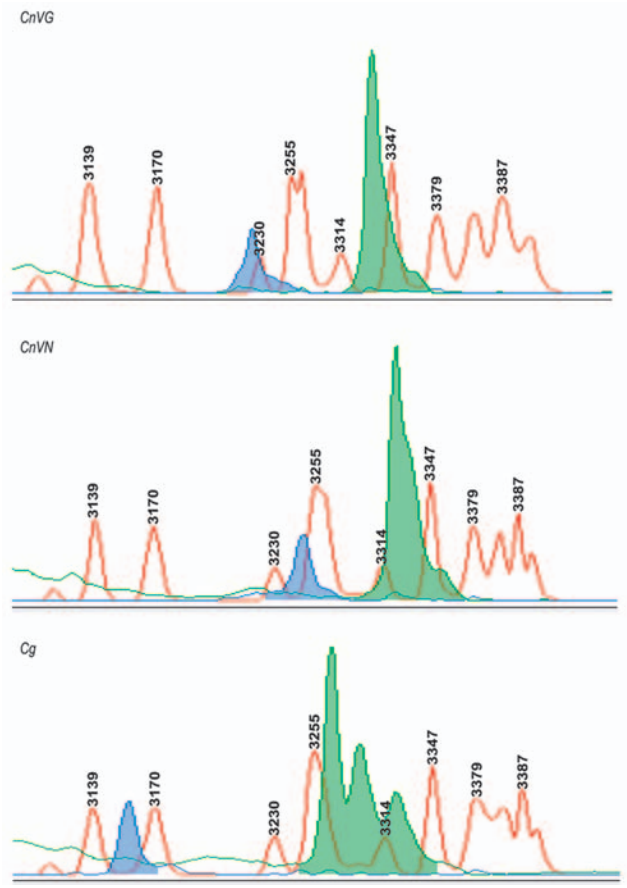

Figure 4. Capillary electrophoresis–single strand conformation polymorphisms (CE-SSCP) for the identification of varieties and species. The ABI PRISM 310 Genetic Analyzer and GeneScan analysis software were used for variety and species determination with the *MFα1* pheromone gene. The *MFα1* sense and antisense primers were labeled with fluorescent probes FAM (blue) and TET (green), and and polymerase chain reaction amplicons were analyzed with 3% polymer at 30°C under nondenaturing conditions. The blue and green peaks depict characteristic peak pattern for *Cryptococcus neoformans* var. *grubii* (*CnVG*), *Cryptococcus neoformans* var. *neoformans (CnVN*), and *Cryptococcus gattii* (*Cg*). These peaks were aligned by using an internal size standard.

highly reproducible results. Therefore, a sample of each of the *Cryptococcus* strains was injected on 4 separate occasions into the same capillary, and the precision of the sizing was calculated. The low standard deviations associated with each mean peak value indicated that the assignment of variety or species for an unknown sample, based on pattern sizing information alone, would be highly reliable (Table 3).

Based on our success with *MFα1* sense primer in the detection of characteristic peaks for *CnVG*, *CnVN*, and *Cg*, we labeled *MF**a**1* sense strands and analyzed *MAT**a*** strains. In this case, we had to use individual sets of *MF**a**1* primers because of the substantial polymorphism observed at the 5′ and 3′ end of this gene between *Cg* and *Cn*, and within *Cn* vatieties (Figure 1). Again, the *MF**a**1* peak pattern was unique to each *Cn* variety and *Cg* (Table 3). Overall, our results indicated that either *MFα1* or *MF**a**1* gene products yielded unique SSCP patterns and could be used for identifying *Cn* varieties and *Cg* strains.

California Isolates

We used standardized CE-FLA and CE-SSCP techniques to analyze 276 isolates of *Cryptococcus* that were obtained from AIDS patients and that were stored at the Infectious Diseases Laboratory, Los Angeles County Hospital, Los Angeles, California. The investigations were fully compliant with Los Angeles County Hospital–University of Southern California (USC) Institutional Review Board guidelines (proposal #924008). CE-FLA showed that all 276 isolates were *MATα* strains, and no *MAT**a*** or hybrid strains were found in our samples. CE-SSCP found that among the total 276 clinical isolates, 219 (79.3%) were *CnVG*, 23 (8.3%) were *CnVN*, and 34 (12.3%) were *Cg*. For corroborations, all of these isolates were also tested by growth on CGB agar, and by serotyping with the Crypto Check Kit, both of which yielded results in agreement with those obtained with pheromone typing.

Discussion

The relatively high prevalence of *Cg* in this survey is noteworthy for several reasons (Table 4). First, we believe it is the first instance in which a large number of *Cg* clinical isolates from AIDS patients have been identified in the United States. Second, *Cg* has never been considered a substantial cause of cryptococcosis among US AIDS patients, including those in the southern California. Third, the presence of *Cg* in HIV-AIDS patient samples in the USC collection is similar to the prevalence recently reported from some countries in Central and South America (29), and it contrasts with the rare occurrence of *Cg* in immunocompromised patient populations in Australia, Southeast Asia, and Africa (30–32). The prevalence of *CnVN* (8%) in our samples closely matches its recently reported prevalence in New York City (33). Thus, cryptococcosis due to

Table 3. Calibration of SSCP peak positions for *CnVG*, *CnVN*, and *Cg**

Cn strains	Sense strand peak†
CnVG (KN99*α*; *MATα*)	3230.57 ± 0.37
CnVG (KN99***a***; *MAT**a***)	4501.35 ± 1.29
CnVN (NIH12; *MATα*)	3252.77 ± 1.29
CnVN (NIH340; *MAT**a***)	4643.75 ± 1.12
Cg (NIH 444; *MATα*)	3161.54 ± 0.95
Cg (NIH198; *MAT**a***)	4593.35 ± 1.2

*SSCP, single-strand conformation polymorphisms; *Cn*, *Cryptococcus neoformans*; *Cg*, *Cryptococcus gattii*; *VN*, var. *neoformans*; *VG*, var. *grubii*.
†Mean ± SD of 4 independent experiments.

Table 4. Relative distribution of *CnVG*, *CnVN*, and *Cg* in HIV-AIDS patients from southern California

Isolates*	n (%) (N = 276)
CnVG	219 (79.3)
CnVN	23 (8.3)
Cg	34 (12.3)

**Cn, Cryptococcus neoformans; Cg, Cryptococcus gattii; VN,* var. *neoformans; VG,* var. *grubii.*

CnVN in AIDS patients is not a rare clinical entity in the United States, and its pattern of distribution on the East and West Coasts does not differ. The absence of *MAT**a*** strains in our samples is not surprising, in view of the rare occurrence of this mating type among clinical and environmental specimens (34). This finding is consistent with the results of other recent clinical and environmental surveys in the United States, Europe, and Australia. The *Cg* outbreak on Vancouver Island also yielded only *MATα* isolates (8,35). Overall, our inferences are based on limited data, since most published studies on cryptococcosis do not include detailed characterization of *Cryptococcus* strains. Future epidemiologic studies will likely yield a more complete picture of the causative varieties or species of *Cryptococcus* across the United States.

As previously noted, *CnVG* infections are predominant in AIDS patients around the world, except in Europe, where *CnVN* is seen in sizable numbers. One explanation for this phenomenon is that *CnVG* is best adapted for the colonization of soil and pigeon droppings. However, *CnVN* may dominate the same ecologic niche in parts of Europe for undetermined causes (10). The lower incidence of *Cg* infection in AIDS patients could be due to the paucity of regions around the world in which *Cg* is endemic and the reported association of *Cg* disease with the flowering of *Eucalyptus camaldulensis* trees in certain areas (6). The unprecedented outbreak of *Cg* infection in Vancouver Island already comprises 66 human and 50 animal cases of cryptococcosis. Ongoing investigation in Vancouver Island indicate that the numbers of human and animal cases are increasing (130 human cases and >200 animal cases), which led to the recent change in the definition of *Cg* outbreak to *Cg* endemicity in this region (7). Additionally, *Cg* was isolated from swab samples from the bark of trees of many species (alder, arbutus, bitter cherry, cedar, fir, garry oak, maple, spruce), as well as from soil and air samples near these trees (7). These investigations have added a new dimension to our understanding of *Cg* ecology and suggest that this pathogen is neither restricted in its geographic distribution nor to its presumed natural host, *Eucalyptus* trees.

Our results indicated that both CE-FLA and CE-SSCP of pheromone genes are amenable to semi-automation and large-scale analyses of pathogenic *Cryptococcus* species, varieties, mating types, and hybrids. Each step of this analysis, namely, PCR, heat denaturation with formamide, and subsequent loading of samples, can be carried out in 48- or 96-well trays, which allow the use of multichannel or automated pipettors. Both CE-FLA and CE-SSCP individual runs are completed in ≤20 min, and the instrument can be programmed for multiple runs, thereby giving a high throughput. Thus, analyzing hundreds to thousands of strains is a good possibility, especially in reference laboratories. Moreover, the electrophoretic runs are saved as electronic files for easy portability over the Internet and to facilitate interlaboratory comparisons. This study reports a logical improvement over our earlier published method on pheromone PCR-RFLP for characterizing *Cryptococcus* isolates. The use of CE-FLA and CE-SSCP allowed us to dispense with running specialized gels as well as the use of unique restriction digestion schemes (21). Thus, CE-FLA alone leads to visualization of size differences in *MFα1* versus *MF**a**1* pheromones, which would distinguish mating types and hybrids. The species and varieties could be distinguished by CE-SSCP on the basis of polymorphisms in nucleotide sequences of *MFα1* and *MF**a**1* in *Cg*, *CnVG*, and *CnVN*. Thus, 1 typing method had the potential to replace multiple tests, such as specialized media and serotyping kits for species/variety determination, crossing with tester strains on mating agar, and flow cytometry for hybrid determination. The current limitations of this approach include the use of 2 polymers and run temperatures, which makes it necessary to run CE-FLA and CE-SSCP as batch applications on ABI 310 Genetic Analyzer. Since individual electrophoretic runs are completed in ≈20 min, ≈16 hours will be necessary to analyze ≈45 samples (one 48-sample tray) by CE-FLA, followed by change of polymer and run conditions, and another 16 hours to complete CE-SSCP analyses. However, these limitations could be easily overcome in the upgraded model of this instrument (ABI 3130), which has 4–16 capillaries and hands-free, 24-hour operation capabilities for simultaneous analyses of multiple samples, thereby considerably decreasing turnaround time.

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Rift Valley Fever in Small Ruminants, Senegal, 2003

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During the 2003 rainy season, the clinical and serologic incidence of Rift Valley fever was assessed in small ruminant herds living around temporary ponds located in the semi-arid region of the Ferlo, Senegal. No outbreak was detected by the surveillance system. Serologic incidence was estimated at 2.9% (95% confidence interval 1.0–8.7) and occurred in 5 of 7 ponds with large variations in the observed incidence rate (0%–20.3%). The location of ponds in the Ferlo Valley and small ponds were correlated with higher serologic incidence ($p = 0.0005$ and $p = 0.005$, respectively). Rift Valley fever surveillance should be improved to allow early detection of virus activity. Ruminant vaccination programs should be prepared to confront the foreseeable higher risks for future epidemics of this disease.

Rift Valley fever (RVF) is an arbovirosis caused by a phlebovirus (*Bunyaviridae*). In ruminants, RVF causes mass abortions and deaths in newborn kids and lambs. Human disease is often limited to a flulike syndrome, but severe forms have been reported (1). In West Africa, domestic ruminants are the main hosts of the virus, which is transmitted between animals by mosquitoes, particularly those belonging to the *Culex* and *Aedes* genera (2,3). Transmission is mostly horizontal, but a vertical mode was described for some *Aedes* species. Human cases are mainly caused by virus exposure after abortion or slaughtering of viremic animals (1).

A large RVF epidemic occurred in 1987 in southern Mauritania, with >200 reported human deaths (4). In the following years, several animal and human outbreaks occurred in Mauritania, Senegal, which emphasizes the need for understanding and modeling the risk for RVF in this region before implementing more efficient surveillance and control measures (5–7). For this purpose, a survey was conducted in the pastoral area of the Ferlo in northern Senegal.

During the rainy season, this agro-ecosystem depends on the availability of surface water in temporary ponds that are flooded after the first rainfalls. These ponds also constitute a favorable habitat for RVF vectors. Previous studies showed that Barkedji, a village located in the central part of the Ferlo, was an area with active viral circulation (5,6). The purpose of this study was to assess RVF activity in the area of Barkedji during the 2003 rainy season and to identify risk factors for its transmission to livestock.

Materials and Methods

Study Area

The survey area (Figures 1 and 2) was a 40-km diameter circle centered on the village of Barkedji (14°52′W, 15°16′N). The shrubby vegetation and hot, dry climate were typically Sahelian, with annual rainfall ranging from 300 to 500 mm, which occurred from July to October. The soil was made of a lateritic crust partially covered by flattened sandy dunes, stabilized by the vegetation. This plateau was eroded by a former effluent of the Senegal River, the Ferlo, which stopped flowing at the end of the last humid Saharan period (Neolithic era). The erosion left a large, fossil valley that crosses the study area from east to west with former effluents coming from the north and the south.

A low-input, extensive livestock-production system was adopted by both settled and transhumant farmers in the Ferlo. They used natural grasslands and surface water as much as possible. During the rainy season, temporary ponds—many in the Ferlo Valley—were flooded. These water resources enabled ruminants to use the surrounding grasslands. Transhumant farmers left the crop-farming

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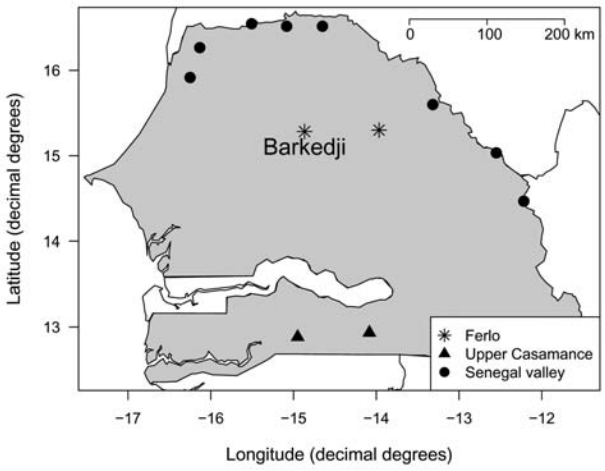

Figure 1. Location of the study of Rift Valley fever serologic incidence (Barkedji) and sentinel herds of the national surveillance system during the 2003 rainy season in Senegal.

regions of Senegal, where they spent the dry season, and converged on the Ferlo to benefit from these resources. The farmers gathered in compounds on the basis of family and ethnic relationships. They left the Ferlo at the end of the rainy season, as the temporary ponds progressively dried up.

Disease Surveillance by the National Veterinary Services

The National Veterinary Services had been surveying the occurrence of RVF in Senegal since the 1987 epidemic. Coordinated by the National Veterinary Services' epidemiologic unit, the surveillance system involved the National Veterinary Research Laboratory (ISRA-LNERV), the Pasteur Institute of Dakar, and field veterinary services (8).

Disease surveillance was activated during the rainy season. Farmers were asked to report a high incidence of abortions in ruminants (cattle, sheep, and goats) to veterinary officers and private veterinarians. When such alerts arose, veterinary officers had to visit the suspected herds, carry out an epidemiologic survey, sample blood of females who had aborted, and collect fetuses. Biological samples were sent to ISRA-LNERV, where serologic tests were conducted. When relevant, reverse transcription–polymerase chain reaction (RT-PCR) and virus isolation were conducted at Pasteur Institute of Dakar.

Twelve veterinary posts were selected to perform serologic surveillance along the Senegal River Valley, in the pastoral area of Ferlo, including Barkedji, and in Upper Casamance (the southern, forested area), because of the previous RVF outbreaks in these places (Figure 1). The goal of this survey was to detect the background incidence of RVF. Sentinel herds (sheep and goats) were identified within the influence area of the selected posts. In the sentinel herds, 30 animals were ear-tagged, and their blood was sampled before the beginning of each rainy season and 2 or 3 times during the rainy season, depending on the length of the rainy season and observed activity of mosquitoes. A serum neutralization test was performed to detect anti-RVF neutralizing antibodies by using Vero monolayer cells infected with a viral suspension of 106.5 PFU/mL of the attenuated RVF virus Smithburn strain. A positive result was defined as a serum sample that showed a lack of cytopathogenic effect at a dilution of 1:160 (9).

Assessment of Transmission Risk

The serologic incidence of RVF was estimated around selected temporary ponds of the Barkedji area. Incidence was measured by the frequency of seroconversions (change from negative to positive status) in small ruminants from the beginning to the end of the rainy season. Interviews with the farmers showed that their criteria for choosing the pond were related to its size. Large ponds were preferred because they remained flooded longer than smaller ponds. Moreover, farmers tended to settle close to the ponds because they also used the water for family needs.

Seven ponds were selected according to their location (inside or outside the Ferlo riverbed) and their size. Size was computed from their perimeters, recorded with a 12-channel global-positioning-system (GPS), after a series of heavy rainfalls, i.e., when the watered surface of each pond was at its maximum.

At the beginning of the rainy season, meetings were organized with the farmers settled around each of the 7 ponds to explain the goal of the study. The decision to participate in the survey was made at the compound level, which comprised several families and herds. Sixteen compounds were selected, and their geographic position was

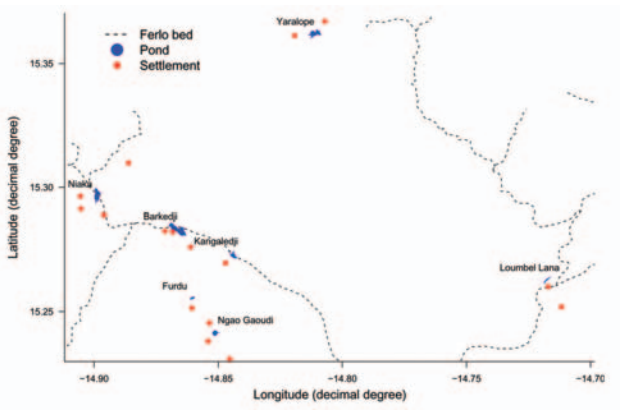

Figure 2. Location of ponds and settlements for the study of Rift Valley fever serologic incidence in 610 small ruminants during the 2003 rainy season in the Barkedji area, Senegal.

recorded with a GPS to compute their distance from the pond, defined as the minimum distance between the compound and the perimeter of the related pond. This risk factor was chosen because ruminants spent the night in pens located in the compounds. Because *Aedes* and *Culex* vectors of RVF virus have a crepuscular or night activity, RVF transmission probably occurs within these pens. The location of ponds and compounds is shown in Figure 2.

The minimum number of animals to be tagged and sampled was set at 30 in each compound, to detect at least 1 seroconversion, with a 95% confidence level, in the case of a 10% serologic incidence. Sampling was performed in August for the first occasion and from mid-November to mid-December for the second (Table 1). Blood samples were centrifuged and serum specimens were stored at –20°C until they were tested at ISRA-LNERV for anti-RVF antibodies with the serum neutralization test described above. Farmers who participated in the survey were asked to report abortions that occurred in ruminants, whatever their involvement in the serologic study.

Data Analysis

Serologic incidence data were analyzed by using logistic-regression mixed models (LRMM) (10). Incidence was the response, aggregated at the compound level (i.e., 1 line per compound in the dataset). The pond was included as a random effect in the models. This strategy allowed estimates of both population-level mean (overall incidence) and pond-specific means.

Three main effects and their interactions were considered in the fixed part of the model: 1) the location of the pond (inside or outside the Ferlo riverbed), 2) its size expressed in hectares (ha) and centered on the surface of the smallest pond, and 3) the distance between the pond and the compound, expressed in hectometers (hm) and centered on the smallest observed distance. The explanatory variables are displayed in Table 2. For surface and distance, only linear effects were considered.

No prior information was available to determine the most plausible model. Therefore, all the possible models with these 3 main effects and their 2- and 3-way interactions were fitted. To avoid the problem of multiple model comparison (e.g., with the likelihood ratio test), the Bayesian information criterion (BIC) was used to select the most plausible model (11,12): $BIC = -2 \log(ML) + k \log(n)$, where ML was the maximized likelihood, k was the number of parameters in the model, and n was the number of observations (number of compounds). For this information criterion, the best model was the one with the lowest value. A database management system designed for herd follow-up was used to enter and store the data (13). R software was used for data analysis and graphs (14).

Table 1. Timeline (month/day) of the study of serologic incidence of Rift Valley fever in small ruminants, Barkedji area, Senegal, 2003 rainy season*

Pond name	First sampling date	Second sampling date
Niaka	08/29–08/30	12/12–12/18
Barkedji	08/02–08/08	12/04–12/09
Loumbel Lana	08/06–08/09	12/06–12/07
Furdu	08/28	11/11–11/12
Ngao Gaoudi	08/10–08/28	12/12–12/15
Kangaledji	07/31–08/01	12/06–12/12
Yaralope	07/29–08/01	12/14–12/17

*At the pond level ($n_1 = 7$) and at the compound level ($n_2 = 16$); N = 610 small ruminants.

Results

Disease Surveillance

During the 2003 rainy season, no outbreak of RVF was confirmed in the Barkedji area by the national surveillance system. However, 76 abortions were reported in small ruminants by farmers in this area, either to the Barkedji veterinary officer or to research staff. Eleven abortions occurred among animals included in the serologic survey. The sera of 2 of these ewes, which lived near the Loumbel Lana pond (Figure 2), were positive for RVF with the serum neutralization test. In Furdu (Figure 2), farmers reported 7 abortions in ewes that were not involved in the serologic survey. Blood samples were taken from these ewes, and 2 serum samples were positive for RVF. In both cases (the national surveillance system and the farmers involved in the serologic survey), abortions were reported late. Consequently, no sample was obtained from the fetuses or from the fetal envelopes for RT-PCR test or virus isolation.

Assessment of Transmission Risk

A total of 610 sheep and goats were sampled on the first occasion. Three ewes' serum specimens were positive with the serum neutralization test (Furdu, Yaralope, and Niaka). They were discarded from the incidence analysis. On the second occasion, 379 animals were sampled (38% of the initial samples were lost to follow-up) (Table 3). At the pond level, the maximum rate of missing data was observed in Niaka (62.7%), and the minimum rate was found in Furdu (2.5%). At the compound level, the lost-to-follow-up rate ranged from 2.5% (Furdu) to 100.0% (Niaka). In this case, the whole compound left the area before the second sampling occasion. For all subsequent analyses, the denominator of incidence probabilities was computed as the initial number of sampled animals minus half the number that were lost to follow-up. This correction assumed that lost-to-follow-up processes were independent from RVF incidence. This assumption was assessed both graphically (graph not shown here) and by computing

Table 2. Variables selected to explain serologic incidence of Rift Valley fever in small ruminants, Barkedji area, Senegal, 2003 rainy season*

Pond name	Pond location	Surface (ha)	Compound code	Distance: pond-compound (hm)
Niaka	Ferlo bed	10.1	BEL	17.8
			NIA	6.7
			NIK	6.1
Barkedji	Ferlo bed	17.9	BK1	0.6
			BK2	2.8
			BK3	5.1
Loumbel Lana	Ferlo bed	1.8	DIA	12.6
			LOU	2.1
Furdu	Outside Ferlo bed	1.6	FUR	3.9
Ngao Gaoudi	Outside Ferlo bed	4.2	GAW	4.0
			NG2	3.4
			NGA	11.9
Kangaledji	Ferlo bed	4.7	KAN	3.9
Yaralope	Outside Ferlo bed	9.4	YA1	5.8
			YA2	4.8

*At the pond level (n_1 = 7) and at the compound level (n_2 = 16); N = 610 small ruminants; hm, hectometers.

a logistic regression of the incidence rate against the proportion of ruminants lost to follow-up. A weak positive trend was found, but the slope coefficient was not significantly greater than zero (p = 0.16).

The observed serologic incidence rate of RVF was 5.4%, with large within- and between-pond differences (Tables 3 and 4), ranging from 0.0% in Barkedji and Yaralope ponds to 20.3% in Kangaledji pond (Table 4). The average incidence rate, estimated from the intercept-only LRMM, was 2.9% (95% confidence interval 1.0–8.7). Observations and model predictions both indicated that RVF transmission occurred in 5 of 7 ponds in the study area during the 2003 rainy season and that the transmission probability differed widely from pond to pond.

Table 3. Sample size, lost to follow-up, and observed serologic incidence of Rift Valley fever in small ruminants, Barkedji area, Senegal, 2003 rainy season*

Pond	Compound code	Initial size	Lost to follow-up	Serologic incidence
Barkedji	BK1	20	0	0
	BK2	50	28	0
	BK3	30	15	0
Furdu	FUR	40	1	1
Kangaledji	KAN	86	34	14
Loumbel Lana	DIA	12	7	2
	LOU	89	69	1
Ngao Gaoudi	GAW	40	10	1
	NG2	30	3	1
	NGA	20	2	1
Niaka	BEL	17	1	0
	NI4	20	20	–
	NIA	37	10	4
	NIK	20	18	1
Yaralope	YA1	20	11	0
	YA2	79	2	0

*For 7 ponds and 16 compounds; N = 610 small ruminants.

Comparison of the 19 possible models is shown in Table 5. The best model according to BIC was the incidence as a function of surface and pond location for the fixed effects. The coefficients of the intercept-only and the best BIC model are shown in Table 6. Fixed-effect coefficients of this model were significantly different from zero (surface p = 0.04; Ferlo p = 0.03). The 3-fold reduction of the variance of the random effect between the intercept-only (variance = 1.75) and the best BIC model (variance = 0.57) indicated that the within-pond correlation of the results was well accounted for by the fixed effects. The population mean of the RVF serologic incidence, as predicted by the location and the surface of the ponds, is displayed in Figure 3. This figure shows that the serologic incidence was higher inside the Ferlo riverbed than outside, and that smaller ponds encountered a higher RVF incidence than larger ponds.

Discussion and Conclusion

Disease Surveillance

In Senegal, 5 outbreaks were reported by the national RVF surveillance network in 2003 (15). They occurred in the Senegal River Valley; none was reported in the Ferlo. However, our serologic results showed that RVF virus actively circulated in the Barkedji area in 2003 and that clinical cases probably occurred in small ruminants.

RVF was detected at the national level. However, the surveillance system was not sensitive enough to detect all outbreaks of RVF. The disease warning was issued in November, i.e., at the end of the rainy season, when ponds dried up. At this time, most transhumant farmers had already left the Ferlo (and other pastoral areas) to join their dry-season settlement. Therefore, a high risk for virus dissemination existed before the warning was given.

Table 4. Observed and fitted serologic incidence rate of Rift Valley fever at the pond level in small ruminants, Barkedji, Senegal, 2003 rainy season

Pond	Observed	Fitted*
Barkedji	0.0	0.9
Furdu	2.5	2.7
Kangaledji	20.3	18.6
Loumbel Lana	4.8	4.4
Ngao Gaoudi	3.6	3.5
Niaka	8.4	7.5
Yaralope	0.0	0.8

*Serologic incidence was fitted with an intercept-only mixed-effect logistic regression model, with the pond as the random effect. N = 610 small ruminants.

A more efficient system should provide evidence of virus circulation at the beginning of the rainy season (July or August). Because Senegal has a long experience in RVF surveillance and outbreak investigations, defining a few hot points, e.g., along the Senegal River valley and in the Ferlo, should be possible; more stringent surveillance could be implemented in these locations, with RT-PCR, virus isolation on entomologic and ruminant samples, or both.

In addition, preventive measures should be considered, such as the vaccination of ruminants to break the amplification cycle of the virus. In July, the beginning of the rainy season, cattle, sheep, and goats are not pregnant. Births occur before the rainy season (May–June for cattle, earlier for small ruminants), and their reproduction cycle is stopped during the hot, dry season because of lack of energy and protein in their diet. Therefore, the residual pathogenicity of the Smithburn vaccinal strain (i.e., a risk for abortion in pregnant ewes) should not be a problem. Moreover, July is the period when farmers usually vaccinate ruminants against anthrax, black leg, botulism, and pasteurellosis. The addition of RVF to this list of recommended vaccines should thus serve the interests of farmers.

These prevention measures will become more important to consider during coming years. Like each Muslim feast, Aïd El Kebir is determined according to the lunar calendar. Therefore, for a given year, this date occurs 10 or 11 days earlier than in the former solar year. At the occasion of this feast, tens of thousands of sheep are slaughtered on the same day, which implies massive animal movements and potential spread of the disease. In addition, slaughtering happens at home, with a high risk of spreading of the virus to humans if the sheep are viremic. In 2005, Aïd El Kebir occurred on January 19. In coming years, the feast will occur during the high-risk period of RVF occurrence.

Assessment of Transmission Risk

The use of the serum neutralization test limited the risk for cross-reaction with other phleboviruses, but the sensitivity of the analyses was low. This feature of the serum neutralization test probably resulted in underestimates of the incidence rate. Moreover, the incidence results were difficult to compare with those of other prevalence surveys undertaken in Mauritania or Senegal (7,16).

Table 5. Bayesian information criteria for 19 mixed-effect logistic regression models of Rift Valley fever serologic incidence at 15 compounds, in small ruminants,* Barkedji area, Senegal, 2003 rainy season

Fixed model	BIC†
Surface‡ + Ferlo§	32.3
Surface + distance¶ + Ferlo + surface × distance	33.0
Intercept-only model	33.2
Surface + distance + surface × distance	33.6
Surface	33.6
Ferlo	34.2
Surface + Ferlo + distance	34.5
Surface + Ferlo + surface × Ferlo	34.6
Surface + Ferlo + distance + surface × Ferlo + surface × distance	35.3
Distance	35.7
Surface + distance + Ferlo + surface × distance +distance × Ferlo	35.7
Surface + distance	36.0
Distance + Ferlo	36.7
Surface + Ferlo + distance + surface × Ferlo	36.7
Ferlo + distance + surface + distance × Ferlo	37.1
Surface + Ferlo + distance + surface × Ferlo + surface × distance +distance × Ferlo	38.0
Distance + Ferlo + distance × Ferlo	39.3
Surface + Ferlo + distance + surface × Ferlo + distance × Ferlo	39.3
Surface + Ferlo + distance + surface × Ferlo + surface × distance + distance × Ferlo + surface × Ferlo × distance	39.8

*With the pond as the random effect. N = 610 small ruminants.
†Bayesian information criterion.
‡Surface (hectare) centered on the smallest observed surface (1.6 hectare).
§Location of the pond: in the Ferlo bed (reference category) or outside the Ferlo bed.
¶Distance from the compound to the pond (hectometers) centered on the shortest observed distance (0.6 hectometers).

Table 6. Parameters of the intercept-only and the best BIC mixed-effect binomial model of Rift Valley fever serologic incidence in small ruminants, Barkedji area, Senegal, 2003 rainy season*

Term	Parameter	Standard error	*Z**	p†
Intercept-only mixed-effect model				
Intercept	–3.49	0.58	–5.99	2.1×10^{-9}
Variance of the random effect‡	1.75	–	–	–
Best mixed-effect model according to information criteria				
Intercept	–1.62	0.67	–2.41	0.02
Surface§	–0.19	0.09	–2.08	0.04
Ferlo¶	–1.95	0.88	–2.21	0.03
Variance of the random effect‡	0.57	–	–	–

*Ratio of the parameter over its standard error. N = 610 small ruminants; BIC, Bayesian information criterion.
†P(x > |Z|) assuming a standard-normal distribution for *Z;* straight vertical bars indicate absolute value of *Z*.
‡Pond-related random effect associated with the intercept.
§Surface (hectare) centered on the smallest observed surface (1.6 hectare).
¶Location of the pond: in the Ferlo bed (reference category) or outside Ferlo bed.

The overall serologic incidence was rather low (5.4%), but RVF transmission occurred in a large proportion of the temporary ponds (5/7) in the study area during the 2003 rainy season. Earlier works suggested that Barkedji was an area of endemic activity for RVF virus (6). The serologic results observed in this study were compatible with this hypothesis. Vertical transmission of the virus in *Aedes* mosquitoes might explain the maintenance of RVF infection in this region. The alternative, and nonexclusive, hypothesis is that RVF virus is introduced in Barkedji by ruminants that are seasonally moved. Confirmatory studies should involve a follow-up survey of transhumant cattle in their dry- and rainy-season settlements, to assess where transmission primarily occurs.

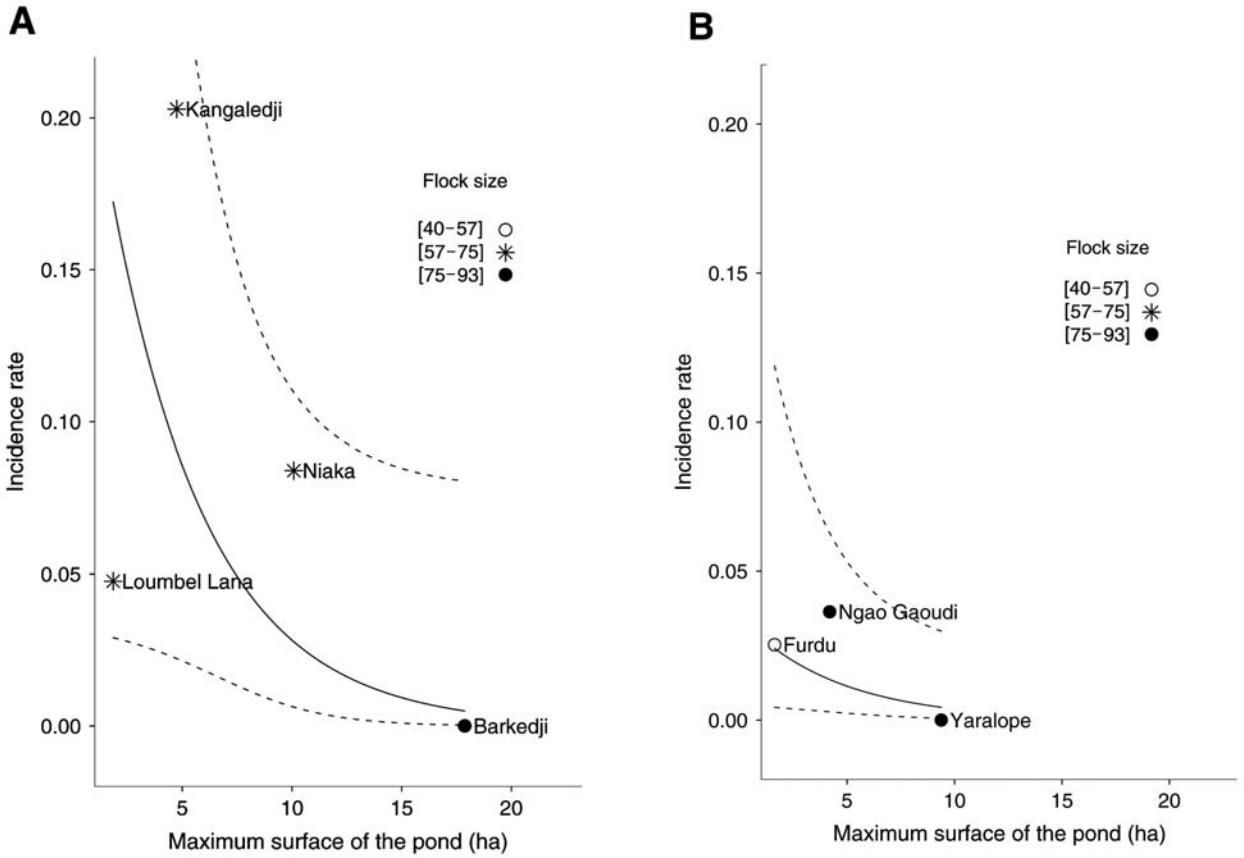

Figure 3. Serologic incidence rate of Rift Valley fever in small ruminants (N = 610), according to the location of the pond (A, in Ferlo River bed; B, outside Ferlo River bed) and its maximum surface during the 2003 rainy season in the Barkedji area, Senegal. Points indicate observed pond-level serologic incidence. Solid line indicates population mean of the serologic incidence estimated with the best Bayesian information criterion mixed-effect logistic regression model. Dashed lines indicate pointwise 95% confidence interval corresponding to these estimates. ha, hectares.

Serologic incidence differed from pond to pond: Barkedji and Kangaledji ponds (Figure 2, Table 4) had different RVF transmission rates, although they were close to each other. This result corroborates previous findings from the same area, which showed that the exposure to *Aedes vexans* bites, and consequently the risk for RVF transmission, was spatially heterogeneous (17). This previous study also suggested that because very few *Aedes* mosquitoes were captured near the Barkedji pond, it had a low risk for RVF transmission. We confirmed this finding.

The lack of protective effect of distance between the pond and compound was probably related to the low range of investigated distances. This range reflects the actual situation, i.e., that farmers like to settle close to ponds. This finding offers few practical recommendations. Even when farmers increased the distance (within the observed distance range) between settlements and ponds in the Ferlo, their herds were not protected against mosquito bites and RVF.

The Ferlo Valley was densely populated by RVF hosts during the rainy season. Moreover, the rather dense tree and grass cover offered a large choice of resting sites for mosquitoes. These favorable conditions for the amplification of RVF virus probably explain why the incidence rate was higher in the Ferlo bed than outside it. Although most ponds of interest for the livestock were located in the Ferlo bed, some outer ponds, like Yaralope and Furdu (Figure 2), were used by farmers because of the large available space and, according to them, the lower risk for sheep schistosomiasis. The optimal use of these outer ponds should thus be encouraged.

The lower incidence observed around large ponds might be related to the predominance of *A. vexans* in the transmission of RVF during the 2003 rainy season. The eggs of this species are laid on the wet soil of the pond banks, and their desiccation is needed before they hatch, when they are watered again. They can survive for several years in the dried mud (18). When the ponds are flooded again, a mass hatching of mosquito eggs occurs, and adult neonates appear 4–8 days later (19). In the study area, larger ponds were also deeper than smaller ones. Once watered, these ponds exhibited slower and more limited changes in the flooded surfaces than did the smaller and shallow ponds, which resulted in fewer mosquitoes hatching and a lower transmission risk. However, the relationship between pond size and incidence might be reversed in the case of RVF transmission by *Culex* mosquitoes, which need water all during their development cycle (20). Previous studies have shown that *Culex* species were sometimes predominant in the Barkedji area, depending on the rainfall patterns during the rainy season (B. Mondet et al., unpub. data). Therefore, care should be taken before advising the farmers to avoid small ponds. Beyond the possibly lower risk for RVF, large ponds might be more dangerous for other human and animal diseases such as West Nile fever or schistosomiasis, which are highly prevalent in the Ferlo (21–23).

Artificial ponds, arranged for their use by livestock, appear to act like large temporary ponds with respect to water-level changes and watering duration. During past years, such ponds were implemented near Barkedji, among other places, and the Senegalese government plans to develop them in the Ferlo. Their impact on human and animal health should be investigated to identify advantages and drawbacks of this possible alternative to the use of natural temporary ponds. Further studies are also needed to assess the influence of ecologic factors on *Aedes* abundance and their relationships to the risk for RVF transmission around the Ferlo temporary ponds.

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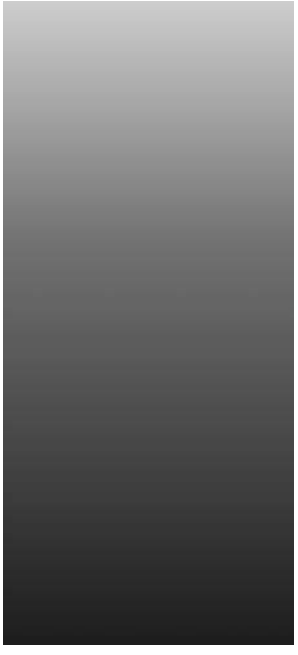

Toscana Virus in Spain

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Toscana virus (TOSV, *Phlebovirus*, family *Bunyaviridae*) infection is one of the most prevalent arboviruses in Spain. Within the objectives of a multidisciplinary network, a study on the epidemiology of TOSV was conducted in Granada, in southern Spain. The overall seroprevalence rate was 24.9%, significantly increasing with age. TOSV was detected in 3 of 103 sandfly pools by viral culture or reverse transcription–polymerase chain reaction from a region of the L gene. Nucleotide sequence homology was 99%–100% in TOSV from vectors and patients and 80%–81% compared to the Italian strain ISS Phl.3. Sequencing of the N gene of TOSV isolates from patients and vectors indicated 87%–88% and 100% homology at the nucleotide and amino acid levels, respectively, compared to the Italian strain. These findings demonstrate the circulation of at least 2 different lineages of TOSV in the Mediterranean basin, the Italian lineage and the Spanish lineage.

Within the last decade, the emergence and reemergence of arthropodborne virus (arbovirus) infections has been a health problem worldwide. West Nile virus (WNV) infection is a seasonal epidemic in North America (1). In southern Europe, WNV infections (2–4), tickborne encephalitis (5), sandfly fever Sicilian virus (SFSV), sandfly fever Naples virus (SFNV), and Toscana virus (TOSV) infections have been reported in Mediterranean countries (6,7). In Spain, a multidisciplinary network, EVITAR, has been recently created to study arthropod- and rodentborne viral diseases. One of the objectives of the network is to study TOSV infections in Spain.

TOSV (genus *Phlebovirus*, family *Bunyaviridae*) is an important agent of acute meningitis and meningoencephalitis in residents and visitors from Mediterranean countries (7–13). Aside from TOSV, other sandfly fever viruses, i.e., SFSV and SFNV, cause a brief, self-limiting febrile illness (6). Although TOSV is not normally associated with mild disease, serologic studies report high seroprevalence rates in areas of confirmed TOSV infections (7,14,15). Furthermore, a case of influenzalike illness caused by TOSV has recently been reported (16). In Spain, the first TOSV infections involving the central nervous system were reported in Granada in 1988 (7). Later, cases of TOSV infections were detected in other areas of Spain (15). Phylogenetic analysis of short polymerase chain reaction (PCR) products from the L segment showed that nucleotide sequences of TOSV isolates from Granada differ significantly from the Italian strain ISS Phl.3 (17).

TOSV was first isolated in Italy from the sandfly *Phlebotomus perniciosus* and later from *P. perfiliewi* (8,18). *P. perniciosus* is the most abundant anthropophilic species of *Phlebotomus* in Spain (19). The maximum activity of sandfly vectors for TOSV occurs during summer, along with most cases of TOSV infection (7). Vector-based TOSV surveillance is useful in reporting virus activity. It provides predictive indicators of transmission activity level associated with elevated human risk. However, no data are available on detection of TOSV from vectors in Spain.

As part of the study of TOSV infection within the aims of the EVITAR network, this work focused on 3 main objectives. First, a seroprevalence study to detect TOSV immunoglobulin G (IgG) antibodies was conducted. Second, by means of viral culture and reverse transcription (RT)-PCR, we investigated the presence of TOSV in pools of phlebotomine sandflies. Finally, positive pools and viral isolates were phylogenetically characterized.

Materials and Methods

Prevalence Study of Anti-TOSV IgG Antibodies

Population Study for Selecting Participants

The seroprevalence study was conducted on study participants from the Granada population. Participants were retrospectively selected on the basis of demographic data

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and estimations of seroprevalence rates to TOSV (7,15). To evaluate differences in seroprevalence rates within Granada, the province was divided into 5 geographic areas: urban, metropolitan, south, west/southwest, and north/northeast (Figure 1). By age groups, 20% were <18 years, 65% were 18–65 years, and 15% were >65 years of age.

Serum Samples

Serum samples were collected from September to December 2003. Specimens from adults 18- to 65-years of age were collected from anonymous healthy blood donors. Specimens from persons <18 and >65 years of age were obtained from 2 laboratories in Granada from persons with noninfectious pathologic features. Only data on age, sex, and geographic area of origin were recorded from the study population. Anti-TOSV IgG was detected by a commercial enzymatic immunoassay, EIA Enzywell Toscana virus IgG (Diesse, Italy), following the manufacturer's instructions.

Investigation of TOSV in Vectors

Capture of Phlebotomine Sandflies

Phlebotomine sandflies were captured with CDC light traps (20) from June to October of 2003 and 2004. Traps were placed in 16 areas of the rural environment where the first cases of meningitis by TOSV appeared (21) (Figure 1). Sandflies were trapped after dusk until dawn. Traps were immediately transported to the laboratory to pool the individual vectors by sex and trapping area. In the 2003 season, pools of male sandflies were used for taxonomic classification, and pools of female sandflies were tested for TOSV by RT-PCR. In the 2004 season, up to 10% of individual sandflies were separated for taxonomic classification. The remaining insects were pooled by sex and trapping area and tested for TOSV by viral culture and RT-PCR. Taxonomic classification of sandflies was carried out according to Gil-Collado et al. (19).

Viral Culture

Phlebotomines were introduced in vials with sterile crystal beads and 0.5 mL minimal essential medium supplemented with 20% fetal bovine serum and antimicrobial mix (0.4 mg/mL gentamicin, 0.5 mg/mL vancomycin, and 2.5 μg/mL amphotericin B). Vials were vortexed and centrifuged at 13,000 rpm for 5 min. A 200-μL aliquot of the supernatant was injected into tubes with African green monkey kidney cells; the remaining supernatant was frozen at –80ºC. The pellet with the phlebotomines was used for RT-PCR. Tube cultures were incubated at 37ºC and examined daily for the appearance of cytopathic effect (CPE). Tubes with positive CPE were tested for TOSV by RT-PCR.

Figure 1. Upper map, geographic situation of the study population (Granada province) in Spain; lower, distribution of geographic areas in Granada province for the seroprevalence study of anti–Toscana virus immunoglobulin G antibodies.

RT-PCR for Testing TOSV in Sandfly Pools

Viral RNA from cell cultures was extracted by using QIAamp viral RNA kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Viral RNA from sandflies was isolated by using the same kit, with minor modifications. Briefly, 500 μL lysis buffer was added to the tubes containing the phlebotomine sandflies, tubes were vortexed and centrifuged at 13,000 rpm for 10 min, and 250 μL of the supernatant was used for RNA extraction. A generic RT-nested-PCR method was used to detect TOSV RNA as described previously (17), which amplifies a 244-bp fragment of the L gene of phleboviruses. Sequences of the primers are shown in Table 1.

Sequencing Reactions on RT-PCR–Positive Pools

Specific TOSV detection was achieved by sequencing PCR products, as described previously (17). Briefly, PCR products were purified from a 2% low-melting-point agarose gel with the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed by using the ABI Prism Big Dye Terminator Cycle Sequencing

Table 1. Primers used for RT-PCR amplification of the L (partial) gene and the N gene of TOSV*

Primer	Sequence	PCR
NPhlebo1+	5′ $_{2047}$ATGGARGGITTTGTIWSICIICC$_{2069}$ 3′	L gene–1st
NPhlebo1–	5′ $_{2600}$AARTTRCTIGWIGCYTTIARIGTIGC$_{2575}$ 3′	L gene–1st
NPhlebo2+	5′ $_{2074}$WTICCIAAICCIYMSAARATG$_{2094}$ 3′	L gene–2nd
NPhlebo2–	5′ $_{2318}$TCYTCYTTRTTYTTRARRTARCC$_{2296}$ 3′	L gene–2nd
TosS1+	5′ $_{4}$CAGAGATTCCCGTGTATTAAAC$_{25}$ 3′	N gene–1st
TosS1–	5′ $_{1052}$GAGTGCTGCCAAGTCTTATGAC$_{1031}$ 3′	N gene–1st
TosS2+	5′ $_{4}$CAGAGATTCCCGTGTATTAAACAAAAGC$_{31}$ 3′	N gene–2nd
TosS2–	5′ $_{1004}$TAGAGAAACTGCTCTTTCCACC$_{983}$ 3′	N gene–2nd

*RT-PCR, reverse transcription–polymerase chain reaction; TOSV, Toscana virus.

Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and analyzed by an ABI model 377 automated sequencer (Applied Biosystems). Two sequencing reactions were carried out on each PCR product by using the sense and antisense primers of the nested-PCR step. TOSV-specific sequences were confirmed by BLAST (basic local alignment search tool) search against GenBank databases. Sequences of the L gene from positive pools were compared with the corresponding sequence of the Italian strain ISS Phl.3 and with 1 Spanish TOSV isolate (STI) obtained previously (17) with the ClustalW Multiple Sequence Alignment program (1.82 version; European Bioinformatics Institute, Cambridge, UK).

Molecular Characterization of Spanish TOSV

To achieve a better characterization of STIs, the N gene was targeted. Sequences of the N gene of different phleboviruses were identified by BLAST search and aligned. GenBank accession numbers of the sequences were TOSV ISS Phl.3: X53794; Rift Valley fever virus (RVFV): NC 002045; SFSV: J04418; Punta Toro virus: K02736; and Uukuniemi virus (UUKV): NC 005221. Subsequently, different combinations of primers were selected to amplify 1 STI. Finally, the obtained sequence and the 1 from the Italian strain ISS Phl.3 were aligned, and specific primers were redesigned to amplify STIs recovered from sandflies and previous STIs from patients (17) (Table 1). RT-PCR conditions are available on request. Sequences were obtained and compared with the Italian strain as described above.

Phylogenetic Analysis

Phylogenetic analysis of the sequences from the partial L gene and the complete N gene was carried out with MEGA 3 program (22) by using the Kimura2-parameter model for nucleotides and amino model with Poisson correction for amino acids to calculate distances between sequences with confidence values of 1,000 bootstrapping trials. Phylogenetic trees of the L gene were constructed from TOSV obtained from sandflies and previous STIs and from different phleboviruses. Available GenBank accession numbers of the phleboviruses sequences were TOSV ISS Phl.3: X68414; RVFV: X56464; UUKV: D10759; and phlebovirus Chios-A (Chios): AY293623. Phylogenetic trees of the N gene were constructed from STI sequences obtained from sandflies and patients and from the phleboviruses sequences described above.

Statistical Analyses

To calculate the sample size for the seroprevalence study, we applied the estimation of proportions model for infinite populations. We made the following assumptions: an estimated seroprevalence of 10% in our area based on previous studies (7,15), 95% confidence level, and 5% precision level. With these premises, the minimum sample size was 139. Results were statistically analyzed with the SPSS 12.0.1. Program (SPSS, Chicago, IL, USA). Along with descriptive statistics, univariate analysis was conducted on the results obtained from the seroprevalence study by χ^2 test. A p value <0.05 was considered significant. TOSV infection rate in phlebotomine sandflies by means of RT-PCR was calculated with Pool Screen 2.0 program (23).

Results

Prevalence Study of Anti-TOSV IgG Antibodies

Anti-TOSV IgG was analyzed in 979 human serum samples. The study population was distributed by geographic area proportional to Granada's population (Table 2). By sex, 472 (48.2%) were males, and 507 (51.8%) were females. By age groups, 183 (18.7%) were <18 years, 662 (67.6%) were aged 18–65, years and 134 (13.7%) were >65 years (see Materials and Methods).

The overall prevalence of anti-TOSV IgG was 24.9% (range 9.4% in persons <15 years to 60.4% in those >65 years, p<0.001) (Table 3). No statistical differences were observed by geographic area or sex. However, when the urban area was compared with rural areas, seroprevalence rate was statistically higher in the latter group (20.6% in the urban area vs 26.7% in the rural areas; p = 0.042).

Vectors for TOSV

For taxonomic classification, 1,431 sandflies were studied, 1,286 males and 145 females. The most abundant

Table 2. Demographic data of Granada population and distribution of the study population by geographic areas

Area	Granada population			Study population, n (%)
	Total, n (%)	Males, n (%)	Females, n (%)	
Urban	240,522 (29.2)	111,774 (46)	128,748 (54)	287 (29.3)
Metropolitan	227,994 (27.6)	114,836 (50)	113,158 (50)	282 (28.8)
South	157,803 (19.1)	78,871 (50)	78,932 (50)	146 (14.9)
West/Southwest	70,397 (8.5)	35,429 (50)	34,968 (50)	72 (7.4)
North/Northeast	128,299 (15.6)	64,214 (50)	64,085 (50)	192 (19.6)
Total	825,015 (100)	405,124 (49)	419,891 (51)	979 (100)

species was *P. perniciosus* (68.7%), followed by *Sergentomyia minuta* (16.4%), *P. sergenti* (7.1%), *P. papatasi* (5.7%), and *P. ariasi* (0.5%); 1.6% of the sandflies were classified as *Phlebotomus* spp.

The presence of TOSV was investigated in 103 sandfly pools, 22 pools of females in 2003 and 81 pools in 2004 (42 pools of males and 39 pools of females). Three of the 81 pools obtained in 2004 were positive for TOSV by RT-PCR (2 female and l male pool); 2 were also positive by cell culture. The infection rate for TOSV in phlebotomine sandflies was 0.05% (95% confidence interval 0.1–0.009).

Molecular Characterization of Spanish TOSV

Genetic Analysis of the L Gene

TOSV sequences of the L (partial) gene from vectors and that from a human STI were almost identical. At the nucleotide level, 19%–20% diversity was observed between sequences obtained from Spanish samples and those of the Italian strain, whereas the homology at the amino acid level was almost 100% (Table 4). Phylogenetic analysis yielded the same results (Figure 2). The phylogenetic tree of nucleotide sequences show 1 group containing all TOSV, in which the Spanish ones form, with a bootstrap value of 100, a different cluster. This diversity is not reflected in the amino acid sequences since both the Spanish and Italian TOSV group into a unique cluster.

Genetic Analysis of the N Gene

Nucleotide and amino acid sequence homology within the N gene was ≈98%–100% among STIs from vectors and patients. Compared with the Italian strain, a 100% homology at the amino acid level was observed between STIs and the Italian strain. However, nucleotide sequences showed a 12%–13% difference between the strains (Table 4). The phylogenetic tree of nucleotide sequences shows that, within the TOSV group, STIs form a different cluster. However, at the amino acid level, both STIs and Italian TOSV strain group into a unique cluster (Figure 3).

Discussion

The overall 25% seroprevalence rate found in this study is similar to the rates reported in Mediterranean areas (14,24). Other seroprevalence studies carried out in northern Europe report lower rates (13), which demonstrates that TOSV infection is endemic in Mediterranean countries. The high seroprevalence rates suggest that the diagnosis of TOSV infection is frequently missed since most cases are mild, as occurs with SFSV and SFNV infections (6), a severe illness involving the central nervous system develops in only a few patients. The increasing seroprevalence rates concurrent with age demonstrate that the Granada population is exposed to TOSV throughout life. Thus, a study on outpatients attending primary care services is necessary to assess the role of TOSV in human disease.

TOSV was first isolated from *P. perniciosus* in Italy (8). Although, the sandflies used in this study for detecting TOSV were not used for taxonomic classification, ≈70% of captured insects were *P. perniciosus*, which suggests that this species is the main vector for TOSV in our area. Furthermore, *P. perniciosus* accounted for 50%–90% of the sandflies captured in the area where TOSV-positive pools were detected. The rest were classified as *S. minuta*, *P. sergenti*, and *P. papatasi*, none of which are known vectors for TOSV.

TOSV was detected in 3 of 103 pools. By using the Pool Screen 2.0 program (23), an infection rate of 0.05% was obtained in the sandflies, which is much lower than the infection rate of 0.2% reported in Italy (25), where

Table 3. Results of prevalence study of anti-TOSV immunoglobulin G, Granada

Area	<15 y, no. (pos)	15–40 y, no. (pos)	41–65 y, no. (pos)	>65 y, no. (pos)	No.	Pos (%)
Urban	52 (2)	141 (22)	60 (20)	34 (15)	287	59 (20.6)
Metropolitan	51 (7)	127 (24)	62 (18)	42 (28)	282	77 (27.3)
South	34 (3)	76 (14)	54 (12)	28 (19)	192	48 (25.0)
West/Southwest	3 (0)	40 (7)	20 (10)	9 (6)	72	23 (31.9)
North/Northeast	19 (3)	71 (15)	35 (6)	21 (13)	146	37 (25.3)
Total	159 (15, 9.4%)	455 (82, 18%)	231 (66, 28.6%)	134 (81, 60.4%)	979	244 (24.9)

*TOSV, Toscana virus; pos, positives.

Table 4. Homology of nucleotide and deduced amino acid sequences of L (partial) gene and complete N gene in Toscana viruses from Spain and the Italian strain ISS Phl.3*

L gene	STI1	ISS Phl.3	GR40	GR41	
nt					
ISS Phl.3	0.199				
GR40	0.010	0.199			
GR41	0.015	0.185	0.026		
GR79	0.010	0.205	0.020	0.015	
aa					
ISS Phl.3	0.015				
GR40	0.015	0. 030			
GR41	0.000	0.015	0.015		
GR79	0.000	0.015	0.015	0.000	
N gene	**STI1**	**STI2**	**ISS Phl.3**	**STI6**	**GR40**
nt					
STI2	0.020				
ISS Phl.3	0.116	0.123			
STI6	0.005	0.017	0.118		
GR40	0.015	0.016	0.123	0.012	
GR41	0.020	0.021	0.130	0.017	0.016
aa					
STI2	0.000				
ISS Phl.3	0.000	0.000			
STI6	0.000	0.000	0.000		
GR40	0.000	0.000	0.000	0.000	
GR41	0.000	0.000	0.000	0.000	0.000

*GR40, GR41, and GR79: TOSV obtained from pools of sandflies; STI1, STI2, and STI6: Spanish Toscana virus isolates recovered from patients in previous studies (17); ISS Phl.3: Italian TOSV strain (GenBank accession no. X68414 [L gene] and X53794 [N gene]); nt, nucleotides; aa, amino acids.

more cases of TOSV infection have been recorded (11,26). The fact that the seroprevalence rate is similar to the rates reported in Italy, another endemic area, and that the virus infection rate in sandflies is much lower, could be due to increased exposure to the vector, as occurs in our area, which is mainly rural.

To investigate the genetic relationship of TOSV detected in sandfly pools with the Italian strain and STIs recovered previously, sequence analysis of a fragment of the L gene and the complete N gene was performed. Similar results were obtained with both regions of the genome. Nucleotide and amino acid sequence homology of TOSV from vectors and patients was ≈100%. The differences between TOSV (from vectors and patients) and the Italian strain within the nucleotide sequences indicate that at least 2 lineages of TOSV, Italian and Spanish, are circulating. These changes are synonymous because almost identical amino acid sequences were found in all analyzed TOSV. This finding suggests that, at least within these regions of TOSV genome, constraints against amino acid changes exist. This fact has already been described with RVFV isolates from different areas (27).

Despite the high seroprevalence rates found in this study, fewer cases of severe disease caused by TOSV occur in our area (21) than in other countries (11). A possible explanation for this could be that Spanish TOSV are less neurovirulent than the Italian. Whether changes in other parts of the genome, such as the noncoding regions, affect the neurovirulence of this virus, as described for tickborne encephalitis virus (28), needs to be investigated.

The finding of this new lineage of TOSV was not an isolated event. Nucleotide sequences of TOSV from sandflies collected during 2003–2004 were almost identical to those

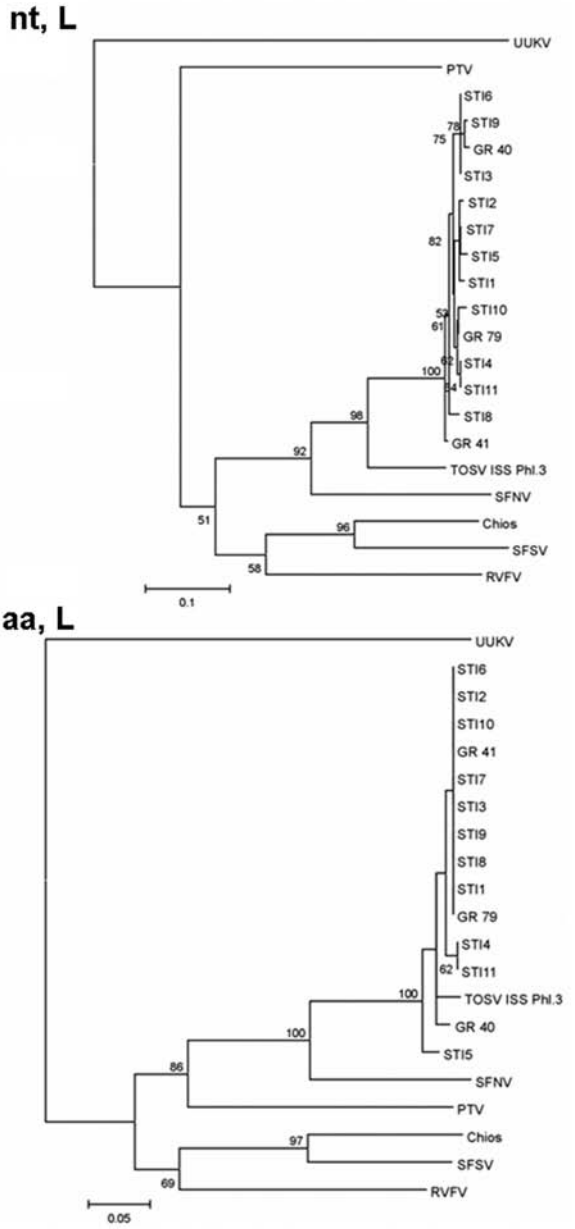

Figure 2. Phylogenetic trees illustrating the relationship between representatives of different phleboviruses and the Spanish Toscana virus (TOSV) within the nucleotide (nt, L) and the deduced amino acid sequences (aa, L) of the L (partial) gene. GR40 and GR41 correspond to TOSV isolates obtained from sandflies. GR79 corresponds to a reverse transcription–polymerase chain reaction–positive pool of sandflies. STI1–STI11 were recovered from patients with aseptic meningitis diagnosed from 1988 to 2002 as described in (17). Abbreviations and GenBank accession numbers are indicated in the text. Bootstrapping values >50 are indicated at the nodes.

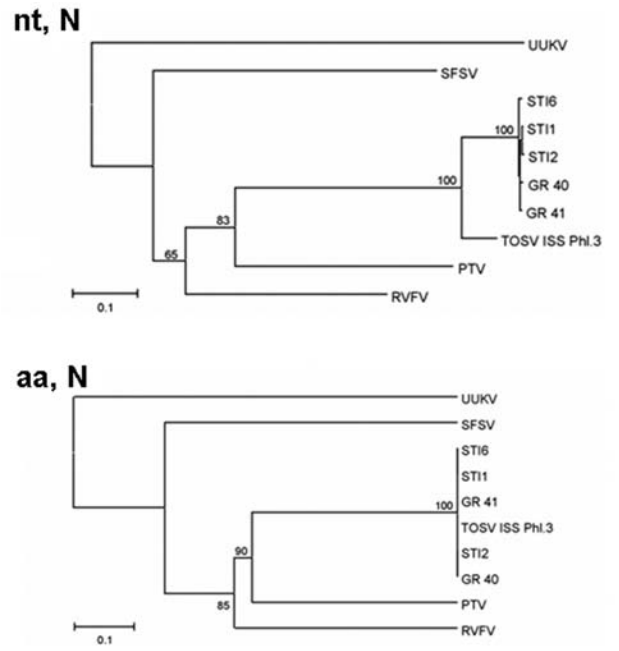

Figure 3. Phylogenetic trees illustrating the relationship between representatives of different phleboviruses and the Spanish Toscana virus isolates within the nucleotide (nt, N) and deduced amino acids sequences (aa, N) of the N gene.

obtained from patients from 1988 to 2002. The genetic diversity between STIs and the Italian strain could be partially explained by vector characteristics. Two lineages of *P. perniciosus* have been reported (29), the typical lineage found in Morocco, Tunisia, Malta, and Italy, and the Iberian lineage. These lineages remain isolated because sandflies move in short hops, flying no more than a few hundred meters from their resting places.

As occurs with WNV, for which several lineages have been reported, the last in central Europe (30), more lineages of TOSV may circulate in other areas. To assess this possibility, further investigation would be necessary. Moreover, sequencing of the S segment of a TOSV isolate recently described in southern France showed 87% and 100% homology with the reference strain within nucleotide and peptide sequences, respectively (31). Although GenBank accession for this sequence is not yet available, this isolate could belong to the Spanish TOSV lineage since differences between this isolate and the Italian strain are similar to the differences that we found in our isolates.

In conclusion, the study and surveillance of arbovirus infections should be considered worldwide since they may cause emergent diseases, many of which may be life-threatening. One part of this study should focus on the vectors and host spectrum of these viruses to control transmission to humans.

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Tickborne Pathogen Detection, Western Siberia, Russia

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Ixodes persulcatus (n = 125) and *Dermacentor reticulatus* (n = 84) ticks from Western Siberia, Russia, were tested for infection with *Borrelia*, *Anaplasma*/*Ehrlichia*, *Bartonella*, and *Babesia* spp. by using nested polymerase chain reaction assays with subsequent sequencing. *I. persulcatus* ticks were infected with *Borrelia burgdorferi* sensu lato (37.6% ± 4.3% [standard deviation]), *Anaplasma phagocytophilum* (2.4% ± 1.4%), *Ehrlichia muris* (8.8% ± 2.5%), and *Bartonella* spp. (37.6% ± 4.3%). *D. reticulatus* ticks contained DNA of *B. burgdorferi* sensu lato (3.6% ± 2.0%), *Bartonella* spp. (21.4% ± 4.5%), and *Babesia canis canis* (3.6% ± 2.0%). *Borrelia garinii*, *Borrelia afzelii*, and their mixed infections were observed among *I. persulcatus*, whereas *B. garinii* NT29 DNA was seen in samples from *D. reticulatus*. Among the *I. persulcatus* ticks studied, no *Babesia* spp. were observed, whereas *B. canis canis* was the single subspecies found in *D. reticulatus*.

Ticks are second only to mosquitoes as vectors of bacterial, viral, and protozoan agents (1). Among tickborne bacteria, extracellular spirochetes of the genus *Borrelia* are widely spread and most studied. Some of these, those that belong to the *Borrelia burgdorferi* sensu lato complex, are causative agents of Lyme borreliosis (1,2). Other known pathogenic bacteria transmitted by ticks are intracellular alpha proteobacteria, which includes the families *Anaplasmataceae*, *Bartonellaceae*, and *Rickettsiaceae* (3). Members of the genera *Anaplasma* and *Ehrlichia*, from the family *Anaplasmataceae*, infect mainly monocytes and granulocytes and cause human and animal anaplasmoses and ehrlichioses (4). Bacteria of the genus *Bartonella* infect erythrocytes and endothelial cells. Different species of *Bartonella* are the etiologic agents of cat-scratch disease, trench fever, and Carrión disease (5,6). The tickborne protozoa of the genus *Babesia* reproduce in erythrocytes, thus causing babesiosis among humans as well as wild and domestic animals (7).

Prevalent tick species for forest-steppe zones of Western Siberia, Russia, are taiga ticks (*Ixodes persulcatus* Schulze) and meadow ticks (*Dermacentor reticulatus*) (Acarina: Ixodidae) (1,2,8). They parasitize many ground-foraging bird species and virtually all the terrestrial mammals (8); both species are able to feed on humans (8,9) and transmit different tickborne infections (2).

The *I. persulcatus* habitat is the southern part of the forest zone of Eurasia (1,2). Until 1987, only tickborne encephalitis, thus causing virus was thought to be associated with taiga ticks, but extensive studies have shown their competence in the transmission of pathogenic spirochetes, *Borrelia garinii* and *Borrelia afzelii* (2). Recently, *Anaplasma*/ *Ehrlichia* was found in *I. persulcatus* ticks (10). The main *Ehrlichia* species found in *I. persulcatus* ticks is a recently characterized species, *Ehrlichia muris*, isolated from a wild mouse in Japan (11–13). The etiologic agent of human granulocytic anaplasmosis, *Anaplasma phagocytophilum*, has also been found in *I. persulcatus* ticks (14–16). Infection of ixodid ticks with *Bartonella* spp. has recently been described in the United States (17), Europe (18,19), and Western Siberia (20). Infection of *I. persulcatus* with *Babesia microti* pathogenic for immunocompromised humans has been shown by polymerase chain reaction (PCR) with genus- and species-specific primers (21), but nucleotide sequences of the specific PCR products remain unknown.

The second tick species, *D. reticulatus*, inhabits meadows and pastures (2), as well as near suburban areas from Europe to central Asia, but not taiga and dry steppes (22).

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D. reticulatus is well known as the vector of a canine pathogen, *Babesia canis canis* (23); *Rickettsia* spp. (13), *Francisella tularensis*, and *Coxiella burnetii* were also found in this tick species (1). *Borrelia* spp. was also detected in different *Dermacentor* species, including *D. reticulatus*, by means of PCR (9) and an indirect immunofluorescence assay (24). Infection of *D. reticulatus* with *Anaplasma*/*Ehrlichia* and *Bartonella* species was previously unknown in spite of the detection of bacterial DNA in other *Dermacentor* species (17,25). Little or nothing was known of the genetic variability of the tickborne pathogens in ixodid ticks from Western Siberia (2); consequently, the aim of the present study was to study prevalence and genetic diversity of *Borrelia*, *Anaplasma*/*Ehrlichia*, *Bartonella*, and *Babesia* among *I. persulcatus* and *D. reticulatus* ticks in Western Siberia, Russia.

Figure 1. Areas where ticks were collected. Omsk, Tomsk, and Novosibirsk regions are shaded.

Materials and Methods

Unfed adult *I. persulcatus* ticks were collected by flagging of lower vegetation in different suburban places of mixed aspen-birch and pine forests of Novosibirsk (55°N, 83°E) (115 ticks) and Tomsk regions (56°N, 85°E) (12 ticks) (Figure 1) in May and June of 2003 and 2004. Questing imago of *D. reticulatus* ticks were collected by flagging in different locations of river valley and forest-steppe zones of Novosibirsk (72 ticks) and Omsk regions (55°N, 73°E) (15 ticks) from May to June of 2003 and 2004 (Figure 1). Nucleic acids were isolated by lysis of 127 individual *I. persulcatus* and 87 *D. reticulatus* ticks in guanidine thiocyanate followed by deproteinization with phenol-chloroform and precipitation with isopropanol.

PCR Assay

To prevent contamination, we performed DNA isolation, PCR master mix assembly, and amplifications in separate rooms. Aerosol-free pipette tips were also used at each stage. We included negative control reactions with bidistillated water in each experiment at both steps of nested PCR. All reactions were performed in 20 μL reaction mixture containing 67 mmol/L Tris-HCl (pH 8.9), 16.6 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 0.01% Tween-20, 200 μmol/L each dNTP, 5% glycerol, 0.5 μmol/L specific primers, 2 U Taq DNA polymerase (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences), and 2 μL tested DNA. Amplification was performed in a Tercic Thermal Cycler (DNA Technology, Moscow, Russia). For the inner reactions, 2 μL of the outer PCR products were added into the reaction mixture. PCR fragments were visualized under UV irradiation after electrophoresis in agarose gels containing ethidium bromide. To control DNA isolation from ticks, we performed PCR on an aliquot of purified DNA with the following universal primers targeted to 18S rRNA gene: forward 5′-AACCTGGTTGATCCTGCCAGTAGTCAT-3′ and reverse 5′-GAATGATCCTTCCGCAGGTTCACCTAC-3′ (26).

To detect tickborne infectious agents, we used nested PCR with specific primers both previously described (6,27,28) and designed in our study. Multiple sequence alignment of nucleotide sequences available in GenBank (http://www.ncbi.nlm.nih.gov) for each tickborne pathogen was performed by using ClustalW (http://www.ebi.ac.uk/clustalw/) (29). The desired specificity of selected primers and absence of cross-reactions were confirmed by BlastN homology search (http://www.ncbi.nlm.nih.gov/BLAST/). Formation of intra- and intermolecular primer dimers was reduced by using OLIGOS (http://www.basic.northwestern.edu/biotools/oligocalc.html).

PCR Detection of *Borrelia*-specific DNA

B. burgdorferi sensu lato DNA was detected by means of nested PCR with primers specific to conservative regions of 5S and 23S rRNA genes to amplify variable intergenic spacer (27). Primers for outer PCR were designed by aligning 5 GenBank nucleotide sequences of rRNA gene clusters for 3 species belonging to *B. burgdorferi* sensu lato complex, including *B. burgdorferi* sensu stricto, *B. garinii*, and *Borrelia lusitaniae*. Other criteria for primer design included the absence of possible cross-reactions with other genera. Outer reactions were performed with NC1 (5′-CCTGTTATCATTCCGAACACAG-3′) and NC2 (5′-TACTCCATTCGGTAATCTTGGG-3′) primers (35 cycles of 60 s at 94°C, 30 s at 58°C, and 30 s at 72°C). Inner reactions were carried out as previously described (27). DNA isolated from *B. burgdorferi* sensu stricto (strain B31), *B. afzelii* (strain Ip-21), and *B. garinii* (strains T6, 2, and 12) was used as positive control. Molecular typing of the PCR-positive samples was

performed by using sequencing and restriction fragment length polymorphism analysis (27) by hydrolysis of the PCR products with the *Tru9*I restriction endonuclease (SibEnzyme, Novosibirsk, Russia) (isoschizomer *MseI*) with subsequent electrophoresis in 15% polyacrylamide gel.

PCR Detection of *Anaplasma*- and *Ehrlichia*-specific DNA

For *Anaplasma*/*Ehrlichia* detection, specific primers were designed by comparing 15 nucleotide sequences of 16S rRNA gene from 12 species (*A. phagocytophilum*, *A. bovis*, *A. platys*, *A. centrale*, *A. marginale*, *E. muris*, *E. ruminantium*, *E. ewingii*, *E. chaffeensis*, *E. canis*, *Wolbachia pipientis*, *Rickettsia rickettsii*). Primers EHR1, EHR2, and EHR3 were identical to the 16S rRNA gene nucleotide sequences of *Anaplasma*/*Ehrlichia* species but differed from *W. pipientis* sequence and markedly distinguished from the sequence of *R. rickettsia*. Outer reactions were performed by using EHR1 (forward, 5′-GAACGAACGCTGGCGGCAAGC-3′) and EHR2 (reverse, 5′-AGTA(T/C)CG(A/G)ACCAGATAGCCGC-3′) primers and inner reactions with EHR3 (forward, 5′-TGCATAGGAATCTACCTAGTAG-3′) and EHR2 primers (35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). DNA isolated from *A. phagocytophilum* and *A. marginale* from spleens of ill cattle were used as positive controls. For PCR-positive samples, nested reactions with *A. phagocytophilum*–specific primers HGE1 (forward, 5′-CGGATTATTCTTTATAGCTTGC-3′) and HGE2 (reverse, 5′-CTTACCGAACCGCCTACATG-3′) were carried out in the same conditions.

PCR Detection of *Bartonella*-specific DNA

For *Bartonella* DNA detection, nested PCR with primers corresponding to *groEL* gene (6) was used. For outer reactions, primers BH1 (forward, 5'-GAAGAAACAACTTCTGACTATG-3') and BH4 (reverse, 5'-CGCACAACCTTCACAGGATC-3') were designed by aligning 31 nucleotide sequences of 13 *Bartonella* species, including *Bartonella henselae*, *Bartonella quintana*, *Bartonella alsatica*, *Bartonella birtlesii*, *Bartonella bacilliformis*, *Bartonella capreoli*, *Bartonella doshiae*, *Bartonella grahamii*, *Bartonella koehlerae*, *Bartonella schoenbuchensis*, *Bartonella taylorii*, *Bartonella tribocorum*, and *Bartonella vinsonii*. The outer reactions were carried out in 45 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. Both primer structures (HSP1, HSP2, and HSP4) and PCR conditions (45 cycles of 30s at 94°C, 30 s at 58°C, and 45 s at 72°C) for inner seminested reactions in the presence of 3 primers were identical to those previously described (6). Full-length genomic DNA samples isolated from *B. henselae* and *B. quintana* were used as positive controls.

PCR Detection of *Babesia*-specific DNA

Babesia DNA was detected by means of nested PCR with primers specific to the 18S rRNA gene. Specific primer PiroA had been previously described (28). Other *Piroplasmida*-specific primers were designed by comparing the 18S rRNA gene nucleotide sequences of 11 species (*B. canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*, *Babesia odocoilei*, *Babesia divergens*, *Babesia caballi*, *Babesia gibsoni*, *B. microti*, *Theileria parva*, *T. equi*, *Plasmodium falciparum*). The chosen primers corresponded to the sequences of most *Piroplasmida* species (including those listed above) but significantly differed from those of *P. falciparum*. Outer reactions were performed with BS1 (forward, 5′-GACGGTAGGGTATTGGCCT-3′) and PiroC (reverse, 5′-CCAACAAAATAGAACCAAAGTCCTAC-3′) primers (36 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C) and inner reactions with PiroA (forward, 5′-ATTACCCAATCCTGACACAGGG-3′) according to Armstrong et al. (28) (with the single nucleotide transition A→T at position 2 from the 5′ end of the primer) and PiroC primers (36 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C). For subsequent sequencing, the 1,304-bp fragment was synthesized in PCR with BS1 and BS2 (reverse, 5′-ATTCACCGGATCACTCGATC-3′) primers (40 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C).

B. canis canis DNA isolated from blood samples of a dog with clinical signs of babesiosis confirmed by microscopic examination (GenBank accession no. AY527064) and *B. microti* DNA from *Clethrionomys rutilus* blood (AY943958) (V. Rar, unpub. data) were used as positive controls.

Sequencing of PCR Products

The PCR products were purified after gel electrophoresis in 1.5%–2% agarose gels with GFX Columns (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Nucleotide sequences of the PCR products were determined by using BigDye Terminator Cycle Sequencing Kit and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Centre of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia. For initial species identification, the nested PCR products were sequenced in 1 direction. Detailed confirmation for each genetic group was performed by sequencing with forward and reverse outer or inner primers as needed.

Nucleotide sequences of PCR products determined in this study were analyzed by BlastN and aligned with ClustalW (29). Phylogenetic analysis was performed with MEGA 3.0 software (30). We used the unweighted pair-group method with arithmetic mean (UPGMA) and

neighbor-joining algorithms with the Kimura 2-parameter model to generate the distance matrix as well as maximum parsimony and minimal evolution with a heuristic search. Bootstrap analysis was performed with 1,000 replications. GenBank accession numbers for the sequences used in the phylogenetic analysis are shown in Figures 2–5.

The nucleotide sequences determined in this study were deposited in GenBank under the following accession numbers: *B. garinii*, AY603350, AY862887, AY862885; *B. afzelii*, AY603351; *A. phagocytophilum*, AY587607; *E. muris*, AY587608; *B. henselae*, AY453166–AY453170; *B. canis canis*, AY527063, AY649326.

Results

Infection of *I. persulcatus* and *D. reticulatus* with 3 bacterial and 1 protozoan tickborne pathogens in Western Siberia, Russia were studied by nested PCR with genus-specific primers. To control DNA suitability for PCR analysis, we amplified the 18S rRNA gene in 125 of the 127 *I. persulcatus* samples tested and in 84 of the 87 *D. reticulatus* ticks studied. Therefore, the 5 samples in which we were unable to amplify tick DNA were excluded from further analysis. Both tick species contained *Borrelia* and *Bartonella* DNA, whereas *Anaplasma*/*Ehrlichia* DNA was detected only in *I. persulcatus*, and *Babesia* DNA was detected only in *D. reticulatus* ticks (Table).

In 37.6% ± 4.3% (standard deviation) of samples isolated from *I. persulcatus* and in 3.6% ± 2.0% of samples from *D. reticulatus*, DNA of *B. burgdorferi* sensu lato complex was found (Table). The nucleotide sequences of the 5S-23S intergenic spacer (216–237 bp) determined in this study were compared to those of other *B. burgdorferi* sensu lato sequences. The sequences from *I. persulcatus* ticks were placed in 2 clades of monophyletic origin, which corresponded to *B. garinii* and *B. afzelii* with excellent bootstrap support (99% and 100%, respectively), whereas samples from *D. reticulatus* were more closely related to *B. garinii* (Figure 2). Thirty-four PCR-positive samples contained DNA of *B. garinii* (23 samples of *B. garinii* group NT29 and 11 of *B. garinii* group 20047), and 11 samples contained *B. afzelii* DNA (Table). For 2 PCR-positive samples from *I. persulcatus*, the hydrolysis of the PCR products with the *Tru9*I restriction endonuclease resulted in 6 fragments of 108, 68, 57, 50, 38, and 20 bp that corresponded to a mixture of patterns C and D (27) and, consequently, 2 species, *B. garinii* group NT29 and *B. afzelii* (Table). Among samples obtained from *D. reticulatus* ticks, 3 contained *B. garinii* group NT29 DNA, but no other variants were found.

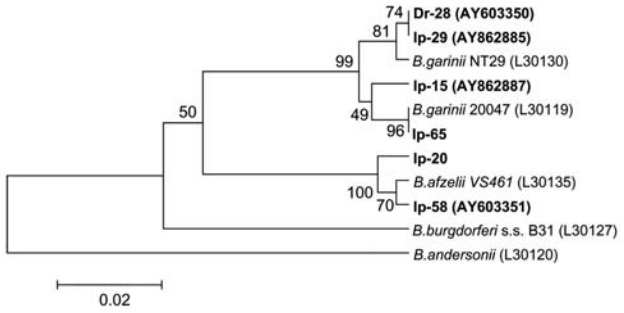

Figure 2. Phylogenetic tree based on the *Borrelia burgdorferi* sensu lato 5S-23S rRNA intergenic spacer fragment sequences. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. *Borrelia andersonii* was used as outgroup. Numbers above the branches indicate bootstrap support indexes. Samples isolated from *Ixodes persulcatus* (Ip) and *Dermacentor reticulatus* (Dr) in this research are in **boldface**.

Anaplasma/*Ehrlichia* DNA was found in 14 *I. persulcatus* ticks but not in *D. reticulatus* ticks from different areas of Novosibirsk region. PCR with primers specific to *A. phagocytophilum* 16S rRNA gene showed the human pathogen DNA in 3 samples, Ip-4, Ip-45, and Ip-68, collected from different areas of Novosibirsk region. The nucleotide sequences of 629 bp of all these samples were identical to each other (GenBank accession no. AY587607) and to the known *A. phagocytophilum* sequence (AF205140). Nucleotide sequences from 11 other DNA samples were identical to each other (GenBank accession

Table. Prevalence of tickborne infectious agents in ticks in Western Siberia, Russia, 2003–2004

Pathogen	Prevalence (% ± SD)*	
	Ixodes persulcatus	*Dermacentor reticulatus*
Borrelia spp.	37.6 ± 4.3	3.6 ± 2.0
Borrelia garinii NT29	18.4 ± 3.5	3.6 ± 2.0
B. garinii 20047	8.8 ± 2.5	0
Borrelia afzelii	8.8 ± 2.5	0
Mixed *B. garinii* NT29 + *B. afzelii*	1.6 ± 1.1	0
Ehrlichia/*Anaplasma* spp.	11.2 ± 2.8	0
Ehrlichia muris	8.8 ± 2.5	0
Anaplasma phagocytophilum	2.4 ± 1.4	0
Bartonella spp.	37.6 ± 4.3	21.4 ± 4.5
Babesia spp.	0	3.6 ± 2.0
Babesia canis canis	0	3.6 ± 2.0

*SD, standard deviation.

no. AY587608) and differed from *E. muris* DNA sequence (U15527) at the single position 91 (C→T). In a phylogenetic tree created by the UPGMA method, both *A. phagocytophilum* and *E. muris* sequences evidently formed the distinctive clusters (Figure 3).

Bartonella DNA was detected by using nested PCR with primers that corresponded to the *groEL* gene in 47 *I. persulcatus* and 18 *D. reticulatus* ticks (Table). Comparative analysis of the *groEL* gene fragment nucleotide sequences of 190 bp showed 2 species, *B. henselae* and *B. quintana*, in both tick species. Part of the data is shown in Figure 4. The evidently separated 2 clades, *B. henselae* and *B. quintana*, were monophyletic with good statistical support (99% and 90%, respectively).

Babesia DNA was found in 3 *D. reticulatus* ticks (Dr-2, Dr-4, Dr-5) by nested PCR and was not detected among *I. persulcatus* studied (Table). The nucleotide sequences of the *Babesia* 18S rRNA gene fragment of 1,203 bp determined in this study were similar to each other and to the single known full-length *B. canis canis* nucleotide sequence (GenBank accession no. AY072926). In the phylogenetic tree, nucleotide sequences from Dr-2 and Dr-5 as well as the *B. canis canis* sequence formed a distinctive cluster that was separated from other *B. canis* subspecies with excellent bootstrap support (Figure 5). Direct sequencing of the PCR fragment from the tick Dr-4 showed a mixture of nucleotide sequences with 2 undetermined bases at positions 609 and 610. Diluting DNA 10 times allowed us to determine 2 nucleotide sequences. The first was identical to those from Dr-2 and the second to a *B. canis canis* sequence found in canine blood from Croatia (AY072926).

UPGMA analysis produced phylogenetic trees (Figures 2–5) that were almost identical to the neighbor-joining trees and results of phylogenetic analysis with maximal parsimony and minimal evolution approaches (trees not shown).

Discussion

I. persulcatus is believed to maintain spirochetes transtadially and to transmit *Borrelia* to animals (31). Previously, the spirochetelike cells were isolated from *I. persulcatus* in Barbour-Stoenner-Kelly-H cultural medium (2) and were observed by indirect immunofluorescence assay (24). The nested PCR with subsequent sequencing showed that *I. persulcatus* contained both *B. afzelii* and *B. garinii* DNA (Table) as was previously shown (2,10,32,33). *B. garinii* appeared to be the prevalent species in *I. persulcatus* in Western Siberia (33). The *B. garinii* NT29 group is widely spread not only in Western Siberia but in the Russian Far East (GenBank accession no. AY429014, AY429015), Japan (34,35), and China (36). The nested PCR with subsequent sequencing allowed us to

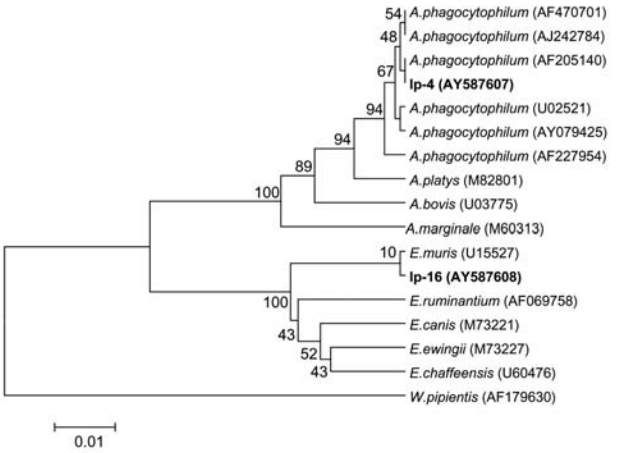

Figure 3. Phylogenetic tree based on the *Anaplasma/Ehrlichia* 16S rRNA gene fragment sequences. Scale bar indicates an evolutionary distance of 0.01 nucleotides per position in the sequence. *Wolbachia pipientis* was used as outgroup. Numbers above the branches indicate bootstrap support indexes. Samples from *Ixodes persulcatus* (Ip-4 and Ip-16) from this study are in **boldface**.

detect DNA of *B. garinii* group NT29 in 3.6% ± 2.0% of *D. reticulatus* ticks. Although *Borrelia*-specific DNA was detected in samples from *D. reticulatus*, numerous previous attempts to cultivate the living spirochetes were unsuccessful (2). Therefore, the ability of *D. reticulatus* to transmit *Borrelia* spp. remains unknown.

E. muris was the prevalent species among *Anaplasma*/*Ehrlichia* and was found in 8.8% ± 2.5% of *I. persulcatus*

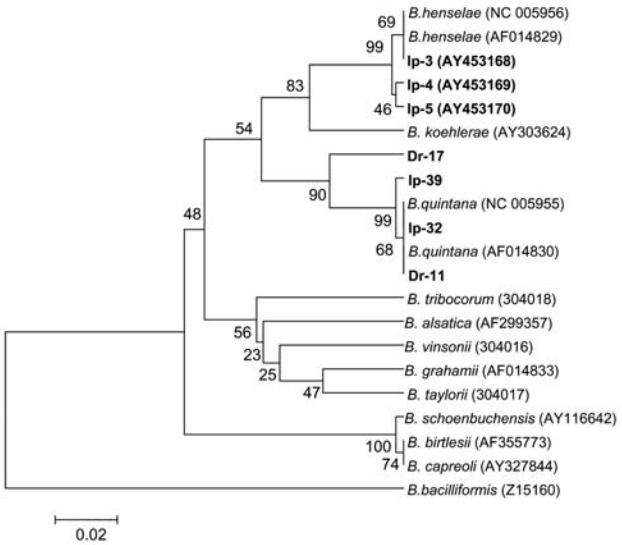

Figure 4. Phylogenetic tree based on the *Bartonella groEL* gene fragment sequences. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. *Bartonella bacilliformis* was used as outgroup. Numbers above the branches indicate bootstrap support indexes. Samples from *Ixodes persulcatus* (Ip) and *Dermacentor reticulatus* (Dr) from this study are in **boldface**.

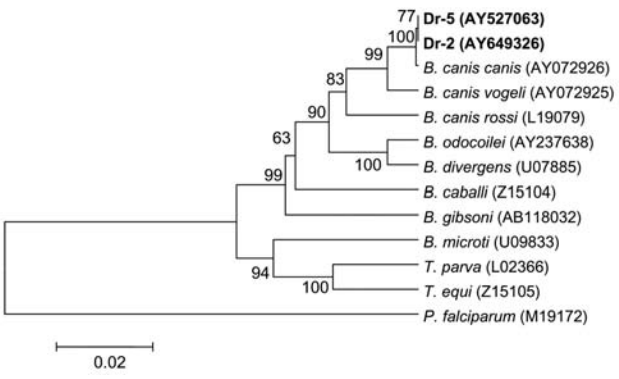

Figure 5. Phylogenetic tree based on the *Babesia* 18S rRNA gene fragment sequences. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. *Plasmodium falciparum* was used as outgroup. Numbers above the branches indicate bootstrap support indexes. Samples from *Dermacentor reticulatus* (Dr-2 and Dr-5) from this study are in **boldface**.

ticks in Western Siberia. This finding coincided with the *E. muris* prevalence (3%–13%) described in Baltic regions of Russia (12) and Siberia (16). The infection rate of *I. persulcatus* ticks with the human pathogen *A. phagocytophilum* (2.4% ± 1.4%) was significantly lower than the rate of infection with *E. muris*. In other regions, the infection rate of *I. persulcatus* with *A. phagocytophilum* varied from 1% to 4% in both China and Russia (14–16,37). The comparison of the *Anaplasma* 16S rRNA gene fragment nucleotide sequences (Figure 3) showed several genovariants of *A. phagocytophilum*. In *I. persulcatus* ticks, 4 types of sequences were found: 3 in China (GenBank accession nos. AY079425, AF205140, AF227954) and 1 in Korea (AF470701). All 3 nucleotide sequences of *A. phagocytophilum* determined in this study coincided with 1 genovariant from China (AF205140) found only in *I. persulcatus* (Figure 3) but not with the *A. phagocytophilum* isolated earlier in West Ural, Russia (GenBank accession no. AY094353) (38). No correlation was seen between genovariant and specific host or location.

Several tick species, such as deer ticks, *I. persulcatus*, *I. ricinus*, and *I. pacificus*, have been found to harbor *Bartonella* spp. (17–20). Thus, PCR with primers specific to the 16S rRNA gene has shown *Bartonella* DNA in >70% of *I. ricinus* ticks in the Netherlands (18). Different *Bartonella* species, including *B. henselae*, have been detected in 19.2% of questing *I. pacificus* ticks in California by amplifying and sequencing the gltA gene fragment (17). More recently, *B. henselae* DNA has been found in 1.5% of *I. ricinus* ticks removed from humans in northwestern Italy (19) and in 38%–44% of *I. persulcatus* in Western Siberia (20). The high *Bartonella* infection rate of *I. persulcatus* in Western Siberia in 2003 and 2004 coincided with our observations from previous years (20). Moreover, both *B. henselae* and *B. quintana* were found not only in the 2 tick species studied (Figure 4) but also in *Aedes* mosquitos (O. Morozova, unpub. data). Only 2 human pathogens, *B. henselae* and *B. quintana*, were found in ixodid ticks in Siberia, despite sample collection for 4 years and phylogenetic analysis of all known *Bartonella* species (Figure 4).

We did not detect *Babesia* spp. in *I. persulcatus*. The only species of *Babesia* detected in *D. reticulatus* was *B. canis canis*, which causes babesiosis in dogs (7). *D. reticulatus* is the only known vector for *B. canis canis* (23,39). Comparison of the previously known *Babesia canis canis* 18S rRNA gene nucleotide sequences showed 3 genetic variants of *B. canis canis* in canine blood from Europe that differed at 2 variable positions 609 and 610 (26,40). Two of these variants were also seen in ticks in Novosibirsk. A new *B. canis canis* genetic variant that differed in a single nucleotide transition from those previously described was found. To our knowledge, this report is the first to identify nucleotide sequences of *B. canis canis* in ticks. *B. microti* was not found among tick samples studied, despite the presence of this human pathogen in small mammals in the same area (V. Rar, unpub. data).

When the 2 tick species were compared, *I. persulcatus* was more likely than *D. reticulatus* to be the host for tickborne bacterial infections examined in Western Siberia, Russia. The *Borrelia*, *Anaplasma*/*Ehrlichia*, and *Bartonella* infection rates for *I. persulcatus* exceeded those for *D. reticulatus* (Table). Moreover, *Borrelia* (10,33) and *Bartonella* (20) DNA from *I. persulcatus* could be easily detected in a single PCR, whereas nested PCR was required to detect DNA in samples from *D. reticulatus*. Neither *Anaplasma* nor *Ehrlichia* spp. were found in *D. reticulatus*. Conversely, *Babesia* spp. were detected only in *D. reticulatus*. The infection of unfed adult *I. persulcatus* and *D. reticulatus* ticks reflected transtadial transmission of tickborne infectious agents.

The experimentally observed and theoretically expected values of mixed infections of ticks with *Borrelia*, *Ehrlichia*, and *Bartonella* were statistically similar and consistent with independent distribution of these pathogens as previously reported (10). Thus, simultaneous coinfection with *Borrelia*, *Anaplasma*/*Ehrlichia*, and *Bartonella* found in 2.9% of *I. persulcatus* ticks slightly exceeded statistical probability of 1.8%. Further studies are required to establish the role of different tick species and biting arthropods as natural vectors of bacterial and protozoan agents.

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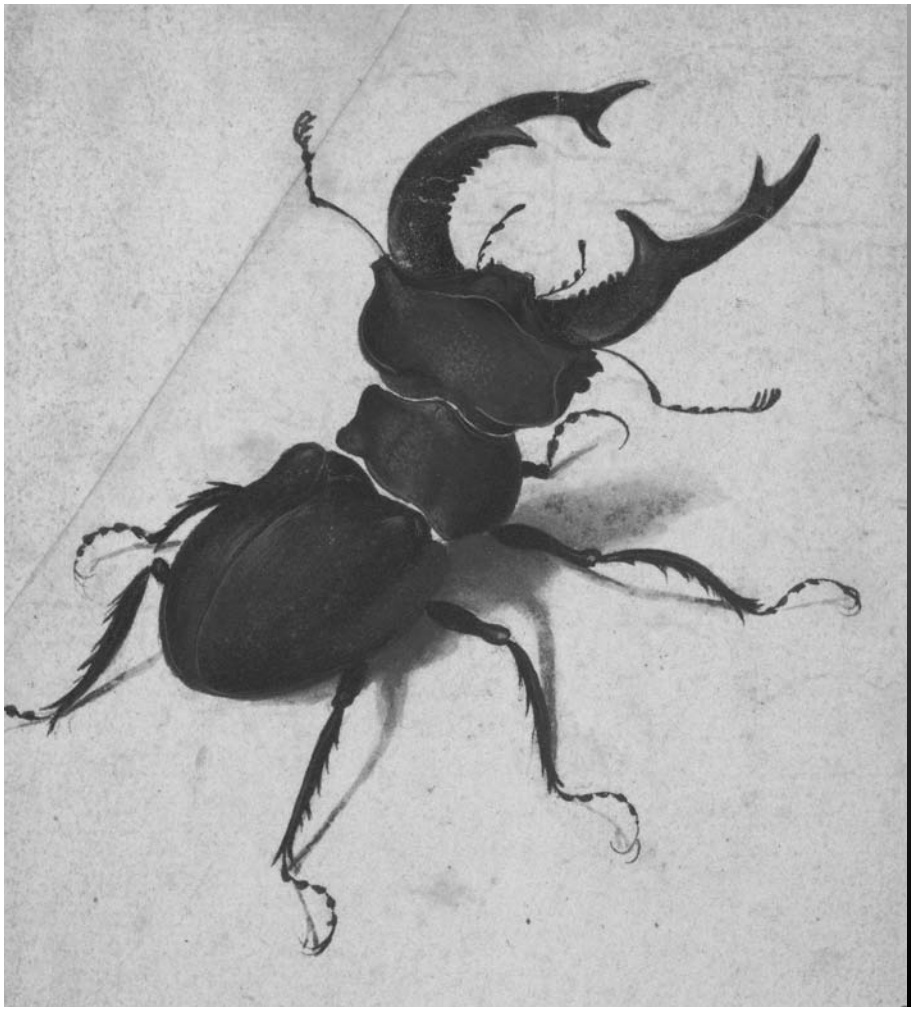

Norovirus Outbreaks from Drinking Water

Leena Maunula,*† Ilkka T. Miettinen,‡ and Carl-Henrik von Bonsdorff*†

As part of an intensified monitoring program for foodborne disease outbreaks in Finland, waterborne outbreaks were investigated for viruses. The diagnostic procedure included analysis of patients' stool samples by electron microscopy and reverse transcription–polymerase chain reaction (RT-PCR) for noroviruses and astroviruses. When these test results were positive for a virus, the water sample was analyzed. Virus concentration was based on positively charged filters from 1-L samples. Of the total 41 waterborne outbreaks reported during the observation period (1998–2003), samples from 28 outbreaks were available for analysis. As judged by RT-PCR results from patient samples, noroviruses caused 18 outbreaks. In 10 outbreaks, the water sample also yielded a norovirus. In all but 1 instance, the amplicon sequence was identical to that recovered from the patients. The ubiquity of waterborne norovirus outbreaks calls for measures to monitor water for viruses.

Water can be a source of disease outbreaks (1). Contamination takes place almost exclusively by sewage that contains enteric pathogens, and enteric viruses that affect humans are mostly species-specific; their abundance may be explained by high concentrations in the stool of patients. Noroviruses (previously called Norwalk-like viruses) cause gastroenteritis in all age groups. Since noroviruses, unlike enteroviruses, do not easily grow in cell culture, their role became evident only in the 1990s, when specific diagnostic methods became available. Only in recent years has the vast genomic variety of noroviruses become apparent (2). A recent report (3) lists 5 genogroups and 22 genetic clusters that include mostly human but also porcine and murine viruses.

In addition to numerous community-based outbreaks, in which transmission is thought to take place from person to person, outbreaks caused by contaminated food have been frequent (4). The dominant role of noroviruses in foodborne and waterborne outbreaks has been estimated by Mead et al. (5). Several waterborne outbreaks have been detected on the basis of epidemiologic evidence (6,7), and only in 1997 did the first report of noroviruses in well water appear (8). The genome-based diagnostic procedure, i.e., reverse transcriptase–polymerase chain reaction (RT-PCR), offers a sensitive and specific tool to identify these viruses. Sequence-based identification is effective for source-tracking outbreaks, especially those caused by noroviruses, which show a highly variable nucleotide sequence even within the short amplicon produced in the polymerase region of the virus (9).

Waterborne viral outbreaks are often difficult to recognize. Illness caused by norovirus is common, and if the contamination level is low, the number of cases remains low. A rather extensive outbreak is usually required for medical personnel and authorities to recognize water as a possible source of infection (10). This report includes virologic analyses of Finnish waterborne outbreaks during a 6-year period. We describe an improved procedure to identify water as the source of viral outbreaks.

Methods

Reporting of foodborne and waterborne outbreaks in Finland was reorganized and intensified in 1997; new regulations emphasized that all suspected cases should be immediately reported to the National Public Health Institute (KTL). Recommendations were given for properly collecting both patient and environmental samples. The functions of local outbreak investigation teams were clarified and included training in conducting epidemiologic surveys. Laboratory performance was improved by including options for viral and protozoan diagnostics from both patient and environmental samples. All cases in which water was suspected as the source of the outbreak were reported to KTL. Sampling recommendations included 3–10 representative patient stool samples. Water samples, raw water, and when appropriate, tap water from different parts of the distribution network were collected immediately. Despite recommendations, not all outbreaks were

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investigated for viruses. The criteria for establishing an outbreak as waterborne were according to the English classification (grades A–D) (11).

In total, 271 patient samples from 25 outbreaks were analyzed for viruses. The range of fecal samples obtained from each outbreak was 2–69 (mean 11). A 10% fecal suspension in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 1 mmol/L $CaCl_2$, pH 7.4, was used for RNA extraction.

A total of 73 water samples from 27 outbreaks were analyzed; 1- to 2-L water samples, collected in clean glass or plastic bottles, were concentrated as described by Gilgen et al. (12). The 1-L samples were run through a positively charged disk membrane filter (diameter 47 mm, pore size 45 μm; AMF-Cuno, Zetapor, Meriden, CO, USA) with or without a fiberglass prefilter. After the elution step in 50 mmol/L glycine buffer, pH 9.5, containing 1% beef extract, the eluate was rapidly neutralized with HCl. The volume was further reduced to ≈100 μL with a microconcentrator (Centricon-100, Amicon, Beverly, MA, USA). This sample was used for RNA extraction and PCR as described (10).

RNA extraction and RT-PCR for the norovirus polymerase region were performed as described (13). Briefly, RNA was extracted by using phenol- and guanidine thiocyanate–containing Tripure reagent (Roche, Indianapolis, IN, USA) and precipitated with ethanol. Viral RNA was transcribed to cDNA, and DNA amplification was performed in separate tubes for norovirus genogroups I and II (GI and GII) by manual PCR with primers Nvp110 (14) and N69 (15), and Nvp110 and NI (16), respectively. From 2002 on, the forward primers for the genogroups were modified as KA1 (5′-GANGGCCTSCCMTCWGGNTT-3′) and KA2 (5′-TGGAATTCNATHGCCCAYTGG-3′). The amplicons were visualized by electrophoresis in an agarose gel, hybridized by a probe panel, and used for nucleotide sequencing.

Sequencing was performed manually (Sequenase, version 2.0 DNA sequencing kit, USB, Cleveland, OH, USA) as described (13). Sequence analysis was performed by programs SeqApp and ClustalW. Our sequences were aligned with the following EMBL/GenBank noroviruses: Southampton/91/UK (L07418), Norwalk/68/US (M87661), Malta (AJ277616), Melksham/94/UK (X81879), Hawaii/76/US (U07611), Lordsdale/93/UK (X86557), GIIb (AY7732101), GIId (AF312728), and murine norovirus (AY228235). For nucleotide sequences for Hillingdon/94/UK and Grimsby/95/UK, see Vinje et al (17); sequence of Lord Harris comes from the sequence database of the European network (9,18). GenBank accession numbers for nucleotide sequences of this study are AY958213–9 for GI and AY958204–12 for GII noroviruses.

Results

Description of Outbreaks and Viral Findings

In total, 41 waterborne outbreaks (3–11 per year) were registered in Finland from 1998 to 2003. Of these, 28 (61%) were investigated for viruses. In 24 outbreaks both water and patient samples were available for analysis; in 3 outbreaks only water was available, and in 1 outbreak only patient samples were available for analysis. Samples for viral analysis were not obtained from the remaining 13 outbreaks. Analysis was performed by RT-PCR. Patient samples were also screened by electron microscopy for other enteric viruses and analyzed by RT-PCR for astroviruses. For water samples, a concentration method according to Gilgen et al. (12) was established, starting from the volume of 1 L. In most cases, water samples were analyzed only for noroviruses. The most prominent viruses that caused waterborne outbreaks were noroviruses (18 outbreaks). Rotavirus caused 1 waterborne outbreak, and no viruses were found in 9 epidemics. Bacterial findings will be published elsewhere.

The 18 waterborne norovirus outbreaks are summarized in Table 1. In every year except 2001, several norovirus outbreaks occurred in Finland. During the study period, 6 large norovirus epidemics with ≥200 cases were encountered. In the largest epidemics, >10,000 persons were exposed, and 2,000–5,500 cases occurred; in addition, 7 medium-sized (40–100 cases) and 5 small outbreaks (<20 cases) were caused by noroviruses.

Most norovirus contaminations occurred in groundwater systems, which are used most commonly in Finland. In 3 instances, surface, lake, or river water was used. Of the ground water epidemics, 8 occurred in public communal systems and 7 in private ground water wells. Typically rental cottages or different kinds of camping grounds with their own wells were affected.

The geographic distribution of the waterborne norovirus outbreaks is shown in Figure 1. Outbreaks occurred all over the country, from the southern archipelago to the northernmost parts of Finland. Seasonal risk for waterborne norovirus outbreak seemed to be approximately equal (Figure 2). Half (20 of 41) of the waterborne epidemics occurred in summer, and norovirus outbreaks (11 of 15) were most common in late winter to spring (February–May). In fact most outbreaks in winter were caused by noroviruses, while in summer they were mainly caused by bacteria.

Detailed Analysis of Noroviruses

Noroviruses from 16 outbreaks (E1–E16) were further characterized by sequence analysis of amplicons, from which the genotype was also deduced (Table 2).

Table 1. Suspected and identified norovirus outbreaks, Finland, 1998–2003

Outbreak	Date	No. exposed/no. ill	Water source
E1, community*	Mar 1998	5,000/2,500	Surface water used as tap water
E2, community	Apr 1998	15,000/2,000	Ground water
E3, rental camp cottage	Jul 1998	45/13	Well
E4, camp on island	Aug 1998	120/40	Communal, well
E5, community	Jan 1999	2,500/200	Ground water
E6, factory area	Feb 1999	250/100	Ground water
E7, community	Apr 1999	160/58	Ground water
E8, spa	Jul 1999	100/60	Well
E9, community*	Mar 2000	10,000/5,500	Ground water
E10, private household	Aug 2000	14/13	Well (drilled)
E11, community	Dec 2000	2,200/300	Ground water
E12, farm (for guests)	Apr 2002	50/25	Well (dug)
E13, community	Oct 2002	960/300	Ground water
E14, guest house	Feb 2003	13/11	Lake water used for drinking
E15, community	May 2003	150/95	Well (drilled)
E16, rental cottage	Aug 2003	25/20	Well
E17, holiday camp	May 2003	56/40	Surface water (river)
E18, community	Apr 2003	90/40	Ground water, broken pipe

*Detailed descriptions of the epidemics E1 and E9 have been published (10,19).

Noroviruses appeared in the patient samples in all 16 outbreaks and in water samples of 10 epidemics. Coliforms were also present in 9 epidemics, whereas in 7 outbreaks, no indication of microbiologic contamination was seen. Most outbreaks were caused by a single norovirus strain/genotype (11 epidemics); >1 virus was found more often in large outbreaks than in small ones. GII noroviruses were only slightly more common than GI (7 vs. 5 outbreaks). Of the 10 epidemics with positive water samples, equal numbers of GI and GII genotypes were detected.

In all but 1 of these outbreaks, the same norovirus genotype found in water samples also appeared in patient samples. The only exception was epidemic E11, in which 2 norovirus sequences, GII.1 and GII.4, were detected in the water sample, but only type GII.4 was detected in the patient samples. Not only the viral genotype but also the entire amplicon sequence were identical in each outbreak (Figure 3). Two norovirus genogroup I types, GI.3 (Birmingham) and GI.6 (Sindlesham, Hesse), were found; 1 GI sequence (outbreak E3) remained undetermined.

In the GII outbreaks, at least 4 different genotypes were found in patient or water samples. The most common genotype was GII.4 (Bristol, Lordsdale), found in water samples of 4 epidemics, and beginning in 2003, it was the new variant type (20). The established genotypes GII.1 (Hawaii) and GII.5 (Hillingdon) were also detected in some outbreaks, along with some potentially new genotypes or sequences that did not cluster well in any of the established genotypes, such as GIId (Upinniemi) and GIIb. As Figure 3 shows, in most outbreaks a virus with a unique amplicon sequence was recovered, even when it belonged to the same genotype as viruses in the other waterborne outbreaks. Norovirus genotype GII.4 was the only exception, and a longer nucleotide sequence likely would have shown some genetic differences (detected between sequences of epidemics E1 and E11; data not shown).

Discussion

As part of the improved and intensified outbreak surveillance system in Finland, we have identified waterborne viral outbreaks since 1998. In a relatively brief period, during which norovirus diagnostics have been available for patient as well as environmental samples, a considerable number of waterborne norovirus outbreaks have been detected.

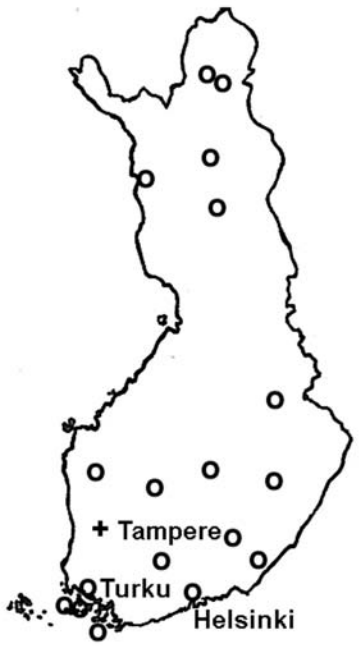

Figure 1. Map of Finland; circles indicate distribution of waterborne norovirus outbreaks, 1998–2003.

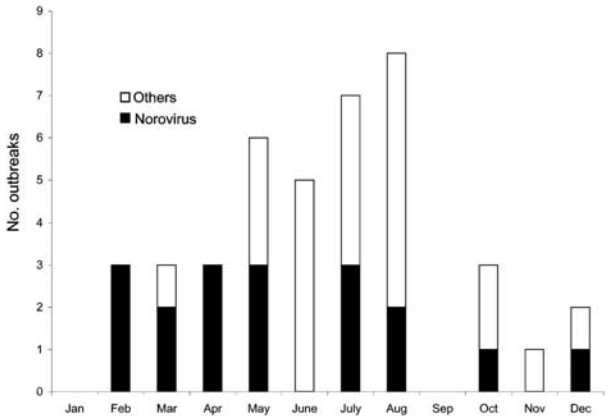

Figure 2. Monthly distribution of waterborne outbreaks, including norovirus outbreaks, Finland, 1998–2003.

That Finland has >1,300 water treatment plants may in part explain the numerous outbreaks. Many of these plants still use surface water (lakes or rivers) as raw water. Inadequate disinfection is then the most common reason for waterborne epidemics, as was the case in outbreak E1 (10). At risk also are water plants that use groundwater and no disinfection. In Finland, snow melts in spring while the ground is still frozen, which leads to surface runoffs and flooding. Breaks in sewer lines in the vicinity of a well caused several large waterborne outbreaks. Poor sewage disposal also caused many small waterborne outbreaks in private homes or rental cottages.

The large number of genetically distinct norovirus genotypes has been advantageous in investigating waterborne epidemics. Although the short amplicon sequence does not definitively show that 2 viruses are identical, for the purpose of source tracing it seems adequate. In this study, a unique viral sequence appeared in most norovirus outbreaks, and viruses from patients and water in a particular outbreak showed identical sequences. The success in most outbreaks in identifying a norovirus with the same sequence from patients and water may be due to the fact that the outbreaks have taken place in small communities. In large waterborne outbreaks, usually >1 norovirus strain and often other viruses and microbes are causative agents.

Both norovirus genogroups occurred in waterborne epidemics. In a 5-year study (1998–2002) in Finland, GII outbreaks clearly outnumbered those caused by GI noroviruses (86.9% vs. 13.1%) (21). In waterborne outbreaks, however, nearly half were caused by GI viruses. Some differences may occur in stability as well as ability to spread from person to person among viruses representing different genotypes. Type GI.3, the most common GI genotype in water samples, was also the most frequent GI type in community outbreaks (21). Viruses of this genotype have caused waterborne outbreaks in the United States in 2001 (22) and in the Netherlands (23).

As might be expected, keeping in mind its ubiquity (24,25), the GII.4 genotype was present in several waterborne outbreaks, and in Finland it has been the most frequent genotype in all outbreaks. The GII.4 new variant emerged in Finland in June 2002, and in the following year 2 waterborne outbreaks were caused by this new variant (20). Another emerging genotype, GIIb, found in Finland in 2001, a year later than in southern parts of Europe, was a causative agent in a waterborne outbreak in 2002. A waterborne outbreak in Sweden caused by this genotype has recently been reported (26).

Environmental virology of human pathogen detection has a rather limited history. A classic case is the monitoring

Table 2. Findings in suspected and identified norovirus outbreaks, Finland, 1998–2003

Outbreak	Date	Presence of coliforms	Microbiologic findings (genotype)*	
			Patients	Water
E1, community	Mar 1998	–	GI.6, GII.4	GII.4
E2, community	Apr 1998	–	GI.6, GIId	–
E3, rental camp cottage	Jul 1998	–	GI, GII	–
E4, camp on island	Aug 1998	–	GII.5	–
E5, community	Jan 1999	–	GII.4	–
E6, factory area	Feb 1999	+	GI.3	GI.3
E7, community	Apr 1999	+	GI.6	GI.6
E8, spa	Jul 1999	+	GI.3	GI.3
E9, community	Mar 2000	–	GI.3, GII	–
E10, private household	Aug 2000	+	GI.3	GI.3
E11, community	Dec 2000	+	GII.4	GII.4, GII.1
E12, farm (for guests)	Apr 2002	+	GII.NA	GII.NA
E13, community	Oct 2002	+	GIIb	–
E14, guest house	Feb 2003	–	GII.4nv	GII.4nv
E15, community	May 2003	+	GII.4nv	GII.4nv
E16, rental cottage	Aug 2003	+	GI.6	GI.6

*nv, new variant.

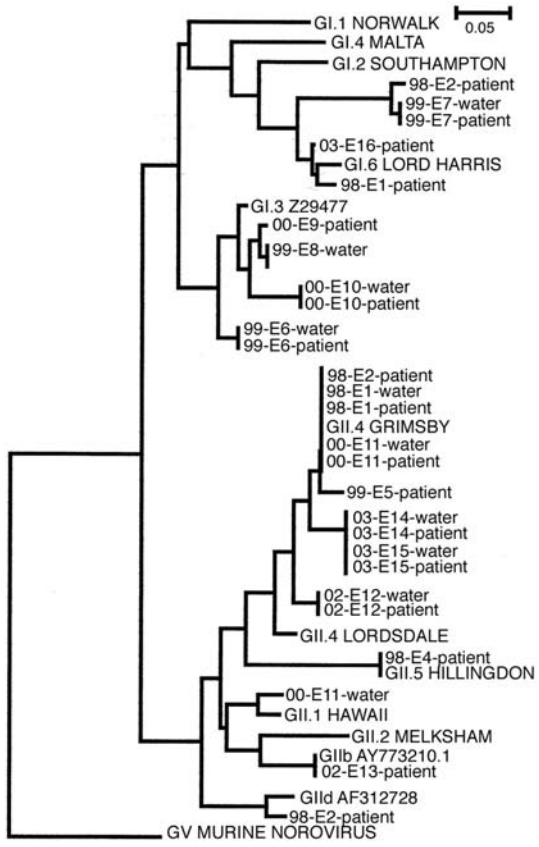

Figure 3. Phylogenetic trees derived from 28 norovirus nucleotide sequences from the polymerase region. The nucleotide sequences were from 10 water and 18 patient samples of 14 outbreaks. Trees were constructed by using the neighbor-joining method with the ClustalW software package. Scale indicated by bars. Branch lengths are related to degree of divergence between sequences.

of polioviruses in sewage (27). This method, based on a cell culture technique, is sensitive in detecting circulating wild poliovirus. Further efforts in environmental virology were lacking for many years, mainly because suitable methods were absent. Only after gene amplification techniques were introduced could a tool be developed to successfully detect norovirus in environmental samples (8,10). In recent years, an increasing number of reports have described waterborne norovirus outbreaks through contaminated drinking or recreational water (22,23,28,29).

National recommendations for volumes of water to be tested vary between tens and hundreds of liters. Such volumes pose a serious practical problem for the testing laboratory. For viral detection by RT-PCR, a smaller volume (1 L) is preferred, as suggested by Gilgen et al (12). Independent of the concentration method, the increase in RT-PCR inhibitors usually sets limits on the water concentration. Sensitive methods are needed to detect viruses in environmental samples. Recent reports on the applicability and sensitivity of real-time RT-PCR (30–32) for noroviruses also offer new possibilities to enhance its sensitivity. Another factor is that the test then becomes more rapid, which is essential in monitoring water quality, particularly in epidemic situations. The third advantage is that a quantitative estimate of the contamination level is obtained.

Microbial risks from water are recognized, with much emphasis on risk assessment (33). Assessment of water, however, depends on indicator organisms, such as coliforms or enterococci, whose survival in water is shorter than that of enteric viruses, especially norovirus and hepatitis A virus. Therefore, viruses can easily be harbored in "microbiologically immaculate" water (34,35). In situations in which a well is contaminated by sewage, coliforms are nearly always found. When sewage is released into lake water that serves as raw water downstream, indicator organisms may no longer be detectable, but noroviruses can still be present and cause illness. This sequence of events probably led to the first outbreak we examined (E1) (10).

When water plants use surface water, the contamination may be short-lived and may have vanished by the time the outbreak is detected. A "rolling sample" system might be used in which samples are collected in water plants at risk for contamination at regular intervals (e.g., daily, weekly) and stored at 4°C. Unless signs of an outbreak appear, the samples can be discarded at the same pace that new ones are collected. In case of contamination, water samples would be available for analyses.

The evidence presented here together with several recent reports mentioned above show the role of viruses as contaminants of drinking water. In Finland, the finding that noroviruses frequently cause waterborne outbreaks has led to authorities' increased awareness of viral risks. As a consequence, laboratory techniques have been improved, and the capacity for analyzing environmental samples, especially water, has increased. Legislative measures for viral monitoring as part of the microbial risk assessment in drinking water production should be seriously considered.

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Typing African Relapsing Fever Spirochetes

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Relapsing fever *Borrelia* spp. challenge microbiologic typing because they possess segmented genomes that maintain essential genes on large linear plasmids. Antigenic variation further complicates typing. Intergenic spacer (IGS, between 16S–23S genes) heterogeneity provides resolution among Lyme disease–associated and some relapsing fever spirochetes. We used an IGS fragment for typing East African relapsing fever *Borrelia* spp. *Borrelia recurrentis* and their louse vectors showed 2 sequence types, while 4 *B. duttonii* and their tick vectors had 4 types. IGS typing was unable to discriminate between the tick- and louseborne forms of disease. *B. crocidurae*, also present in Africa, was clearly resolved from the *B. recurrentis/B. duttonii* complex. IGS analysis of ticks showed relapsing fever *Borrelia* spp. and a unique clade, distant from those associated with relapsing fever, possibly equivalent to a novel species in ticks from this region. Clinical significance of this spirochete is undetermined.

Both tickborne and louseborne forms of relapsing fever, caused by borrelial spirochetes, are substantial causes of illness and death in regions of East Africa. In Tanzania and Ethiopia, for example, these infections are endemic, with much of the population living in close proximity with the disease vectors. Indeed, surveys of traditional Tanzanian "Tembe" dwellings have shown that up to 88% are infested with ticks of the *Ornithodoros moubata* complex (1). These ticks are vectors for *Borrelia duttonii*, the cause of tickborne relapsing fever in East Africa. In disease-endemic regions, tickborne relapsing fever is one of the top 10 diseases associated with deaths in those <5 years of age. The louseborne form of the disease is caused by *B. recurrentis* and is transmitted by the human clothing louse, *Pediculus humanus*. In regions where louseborne relapsing fever is endemic, such as Ethiopia, this disease contributes substantially to human disease prevalence, particularly during the rainy season. Incidence data are largely lacking; the diagnosis typically relies on demonstration of spirochetes in blood films of samples taken from febrile patients. Clinical signs and symptoms overlap with those of other prevalent diseases such as malaria, which may lead to inaccurate diagnoses.

Other *Borrelia* spp. have been associated with relapsing fever in Africa; for example, *B. crocidurae* is associated with this disease in West Africa (2). However, this spirochete has an established reservoir in rodent populations that also serve as hosts for the *Ornithodoros erraticus* ticks. Recent reports have also established additional spirochetes in tick vectors (3) and in human blood samples (4); however, their clinical relevance has yet to be defined.

To determine the population structure within these spirochetes and to assess the role of newly described spirochetes, appropriate microbiologic tools must be used. Many widely used techniques for molecular typing have been applied to *Borrelia* with variable success (5,6). Of concern when these methods are used for characterizing these spirochetes is the organisms' ability to undergo multiphasic antigenic variation, which can be associated with large inter- or intraplasmidic recombinations/duplications (7,8). Indeed, this ability is a likely cause of the variability in genomic organization of these spirochetes, which possess segmented genomes (9–11) composed of giant linear and circular plasmids.

Although conventional approaches, such as sequencing of 16SRNA gene, have provided useful information on population structure among the *Borrelia* spp., this gene only provides low discriminatory resolution among African relapsing fever spirochetes (2,12). Others have used the flagellin gene as a target for population structure (5,13); however, as with 16SRNA, these methods, although useful for interspecies comparisons, are of limited value for discrimination of African relapsing fever spirochetes and for intraspecies analysis. A partial intergenic spacer region (IGS; *rrs* [16S rRNA]-*ileT* [tRNA]) has recently been used for typing Lyme-associated *Borrelia* spp., together with other genetic loci (14). Additionally, IGS fragment typing proved valuable for

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differentiating relapsing fever spirochetes (15). However, these researchers only applied this typing to US strains and to nonpathogenic relapsing fever spirochetes from Sweden. We applied this method to isolates, blood samples, ticks, and lice collected in Tanzania and Ethiopia.

Materials and Methods

Blood Samples

Blood samples from Tanzania were collected into glass capillary tubes after fingerprick of spirochetemic patients attending Mvumi Hospital, Dodoma, Tanzania. In Ethiopia, samples were collected from persons who were hospitalized with louseborne relapsing fever at the Black Lion Hospital in Addis Ababa. Total DNA was extracted from these samples by using a QIAamp DNA blood mini kit (Qiagen, Crawley, UK).

Isolates

Borrelial isolates were obtained from blood samples drawn from patients with clinical cases of relapsing fever as previously described (16–18). *Borrelia* spp. were grown in Barbour-Stoenner-Kelly medium (BSKII) (19) incubated at 33°C to stationary phase. Spirochetes were collected by centrifugation, washed with 0.1 mmol phosphate-buffered saline, and DNA was prepared by using phenolchloroform extraction. A total of 6 cultivated *B. duttonii* isolates (Ly, La, Lw, Ma, Ku, and Wi) and 18 *B. recurrentis* isolates (A1–A18) were investigated. Cultures were diluted in BSKII medium, and the one with the lowest growth was added to media from which DNA was extracted. Because these spirochetes are fastidious, recovery from a single cell cannot be guaranteed. For comparison, other *Borrelia* spp. analyzed included Nearctic relapsing fever strains *B. hermsii* (HS1), *B. turicatae*, and *B. parkeri*; West African *B. crocidurae*; and Lyme borreliosis strain *B. burgdorferi* sensu stricto (B31).

Lice

Lice were collected from the clothing of patients with louseborne relapsing fever, kept in 70% ethanol, and transported to the United Kingdom (import license not required). Total DNA was extracted by using a DNeasy Tissue kit (Qiagen) from pools of 4 to 6 lice.

Ticks

Ticks were collected from traditional dwellings in 4 villages, Mvumi Makulu (MK), Iringa Mvumi (IM), Ikombolinga (IK), and Mkang'wa (MA), in the Dodoma Rural District, central Tanzania. Scoops of earth were collected and passed through a sieve, and ticks were collected into containers containing 70% ethanol. Dead ticks were imported under license (AHZ/2074A/2001/13) and heat inactivated (>80°C for 30 min) before DNA extraction. After manual homogenization of 1 to 6 ticks with a sterile pestle, samples were digested overnight in SNET lysis buffer (20 mmol/L Tris-HCl pH 8.0, 5 mmol/L EDTA, 400 mmol/L NaCl, 1% sodium dodecyl sulfate, 55°C), supplemented with proteinase K (400 μg/mL final concentration). Debris was pelleted, lysate was transferred to a fresh tube, and total DNA extracted by using standard phenolchloroform extraction or the automated MagnaPure DNA extraction robot with the LC DNA isolation kit II for tissues (Roche, Lewes, UK). DNA extracted from 2 purification rounds was pooled, concentrated, and resuspended in sterile distilled water.

PCR

A *Borrelia*-specific nested polymerase chain reaction (PCR) designed to amplify the 16S–23S IGS region was used (14). The outer primers were anchored in the 3′ end of the *rrs* gene and the *ileT* genes, respectively (5′-GTATGTTTAGTGAGGGGGGTG-3′ and 5′-GGATCATAGCTCAGGTGGTTAG-3′ for forward and reverse, respectively, while the inner nested primers were 5′-AGGGGGGTGAAGTCGTAACAAG-3′ and 5′-GTCTGATAAACCTGAGGTCGGA-3′, again for forward and reverse). Amplicons were resolved with 1% agarose gels, bands were excised, and DNA was purified by using a Wizard SV gel and PCR clean-up system (Promega, Southhampton, UK).

DNA Sequencing

Purified DNA was sequenced directly or cloned into pGEMT-easy (Promega) and sequenced. Sequencing reactions were performed according to manufacturer's recommendations by using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and analyzed on an Applied Biosystems Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Nucleotide sequences were analyzed by using Chromas (version 1.45) and DNA Star software (Lasergene 6). Multiple alignments were performed by using ClustalW. Results produced by IGS fragment typing were compared with those obtained using the *rrs* gene with sequences held in GenBank.

Phylogenetic Trees

The phylogenetic relationships of sequence data were compared by using Mega software (version 3) and neighbor-joining methods for compilation of the tree. A bootstrap value of 250 was used to determine confidence in tree-drawing parameters.

Results

Clinical Isolates

Cultivable isolates of *B. duttonii* were identical over the 587–bp portion of the IGS sequenced and, consequently, the Ly strain was selected to represent these isolates. This isolate has been used by others as representative for *B. duttonii* (2,20). When applied to *B. recurrentis*, IGS fragment sequencing was able to group these isolates into 2 types that differed by 2 nucleotides (nt). Of the 18 isolates A1–A10, A17, and A18 comprised type I, while isolates A11–A16 gave a second type II profile. When the 2 *B. recurrentis* types were compared with *B. duttonii* types I–IV, differences appeared negligible (Table 1, Figure 1). The IGS fragment sequences of *B. duttonii* type II and *B. recurrentis* type I were identical.

A comparison of *rrs* gene sequences confirmed the difference between the 2 groups of *B. recurrentis*, in this case, with only a single nucleotide difference (Table 2, Figure 2). Whereas the IGS fragment analysis produced different profiles within species, analysis of the *rrs* gene sequences, with the exception of the *B. recurrentis* types, produced single clusters for each relapsing fever species (Table 2, Figure 2). Differences between species were small, with a 4-nt difference between *B. recurrentis* and *B. duttonii*; a 6-nt difference between *B. recurrentis* and *B. crocidurae*; and only 2 nt differentiating *B. duttonii* and *B. crocidurae* (Table 2).

Lice

Clothing lice collected from 21 febrile, spirochetemic louseborne relapsing fever patients from Ethiopia produced amplicons from 20 samples. Of these, 18 were identified as the *B. recurrentis* type I, and 2 represented type II. Thus, sequence types detected among clinical isolates mirrored those found in the lice.

Ticks

Tick infestation rates in traditional Tembe huts approached that previously described (1), with ticks found in an average of 61% of the 150 huts tested (range 49%–69%). Ten of 14 Makulu village huts contained ticks positive by partial IGS PCR (71.4%), and all were identical to *B. duttonii* Ly strain. Ten of 11 Makang'wa village huts (91%) were similarly positive for IGS DNA. Greater heterogeneity was observed with 3 IGS types detected. The first, type I, was identical to the Ly strain and was found in 8 of the tick extracts (80%). Ticks from hut MA/15 gave a different sequence, diverging by 7 nt from the Ly strain (type II). Notably, the IGS fragment produced from these ticks was identical to that seen in *B. recurrentis* type I. A further profile, type III, was identified in ticks found in another hut, MA/18, which also differed in 8 nt from the Ly strain and in 1 nt from type II. Type II and III sequences were the same size as those in the Ly strain (587 bp).

Nine of 12 Iringa Mvumi village tick extracts (75%) yielded borrelial IGS fragment amplicons; 1 (IM/36) produced 2 different-sized bands (sequence types I and an IGS sequence showing greatest homology with *B. crocidurae*). Sequence analysis showed 4 IGS types. *B. duttonii* type I (Ly strain) was again predominant, identified in 7 (70%) of the 10 sequences, with the smaller (568 bp) detected as a mixed infection in 1 tick extract (IM/36, 10%). The sequence for this smaller band fell outside the *B. duttonii/B. recurrentis* cluster and showed greatest homology with an isolate of *B. crocidurae*. However, this band was surprisingly distant from the IGS *B. crocidurae* sequence held in GenBank AF884004. Because of its highly divergent nature, this latter sequence was excluded from further analysis. Two additional single tick extracts (each representing 10%), yielded larger amplicons of 757 bp and 759 bp, respectively (IM/16 and IM/19). Substantial differences between these sequences and those of *B. duttonii* were evident, and these sequences were assigned *Borrelia* spp. types 1 and 2, respectively (Table 3). Ticks from 9 (75%) of 12 huts from Ikombolinga amplified, yielding 2 distinct IGS fragment types. Ly strain type I was found in most (8 [89%]/9), and a larger amplicon of 762 bp was sequenced from IK/23, which shared little sequence homology with the Ly strain. As a result of this divergence with known *B. duttonii*, this amplicon was assigned *Borrelia* spp. type 3 (Table 3).

Clinical Tickborne Relapsing Fever Patients

DNA was extracted from 6 partially purified blood cultures from tickborne relapsing fever patients and amplified by using IGS fragment primers. Of these, 5 (83%) were identical to those of the Ly strain (type I), and 1 was 568 bp, identical to strains found in ticks (IM/36) and showing greatest homology with *B. crocidurae*. An additional 6 blood samples from spirochetemic tickborne relapsing fever patients yielded amplicons, 5 of which were identical to the Ly strain, while the remainder, *B. duttonii* type IV (patient WM) showed 10–12 nt differences and 1 nt deletion when compared with other sequence types (Table 1).

In summary, *B. duttonii* type I was predominant, found in isolates from patient isolates and blood from patients with clinical cases of tickborne relapsing fever and *O. moubata* ticks. In fact, this was the only type represented among cultivable isolates. Three variants, types II, III, and IV, were occasionally found in both clinical patients (type IV) and ticks (types II, III). A further, smaller, amplicon showed greatest homology with *B. crocidurae* and fell outside the cluster of *B. recurrentis* and *B. duttonii* sequences and was found in both a tick and a patient. Although these variants are an apparent minority, they have been found in

Table 1. Sequence heterogeneity among the 568- to 587-bp intergenic spacer sequence of the *Borrelia duttonii*/*B. recurrentis* group*

Strain	Position															
	108	129	187	215	304	325/326	379	402	438	441	456	467	474	491	525	532
B. recurrentis type I	A	T	T	C	T	GT	T	A	T	G	T	T	A	G	G	C
B. recurrentis type II	A	T	C	T	T	GT	T	A	T	G	T	T	A	G	G	C
B. duttonii type I	G	T	C	C	C	GT	T	A	C	T	T	T	A	G	A	G
B. duttonii type II	A	T	T	C	T	GT	T	A	T	G	T	T	A	G	G	C
B. duttonii type III	A	C	T	C	T	GT	T	A	T	G	T	T	A	G	G	C
B. duttonii type IV	G	T	C	C	T	AC	G	-	T	T	C	C	G	A	G	G
Consensus sequence	A	T	T/C	C	T	GT	T	A	T	G	T	T	A	G	G	G/C

*Only exemplar sequences are included. GenBank accession nos. for *B. recurrentis* are DQ000277–8; those for *B. duttonii* types I–IV are DQ000279–DQ000282.

conjunction with type I in ticks (*B. crocidurae*–like) and as the only *Borrelia* type found in patients with clinical cases (1 patient with *B. duttonii* type IV and 1 with a *B. crocidurae*–like spirochete). Whether these strains can be cultivated remains unresolved.

Novel partial IGS sequence types were found in ticks from huts IM/16 and IM/19, which clustered together with those found in IK/23 and were assigned *Borrelia* spp. types 1, 2, and 3, respectively. Each of these *Borrelia* types showed slight variability in their IGS fragment sequence (Table 3); however, they formed a distinct cluster away from other borrelial species. Insufficient material remained to undertake further PCR assays with other targets such as the *flaB* or *rrs* genes. Greatest homology was with Nearctic species *B. hermsii* and *B. turicatae.* All differ substantially from the Ly *B. duttonii* strain, except over the primer regions, and consequently cannot be considered as belonging to this species.

Accession Numbers

B. recurrentis types I and II were assigned GenBank accession numbers DQ000277 and DQ000278, respectively. *B. duttonii* groups I–IV were assigned DQ000279-DQ000282, respectively. The *B. crocidurae*–like sequence was given DQ000283. *Borrelia* spp. IGS sequences types 1–3 were assigned DQ000284-DQ000286, respectively, while the *B. crocidurae* IGS sequence determined in this study was given DQ000287.

Discussion

Application of gene sequence–based approaches has provided useful information regarding population structure and possible phylogenetic associations among *Borreliae* spp. (21). Of particular value has been sequence-based comparisons of the *rrs-rrlA* IGS region, which (because noncoding DNA shows greater variability than would be expected for coding sequences) is sufficiently conserved to permit phylogenetic conclusions (22,23). Our results demonstrated that the differences between *B. duttonii* and *B. recurrentis* were as great as those within species, raising the question of whether these spirochetes are indeed a different species. This difference is exemplified by the identical IGS fragment sequence shared between *B. recurrentis* and *B. duttonii* type II. Whether these data suggest a common ancestral lineage for these relapsing fever spirochetes can only be fully resolved through sequence analysis of the

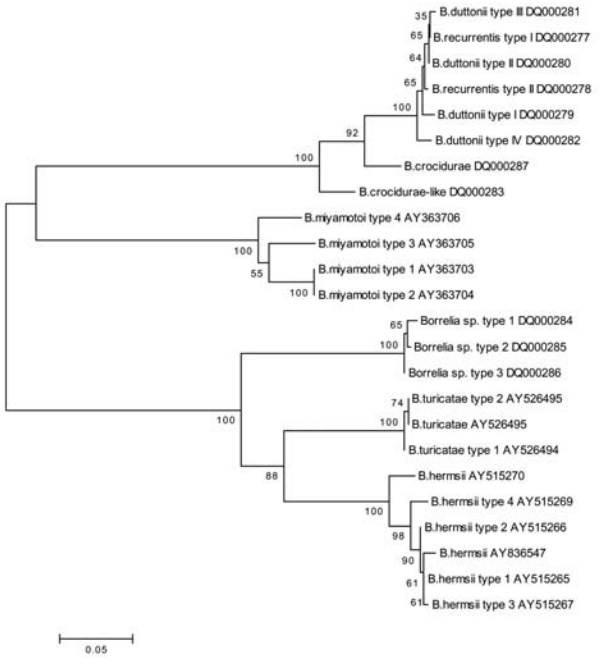

Figure 1. Neighbor-joining phylogenetic tree (bootstrap value 250) showing clustering of intergenic spacer (IGS) fragment generated within this study and compared with IGS downloaded from GenBank. Accession nos. DQ000277–DQ000287 were determined in this study.

Table 2. Sequence heterogeneity within and between *Borellia* spp. using the *rrs* gene

Type or species	Position						
	57	206	373	488	917	1013	1429
B. recurrentis type I	A	T	C	T	T	G	T
B. recurrentis type II	A	T	C	T	T	A	T
B. duttonii	A	C	C	C	C	G	C
B. crocidurae	G	C	–	C	C	G	C

whole genome. An analogous situation was found when IGS typing was used to characterize other microbes such as *Fusobacterium* spp.; in that situation, good resolution of strains was provided; however, with IGS typing, 2 species appeared to be identical (23).

Analysis of *rrs* phylogeny, in contrast, showed distinct clusters for *B. duttonii*, *B. recurrentis*, and *B. crocidurae*; however, a small number of nucleotides differentiated these clusters. Some Old World relapsing fever spirochetes show a high degree of similarity, both in using 16S RNA gene sequences (12) and in flagellin (20,24). Using the 16S RNA gene, we found that only 4 bases differed over the *rrs* gene between *B. duttonii* and *B. recurrentis*; this finding was also reported previously (12). Ras et al. concluded that this small difference did not support these being single species but instead postulated that *B. recurrentis* could be a clone derived from the *B. crocidurae–B. hispanica–B. duttonii* cluster (12). Although our results would support the concept that *B. recurrentis*, *B. duttonii*, and *B. crocidurae* are clonal variants, IGS fragment typing suggested a substantially greater distance between the *B. duttonii/B. recurrentis* complex and *B. crocidurae*. More *B. crocidurae* isolates were not available to further verify phylogenetic inference by using partial IGS sequencing. A further bias within this comparison is that the *rrs* phylogeny presented here used cultivable isolates. Since only *B. duttonii* type I was represented among these isolates, the *rrs* gene phylogeny may not truly represent the diversity found among these spirochetes.

Despite these apparently conflicting datasets, resolution of the IGS fragment typing was clearly greater, enabling a more detailed analysis of clinical or host associations. Failure to discriminate between the *B. duttonii/B. recurrentis* complex, however, would require the use of alternative targets, if they are indeed different species. This apparent paradox may reflect that these are clones derived from a common ancestral origin (12). This hypothesis is strengthened by the finding of a partial IGS sequence (*B. duttonii* type II) in *O. moubata* complex ticks from a disease-endemic area for tickborne relapsing fever, which showed total homology with the predominant sequence type identified among *B. recurrentis.* Further investigation of ticks from this area should be conducted.

To overcome problems associated with use of single gene targets for addressing population structure among microorganisms, multilocus sequence typing (MLST), which is less likely to show linkage disequilibrium than many surface markers, is commonly used to investigate more conserved housekeeping genes. However, a few housekeeping genes possessed by *Borrelia* spp. are located on plasmids, which raises a question about the validity of this approach (25). Others have used surface exposed proteins as targets for MLST (14). More recently, an MLST study focused on 18 different, highly polymorphic loci to show the role of recombination in creation of diversity among Lyme disease–associated spirochetes and their correlation with outer surface protein C alleles (25). Despite extensive application of these methods to Lyme

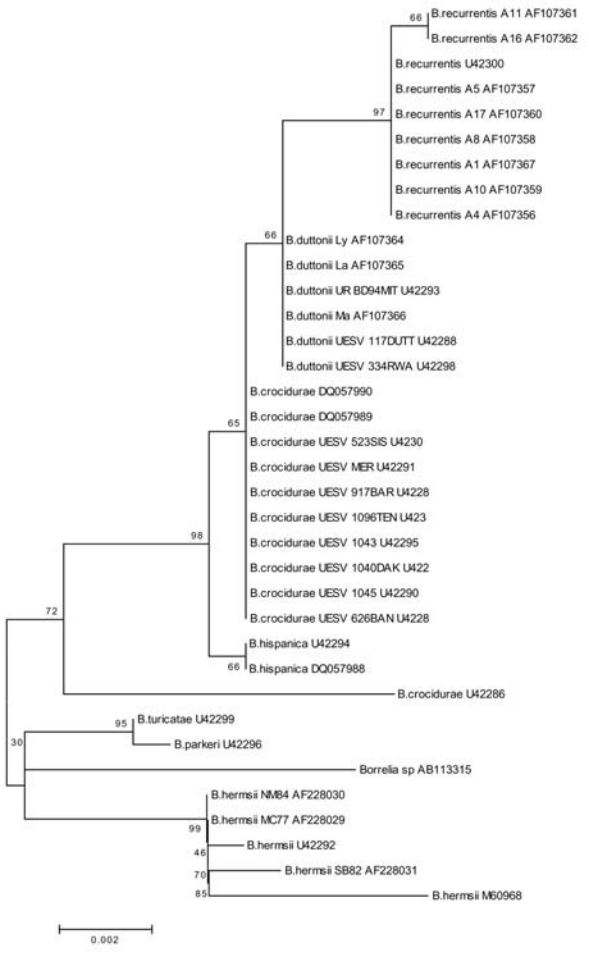

Figure 2. Neighbor-joining phylogenetic tree (bootstrap value 250) showing clustering of the *rrs* gene between *Borrelia duttonii/ B. recurrentis* and *B. crocidurae*.

Table 3. Intergenic spacer sequence diversity among 757–762 bp *Borrelia* spp., DQ000284–DQ000286

	Location										
Strain	133	224	255	290	336	337	347	402–5	424	512	520
Type 1 (IM/16)	C	G	G	A	–	–	T	–	C	A	C
Type 2 (IM/19)	T	A	A	G	T	A	T	–	C	G	T
Type 3 (IK/23)	C	A	G	G	T	G	–	TAGA	T	G	T
Consensus sequence	C	A	G	G	T	X	T	–	C	G	T

disease–associated *Borrelia* spp., the relapsing fever spirochetes have been largely neglected. However, recently a MLST-based approach, in which *rrs*, *flaB*, *gyrB*, and *glpQ* chromosomal genes were used to examine the phylogenetic relationship among *B. parkeri* and *B. turicatae*, confirmed their species status and demonstrated that the Florida canine *Borrelia* isolate clustered among *B. turicatae* (26).

Investigations have been limited by the lack of available strains for assessing and identifying suitable markers. Cultivating these fastidious microbes is challenging, with some, until recently, considered noncultivable (16–18). Furthermore, the complexity of coexistent mechanisms for antigenic variation with associated potential for major genomic reorganization presents a problem for many microbiologic typing approaches. Isolates that are homogeneous by gene sequence–based approaches may present vastly different pulsed-field electrophoretic profiles (16,17,26), possibly through extensive genetic duplication associated with antigenic variation of major outer membrane proteins, whose genes are carried on large linear plasmids (7,11).

Although using the more variable IGS region for molecular typing of relapsing fever spirochetes has been reported as valuable (15), only a limited selection of relapsing fever species, *B. miyamotoi* and *B. lonestari*, have been assessed. When we used this approach to characterize clinical isolates from patients with tickborne relapsing fever and louseborne relapsing fever, blood samples from patients, and ticks and lice collected in East Africa, we demonstrated that this method could successfully be applied to all sample types. We also demonstrated 3 distinct groups: a) 1 comprising cultivable strains of *B. duttonii/B. recurrentis* and amplicons from tick, lice, and human blood samples; b) 1 from both a patient and a tick showing greatest homology with *B. crocidurae*; and c) a further group containing unique amplicons from ticks collected in Tanzania. This last group was distinct in its IGS sequence and is likely compatible with the novel *Borrelia* species from this region (3,4,20). Three sequence types were identified that clustered within this novel IGS sequence clade, separate from the *B. duttonii-B. recurrentis* complex. This clade fell among those containing *B. hermsii* and *B. turicatae* (Figure 1), thus supporting earlier reports of a new *Borrelia* spp. characterized by using flagellin and 16S RNA sequences (*Borrelia* species AB113315 [3], Figure 2), and reported to show greater resemblance to Nearctic isolates such as *B. hermsii* and *B. turicatae*, rather than Old World tickborne relapsing fever and louseborne relapsing fever isolates (3,4,20). Insufficient material remained to allow repeat analysis for either *flaB* or *rrs* genes to confirm that *Borrelia* species types 1–3 were indeed the same spirochete described above. These *Borreliae* spp. were not detected in any relapsing fever patient samples; consequently, their clinical significance has yet to be established.

Of further interest was the identification of a *B. crocidurae*–like sequence in a patient and *O. moubata* tick. *B. crocidurae* is not believed to be present in East Africa but is endemic to the West African coast. Although the patient may have traveled from the west coast, finding this sequence type in a tick that was co-infected with the local endemic *B. duttonii* suggests that this spirochete may coexist in this area and may have extended vector compatibility. These findings were facilitated by methods able to give substantial resolution between strains, such as partial IGS typing, and would not have been possible by using clinical criteria or customary diagnostic approaches.

Traditionally, relapsing fever nomenclature was assigned according to the spirochete's arthropod vector and the host species susceptibility. Clinical differences have also been documented: the louseborne form of the disease has been considered the most severe (27,28), although more frequent relapses are associated with the tickborne form. Such differences have lately been questioned because more recent studies of louseborne relapsing fever suggest less severe outcomes (29). *B. duttonii* has been correlated with higher perinatal death rates (30–32), while *B. recurrentis* is associated with a higher incidence of epistaxis (28). These differences suggest that the diseases are associated with different clinical etiologic agents; however, these differences may actually be associated with differences in transmission or even in host response. Alternatively, different clinical symptoms may be serotype associated. Indeed, serotype switching has been associated with different clinical symptoms in rodent models infected with the American relapsing fever spirochete, *B. turicatae* (33). The lack of animal models for East African isolates precludes similar studies among these spirochetes. However, different isolates of the same species (*B. recur-*

rentis), which express different variable membrane proteins, have been shown to induce tumor necrosis factor release to different degrees (34).

Unlike many relapsing fever spirochetes, *B. duttonii* and *B. recurrentis* infections are believed to be diseases of humans with as yet unidentified animal reservoirs. This restricted host range may impose constraints on these species compared with their less host-specific relatives. Indeed, this could account for greater diversity among other tickborne *Borrelia* spp. than among *B. duttonii* and the louseborne *B. recurrentis*.

In summary, 38 *B. recurrentis* sequences and 47 *B. duttonii* IGS fragments were studied, showing 2 sequence types differing by 2 nt in the former and 4 types differing in from 1 to 12 nt (and a further deletion in 1 type) in the latter. These observations suggest more recent evolution of *B. recurrentis*, if one assumes that both spirochetes acquired changes in their IGS region at the same rate. IGS fragment sequencing proved to be a valuable typing tool for these spirochetes, allowing discrimination between relapsing fever and other borrelial species carried by ticks and lice prevalent in the same geographic region. However, this method was unable to separate *B. duttonii* and *B. recurrentis.* This typing approach appeared sufficiently robust to work on multiple sample types, including isolated strains, patient blood samples, and ticks and lice, thus negating the requirement for cultivation.

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Neutralizing Antibody Response and SARS Severity

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Using the Taiwan nationwide laboratory-confirmed severe acute respiratory syndrome (SARS) database, we analyzed neutralizing antibody in relation to clinical outcomes. With a linear mixed model, neutralizing antibody titer was shown to peak between week 5 and week 8 after onset and to decline thereafter, with a half-life of 6.4 weeks. Patients with a longer illness showed a lower neutralizing antibody response than patients with a shorter illness duration ($p = 0.008$). When early responders were compared with most patients, who seroconverted on and after week 3 of illness, the small proportion (17.4%) of early responders (antibody detectable within 2 weeks) had a higher death rate (29.6% vs. 7.8%) (Fisher exact test, $p = 0.004$), had a shorter survival time of <2 weeks (Fisher exact test, $p = 0.013$), and were more likely to be > 60 years of age (Fisher exact test, $p = 0.01$). Our findings have implications for understanding the pathogenesis of SARS and for SARS vaccine research and development.

Severe acute respiratory syndrome (SARS) is a newly emerged infectious disease. Its etiologic agent is a novel coronavirus (SARS-CoV) (1,2), which can readily infect a variety of wild and laboratory animals without causing apparent clinical symptoms (3,4), making the existence of an animal reservoir possible. In humans, SARS appears with a wide clinical spectrum, ranging from self-limited pneumonia to acute respiratory distress syndrome (ARDS) and death (5,6). Anecdotally, asymptomatic infection has also been reported (7).

Autopsies of SARS patients have found the virus to be widespread throughout a variety of tissues and organs (8). During the acute phase, the virus is found in the excreta of infected persons (9,10) and is thought to be transmitted by direct contact, droplets, or contaminated environmental surfaces. Infection can be prevented largely by good hand hygiene, although some healthcare settings and communities may be prone to the aerosolization of contaminated human excreta, and in these cases, precautionary measures should be instigated accordingly (11,12). The chain of human transmission has been successfully interrupted by public health measures, but potential reintroduction of the virus from an unidentified natural reservoir remains a concern. A wealth of clinical and epidemiologic observations have emerged and contributed to the successful control of the SARS epidemic (see Peiris et al. [13] for a review). However, information on immunity and pathogenesis is insufficient to provide a comprehensive basis for specific drug or vaccine design. Nor have animal pathogenic models been established that adequately resemble the pathogenesis of SARS in humans. Without a good experimental model to study the biologic basis for human disease, the observational data collected from reported SARS case-patients, along with the associated laboratory diagnostic tests, will continue to provide essential leads in controlling a possible reemergence of SARS. To gain a better insight into the humoral responses in the context of epidemiologic and clinical settings, we analyzed the neutralizing antibody data, along with a variety of epidemiologic elements in the database.

Material and Methods

This retrospective analysis is based on Taiwan's nationwide database on SARS cases reported from March to July 2003 to the Center for Disease Control in Taiwan (Taiwan-CDC). The criteria for reporting SARS patients evolved over time but were principally adopted from the World Health Organization, and the total reported probable SARS patients in Taiwan were 665.

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Data

The epidemiologic database contains basic demographic information (age, sex, city/county of residence); symptoms at onset; date of onset of first symptoms; date of diagnosis; dates of hospitalization, discharge, or death; results of all epidemic investigations on contact tracing; travel history; and results of laboratory tests of reverse transcription–polymerase chain reaction (RT-PCR) on SARS-CoV and other pathogens in the differential diagnosis of atypical pneumonia. The analysis of epidemiologic data has been reported previously (14,15). The detailed laboratory data taken from molecular and serologic tests of SARS-CoV infection were compiled in a separate file that could be linked to the epidemiologic data. The concordance and discordance between various serologic tests and molecular diagnostic methods of SARS have also been reported previously (9). The serum neutralizing antibody was measured by microtiter assay and by enzyme-linked immunosorbent assay (ELISA) (Centers for Disease Control and Prevention, Atlanta, GA, USA) as described (9).

Severity of Illness

Hospitalization served the dual purposes of isolating patients and providing health care; therefore, criteria for discharging patients, i.e., being afebrile for 5 days and clinical improvement, were stringently adhered to by the clinicians as a part of public health practice. Since no antiviral drug was known to effectively shorten the clinical course of SARS, the duration of illness, defined as the number of days between onset of fever and time of discharge from the hospital, can be assumed to reflect the clinical severity of SARS manifested by the patient. To validate the consistency of the interhospital practices in patient care in relation to the severity of patients, we collected and analyzed anonymous and computerized clinical data, focusing on oxygen supplementation and respiratory therapy, on a sample of SARS patients from 3 hospitals that represented 3 healthcare accreditation levels in Taiwan: a major medical center (National Taiwan University Hospital), a regional teaching hospital (Taipei Mackay Memorial Hospital), and a district hospital (Taipei Hospital). Regardless of hospital, duration of illness correlated highly with the supplementation of oxygen, which is a good surrogate for the level of pulmonary dysfunction (p for trend <0.001) (Table 1). Thus, in our analysis, duration of illness was used as a surrogate for clinical severity among the surviving SARS patients, and death rate was also used as severity index. For the convenience of discussion, a duration of illness ≤2 weeks was considered mild, 2–4 weeks as intermediate, and >4 weeks as severe. A fatal case, regardless of the length of survival, was considered severe. Our data corroborate the report that SARS patients with a severe clinical course mainly had a slower and prolonged recovery (16).

Statistical Analysis

Data were analyzed with SAS software (Version 8, SAS Institute Inc, Cary, NC, USA). Differences in frequencies or proportions were tested using a χ^2 test and by risk ratios. The continuous variables, i.e., age distribution or titers of neutralizing antibody, were compared by using the Wilcoxon rank-sum nonparametric method. Multivariate logistic regression (17) was used to analyze factors that can affect seropositivity, including demographic information, source of infection, and duration of illness. To adjust the time effects and other covariates of interest, the relationship between antibody titer, based on logarithmic transformation of base 2 (serum dilution) and other potential factors, i.e., age, sex, infection source, and duration of illness, was quantified by linear mixed models (18), which took into account the correlation between repeated measurements of each study participants.

Results

Participants

Specimens from all patients with probable SARS in Taiwan were serologically tested for case confirmation, with a particular focus on the convalescent-phase serum specimens, as previously reported (9). Positive neutralizing antibody results, which correlated well with those of ELISA (Table 2), were used as the standard assay for case confirmation. Thus, 347 of 665 reported probable SARS

Table 1. Requirement of oxygen supplement in relation to the duration of illness among SARS-CoV–infected patients*†

	Oxygen supplement		
Outcome	None or ≤2 L, % (n)	>2 L by mask, % (n)	By assisted ventilation, % (n)
Duration of illness (d)			
≤14	100.0 (5)	0	0
15–21	100.0 (16)	0	0
22–28	86.9 (20)	13.0 (3)	0
>28	23.5 (4)	23.5 (4)	52.9 (9)
Death	0	0	100 (11)

*N = 81; SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
†p value for trend <0.0001.

Table 2. Comparison of SARS antibody results of ELISA and neutralization assay*

	Neutralizing antibody‡	
ELISA†	Positive	Negative
Positive	207	1
Negative	10	206
Total	217	207

*SARS, severe acute respiratory syndrome; ELISA, enzyme-linked immunosorbent assay.
†Reagents were supplied by Thomas Ksiazek, Centers for Disease Control and Prevention, Atlanta, GA, USA (2 reagents).
‡Using ELISA as the reference assay, the sensitivity of the neutralizing antibody (NT) test = 99.5%, the specificity of neutralizing antibody = 95.4%.

cases were confirmed. These included cases in 126 patients whose diagnoses were based on serologic testing alone, 121 whose results were positive by both tests, and 100 patients whose diagnoses were based on the RT-PCR alone. Of these 100 diagnoses based on RT-PCR alone, 32 had convalescent-phase serum specimens that tested negative, and 68 did not have appropriate convalescent-phase serum specimens for antibody testing because of death, loss to follow-up, or inappropriate timing of serum collection (Table 3).

Seronegative Results

We examined whether the seronegative results of the 32 patients were false-positive instances of virus detection by RT-PCR, most commonly caused by laboratory error or contamination. Cross-contamination in the laboratory should occur without any correlation with the patients' demographic or clinical parameters. The seronegative rate (19.1%, 18/94) was significantly higher in men than in women (7.5%, 14/185) (Mantel-Haenszel test, $p = 0.004$), but the effect of age was not statistically significant ($p = 0.07$ in men, χ^2 test) (Figure 1). Based on the transmission risk of known or unknown sources, patients whose sources could not be ascertained, i.e., had no apparent history of having contact with SARS patients, were significantly more likely to be seronegative (45.1%, 23/51) than those with known sources of infection (3.7%, 8/212) (χ^2, $p<10^{-7}$) (Table 4). Patients with a shorter duration of illness were more likely to be seronegative; 14 (30.4%) of 46 patients with a duration of illness ≤14 days, 8 (9.8%) of 81 patients with illness durations of 15 and 21 days, 5 (7.8%) of 64 patients for those with 22 and 28 days, and none of those who survived for >28 days (χ^2 for trend = 20.5, $p = 0.00001$). A logistic regression model confirmed that patients with a known source of infection (odds ratio [OR] = 15.6, $p<0.0001$) and a longer duration of illness (OR = 1.08 for each additional day of illness, $p = 0.004$) were more likely to possess a detectable level of neutralizing antibody than those with no discernible infection source and shorter duration of illness (Table 5).

Antibody and Duration of Illness

The total number of serum specimens collected from each patient ranged from 1 to 4, including ≥1 convalescent-phase serum sample collected after week 4. Of the 247 seropositive SARS patients, 217 (87.8%) of the patients were seropositive by week 3; 27 (17.4%) of 155 patients who were seropositive within the first 2 weeks of illness and are called early responders hereafter. On rare occasions (1.21%, 3/247), seroconversion occurred after week 6 of symptoms onset.

Because of the differences in the distribution of age and sex among patient groups and the differences in the number and timing of specimens collected for antibody measurement, we used regression-based modeling approach to examine these factors simultaneously and tried to analyze the relationship between their potential interactions and antibody titers (Table 5, Figure 2A). This model was based on the neutralizing antibody titer of the 312 convalescent-phase serum assays, representing 194 patients who had had 1 convalescent-phase serum sample collected between weeks 3 to 12 after onset of fever, 41 patients who had 2 convalescent-phase samples, and 12 patients who had 3. The number of serum specimens collected ranged from 21 to 43 per week from week 4 of illness through week 12. The model suggested that neutralizing antibody rose and diminished during the follow-up period between weeks 3 and 13 after onset of illness ($p = 0.026$ for linear term and $p = 0.042$ for quadratic term); the estimated half life was ≈6.4 weeks. Patients with a more protracted clinical course tended to have a higher antibody titer than patients with a shorter clinical course ($p = 0.008$). Antibody in patients with more severe clinical courses tended to decay at a faster rate than in patients with shorter clinical course (the interaction between duration of illness and time of serum collection, $p = 0.037$). This pattern of decay followed a half-life of ≈6.4 weeks after reaching the peak, which occurred between weeks 5 and 8 after infection (Figure 2B). The time that the blood was collected for each patient was examined, and an equally dispersed pattern of blood collection was found in all clinical groups (Figure 3).

Table 3. Laboratory confirmation of the reported 347 SARS-CoV–infected patients by neutralizing antibody (NT) or by RT-PCR*

NT†/RT-PCR	No. (%)
Positive/positive	121 (34.6)
Positive/negative or ND‡	126 (36.7)
Negative/positive	32 (9.2)
ND‡/positive	68 (19.7)

*SARS-CoV, severe acute respiratory syndrome–associated coronavirus; RT-PCR, reverse transcription–polymerase chain reaction; ND, data not available.
†Positive = NT titer of ≥1:16 dilution; negative = NT titer <1:16 dilution
‡Data not available because of loss to follow-up or insufficient number of specimens (n = 8), death (n = 55), or serum collected on the 3rd week (n = 5) of fever onset.

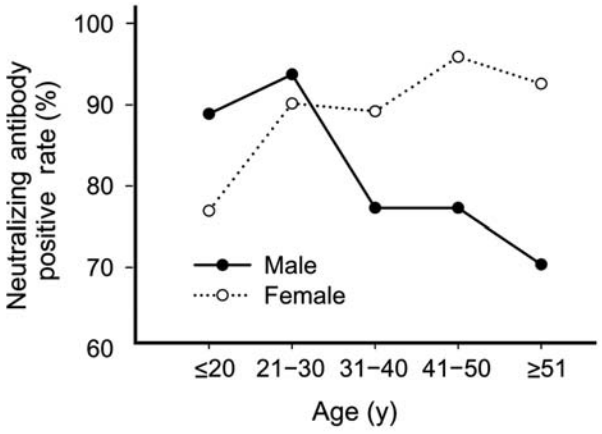

Figure 1. Positive rate of severe acute respiratory syndrome–associated coronavirus titer by sex and age.

Of the 53 patients who had a second convalescent-phase specimen collected after 6 weeks, 16 (31.2%) of 53 showed a 4-fold (2 dilution) drop by week 12 postinfection. Three patients had a negative seroconversion during the same period. Conversely, a measurable neutralizing antibody persisted in 12 (85.7%) of 14 patients who had been followed up between weeks 13 and 16. To better examine the dynamics of the antibody profile, cross-sectional views of Figure 2A were extracted (Figure 2B). The model suggested that antibody response was higher and occurred earlier in patients with a more severe clinical course than in those with a shorter clinical course.

Early Responders and Deaths

In the model, patients with a more severe clinical course had earlier and higher antibody responses; we then examined the death rate of the early responders (Table 6). These early responders had a significantly higher mortality rate (29.6% vs. 7.8%) than others who did not undergo seroconversion until week 3 of illness or later ($p = 0.004$, χ^2 test). These early responders also tended to die early during the acute phase: 6 of 8 died during the first 2 weeks of illness, and the other 2 died on days 15 and 17 of illness, respectively (Fisher exact test, $p = 0.028$). Of the 10 patients who died and who seroconverted after the second week of symptom onset, only one died during the first 2 weeks of illness (Wilcoxon rank sum, $p = 0.007$). Among the 27 early seroresponders, the antibody titer of those who died ($n = 8$) (median titer = 48) was not significantly higher than that of those who survived (median titer = 32) (Wilcoxon rank sum, $p = 0.79$). However, the early seroresponders were significantly older (mean age 43.7 years) than the 128 case-patients who seroconverted after week 2 of illness (mean age 37.3 years) (Wilcoxon rank sum, $p = 0.028$); i.e., older patients were more likely to be early responders, 60% of patients >60 years of age versus 16.3% of patients ≤60 years (Fisher exact test, $p = 0.004$).

Discussion

Neutralizing antibody plays an integral role in immunoprotection from viral diseases, and serologic tests are important to their diagnosis. This report relates SARS neutralizing antibody profiles to clinical outcomes. The lack of a readily available, well-characterized diagnostic assay that could be used as a standard and the additional lack of a well-established typical clinical description that encompasses all clinical syndromes are intrinsic difficulties of working with a new infectious disease. Therefore, analysis of the interrelation between clinical, epidemiologic, and laboratory data might provide further insight into these elements. Results of our analysis, although they passed a certain level of statistical scrutiny, should be interpreted with caution.

Antibody Titer and Seronegativity

The 32 seronegative SARS patients whose diagnoses were based on positive RT-PCR results of nasopharyngeal swab specimens warrant further discussion concerning whether they were indeed SARS patients or were merely misdiagnosed by the false-positive RT-PCR of SARS-CoV. The false-positive RT-PCR is most commonly due to cross-contamination, which pertains to the nature and quality of a laboratory procedure and should be independent of patient's profile. However, we found that seronegative patients were more likely to have a short duration of illness and no clear source of infection. Lack of specificity of the test is another reason for having a false-positive RT-PCR, but none of the commercial tests we used have been reported to have nonspecific cross-reactivity with other known pathogens. Furthermore, after May 1, SARS diagnosis required positive results of ≥2 specimens collected at different time or from different sites, or tested by

Table 4. Seronegative rate of SARS-CoV patients, by source of infection*

Source of infection	Total no.	Seronegative rate, n (%)
Known		8/212 (3.7)
Hospital-associated		
Patient†	81	3/44 (6.8)
Patient's close contacts	55	1/43 (2.3)
Healthcare worker	82	2/71 (3.1)
Other worker‡	27	1/19 (4.0)
Family and social contacts	43	1/35 (2.9)
Unknown		23/51 (45.1)
Imported	22	11/20 (55.0)
Indigenous	36	12/31 (38.7)

*Seronegative rates of the 2 groups with known and unknown source of infection were significantly different (χ^2, $p<0.10^{-7}$).
†In-hospital patients experiencing nosocomial SARS infection.
‡Including laundry workers, cleaners, clerks, and ambulance drivers.

Table 5. Multivariate analysis of factors affecting seropositivity and neutralizing antibody titer of severe acute respiratory syndrome (SARS) patients

Variables*†	Seropositivity*		Antibody titer†	
	OR (95% CI)	p value	Parameter estimates ± SE	p value
Age (y) (n + 1 vs. n)	0.97 (0.94–1.00)	0.065	0.0056 ± 0.0079	0.478
Women vs. men	1.24 (0.47–3.3)	0.67	–0.417 ± 0.235	0.081
Infection source, known vs. unknown	15.6 (5.9–41.4)	<0.0001	0.248 ± 0.313	0.431
Duration of illness (d) (n+1 vs. n, n = 1 through 44 d)	1.08 (1.025–1.143)	0.004	0.0638 ± 0.0233	0.008
Time of convalescent-phase serum sample (weeks after fever onset) (n + 1 vs. n, n = 3 through 15 wk)	–	–	0.449 ± 0.198	0.026
(Duration of illness) × (Time of convalescent-phase serum sample)	–	–	–0.005 ± 0.0024	0.037
(Time of convalescent-phase serum sample)^2	–	–	–0.025 ± 0.012	0.042

*Logistic model: age, with every additional year of age, the odds of seropositivity is 0.97 (odds ratio, OR) (see Figure 1); sex, the odds for women to be seropositive is 1.24 (OR) when compared with men; infectious source, the odds of patients with known infection source to be seropositive is 15.6 times that of the patients without known source of infection; duration of illness, for every additional day of illness, the odds of seropositivity increases by 1.08.
†Linear mixed model: $\log_2$ (neutralizing antibody titer) = $\beta_0 + \beta_1$ (age) – β_1 (sex) + β_3 (infection source) + β_4 (duration of illness) + β_5 (time of convalescent-phase serum sample) –β_6 (duration of illness × time of convalescent-phase serum sample) –β_7 (time of convalescent-phase serum sample)^2. In results above, the model estimates are based on $\log_2$ (titers), to which the time of convalescent-phase serum collection (in weeks postonset of illness, starting from week 3) contributed in 3 terms; the antibody rise follows the first order of weeks postonset, and decay follows the second order of weeks postonset and an interactive term between duration of illness and weeks postonset.

>1 RT-PCR method (Centers for Disease Control and Prevention, Atlanta, GA, USA; Roche Diagnostics GmbH, Mannheim, Germany; Artus GmbH, Hamburg, Germany) if only 1 specimen was available. Therefore, a specimen that yields false-positive results with 2 different test methods is deemed unlikely.

Alternatively, these 32 patients were indeed SARS patients, but the negative neutralizing antibody reading was due to patent's low antibody level in combination with the low sensitivity of the antibody test. The sensitivity of our neutralizing assay is comparable to that of ELISA (9), but the possibility that our assay had a low sensitivity remains because the neutralizing antibody test is based on the reading of a complete inhibition of cytopathic effect. Thus the absolute titer is expected to be lower than the results, based on reading of 50% inhibition. The neutralizing antibody of SARS patients has been reported in only 1 other study, in which a pseudovirus containing the S protein of SARS-CoV was used; antibody titers were found to be low (19). A low antibody response may be associated with a primary infection of SARS-CoV, as seen with primary infection of respiratory syncytial virus (RSV) (20), and infection through the respiratory tract was shown to stimulate a less vigorous immune response than infection by an invasive intravenous rejection of RSV (21). Furthermore, a robust humoral immune response requires antigen in sufficient doses through a proper route; this fact has been demonstrated in vaccine studies, including research on several live vaccines (22–24). The lack of detectable antibody among patients without history of contact with a known SARS

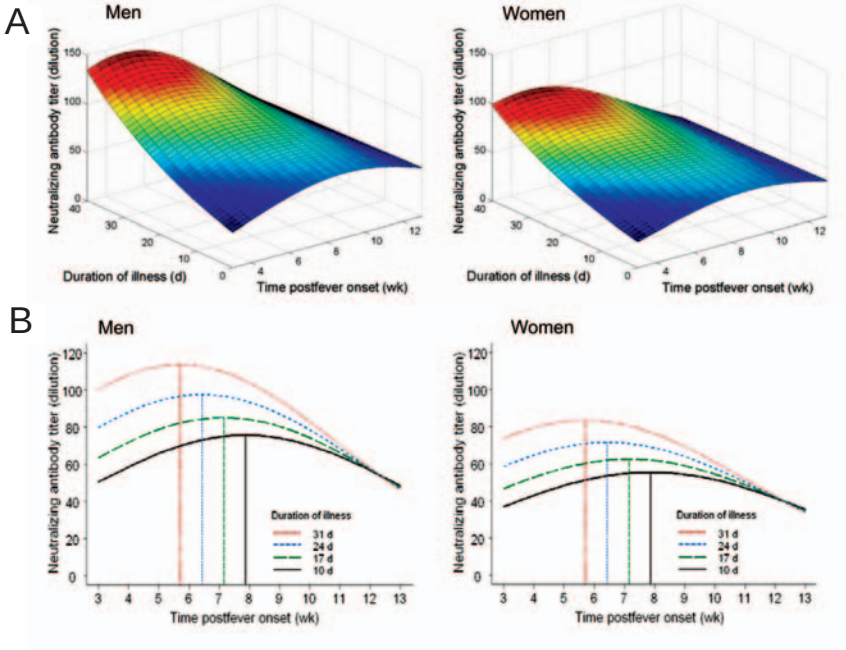

Figure 2. A) Perspective surfaces of neutralizing antibody titer (dilution) based on the fitted linear mixed model in Table 5. The median age was 36 years for men (left panel) and for women (right panel). B) Cross-sectional curves of neutralizing antibody titer (dilution) extracted from panel A with duration of illness set at 10, 17, 24, and 31 days, respectively, for men (left panel) and women (right panel); the vertical lines mark peak titer times.

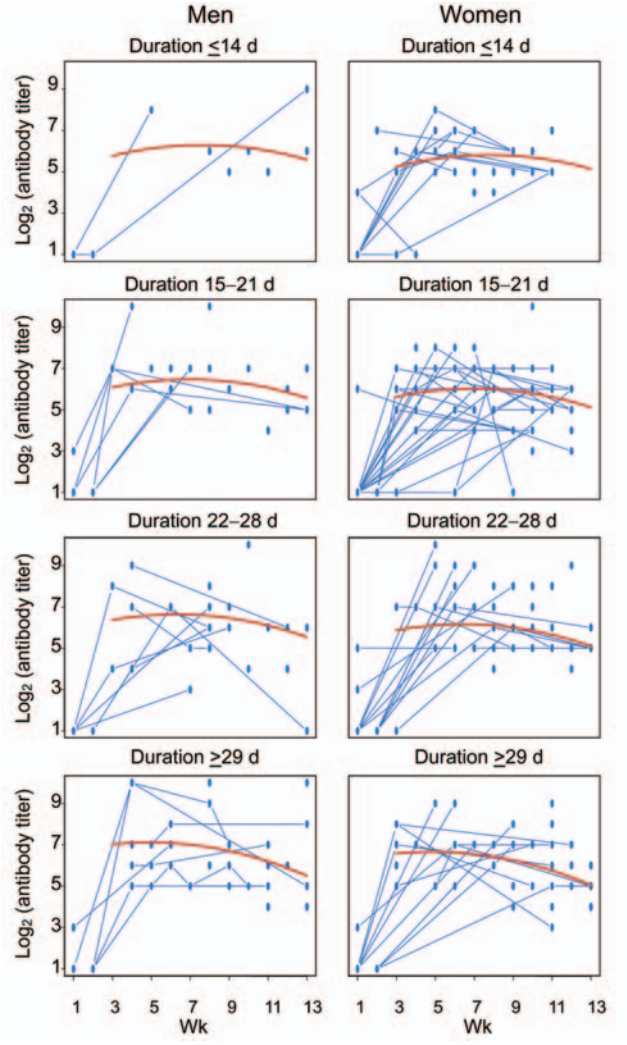

Figure 3. Scatterplot of antibody titers of the 247 seropositive study participants (titers of the same participant measured at different times are connected); superimposed is the fitted mean curve (in red) of $\log_2$ (antibody titer) between weeks 3 and 13 postinfection based on the linear mixed model by severity (duration of illness) and sex at the median age of 36 years. Each dot represents ≥1 titer; no distinction is made between single values and those with >1 value.

patient might be associated with a low inoculum of the virus because of incidental exposures, in contrast to patients who acquired SARS in hospitals under circumstances assumed to have a high virus density. When systematically screened during the SARS outbreak, some healthcare workers and public health personnel who had a history of direct contact with patients were shown to harbor nasopharyngeal SARS-CoV. Subsequently, however, they did not show seroconversion (Y.-T. Lu et al., unpub. data), which raises the possibility of asymptomatic mucosal epithelial colonization by SARS-CoV. Our seronegative patients with mild symptoms might fit into the spectrum between the seronegative asymptomatic colonizers and the severe SARS patients with high neutralizing antibody response. All considerations appear to favor the possibility that seronegative patients indeed had acquired SARS. Since the natural reservoir of SARS-CoV has not been clearly identified, and reintroduction of SARS-CoV to humans is possible, the short duration of having detectable antibody should be considered when a vaccine against SARS-CoV is developed.

Severity and Pathogenesis

The clinical course of SARS patients with severe infection is described as follows: pulmonary functions worsen during week 2 of illness (5,25–27), while the virus load in the airway decreases (5), and patients with mild disease would begin to stabilize clinically. Those in whom ARDS later develops usually show pulmonary decompensation during week 2. Severity was intensified by a slower and prolonged recovery with complications of pulmonary fibrosis occurring in week 3 in some patients (16). Results of a high-resolution computed tomographic scan in follow-up of SARS patients corroborates this observation by showing a high correlation between bilateral fibrotic lung changes and clinical severity (28). Findings of these studies, in conjunction with clinical study on cytokines during the acute phase (29–31), suggest that activation of Th1 cell–mediated immunity and a hyperinnate inflammatory response, rather than direct damages from uncontrolled virus growth, are responsible for the pathogenic process in severe infection (5). In previous vaccine studies, immunization conditions that could induce a stronger activation of Th1 response would concurrently result in a higher antibody response (24,32). Thus, the high antibody response and a strong cell-mediated Th1 response may reasonably be understood as concurrent events, and the latter may be causally related to a severe clinical course of SARS. Neutralizing antibody is unlikely to be causally related to the pathogenesis of SARS because treating SARS patients with convalescent-phase serum collected from patients who had recovered from SARS showed no adverse effect and probably had beneficial effects (33). Thus, for all the reasons stated above, our finding that high neutralizing antibody correlating with clinical severity should not be interpreted to mean that neutralizing antibody is harmful.

Death Rate and Early Responders

Having a detectable neutralizing antibody during the first 2 weeks of illness, in our analysis, coincides with a high and an early SARS mortality rate. The basis for early antibody response is not apparent, but 1 possibility is the priming effect of a previous non–SARS-CoV infection. Indeed, antibody against SARS-CoV has been shown to cross-react with human coronavirus 229E (2). The finding that early responders are older than other SARS patients is

Table 6. Comparison of early seroresponders and other SARS patients, by death rate, time of death, and age*

Factor	Early seroresponders†		OR	p value‡
	Yes, n (%)	No, n (%)		
Died				
Yes	29.6 (8)	7.8 (10)	4.97	0.004
No	70.4 (19)	92.2 (118)	1.00	
Died within 2 wk				
Yes	75.0 (6)	10.0 (1)		0.013
No	25.0 (2)	90.0 (9)		
Age (y)				
>60 (n = 14)	42.9 (6)	57.1 (8)		0.01
≤60 (n = 141)	14.9 (21)	85.1 (120)		

*SARS, severe acute respiratory syndrome; OR, odds ratio.
†Early seroresponders are SARS patients who were seropositive for SARS neutralizing antibody during the first 2 weeks of illness.
‡p value based on Fisher exact test.

in agreement with the priming effect since cumulative infection rate increases with increasing age. The priming effect of a previous viral infection can induce cross-reactive but nonneutralizing antibody, as well as neutralizing antibody to SARS. Furthermore, the nonneutralizing antibodies are known to facilitate viral infection, termed antibody-dependent enhancement (ADE), which is the pathogenic basis of feline infectious peritonitis virus (FIPV, a type II coronavirus), dengue hemorrhagic fever, and other viruses (34–36,37). In the case of FIPV, ADE can occur even with neutralizing antibody (38). However, this type of ADE resulted directly from neutralizing antibody is unlikely to occur with SARS-CoV infection because a number of SARS patients have been treated with convalescent-phase serum of SARS patients and show no adverse effect (33).

The hypothesis that the early responders may have experienced a priming effect could be verified by demonstrating that a significantly higher proportion of early responders than other SARS patients possess antibody against non-SARS coronavirus during the acute phase. Early death occurring within the first 2 weeks of illness is also associated with high nasopharyngeal virus load among a subset of SARS patients with information on nasopharyngeal virus load (J.-Y. Yang et al., unpub. data). Unfortunately, the number of early responders for whom information on virus load was available was too few to yield a meaningful statistical analysis on whether high virus load is correlated with an early humoral response. While antibody induced by a variety of SARS-CoV antigen preparations protects against SARS-CoV infection in mice and ferrets (39,40), these animals do not develop clinical symptoms resembling that of SARS-CoV infection in humans and thus are not models of pathogenesis. Since ADE can occur through a number of mechanisms and is not completely understood, clinical trials of vaccine against SARS-CoV should be conducted with caution.

In summary, SARS neutralizing antibody level is positively correlated with clinical severity, and in a portion of the patients with mild infection, a detectable neutralizing antibody response may not develop. All published clinical and immunologic data on SARS patients suggest that a strong cell-mediated Th1 response is causally related to a severe clinical outcome, whereas high neutralizing antibody is probably a concurrent event of a strong Th1 activation. Early neutralizing antibody responders are more likely to be older, to have a higher case-fatality rate, and to survive for a shorter time. These observations, if corroborated with further analysis of data collected in other countries, should raise the concerns of possible ADE in the pathogenesis of SARS-CoV infection in humans and should be considered in the process of vaccine development.

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Respiratory Infections during SARS Outbreak, Hong Kong, 2003

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The effect of community hygienic measures during the outbreak of severe acute respiratory syndrome in Hong Kong was studied by comparing the proportion of positive specimens of various respiratory viruses in 2003 with those from 1998 to 2002. Community hygienic measures significantly reduced the incidence of various respiratory viral infections.

Severe acute respiratory syndrome (SARS) is an infection caused by a novel coronavirus that is transmitted primarily through direct mucous membrane contact with infectious respiratory droplets and through exposure to fomites. In 2003, Hong Kong reported SARS cases from March 11 to June 2. During the height of the outbreak, schools were suspended, social activities were curtailed with the closure of various public places, and the community was engaged in a sustained and intense hygiene campaign (1–3). Population education on personal hygienic measures was spearheaded by the government with concerted efforts from various organizations and the community. Surveys conducted in April and May 2003 showed that most of the population wore a face mask (76%), washed their hands after contact with potentially contaminated objects (65%), used soap when washing hands (75%), covered their mouths when sneezing or coughing (78%), and used diluted bleach for household cleaning (>50%) (4,5). Another survey on health-seeking behavioral traits conducted in June 2003 showed that >70% of respondents practiced some of these hygienic measures more frequently during the SARS outbreak than during the pre-SARS period (6).

The Study

We postulated that these populationwide anti-SARS measures would have effects on other infections spread by the respiratory route. In this study, we examined whether such measures also affected the incidence of some common acute viral respiratory infections.

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The study period was January 1, 1998, to December 31, 2003. Data were obtained from the Government Virus Unit (GVU), a public health and diagnostic virology laboratory serving public and private hospitals and outpatient clinics in Hong Kong. At GVU, all respiratory specimens are routinely cultured using 4 continuous cell lines, Rhesus monkey kidney, Madin-Darby canine kidney, rhabdomyosarcoma, and human laryngeal epithelium, which could support the growth of various viruses including influenza, parainfluenza, respiratory syncytial virus (RSV), and adenovirus. On detection of specific cytopathic effects, the viruses are identified with standard protocols (7).

For each month of the study period, we obtained the number of respiratory virus isolates as a proportion of the total number of respiratory specimens processed by GVU. We computed the percentage change in the proportion of positive specimens (PPS) for each virus between each month of 2003 and the mean PPS in the same month of the preceding 5 years (1998–2002), which served as the reference period. For comparison purposes, we obtained the monthly number of positive tests for immunoglobulin M (IgM) antibody against hepatitis B core antigen (anti-HBc) and the corresponding total number of tests performed in the study period. The percentage change in PPS was calculated as above. Although a positive IgM anti-HBc test result indicates acute hepatitis B infection or an exacerbation of chronic hepatitis B infection, as we were testing the same catchment of population throughout 1998 to 2003, the proportion of exacerbations of chronic hepatitis B infection is assumed to have remained unchanged during the study period.

The Table shows the change in PPS in 2003 for the various viruses in comparison with the reference period. In 2003, the monthly number of respiratory specimens ranged from 665 to 5,432 (mean 1,399), in comparison with a range throughout the years 1998–2002 of 757 to 3,162 (mean 1,334.5). A surge in the number of specimens was noted during March and April 2003 (5,432 and 3,758, respectively). During March to July 2003, marked reductions in PPS occurred compared with the reference period for influenza virus, parainfluenza virus, RSV, and adenovirus, particularly in the months of April, May, and June. This reduction corresponded to the period when anti-SARS measures in the community were most rigorous. In contrast, similar changes in PPS were not observed for hepatitis B, which is caused by a bloodborne virus with a different mode of transmission than that of the 4 respiratory viruses (Figure 1). Since August 2003, instead of reductions in PPS, a rebound in isolation rates was observed for the 4 viruses.

The 2003 SARS outbreak overlapped with the traditional seasonal peak from March to September for RSV in Hong Kong (8). In 2003, the RSV peak season shifted to

Table. Change in proportion of positive specimens (PPS) in 2003 for various viruses with reference to the period 1998–2002*

Virus	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Influenza												
No. isolates	327	419	786	105	22	58	96	54	121	50	10	35
No. tests performed	1,166	1,530	5,432	3,758	1,495	974	776	665	1,283	1,193	1,008	1,407
PPS (03)	0.28	0.27	0.14	0.03	0.01	0.06	0.12	0.08	0.09	0.04	0.01	0.02
Average PPS (98–02)	0.235	0.368	0.329	0.154	0.127	0.143	0.186	0.153	0.063	0.022	0.019	0.044
Range of PPS (98–02)	0.067–0.321	0.143–0.519	0.121–0.517	0.065–0.203	0.043–0.194	0.072–0.216	0.145–0.232	0.123–0.173	0.040–0.105	0.009–0.044	0.008–0.030	0.022–0.110
% change†	+19	–26	–56	–82	–88	–58	–33	–47	+49	+87	–49	–44
Parainfluenza												
No. isolates	29	35	75	12	2	1	11	26	29	68	75	112
No. tests performed	1,166	1,530	5,432	3,758	1,495	974	776	665	1,283	1,193	1,008	1,407
PPS (03)	0.02	0.02	0.01	0.00	0.00	0.00	0.01	0.04	0.02	0.06	0.07	0.08
Average PPS (98–02)	0.035	0.025	0.022	0.036	0.037	0.034	0.023	0.017	0.027	0.061	0.081	0.065
Range of PPS (98–02)	0.014–0.106	0.014–0.053	0.008–0.046	0.021–0.046	0.024–0.054	0.026–0.046	0.017–0.034	0.010–0.028	0.014–0.043	0.025–0.102	0.031–0.149	0.035–0.156
% change†	–28	–8	–38	–91	–96	–97	–38	+135	–16	–7	–9	+22
RSV												
No. isolates	49	53	66	24	6	3	8	53	181	48	12	5
No. tests performed	1,166	1,530	5,432	3,758	1,495	974	776	665	1,283	1,193	1,008	1,407
PPS (03)	0.04	0.03	0.01	0.01	0.00	0.00	0.01	0.08	0.14	0.04	0.01	0.00
Average PPS (98–02)	0.014	0.021	0.055	0.104	0.081	0.066	0.072	0.094	0.092	0.036	0.022	0.032
Range of PPS (98–02)	0.003–0.054	0.010–0.065	0.037–0.090	0.055–0.155	0.049–0.112	0.042–0.098	0.038–0.113	0.046–0.126	0.057–0.118	0.018–0.051	0.001–0.060	0.001–0.072
% change†	+203	+64	–78	–94	–95	–95	–86	–15	+54	+13	–47	–89
Adenovirus												
No. isolates	61	78	84	41	5	0	1	2	4	7	8	17
No. tests performed	1,166	1,530	5,432	3758	1,495	974	776	665	1,283	1,193	1,008	1,407
PPS (03)	0.05	0.05	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01
Average PPS (98–02)	0.034	0.030	0.035	0.056	0.055	0.058	0.068	0.044	0.038	0.049	0.052	0.074
Range of PPS (98–02)	0.012–0.058	0.019–0.052	0.017–0.071	0.012–0.121	0.022–0.110	0.028–0.105	0.032–0.091	0.029–0.058	0.026–0.054	0.045–0.055	0.042–0.062	0.058–0.093
% change†	+52	+72	–55	–81	–94	–100	–98	–93	–92	–88	–85	–84
Hepatitis B												
No. of positive test results	16	11	13	18	20	13	19	20	22	21	15	20
No. of tests performed	143	98	135	99	97	118	139	135	120	111	102	131
PPS (03)	0.112	0.112	0.096	0.182	0.206	0.110	0.137	0.148	0.183	0.189	0.147	0.153
Average PPS (98–02)	0.147	0.132	0.127	0.129	0.125	0.156	0.154	0.156	0.168	0.144	0.167	0.148
Range of PPS (98–02)	0.083–0.228	0.079–0.190	0.093–0.153	0.070–0.168	0.085–0.162	0.096–0.188	0.104–0.186	0.068–0.228	0.086–0.250	0.081–0.233	0.099–0.249	0.082–0.197
% change†	–24	–15	–24	+41	+65	–29	–11	–5	+9	+32	–12	+3

*RSV, respiratory syncytial virus.
†Percentage change of PPS in 2003 with respect to the average PPS from 1998 to 2002.

August–October. The accumulation of susceptible infants offset the infection control measures instituted against respiratory infections as well as the normal seasonality; as a result, RSV activity increased in the late months of 2003. Figure 2 illustrates the usual seasonal variation of the 4 respiratory viruses and their pattern from 1998 to 2003.

Discerning whether the observed effects in our study were real or apparent is important. The surge in specimens during March and April 2003 suggested that physicians were more inclined to order tests for patients with respiratory symptoms at the height of the SARS outbreak. This fact could conceivably dilute PPS for respiratory viruses. However, since May 2003, the number of tests has returned to normal levels; however, PPS remained significantly decreased during May to July 2003. Thus, PPS reductions cannot be explained by a dilution effect caused by an increased number of specimens processed. Furthermore, after we controlled for the patients' age group differences, PPS for influenza virus remained depressed when compared to PPS in the reference period (data not shown). The same pattern was true for adenovirus.

Population coverage for influenza vaccination in Hong Kong has been <15% throughout the study period (A. Chan, pers. comm.), so vaccination was unlikely to have resulted in reduced influenza circulation in the community. The concomitant significant reduction in PPS for all 4 respiratory viruses in the same period argues against 2003's being a milder year for influenza. Temporally, the moderation of PPS reductions since August 2003 (the last SARS case was reported on June 2) supported the hypothesis that the effects of populationwide anti-SARS measures on the incidence of respiratory viruses were real.

With the recent outbreaks of highly pathogenic avian influenza among poultry in Asian countries, and the associated human infections, pandemic planning for influenza

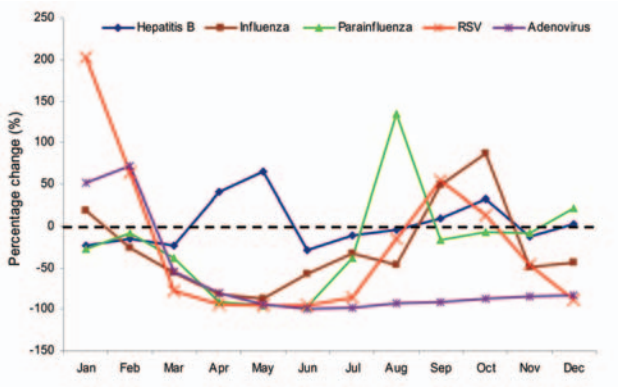

Figure I. Change in proportion of positive specimens in 2003 for various viruses with reference to period 1998–2002. RSV, respiratory syncytial virus.

has been undertaken with renewed efforts on a worldwide basis (9). In pandemic preparedness planning, control measures have traditionally focused on the use of antiviral chemotherapy and the expedient development of an effective vaccine. However, such strategies may not be feasible, especially in countries with limited resources. An effective vaccine would probably become available only during the latter phase of the pandemic. Information concerning the effects of increased social distance and communitywide hygiene measures on the incidence of common viral respiratory infections at a population level has been lacking. The SARS outbreak offers a unique opportunity to study the association. Although our study was observational and thus could not establish a causal relationship, it suggests a possible association between population-based hygienic measures and the reduced incidence of influenza and other acute viral respiratory infections. However, the relative contribution of each of these measures could not be estimated in our study. The effective implementation of such measures requires determined and sustained educational efforts from health authorities with collaboration of the public. We thus propose that stockpiling personal protective equipment and having public education campaigns on infection control practices should form an integral component in pandemic planning for the emergence of novel influenza virus strains in humans.

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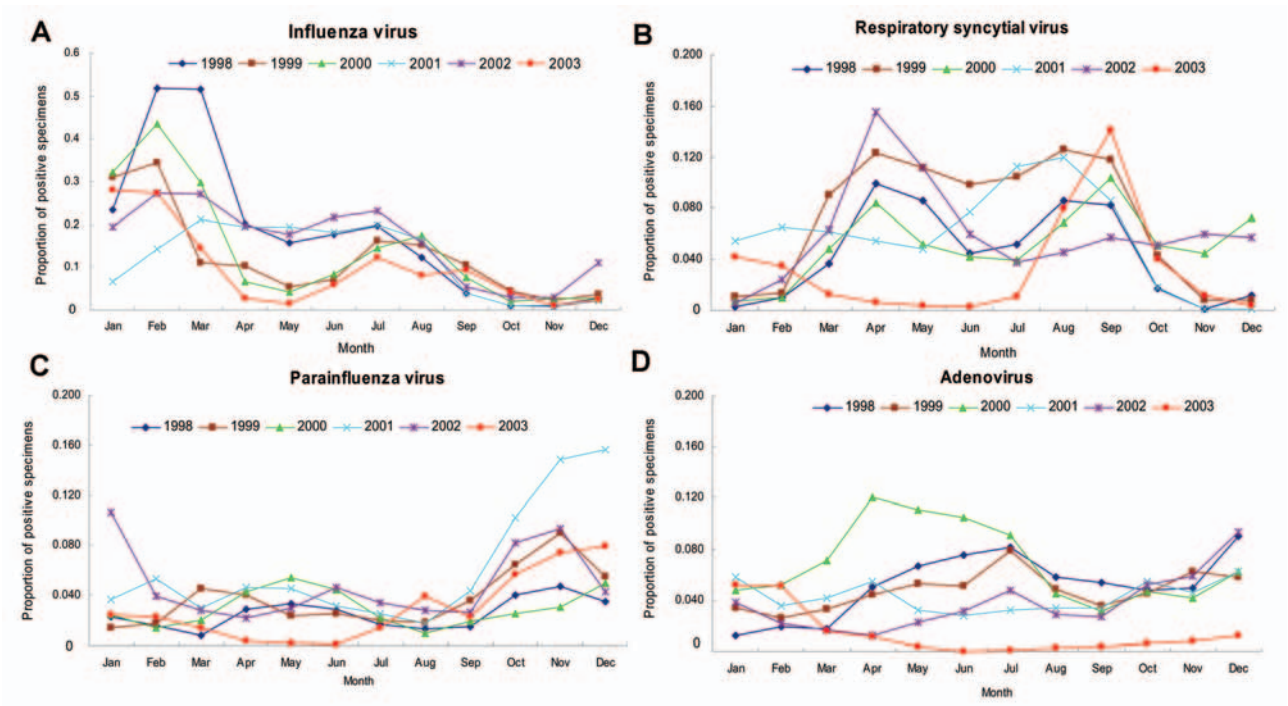

Figure 2. Proportion of positive specimens by month, 1998–2003, for A) influenza; B) respiratory syncytial virus; C) parainfluenza virus, and D) adenovirus.

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Bartonella quintana and *Rickettsia felis* in Gabon

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We detected *Rickettsia felis* DNA in *Ctenocephalides felis* and *Bartonella quintana* DNA in 3 *Pulex irritans* fleas taken from a pet *Cercopithecus cephus* monkey in Gabon, sub-Saharan Africa. This is the first report of *B. quintana* in the human flea.

Bartonellae are gram-negative bacteria that cause several human diseases and are transmitted by various arthropods, such as lice, ticks, and fleas (1). *Bartonella quintana* is a worldwide fastidious bacterium that infects humans and belongs to the alpha subgroup of the *Proteobacteria*. Recent reports suggest that humans are the natural reservoir of *B. quintana* and that the human body louse is the vector (1). However, we have recently reported molecular detection of several *Bartonella* species, including *B. quintana,* in *Ctenocephalides felis* fleas from France, which suggests that fleas may be important vectors of human disease (2). Fleas are found worldwide on mammals and are vectors of several major zoonoses, including plague caused by *Yersinia pestis* (3), murine typhus caused by *Rickettsia typhi*, and fleaborne spotted fever caused by *R. felis* (3). More than 2,000 species of fleas exist worldwide. While some species are highly host specific, others are more catholic and will feed on numerous hosts, especially in the absence of their preferred host (3). Several flea species, including *Pulex irritans, C. canis, C. felis, Ceratophyllus gallinae, Ceratophyllus columbae*, and *Archaeopsylla erinacei*, may infest humans. In this study, we collected *P. irritans* (human fleas) and *C. felis* fleas on a pet monkey in Gabon and report for the first time the molecular detection of *B. quintana* in *P. irritans*.

The Study

Four fleas collected from a pet monkey (*Cercopithecus cephus*) in Franceville, Gabon, were stored in 70% alcohol and sent to the World Health Organization (WHO) Collaborative Center for Rickettsial Reference and Research in Marseille, France, where molecular studies were performed in April 2005. Fleas were rinsed with distilled water for 10 min and dried on sterile filter paper in a laminar flow hood. Preliminary entomologic identification was performed by using reference taxonomic keys as previously reported (4).

Fleas were crushed individually in sterile Eppendorf tubes with the tip of a sterile pipette. DNA was then extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Rickettsial DNA was detected by polymerase chain reaction (PCR) with primers targeting the citrate synthase gene (*gltA*) as previously described (4). *R. montanensis* DNA was used as positive control, and negative controls consisted of laboratory uninfected flea DNA. *Bartonella* DNA was detected by PCR with 3 sets of primers targeting the intergenic spacer (ITS) gene, and the *B. quintana* spacers 336 and 894 as previously described (4,5). *B. elizabethae* DNA was used as positive control and uninfected fleas as negative controls. Additionally, fleas were identified at the species level after amplification and sequencing of a portion of the 18S rDNA gene as previously described (4). PCR products were purified, and DNA sequencing was carried out by using the d-Rhodamine Terminator cycle sequencing ready reaction kit with Amplitaq Polymerase FS (Perkin-Elmer, Coignieres, France) as described by the manufacturer. For all PCR products, sequences from both DNA strands were determined twice. Sequencing products were resolved by using an ABI 3100 automated sequencer (Perkin-Elmer). Sequence analysis was performed by using the software package ABI Prism DNA Sequencing Analysis Software version 3.0 (Perkin-Elmer). All obtained sequences were compared with those available in GenBank by using the nucleotide-nucleotide BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLAST/).

Using morphologic taxonomic keys, 3 fleas were identified as *P. irritans* and 1 flea as *Ctenocephalides felis*. These findings were unambiguously confirmed when a 331-bp fragment of the 18S rDNA gene showed 99.7% and 99.4% homology with previous sequences of *C. felis* (GenBank accession no. AF423914) and *P. irritans* (GenBank accession no. AF423915). When *gltA* primers were used, *R. felis* (GenBank accession no. AF516333, 100% homology) was detected in the *C. felis*, whereas the *P. irritans* as well as negative controls were negative. Using the ITS primers for *Bartonella* spp., we detected PCR products in the 3 *P. irritans* fleas, whereas the *C. felis* flea and negative controls were negative. By sequencing the ITS gene–amplified fragments from these 3 fleas, we identified *B. quintana* (GenBank accession no. AF368396, 100% homology). Two PCR procedures targeting specific *B. quintana* spacers previously described (5) were carried out to confirm the results. By using these primers, *B. quintana* type 1 sequence was obtained for the spacer 336 (GenBank accession no. AY660705, 100% homology) and

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B. quintana type 2 sequence for the spacer 894 (GenBank accession no. AY660713, 100% homology). Thus, according to current guidelines for *B. quintana* typing (5), we have amplified genotype 2 of *B. quintana*.

Conclusions

We present here the first molecular detection of *R. felis* in sub-Saharan Africa, Gabon (Figure). To date, 4 species of fleas have been associated worldwide with *R. felis* including *C. felis* (3,6), *C. canis* (6), *P. irritans* (3), and *Archeopsylla erinacei* (4). Thus, the amplification of *R. felis* in the *C. felis* flea from the monkey was not surprising but suggests that nonhuman primates may be infected as well as humans and may represent a reservoir of *R. felis*. The role of mammals, including rodents, hedgehogs, cats, dogs, and monkeys, in the life cycle and circulation of *R. felis* remains unclear and warrants further epidemiologic studies.

We report for the first time that the human flea *P. irritans* can be infected with *B. quintana*. Apart from the body louse, the natural vector of *B. quintana* in humans, we have previously detected *B. quintana* in *C. felis* fleas with a prevalence of 4.5% in a series of 309 fleas collected in various regions of France (2). Thus, our results confirm that *B. quintana* may be found in the human flea and may explain 2 clinical reports of chronic adenopathy attributed to *B. quintana* infection for which the only epidemiologic risk factor identified was the presence of fleas (7,8). Few reports of detection of other bartonellae in fleas have been made (Table). Recently, the rodent flea *Ctenophthalmus nobilis* has been found to be a competent vector of at least 2 *Bartonella* species, *B. grahamii*, which has previously been associated with human infection, and *B. taylorii* (9). In contrast, no evidence of either horizontal or vertical transmission was seen in bank voles (*Clethrionomys glareolus*) injected with *B. taylorii* maintained in an arthropod-free environment, which suggests that fleas may be essential for transmitting some *Bartonella* spp. (9). In the study of Stevenson et al, *Bartonella* spp. were detected in 38 *Oropsylla hirsuta* and 3 *Oropsylla tuberculatus cynomuris* prairie dog fleas in United States (10). In addition, new *Bartonella* genotypes, whose medical importance is not yet known, were detected in *Pulex* fleas in Peru (11), in 5 *C. felis* collected from cats, and in a *Nosopsyllus fasciatus* collected from a *Rattus surifer* specimen in Thailand (6).

Figure. Detection of *Rickettsia felis* in fleas worldwide. *This study.

Although detection of *Bartonella* DNA is often reported from several sources, including fleas, mammals, and human samples, isolation of bartonellae by culture remains infrequent (12). Culture media and procedures used for *Bartonella* spp. have been highly variable and have questionable sensitivity (12). A novel chemically modified liquid medium that will support the growth of several *Bartonella* spp. has been recently developed and may provide an advantage over conventional blood agar culture for the isolation of *Bartonella* spp. (13). The prevalence of *B. quintana* as well as other bartonellae in human fleas remains unknown, and this subject needs to be addressed to better define possible sources of *Bartonella* infections in humans.

Table. *Bartonella* species detected in fleas worldwide

Species	Species of *Bartonella* detected	Country/animal (ref.)
Ctenocephalides felis	*B. clarridgeiae*	France/cat (2,14)
		Thailand/cat (6)
		New Zealand/cat (15)
	B. koehlerae	France/cat (2)
	B. quintana	France/cat (2)
Ctenophthalmus nobilis	*B. grahamii*	United Kingdom/bank vole (9)
	B. taylorii	United Kingdom/bank vole (9)
Nosopsyllus fasciatus	*Bartonella* spp.	Thailand/rodent (6)
Oropsylla hirsuta	*Bartonella* spp.	United States/prairie dog (10)
O. tuberculatus cynomuris	*Bartonella* spp.	United States/prairie dog (10)
Pulex spp.	*Bartonella* spp.	Peru/human (11)
Pulex irritans	*B. quintana*	Gabon/monkey (this study)

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African Trypanosomiasis Gambiense, Italy

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African trypanosomiasis caused by *Trypanosoma brucei gambiense* has not been reported in Italy. We report 2 cases diagnosed in the summer of 2004. Theses cases suggest an increased risk for expatriates working in trypanosomiasis-endemic countries. Travel medicine clinics should be increasingly aware of this potentially fatal disease.

Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by a flagellated trypanosome protozoan and transmitted by *Glossina* (tsetse) flies. It is classified into 3 subspecies: *Trypanosoma brucei gambiense*, *T. brucei rhodesiense*, and *T. brucei brucei* (the third subspecies is not pathogenic to humans). These subspecies cannot be distinguished morphologically. *T. b. gambiense*, which is found in western and central Africa, causes chronic disease, while *T. b. rhodesiense*, which is found in eastern and southern Africa, causes acute severe disease. The epidemiology of these subspecies also differs and follows distribution of their main vectors, *Glossina palpalis* and *G. morsitans*, respectively. *G. palpalis* prefers areas of vegetation near rivers and cultivated fields, and *G. morsitans* feeds on wild animals in savannah areas, far from human settlements.

Trypanosomiasis rhodesiense is a zoonosis, and humans visiting affected areas (usually for hunting or tourism) are accidental hosts. Humans are the only meaningful reservoir of *T. b. gambiense*. Untreated infections may persist for years. This disease is highly prevalent in Africa; ≈500,000 people are infected in 36 countries because of poor health systems in regions of civil and military turmoil (1). Despite its sporadic occurrence among travelers, *T. b. rhodesiense* has been reported more often in European (2) and American tourists (3) than *T. b. gambiense* because *T. b. rhodesiense* is present in areas not visited by expatriates. We report 2 cases of imported trypanosomiasis gambiense in Italy during the summer of 2004.

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Patient 1

On July 2004, a previously healthy 44-year-old man who lived in Gabon was admitted to the outpatient clinic of the university hospital in Udine, Italy, with a 6-month history of recurrent fever, headache, fatigue, weight loss, leg paresthesias, gait difficulties, and daytime somnolence. He had been living in Libreville, Gabon, since 1961, made yearly visits to Italy, and had never visited other African countries. He reported frequent tsetse fly bites while sailing on the Como River or walking in the forests in Gabon. He recalled several febrile episodes that had been presumptively treated as malaria; the last occurred in February 2004. The fever pattern then changed and became recurrent. The patient also had headaches and cutaneous hyperesthesia in the lower extremities. He subsequently had bilateral peripheral edema of the leg and progressive weakness, reversal of his sleep pattern with daytime somnolence and insomnia at night, loss of appetite, and a marked weight loss (20 kg). One month before admission to the hospital, he was examined in an emergency room and by a general practitioner, but a diagnosis was not made. Laboratory findings at that time were an erythrocyte sedimentation rate (ESR) of 122 mm/h and hypergammaglobulinemia (4.5 g/dL).

Upon examination, he was oriented but irritable and apyretic. He had a blood pressure of 110/70 mm Hg and a pulse rate of 104/min. Enlarged lymph nodes were found in the axillae, groin, supraclavicular region, and posterior neck triangle. The liver and spleen were enlarged (spleen diameter 20 cm by ultrasound). Neurologic examination showed walking ataxia, decreased sensitivity to light touch in both legs, and no deep tendon reflexes. Laboratory tests showed pancytopenia, an increased ESR, and hypergammaglobulinemia with increased levels of immunoglobulin M (IgM) (Table). Giemsa-stained blood films showed trypomastigotes. Lumbar puncture showed clear cerebrospinal fluid (CSF) with increased leukocyte counts, protein and IgM levels, and a low glucose level (Table). Trypanosomes were also found in the CSF (Figure 1). An indirect hemagglutination (IHA) test result was positive for *T. brucei* (titer 1:64). Second-stage sleeping sickness (stage 2 HAT) was diagnosed, but treatment with eflornithine (obtained from the World Health Organization [WHO]) could not be initiated until 9 days after the diagnosis because of getting medication through customs. In this 9-day period, daily peripheral blood smears were negative, except on day 5. The patient was then given a standard dose of eflornithine (100 mg/kg intravenously 4× /day for 14 days), and his condition improved rapidly, lymphadenopathy resolved, and neurologic status normalized within 2 weeks. Lumbar puncture on day 14 of treatment did not show any trypanosomes, and all CSF parameters improved. Repeat peripheral blood smears were also

Table. Laboratory test results for the 2 patients at admission*

Test (normal range)	Patient 1	Patient 2
Blood		
Leukocytes/μL (4.2–9.5 × 10^3)	2.7	3.2
Hemoglobin, g/dL (13–17)	8.3	9.5
Platelets/μL (130–400 × 10^3)	100	127
ESR, mm/h (<15)	131	46
CRP, mg/L (<5)	44.5	128
γ-globulins, g/dL (0.7–1.7)	3.8	1.5
Total protein, g/dL (6–8.5)	8.7	6.7
Total IgM, mg/dL (40–230)	1,161	681
Cerebrospinal fluid		
Protein, mg/dL (200–500)	839	330
Glucose, mg/dL (40–70)	49	68
Leukocytes/μL (0–2)	40 (90% lymphocytes)	0
IgM, mg/dL (0.5–1.5)	220	0.24
Trypanosomes	Present	Absent

*ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IgM, immunoglobulin M.

negative, and he was discharged. Two weeks later he was still healthy. He was advised to remain in Italy for further follow-up, but he went back to Gabon and has not provided any subsequent medical information.

Patient 2

A 54-year-old woman was admitted to the Centre for Tropical Diseases of Sacro Cuore Hospital of Negrar in Verona, Italy, in late September 2004 with a 3-month history of recurrent fever, headache, insomnia, and increased fatigue. She had lived for 30 years in the Central African Republic and had not visited any other African countries during that time. At admission, she was afebrile, and physical examination showed diffuse cutaneous hyperesthesia and splenomegaly (main spleen diameter 19.5 cm by ultrasound). Blood cell counts and biochemical tests showed anemia (hemoglobin level 8.3 g/dL) and leukopenia (leukocyte count 2,700/μL). A quantitative buffy coat test result for malaria was negative, and she was discharged.

Three days later she returned with a fever. A quantitative buffy coat test result was negative for malaria, but this test showed viable trypomastigotes. They were also found in peripheral blood smears (Figure 2). Serologic results for *T. brucei* (IHA test) were positive (titer 1:128). Other relevant laboratory findings are shown in the Table. Results of CSF examination were normal. Since we could not treat this patient with eflornithine (WHO provides this drug only for stage 2 HAT), intramuscular pentamidine was administered at the dose of 4 mg/kg for 10 days. Tests to detect trypanosomes in blood were conducted daily for 8 days after treatment was initiated, but no trypomastigotes were found. Her clinical course was uneventful, except for a sterile abscess at the injection site. All laboratory findings improved markedly. She was afebrile and was discharged on day 25 in good condition, although she still had insomnia and headaches.

In February 2005, she returned for a follow-up examination. The headaches and insomnia continued (she did not sleep >2 hours per night). Laboratory findings, including serum IgM levels, were within normal ranges. Total protein levels in CSF increased to 570 mg/dL, but cells in CSF were within normal ranges. Based on these findings, treatment with intravenous eflornithine (100 mg/kg 4×/day for 14 days) was initiated. The patient had generalized tremors (without fever) during the third infusion (no electroencephalographic signs of convulsions), but subsequent findings were uneventful. She was discharged after completion of treatment. At a follow-up visit in April 2005, she reported a nearly normal sleeping pattern.

Conclusions

Eighty-four imported cases of trypanosomiasis caused by *T. b. gambiense* were reported in Europe before 1963. From 1966 to 1979, 12 cases were reported in France, which reported the most cases in Europe (4). During this period, incidence in trypanosomiasis-endemic countries decreased after intensive control activities. Eight imported cases of infection with *T. b. gambiense* in persons from Europe have been reported since 1985 (4–11), and 2 additional cases were recorded in France by the Centre National de Référence de l'Epidémiologie du Paludisme d'Importation et Autochtone (F. Legros, pers. comm.). To our knowledge, *T. b. gambiense* infection has not been reported in Italy (C. Mauro, Ministry of Health, pers. comm.).

Both patients denied visiting African countries where *T. b. rhodesiense* was present. A sporadic case of infection with *T. b. gambiense* in an Italian expatriate in Zaire (now the Democratic Republic of Congo) was reported in Belgium in 1996 (12). The simultaneous occurrence of

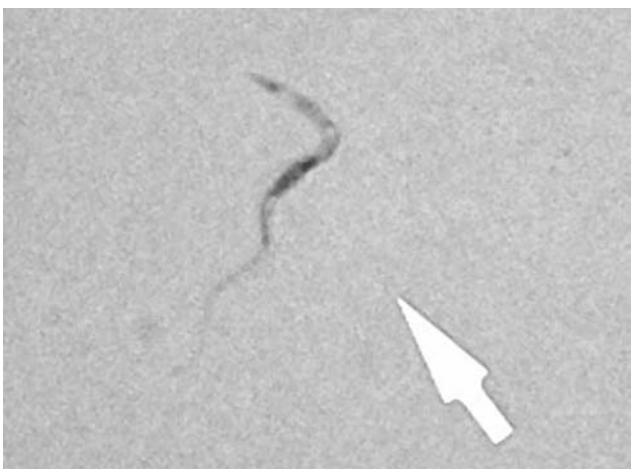

Figure 1. Trypomastigote (arrow) in a Giemsa-stained cerebrospinal fluid smear of patient 1 (original magnification ×1,000).

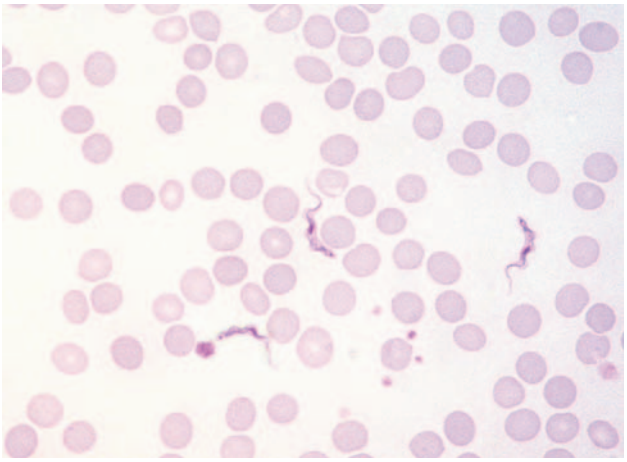

Figure 2.Trypomastigotes in a Giemsa-stained thin blood film of patient 2 (original magnification ×1,000).

2 cases in Italy suggests an increased risk for infection with *T. b. gambiense* in expatriates working in disease-endemic areas. This increased risk is not surprising if one considers the increased incidence of this infection in African countries (1).

In countries not endemic for this infection, diagnosis of imported cases of infection with *T. b. gambiense* is challenging because of variations in clinical signs and symptoms and low sensitivities of diagnostic tests (13). The first patient in our study was misdiagnosed and not treated for several weeks because he had no fever and clinical manifestations were limited to neurologic symptoms. The second patient was also initially misdiagnosed. High levels of IgM in blood and an enlarged spleen should suggest the possibility of trypanosomiasis (the main differential diagnosis is hyperreactive malarial splenomegaly). Techniques for concentrating parasites should be used, and blood films should be examined daily in patients with these symptoms.

Involvement of the central nervous system in trypanosomiasis has been confirmed by increased lymphocyte counts (>5 cells/μL) or trypanosomes in CSF (14). However, as in the second patient, neurologic involvement cannot be ruled out even in those in whom CSF is normal. Better indicators of infection in blood and CSF are needed. Leukocyte counts >20 cells/μL in CSF and intrathecal IgM synthesis independent of trypanosomes in CSF have been proposed as modified criteria for diagnosis of stage 2 HAT (14,15).

Eflornithine, the preferred treatment for stage 2 HAT, was obtained from WHO to treat 1 of the patients. However, custom formalities in Italy, which resulted in a delay in receiving this drug, are inappropriate for emergency drug treatment. Thus, our experience with the 2 patients and recent outbreaks of infection with *T. b. rhodesiense* emphasize the need for readily available trypanocidal drugs.

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Australian Public and Smallpox

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A national survey of 1,001 Australians found that most were concerned about a bioterrorist attack and were ill-informed about smallpox prevention and response. Since general practitioners were commonly identified as the initial point of care, they should become a focus of bioterrorism response planning in Australia.

Australia has identified protection against bioterrorism as a national research priority, and the Commonwealth Chief Medical Officer emphasizes the "need to be prepared for a bioterrorism incident" (1). Preparations have focused on central public health surveillance, with little attention to public understanding of bioterrorism (2).

Smallpox and anthrax are considered potential bioterrorism agents (1,3,4). Although smallpox response guidelines have been prepared for Australia (http://www.health.gov.au/internet/wcms/Publishing.nsf/Content/health-pubhlth-publicat-document-metadata-smallpox.htm), the level of community awareness of these recommendations is unknown. In the event of an attack, the response of the public will be based on persons' current knowledge, beliefs, and patterned behavior (5,6). We conducted a cross-sectional national survey in Australia to assess knowledge and views about smallpox, vaccination, and other mitigation strategies.

The Study

A list of private telephone numbers was randomly selected for each of Australia's 8 states and territories that was proportional to their contribution to the adult population. Participants were recruited to provide a sample size of 1,000, which allowed a precision of 2%–3% when calculating a 95% confidence interval of a dichotomous variable with a base proportion ranging from 10% to 50%.

Eight experienced telephone interviewers conducted the survey during July 2004. Repeat calls were conducted when persons indicated interest in participating but were unable to do so during the initial contact. The questionnaire was administered upon agreement to participate, after introducing the survey's purpose, providing a guarantee of confidentiality, and giving reassurance of freedom to withdraw consent.

The questionnaire was pretested for length and comprehensibility in a pilot study during May 2004. The final instrument took 10–15 minutes to administer and contained 22 questions. Data were analyzed by using SPSS for Windows version 11 (SPSS Inc., Chicago, IL, USA). Ethical approval was granted by the human ethics subcommittee at James Cook University (Nr. H1745).

A total of 1,001 Australian adults completed the survey. Two hundred thirteen were excluded (38 children, 91 adults with limited English ability, 9 incoherent adults, and 75 adults contacted at their workplace), and 582 refused to participate (response rate 63.2%). Respondents were geographically representative of the Australian population.

Respondents' ages were normally distributed (mean 52.2 years, standard deviation 17 years) and 62.8% (629) were female. Most (58.6%, 587) lived in cities, which reflected the situation in Australia, where 66.3% of the population live in urban areas. The level of education of respondents reflected that of the Australian population.

Concern about the risk of a bioterrorist attack in Australia was perceived as high by 182 (18.2%), medium by 392 (39.2%), low by 339 (33.9%), and nonexistent by 14 (1.4%); 72 (7.2%) did not know and 2 (0.2%) did not answer. Logistic regression modeling showed that age was the only demographic feature significantly associated with perceiving high risk of a bioterrorist attack (compared with low, medium, or none), with an odds ratio of 1.016 per year ($p<0.001$).

Most respondents (60.6%, 606) believed that human smallpox cases had occurred in the past 5 years and that effective medical treatment existed for smallpox (Table 1). The likelihood of contracting smallpox by working in close contact with someone with the disease (e.g., in the same office) was considered low by 157 (15.7%), medium by 163 (16.3%), and high by 419 (41.9%); 261 (26.1%) did not know and 1 (0.1%) did not answer.

A total of 583 (58.2%) respondents stated that they had been vaccinated against smallpox; 346 (34.6%) indicated no prior vaccination against smallpox, 71 (7.1%) did not know, and 1 (0.1%) did not answer. Among 61 respondents born since 1979, the year that smallpox was eradicated and worldwide childhood vaccination terminated, 32 (52.5%) indicated that they had not been vaccinated against smallpox, 20 (32.8%) reported that they had been vaccinated, and 9 (14.8%) did not know. Of 841 respondents born before 1980, 502 (59.8%) reported that they had been vaccinated against smallpox.

The acceptance of vaccination against smallpox under specific hypothetical scenarios was explored. Vaccination would be accepted as an immediate precautionary measure

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Table 1. Responses to questions on general knowledge of smallpox, Australia, 2004

In the past 5 years, do you think …	Yes, n (%)	No, n (%)	Do not know, n (%)	No answer, n (%)
Human cases of smallpox have occurred in Australia?	127 (12.7)	538 (53.7)	336 (33.6)	0
Human cases of smallpox have occurred somewhere in the world?	606 (60.6)	233 (23.3)	161 (16.1)	1 (0.1)
An effective medical treatment exists for smallpox?	524 (52.3)	114 (11.4)	363 (36.3)	0

by 41.7% of respondents, while 42.3%, 48.9%, and 56.3% would accept vaccination if cases were reported somewhere in the world, Australia, or their own community, respectively. Among respondents who did not report previous vaccination, 44.5% would accept vaccination as a precautionary measure (Table 2).

Modeling the readiness to accept vaccination showed that older persons were less likely to accept smallpox vaccination (odds ratio 0.977 per year, $p<0.001$). Respondents with more education were also less likely to accept vaccination under any scenario (odds ratio 0.845 per education category, $p<0.01$).

When asked in an open-ended question where they would first seek diagnosis or care if they thought they had contracted smallpox, 591 (59.0%) respondents mentioned their general practitioner (family physician). Hospital emergency departments were indicated by 330 (33.0%), a public health department by 43 (4.3%), and other sources by 18 (1.8%); 16 (1.6%) did not know and 3 (0.3%) did not answer. Overall, 418 (41.8%) indicated a high level of confidence in their physicians' ability to recognize symptoms of smallpox, 291 (29.1%) a medium level of confidence, 177 (17.7%) a low level of confidence, and 42 (4.2%) no confidence; 68 (6.8%) did not know, and 5 (0.5%) did not answer.

Conclusions

Most Australian adults interviewed in this national survey reported medium-to-high concern about the risk of a bioterrorism attack in Australia (57.4%) and believed that human smallpox cases had occurred in the past 5 years (60.6%). This finding may explain the general willingness to accept vaccination as a precautionary measure in the absence of a bioterrorism event (7). This finding is similar to that of a US survey, which indicated a strong community desire for precautionary vaccination against smallpox (5). However, the general public is unlikely to be sufficiently informed to balance the risks of a bioterrorism event against the potential for harm from vaccination (8). Given that the currently available smallpox vaccine must produce a significant lesion to be considered effective and commonly results in other adverse events, some severe, mass vaccination as an antiterrorism strategy must be epidemiologically justified by a substantial risk (9–11). Accurate information on smallpox vaccine adverse effects must be made available to the Australian public, although this information may affect acceptance of vaccination, as was documented among potential medical first responders in the United States (12).

Participants were unclear about their personal smallpox vaccination status. Although respondents born after smallpox vaccination was stopped in Australia were incorrect if they believed that they had been vaccinated against smallpox, 33% of this group falsely indicated that had been vaccinated. This belief may lead to a false sense of security in the event of an actual bioterrorist attack with smallpox virus.

Despite the desire for precautionary vaccination, only 259 (62%) respondents who believed they were unvaccinated would accept smallpox vaccination if cases were reported in Australia. A false belief that effective medical treatment exists for smallpox, which was held by more than half of the respondents, may influence decisions to accept vaccination in response to locally occurring cases (13). Public health authorities have a clear mandate to improve the community's knowledge of smallpox and bioterrorism. These efforts must involve groups, particularly the elderly and those with more education, who appear more unwilling to accept indicated public health measures.

General practitioners emerged as a pivotal group should a bioterrorism event occur in Australia; respondents identified these medical professionals as the preferred source of initial diagnosis and management and expressed a high level of confidence in their ability to correctly diagnose smallpox. This central role for general practitioners in optimizing biopreparedness in Australia has previously been hypothesized (14). Whether the community's belief in the ability and skills of general practitioners is justified is unknown, and this aspect clearly warrants investigation (15). Specific training courses for general practitioners that heighten their clinical index of suspicion, introduce public

Table 2. Responses of 418 persons who did not report being vaccinated against smallpox who would accept vaccination under various hypothetical conditions, Australia, 2004

Would accept vaccination …	Yes, n (%)	No, n (%)	Do not know, n (%)	No answer, n (%)
As a precautionary measure?	186 (44.5)	202 (48.3)	15 (3.6)	15 (3.6)
If cases were reported in the world?	193 (46.2)	199 (47.6)	11 (2.6)	15 (3.6)
If cases were reported in Australia?	259 (62.0)	132 (31.6)	12 (2.9)	15 (3.6)
If cases were reported in your community?	332 (79.4)	62 (14.8)	9 (2.2)	15 (3.6)

health containment and surveillance principles, and emphasize effective communication strategies should be developed in Australia and accredited for continuing professional development.

Findings in this Australian survey are similar to those in a survey in the United States, even though Australia has not experienced a bioterrorism event. In the US study, a similar proportion of respondents (63%) believed that smallpox cases had occurred in the past 5 years, but a greater proportion would accept precautionary vaccination (61%) and a slightly lower proportion (52%) would go to their own physician for diagnosis and care (5). The participation rate of 63% for this survey was similar to that in the US study (65%).

This national survey found that the Australian public holds many inaccurate beliefs about smallpox and smallpox vaccination, and this misinformation could negatively affect response to a bioterrorist event. General practitioners were identified as the primary point of care and should become an important focus of bioterrorism response planning in Australia.

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Evaluation of West Nile Virus Education Campaign

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We evaluated the 2003 Kansas West Nile virus public education campaign. Awareness was widespread but compliance was low. Spanish-speaking persons were poorly informed. Relevant factors included population segment variability, campaign content, media choice, and materials delivery methods.

West Nile virus (WNV) is a public health problem throughout the United States (1). While recovery without sequelae often occurs, the illness can be debilitating and fatal; substantial cost is associated with the illness (2). Because no cure or vaccine exists, prevention through education and behavioral change is essential (3). Many states have undertaken such prevention efforts, yet their efficacy is unknown, despite the Centers for Disease Control and Prevention's (CDC) request for evaluations (4). Although some states have assessed residents' knowledge, attitudes, and behaviors about WNV (5–9), Kansas is the first to report a process and outcome evaluation of its education campaign.

In 2003, the Kansas Department of Health and Environment (KDHE) implemented an extensive WNV prevention education campaign. Based on CDC recommendations, the campaign focused on education and encouraged 4 preventive behavior measures: use insect repellent with DEET (N, N-diethyl-meta-toluamide), wear long-sleeved shirts and long pants when outside at dawn or dusk, eliminate standing water, and maintain window screens.

Campaign materials were developed for television, radio, newspapers, and a web site. Public service announcements (PSAs) for print and broadcast media and brochures in English and Spanish were produced. Materials were distributed by email and the US Postal Service well before expected summer WNV outbreaks.

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The Study

Evaluation focused on a sample of 10 representative counties and consisted of personal and media surveys. The University of Kansas Medical Center Human Subjects Committee approved the surveys. The personal survey assessed knowledge, behavior, and attitudes about WNV (8). Knowledge was measured by asking respondents how WNV is transmitted, who is most vulnerable, and what self-protection measures are available. Behavior was measured by asking what respondents had done during the past week to protect themselves from mosquito bites. Attitude was measured by asking respondents their perceived risk of contracting WNV and for concerns or comments about recommended protective measures.

Surveys were conducted from August through October. Respondents were chosen from randomly generated telephone numbers. Telephone calls were made from 9:00 a.m. to 8:00 p.m. Monday through Saturday. The survey was administered in Spanish if respondents preferred to speak Spanish. A total of 2,329 calls were made reaching 779 eligible respondents; 534 (69%) respondents participated in the survey. Compared to Kansas' Census 2000 data, the sample's demographics were comparable to the general population.

Ninety seven percent of the sample had heard of WNV, 94% knew that it is transmitted through mosquitoes, and 70% knew that persons >50 years of age were most likely to become severely ill from it. Among the 17 Spanish-speaking respondents, there was significantly less awareness ($p<0.001$, chi-square test); only 7, (41%) had heard of WNV.

Among respondents who had heard of WNV, 89% knew $\geq$1 personal protective measure, 59% knew to avoid mosquitoes, 47% knew to use insect repellent, and 21% specified the use of repellent with DEET (Table 1). Reported behavior (Table 1) did not reflect knowledge. Fewer respondents used repellent than those who had cited this measure ($p<0.001$, chi-square test). More respondents wore protective clothing ($p<0.001$, chi-square test), eliminated standing water ($p<0.001$, chi-square test), and maintained window screens ($p<0.001$, chi-square test) than those who had cited these measures.

To understand protective measures attitudes, respondents were asked for comments or concerns. More than one third expressed concerns about DEET, mostly about health and safety; 26% stated that wearing long clothing outside at dawn and dusk was uncomfortable, and 16% cited difficulties eliminating standing water. Respondents estimated the risk of contracting WNV; 55% considered the risk to be low and 8% considered it to be high. These data are consistent with other studies (5–9).

To assess the process and outcome of the KDHE campaign, respondents were asked to list their most recent

Table 1. Comparison of respondents' knowledge and behavior about West Nile virus (WNV) protective measures*

WNV personal protective measures	% of respondents who knew specific WNV personal protective measures	% of respondents who took specific WNV or mosquito-bite protective measures in past week
Use repellent	47.2	27.5†
Use DEET repellent	21.3	17.6
Wear long clothes	26.6	36.9†
Eliminate standing water	34.3	54.1†
Repair window screens	0.6	33.0†
Other measures‡	59.2	NA

*DEET, N, N-diethyl-meta-toluamide; NA, not available.
†p<0.001, chi-square test.
‡Mostly general mosquito avoidance.

sources of WNV information. Most respondents cited mass media and word-of-mouth, few cited magazines or web sites, and even fewer cited healthcare providers or brochures (Table 2).

Process and outcome were also evaluated through the media survey. Every newspaper, radio station, and television station in the 10-county sample was contacted by phone or email. All were asked about receipt of KDHE WNV materials and if and how materials were used. Results indicated minimal use of the materials. No television station broadcasted PSAs or scripted stories provided by KDHE; only half recalled receiving the materials. Fifteen of 40 radio stations recalled receiving materials, although none aired them. Ten of 23 newspapers recalled receiving materials; 5 used the materials in publication.

Conclusions

Most persons were knowledgeable about WNV and cited mass media as their information source. KDHE campaign materials were used minimally by local media; persons were likely informed from national news, CDC, or news releases when local cases of WNV were reported. Low awareness levels in Spanish-speaking respondents indicated that prevention messages from any source were not reaching this population segment.

Three factors appeared to influence the degree to which WNV prevention messages affected respondents' knowledge, attitudes, and behavior: message content, media used, and method of delivery. More respondents knew they should be using repellent than actually used it. Knowledge and awareness are insufficient to impact behavior. Risk perceptions may be a moderating factor. Despite 90 confirmed cases of WNV, a 4-fold increase from 2002, and 731 reported presumed cases, most respondents perceived little risk of acquiring the illness. Thus, they likely were not motivated to use protective measures, especially those seen as deleterious or unpleasant. More respondents took other protective measures (wore protective clothing, removed standing water, maintained screens) than cited them. Clearly some took these measures for other reasons, unaware that they provided protections from WNV.

Table 2. Percentage of respondents citing various media as sources of West Nile virus information

Source	%
Television	88
Newspaper	72
Word-of-mouth	65
Radio	44
Magazine	17
Website	16
Veterinarian	9
Healthcare provider	8
Brochure	6
Other	5

Mass media and word-of-mouth were the most successful methods of providing WNV information to respondents. Healthcare providers, veterinarians, magazines, and the Internet were less successful methods. Brochures were least successful, indicating that they may be ineffective for this type of communication, or that difficulties with their distribution occurred.

Timing and campaign material delivery methods were critical factors. Email delivery was problematic; some attached files could not be opened. Some broadcast stations had policies prohibiting opening unsolicited email attachments. Timing of materials distribution also contributed to their minimal use. Materials were sent in the spring before the peak of WNV incidence. By the time our survey was conducted during peak WNV season, news media considered WNV a "hot topic." Media respondents often did not recall receiving WNV materials, yet asked our surveyors for materials. News media look for current materials and do not likely store information for later use.

We recommend the following practices for public health disease prevention campaigns for WNV and other emerging diseases: 1) Reduce barriers to desired behavioral changes. For WNV, this includes greater focus on the safety of DEET. 2) Distribute materials by email with provisions to assure recipients that materials are virus-free. Send explanatory letters by the postal service before and after any email. Provide a web site for direct access to campaign materials. 3) Provide information close to the time it will be used. 4) Research ways to influence and promote word-of-mouth, an important source of information.

5) Research how media differentially impact population segments. 6) Increase PSA exposure; consider purchasing broadcast time. 7) Design campaigns for a linguistically and culturally diverse population (10) and to reach vulnerable population subgroups such as, for WNV, the elderly and immunocompromised (11–15).

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Long-term Death Rates, West Nile Virus Epidemic, Israel, 2000

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We studied the 2-year death rate of 246 adults discharged from hospital after experiencing acute West Nile Virus infection in Israel during 2000. The age- and sex-adjusted death rates were significantly higher than in the general population. This excess was greater for men. Significant adverse prognostic factors were age, male sex, diabetes mellitus, and dementia.

West Nile virus (WNV) was first isolated in 1937 (1,2) and has since been found in Europe, Africa, Asia, and North America (1). Human WNV infection was first documented in Israel in 1951 (3). Several outbreaks occurred during the 1950s and during the following 40 years, but mainly sporadic cases occurred (4). In 2000, an epidemic of 428 serologically confirmed cases led to 42 deaths during the acute phase of the illness (5). Little has been reported in the literature on the long-term sequelae of WNV infection. In 1953, 70 patients 18–20 years of age were followed up for ≤11 months, and no serious sequelae or deaths were observed (6). Although 12% of the patients in the New York outbreak in 1999 died during the acute phase of the illness, no deaths occurred among the 35 patients followed up for 1 year after hospitalization (7). The aim of the present study was to determine whether infection with WNV has long-term effects on death rates and to evaluate variables such as age, sex, symptoms and signs, and coexisting conditions as potential prognostic factors.

The Study

Between July 1 and November 30, 2000, 428 patients with serologic diagnoses of WNV infection were reported to the Ministry of Health (MOH) Department of Epidemiology. Diagnosis of WNV infection was made on the basis of symptoms, signs, and laboratory confirmation of the presence of immunoglobulin M (IgM) antibodies. All serologic tests were performed in the MOH's Central Virology Laboratory by using an IgM-capture enzyme-linked immunosorbent assay in serum or cerebrospinal fluid samples of patients (8). At the time of the epidemic, this was the working definition of a case of WNV infection. While WNV IgM antibodies can persist >1 year (9), a seroepidemiologic study in a healthy population in Israel at the time of the outbreak, using the same laboratory methods, found only 1.2% had IgM antibodies (M.Y. Chowers et al., unpub. data). Thus, few, if any, false-positive results would be expected.

The study population was limited to the 326 hospitalized patients. After excluding 34 patients <20 years of age and the 46 patients who died during hospitalization or within 1 week of discharge, 246 survivors were eligible for follow-up. Demographic data on the cases and date of onset of the disease were obtained from the Department of Epidemiology. The mean ages of the patients were 57.8 years for men and 62.8 years for women. Clinical data on symptoms and coexisting conditions were obtained from the hospital discharge letters. Of eligible survivors, 48.3% had diagnoses of encephalitis or meningoencephalitis, 13.5% had a diagnosis of meningitis, 28.2% had fever, rash, or both, and the other 10% of the survivors had other clinical symptoms.

The patients were monitored to determine whether any deaths occurred from date of hospital discharge until November 30, 2002. Deaths were determined by matching the identity numbers of the case-patients with the data in the national population registry maintained by the Ministry of Interior. We were able to confirm all deaths in the cohort during this period. By November 30, 2002, after an average follow-up period of 24 months (median 26 months, range 1–29 months), 30 of 246 patients had died.

Death certificates were obtained for all case-patients, and all causes of death were recorded and coded by using the International Classification of Disease, Ninth Revision. The coding was reviewed independently by another coder, and the underlying cause of death was determined by using World Health Organization criteria.

Bivariate comparisons between characteristics of the patients who died and the survivors were carried out by using the χ^2 and *t* tests. Kaplan-Meier survival curves were constructed for all patients and for men and women separately, and the difference between men and women was compared by using the log-rank test. The age-standardized mortality ratios (SMRs) were computed by using the person-time method with the PAMCOMP program (Institute of Epidemiology and Social Medicine, University of Muenster, Muenster, Germany) (10). SMRs were calculated by dividing the observed number of deaths by the

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expected number. Person-months of risk were calculated from the reported onset of the disease until either death or November, 30, 2002, whichever occurred first. The expected number of deaths in the study cohort was calculated by multiplying person-months at risk by the national death rates in 1997 (the last year for which detailed published data are available) for the same age and sex categories. Exact 95% confidence intervals (CIs) and p values were calculated by using the Poisson distribution. Attributable risk was computed by the formula (SMR-1)/SMR. Prognostic factors were evaluated by using Cox proportional hazards regression analysis to estimate adjusted rate ratios while adjusting for other potential determinants of death. The hazard rate ratio (RR) is defined as the ratio of the mortality rate in the group of patients with a given study factor to the rate in those without the factor. We calculated 95% CIs for each adjusted RR. The SAS package version 8.2 (SAS, Cary, NC, USA) was used for all calculations other than the SMRs.

The Kaplan-Meier survival curves are shown for the total cohort and for men and women separately (Figures 1 and 2). Overall, after 1 year, 7.7% had died and after 2 years, 12.2%. After 1 year the death rate was 6.4% for women compared with 9.1% for men (p = 0.025), and after 2 years it was 10.4% for women and 14.1% for men (p = 0. 021).

Age-adjusted SMRs, both sex-specific and sex-adjusted, at 6 months, 1 year, and the end of the follow-up, are shown in Table 1. The overall SMR at 1 year was nearly 2.5 times higher than expected (SMR = 2.49, 95% CI 1.50–3.89). Among men, the death rate was >3 times higher than expected, whereas among women the death rate was less than twice as high and not significant. The excess risk at the end of the second year was lower and not significant**.** Overall, in 50% of the deaths the underlying cause was ascribed to cardiovascular disease. In only 20% was WNV infection given as the underlying cause of death, although this result is clearly influenced by coding practices.

In the Cox regression analyses for evaluating demographic factors and the symptoms and signs as prognostic factors, while simultaneously controlling for each, only age, and especially the oldest age group tested (≥85 years), was a significant adverse prognostic factor (data not shown). Possible interactions between the symptoms and signs with age and sex were not significant. A second Cox regression analysis for evaluating coexisting conditions as prognostic factors is shown in Table 2. After age, sex, and the other coexisting conditions were controlled for, only diabetes mellitus and dementia remained as significant, independent predictors of death (RR = 2.74 and 2.94 for diabetes mellitus and dementia, respectively). Again, interaction effects were not significant.

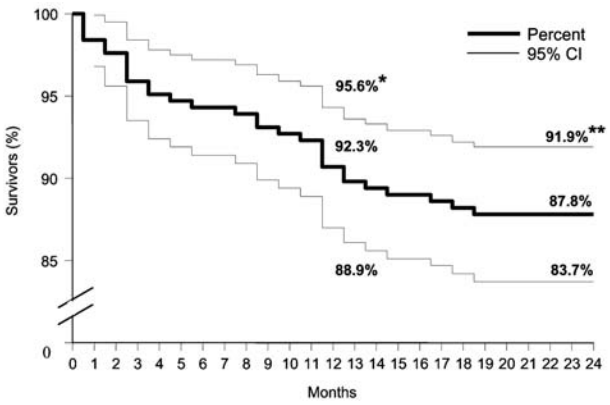

Figure 1. Kaplan-Meier survival curves for 2-year mortality follow-up of 246 patients discharged from hospital after West Nile virus infection during the epidemic in Israel in 2000. *Survival after 1 year; **survival after 2 years; CI, confidence interval.

Conclusions

In this study, excess deaths occurred among survivors compared with the general population. This excess appeared to occur mainly in the first year after the acute illness and was more prominent among men than women. Older age, and especially the oldest age group tested (≥85 years), diabetes mellitus, and dementia were other significant independent predictors of death.

To demonstrate the magnitude of this excess risk, we compared these findings with an Israeli study of deaths within 1 year after hospital discharge among patients with acute myocardial infarction of whom ≈30% had diabetes (S. Bachar, Acute Coronary Syndrome in Israel 2000 study, unpub. data). After age and sex were controlled for, the death rate within 1 year for WNV-infected patients in

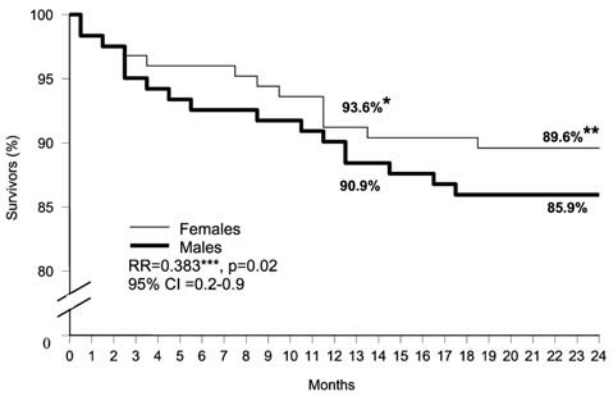

Figure 2. Kaplan-Meier survival curves for 2-year mortality follow-up of 246 patients discharged from hospital after West Nile Virus infection during the epidemic in Israel in 2000, by sex. *Survival after 1 year; **survival after 2 years; ***relative risk (RR) for women compared with men, adjusted for age, diabetes, ischemic heart disease, immunodeficiency, cerebrovascular disease, hypertension, and dementia.

Table 1. Standardized mortality ratios (SMRs) for 246 patients ≥20 years of age discharged from hospital after West Nile virus infection, Israel, 2000, compared with those for the general population during 2 years of follow-up*

Follow-up period	No.	Observed deaths	Expected deaths	SMR	95% CI
First 12 mo					
Men	121	11	3.47	3.17†	1.58–5.67
Women	125	8	4.16	1.92†	0.83–3.79
Total	246	19	7.63	2.49‡	1.50–3.89
12–24 mo					
Men	110	6	2.91	2.06†	0.75–4.49
Women	117	5	3.74	1.34†	0.43–3.12
Total	227	11	6.64	1.66‡	0.83–2.96

*CI, confidence interval.
†Adjusted for age.
‡Adjusted for age and sex.

the present study was similar to that of patients after myocardial infarction (7.7% vs. 9.5%, p = 0.881). This finding indicates that the 1-year postdischarge death risk in the WNV-infected patients is of a similar magnitude to that of patients with a severe, acute, noninfectious illness.

The main negative prognostic factors in the present study were older age, male sex, diabetes mellitus, and dementia. Similar associations with deaths in the acute phase of the illness have been observed for age (5,11) and diabetes mellitus (5). Thus older age and possibly diabetes mellitus influence both acute-phase and long-term mortality rates. Why long-term mortality rates, not related to preexisting illnesses or age, are higher in men than in women is unknown. In several studies of clinical WNV infection, men appeared to have a higher incidence of disease, but this difference has not been observed in Israel (5).

Survivors of herpes simplex encephalitis have a shorter life expectancy (12,13), and to a lesser extent, so have patients with other viral encephalitides (13). Some of these excess deaths could be attributed to severe neurologic impairment and instability, while other deaths were unrelated to the infection.

The reasons for the increased long-term death rate in survivors of acute WNV infection are not clear. The clinical features described in WNV encephalitis have been described as typical for arboviral encephalitides (1). Severe neurologic sequelae have been described in survivors of invasive WNV infection, and a substantial number of patients do not regain their baseline function (8). This deficit could contribute to an increase in deaths.

In this epidemic in Israel, 2 clades of WNV were isolated (8,14). One was closely related to the 1999 New York isolate and the other to a 1999 Russian isolate and a 1997 Romanian isolate (14). The outbreak was characterized by relatively high case-fatality rates among hospitalized patients; 14% of the patients died during the acute phase (5). This rate is similar to that observed in the New York outbreak (15). No evidence suggests that the virus in Israel was any different in virulence.

The results of this study on the potential long-term impact of WNV infection are relevant for hospitalized patients. They may have been more susceptible to clinical WNV infection. The significant excess death rate indicates that the combined case-fatality rate in all hospitalized patients in the acute and convalescent phases could be >10%, and in patients >65, >30% (5). Thus, such patients require long-term monitoring and support. Possible sex differences in death rates require further investigation.

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Table 2. Cox proportional hazards analysis for the association between coexisting conditions and 2-year death rate follow-up of 246 patients ≥20 years discharged from hospital during a West Nile virus epidemic, Israel, 2000*

Variable	B	RR	95% CI	p
Age† (y)				
75–84	2.00	7.38	2.77–17.71	<0.0001
≥85	2.49	12.10	4.24–34.49	<0.0001
Sex (M = 0, F = 1)	–1.09	0.33	0.14–0.79	0.0124
Diabetes (no = 0, yes = 1)	1.01	2.74	1.11–6.81	0.0294
Ischemic heart disease (no = 0, yes = 1)	0.17	1.19	0.52–2.72	0.6794
Immunodeficiency (no = 0, yes = 1)	0.04	1.04	0.13–8.08	0.9696
Cerebrovascular disease (no = 0, yes = 1)	–1.22	0.29	0.07–1.28	0.1023
Hypertension (no = 0, yes = 1)	0.09	1.09	0.48–2.48	0.8365
Dementia (no = 0, yes = 1)	1.08	2.94	1.09–7.91	0.0330

*B, natural logarithm of the hazard rate ratio; RR, hazard rate ratio; CI, confidence interval.
†The age group <75 years old was set as a reference.

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Vibrio cholerae Pathogenic Clones

Anna Salim,* Ruiting Lan,† and Peter R. Reeves*

We resolved the relationships between 2 pandemic clones of *Vibrio cholerae*. Using 26 housekeeping genes, we showed that the US Gulf clone, the Australian clone, and 3 El Tor strains isolated before the seventh pandemic were related to the seventh pandemic clone. The sixth pandemic clone was well separated from them.

Cholera caused by *Vibrio cholerae* is a major disease that has caused great fear since the first recorded pandemic in 1817 because of the frequency of death and the rapidity with which it occurs (1,2). Approximately 200 O antigens have been distinguished serologically (3,4), but only O1 and O139 have been found in epidemic and pandemic cholera isolates (5,6).

The seventh pandemic (1961–present) is still widespread and has a severe impact on 3 continents. The sixth pandemic ended in 1923, but the clone persisted at least until the 1990s (7). Furthermore, several cholera outbreaks were reported after the sixth pandemic retreated but before the start of the seventh pandemic. Isolates from these outbreaks were recognized as different from those of the sixth pandemic and were allocated to the El Tor biotype, while the sixth and fifth pandemics, both of which had been studied microbiologically, were referred to as the classical biotype. These El Tor outbreaks occurred in Indonesia and the Middle East (1926–1960) (5) and are often referred to as prepandemic isolates because they were later seen as forerunners of the seventh pandemic, which was also of the El Tor biotype. However, now that environmental *V. cholerae* has been studied in some detail, major components of the El Tor phenotype are known to be present in most environmental isolates, and the classical biotype is believed to have arisen by loss of characters otherwise widely present in the species (8). Also, cases of sporadic indigenous cholera have been detected in Australia (9) and the United States (10), both of the O1 El Tor biotype. These are generally referred to as the US Gulf and Australian clones. All of the pathogenic forms discussed above had the O1 serotype, but in 1992 a variant of the seventh pandemic appeared with a new O antigen, O139; this variant is known as *V. cholerae* O139 Bengal (11).

The relationships of *V. cholerae* have been studied in several ways, but the most useful insights have come from multilocus enzyme electrophoresis (12) and more recently by multilocus sequence analyses (4,13,14). In this study, we sequenced 26 housekeeping genes from *V. cholerae* isolates representative of the sixth and seventh pandemic clones and other closely related toxigenic strains to determine relationships to better understand the origins of pandemic clones.

The Study

Twenty-six housekeeping genes distributed evenly on both chromosomes (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol11no11/04-1170_app.htm) were studied by using 5 nontoxigenic environmental isolates and 12 toxigenic *V. cholerae* isolates, which comprise 5 sixth and seventh pandemic isolates and 7 pathogenic isolates related to them (Table). One of the 3 seventh pandemic isolates is N16961; the sequence of its genome (15) was used in this study. All 26 housekeeping genes were successfully sequenced from the remaining 11 toxigenic isolates. However, 4 genes could not be amplified by polymerase chain reaction from 1 of the environmental isolates (*sdaA* for 370-94, *hmpA* for 905-93, *glgX* for 928-93, and *pepN* for 370-94) and were not sequenced. The GenBank accession numbers for the nucleotide sequences determined in this study are DQ020969–DQ021380.

The 5 nontoxigenic environmental isolates had different sequences for each gene. These sequences were not found in any of the 12 toxigenic isolates. The average pairwise difference among the 5 environmental isolates in the 22 genes sequenced ranged from 0.67% to 5.29%, an indication that *V. cholerae* as a species has a high level of sequence variation. However 11 of the 26 housekeeping genes (*glyA*, *gppA*, *pntA*, *icd*, *purM*, *plsX*, *ndh*, *glgX*, *adk*, *carA*, and *speA*) were identical in all toxigenic *V. cholerae* isolates, confirming that they are closely related.

There were 18 mutation/recombination events among the 15 genes with sequence variation in the 12 toxigenic isolates. Three (*malP*, *pyrC*, and *gyrB*) of these genes had undergone 2 changes. Seven cases of single base differences, which are attributed to mutation, were clearly distinct from 11 cases of multiple base differences, which are attributed to recombination. Ten of the events attributed to recombination involve changes in 10 to 51 bases (Figure 1). The *metG* gene, in which the 2 sequences differ by only 4 bases, might have undergone 4 mutational events rather than a single recombination event, but we considered this to be most unlikely.

Some of the 11 genes believed to have undergone recombination may have undergone >1 recombination event. This possibility is likely because 11 of 26 genes have undergone recombination. For example, in *metE* the base changes are at the 2 ends and may represent

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Table. Isolates of *Vibrio cholerae* tested

Original name	Laboratory name	Clone/isolate	Year isolated	Location	Source*	Serogroup	Biotype
#75	M967	6th pandemic	1921	Japan	CDC	O1	Classical
395	M1616	6th pandemic	1965	India	CVD	O1	Classical
E506	M794	US Gulf Coast	1974	Texas, USA	CVD	O1	El Tor
4808	M796	US Gulf Coast	1978	Louisiana, USA	CVD	O1	El Tor
NCTC 9420	M640	Pre-7th pandemic	1954	Cairo, Egypt	NCTC	O1	El Tor
NCTC 5395	M543	Pre-7th pandemic	1938	Baghdad, Iraq	NICED	O1	El Tor
66-2 (Makassar 759)	M802	Pre-7th pandemic	1937	Sulawesi, Indonesia	IP	O1	El Tor
SIMP/77	M2140	Australian	1977	Australia	QH	O1	El Tor
M4287/77	M2141	Australian	1977	Australia	QH	O1	El Tor
2100	M663	7th pandemic	1992	Bali, Indonesia	IMVS	O1	El Tor
E9120	M793	7th pandemic	1961	Indonesia	CVD	O1	El Tor
N16961†		7th pandemic	1971	Bangladesh	GenBank	O1	El Tor
1085-93	M549	Environmental	1993	Germany	NIHJ	O37	
141-94	M553	Environmental	1994	Germany	NIHJ	O70	
905-93	M555	Environmental	1993	Argentina	NIHJ	O97	
928-93	M557	Environmental	1993	Argentina	NIHJ	O6	
370-94	M563	Environmental	1994	South Korea	NIHJ	O81	

*CDC, Centers for Disease Control and Prevention; CVD, Centre for Vaccine Development (Dr James Kaper); NCTC, National Collection of Type Cultures; NICED, National Institute for Cholera and Enteric Diseases; IP, Institute Pasteur (Dr A. Dodin); QH, Queensland Health (Dr Denise Murphy); IMVS, Institute of Medical and Veterinary Science; NIHJ, National Institute of Health, Japan (Dr Tohio Shimada).
†Sequence of the genome of isolate N16961 was used. GenBank accession numbers for chromosomes 1 and 2 are AE003852 and AE003853, respectively.

2 recombination events; the same applies, to a lesser extent, to *malP*.

With regard to the length of DNA involved in recombination events, segments longer than a gene are most common because only in the genes discussed above and in *sdaA* and *gyrB* are the bases that differed clustered within the gene. This is suggestive of recombination within that gene.

The sequences for the 26 genes were used to produce a tree with mutational and recombinational events and given equal weight, which is shown in Figure 2. By sequencing 26 genes, we have observed sufficient variation to determine the relationships of the isolates studied. The tree is unrooted because the environmental isolates share no alleles with each other or toxigenic isolates. The relatively high rate of recombination in *V. cholerae* means that the level of sequence similarity does not indicate relatedness of the isolates unless the sequences are very similar and differences can be attributed to mutation, as in the toxigenic strains. However, other reasons exist for believing that the root is on the long 10-event branch that includes 2 mutational changes. The 10 El Tor isolates on 1 end are, at most, 3 steps from it and the 2 classical sixth pandemic isolates are separated by 1 event. The isolates date from 1937 to 1992 for the former group and from 1921 to 1965 for the latter group. Since 1 group giving rise to the other while undergoing little divergence itself is highly improbable, we believe that the root lies somewhere on that branch. The properties that characterize the El Tor biotype are those of environmental strains, which makes it unlikely that they are derived from the sixth pandemic, and the reverse seems even less likely because the fifth pandemic was also the classical biotype. We therefore treat the tree as being rooted on the long branch, which enables us to follow the sequence of events.

Among the El Tor isolates, the Australian clone is the most closely related of the current clones to the seventh pandemic clone, with 1 and 2 events along the 2 branches separating them. The Australian clone, although not discovered until 1977, must have arisen before the seventh pandemic and spread to Australia. The prepandemic outbreak isolates are located separately among the surviving El Tor pathogenic clones, with the 1937 Indonesian 66-2

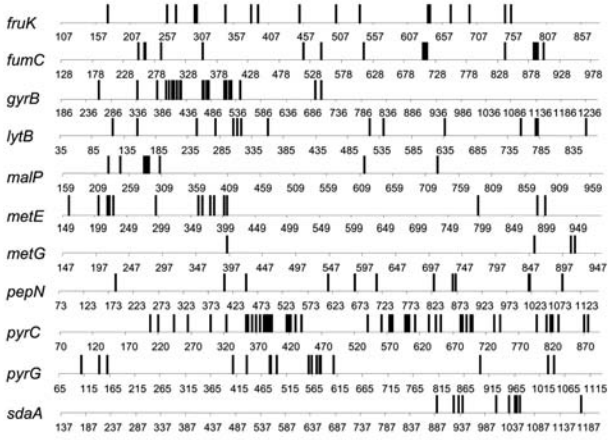

Figure 1. Distribution of base differences in strains of *Vibrio cholerae*. The base positions relative to the start of the ATG codon are shown. Isolates 395 and E9120 were compared for the *fruK*, *lytB*, *metE*, *pepN*, *pyrG*, *fumC*, *malP*, *metG*, *pyrC*, and *sdaA* genes, and isolates 395 and E506 were compared for the *gyrB* gene.

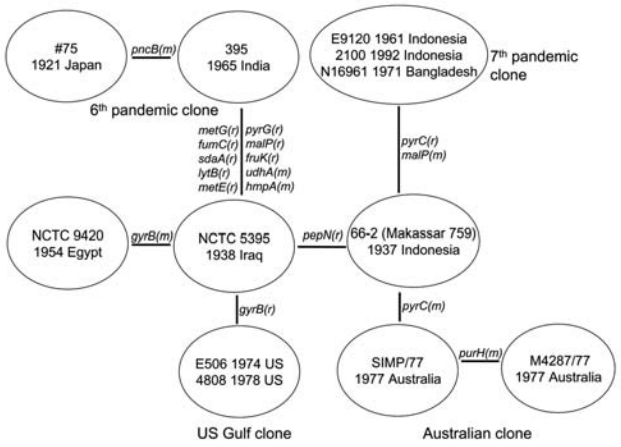

Figure 2. Relationships of toxigenic *Vibrio cholerae* isolates. The mutational (m) and recombinational (r) changes are given equal weight. Shown is a unique (unrooted) tree, with the events indicated on the branches.

(Makassar 759) isolate located closest to the seventh pandemic clone. The US Gulf clone diverged before the Australian clone and the seventh pandemic clones diverged, with a single recombination event on the branch to the common ancestor of the Australian and the seventh pandemic clones.

The 2 sixth pandemic isolates are well separated from the other strains, differing from them at 10 loci, an average of 5 events per branch. These include recombination events affecting 8 genes. If representative, ≈30% of the genes have undergone recombination during divergence of the sixth pandemic clone and the El Tor group of pathogenic clones that includes the seventh pandemic clone. The extensive divergence between the sixth pandemic and other toxigenic isolates studied indicates a long period since divergence from the common ancestor, which presumably occurred well before the sixth pandemic (1899–1923). In the absence of any intermediates, we cannot allocate individual events to either branch but presume that each is equally likely to have occurred on either branch.

Conclusions

This study using sequences of 26 genes has resolved the evolutionary relationship of the 2 major pandemic clones of *V. cholerae* and the relationships of the seventh pandemic clone to other pathogenic El Tor clones and isolates. With the relationships established it is clear that study of the prepandemic isolates and Australian clone in particular could illuminate the events involved in the emergence of the current seventh pandemic clone from this lineage.

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Methicillin-resistant *Staphylococcus aureus* in Taiwan

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We found a virulent closely related clone (Panton-Valentine leukocidin–positive, SCC*mec* V:ST59) of methicillin-resistant *Staphylococcus aureus* in inpatients and outpatients in Taiwan. The isolates were found mostly in wounds but were also detected in blood, ear, respiratory, and other specimens; all were susceptible to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole.

Although most methicillin-resistant *Staphylococcus aureus* (MRSA) illness and death are associated with healthcare facilities (H-MRSA), isolates from community-associated MRSA (C-MRSA) infections have been obtained with increasing frequency in the last few years in different countries, including Taiwan (1–5). The changing epidemiology of MRSA has become an important public health concern worldwide (1,4). MRSA arises when *S. aureus* organisms acquire a large mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) (6). Most H-MRSA strains possess either SCC*mec* II or III; most C-MRSA strains possess SCC*mec* IV (7–9). Recently, a novel type V SCC*mec* type was characterized and found in a C-MRSA isolate from Australia (10). With the exception of variable resistance to erythromycin, C-MRSA strains are generally susceptible to other non-β-lactam antimicrobial agents, in contrast to most H-MRSA, which are typically resistant to many of the non-β-lactam agents (1,4,9). Another characteristic of C-MRSA is the production of Panton-Valentine leukocidin (PVL), an extracelluar cytotoxin involved in primary skin infections and pneumonia (2–4).

We conducted a study to characterize the molecular epidemiology of selected MRSA isolates from the Taiwan Surveillance of Antimicrobial Resistance (TSAR), a national surveillance program of inpatient and outpatient clinical isolates in Taiwan (11). We describe the finding of a virulent closely related clone of MRSA and its prevalence in Taiwan.

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The Study

A total of 398 and 865 nonduplicate *S. aureus* isolates were collected from March to May 2000 from 21 hospitals and from July to September 2002 from 26 hospitals, respectively, as part of the TSAR collection (11). The proportions of isolates for the 2 years were similar for outpatients (27.5% in 2000 and 28.9% in 2002); the rest of the isolates were from inpatients. The most common specimen type was wound, which accounted for 35.4% and 49.2% of all *S. aureus* isolates in 2000 and 2002, respectively. Antimicrobial susceptibility was determined on the basis of results of MICs obtained from a broth microdilution method, following the guidelines of the National Committee for Clinical Laboratory Standards (12) by using custom-designed Sensititre plates (Trek Diagnostics, East Essex, United Kingdom). Overall, MRSA accounted for 238 (59.8%) of 398 *S. aureus* isolates in 2000 and 475 (54.9%) of 865 *S. aureus* isolates in 2002.

To obtain an overall understanding of MRSA throughout Taiwan, we first chose 80 MRSA isolates (68 inpatient and 12 outpatient isolates) collected in 2002 from 4 hospitals located in the north, middle, south, and east regions of Taiwan. Pulsed-field gel electrophoresis was performed according to a published protocol (9), and pulsotypes were assigned to clusters of isolates with >80% similarity from the dendrograms. SCC*mec* typing and PVL gene detection were performed according to published protocols (7,8,10). Multilocus sequence typing (MLST) was performed on randomly selected strains from major pulsotypes, and the sequence type (ST) was assigned by using the MLST database (http://www.mlst.net) (13). Three major clusters (pulsotypes) were found, including 47 (58.8%) pulsotype A, 7 (8.8%) pulsotype B, and 18 (22.5%) pulsotype C (online Appendix Figure 1, available from http://www.cdc.gov/ncidod/EID/vol11no11/05-0367_app1.htm). All 47 pulsotype A isolates had SCC*mec* III; 4 isolates tested by MLST had ST239. In addition to being resistant to clindamycin (94%), erythromycin (100%), and tetracycline (100%), all pulsotype A isolates were resistant to ciprofloxacin (CIP), gentamicin (GEN), and trimethoprim/sulfamethoxazole (SXT). The 7 pulsotype B isolates possessed SCC*mec* IV but were not of the 4 known IV subtypes (IV not a–d); all were CIP- and SXT-susceptible but GEN-resistant. Seventeen of the 18 isolates in pulsotype C possessed SCC*mec* V; the other had SCC*mec* IVa; all 18 were CIP/GEN/SXT-susceptible. Of the 10 isolates tested by MLST from pulsotype B (3 isolates) and pulsotype C (7 isolates), all had ST59. Only pulsotype C isolates were PVL-positive.

Because we found a large percentage (21.3%, 17/80) of SCC*mec* V:ST59, PVL-positive clones, we selected an additional 69 CIP/GEN/SXT-susceptible MRSA isolates (25 from 2000, 44 from 2002) to determine what portion of

them had the same clone. These isolates were from intensive care unit (ICU) and non-ICU inpatients, plus outpatients from 15 hospitals in 2000 and 21 hospitals in 2002 (excluding the 4 hospitals already tested). Fifteen (60%) of the 25 isolates from 2000 and 32 (72.7%) of the 44 isolates from 2002 belonged to pulsotype C, had SCC*mec* V, and were PVL-positive. Of the 4 isolates characterized by MLST, 2 were ST59, 1 was ST388, and 1 was a new ST; the last 2 isolates differed from ST59 by 1 nt each in *gmk* and *arcC* genes**,** respectively.

When the 2 groups of isolates were combined, a total of 90 CIP/GEN/SXT-susceptible MRSA were studied (online Appendix Figure 2, available from http://www.cdc.gov/ncidod/EID/vol11no11/05-0367_app2.htm). These isolates were mostly resistant to clindamycin (89%), erythromycin (92%), and tetracycline (82%). Of these 90 isolates, 68 (75.6%) were PVL-positive and belonged to same pulsotype C; 64 (71.1%) had SCC*mec* V. MLST performed on 12 isolates found 10 to be ST59; the other 2 (ST338 and the new ST) are closely related to ST59. Forty-eight of these 64 isolates were from wounds (75%), but isolates were also found in respiratory (6 isolates), ear (3 isolates), blood (2 isolates), urine (2 isolates), and catheter and other body site (3 isolates) specimens. Only half (32 isolates) were from outpatients; the rest were from ICU (10 isolates) and non-ICU (22 isolates) inpatients. Two ICU isolates were from hospital-acquired infections. Because 189 (26.5%) of the 713 MRSA isolates from the 2000 and 2002 collections were CIP/GEN/SXT-susceptible, and because 64 (71.1%) of the 90 CIP/GEN/SXT-susceptible MRSA we studied were PVL-positive, SCC*mec* V:ST59, and belonged to pulsotype C, an estimated 18.8% (26.5% × 0.711 = 18.8%) of MRSA isolates in Taiwan could be this closely related virulent clone.

The reason for the high prevalence of this virulent clone (pulsotype C:ST59, SCC*mec* V, PVL-positive) of MRSA in Taiwan in both inpatients and outpatients is not known. Data on the prevalence of SCC*mec* V are still limited, and ST59 has been described infrequently. A recent longitudinal study of MRSA isolates in the San Francisco area found that the ST59-SCC*mec* IV has increased steadily from 1999 to become 1 of the 4 major clones associated with C-MRSA (14).

Production of the PVL cytotoxin is considered a genetic marker for C-MRSA, and although PVL-positive MRSA have usually been associated with skin and soft tissue infections, severe and fatal infections, such as necrotizing pneumonia, have been reported (2–4). Thus, PVL may confer an additional virulence advantage for this particular clone of MRSA in Taiwan. Other possible explanations are that the less resistant C-MRSA can grow faster than multidrug-resistant H-MRSA and that SCC*mec* IV and V carried by C-MRSA may have the advantage over SCC*mec* I–III carried by H-MRSA because they are smaller and more transferable; both of these putative advantages may contribute to their propagation (10,15).

The close relatedness and high prevalence of this virulent pathogen argue for a clonal expansion advantage of this particular clone. Outbreaks of C-MRSA infections caused by SCC*mec* IV (IVa) have been reported in several countries (4). Since our genotyping results showed that MRSA isolates possessing SCC*mec* V and PVL in Taiwan are clonally related, we cannot rule out the possibility of outbreaks due to this particular clone in some areas. However, our isolates came from multiple hospitals throughout the 4 geographic regions of Taiwan. Our data also showed that this particular clone was already present in 2000. In addition, this particular clone was found not only in outpatients but also in ICU and non-ICU inpatients, including in hospital-acquired infections. These findings indicate that this clone has migrated into the hospital environment; moreover, it can cause more severe infections, as shown by its presence in blood, respiratory, ear, and other specimens.

Conclusions

Our analysis of MRSA isolates collected in 2000 and 2002 indicated that a virulent clone of MRSA (pulsotype C:ST59, SCC*mec* V and PVL-positive), which caused wound infections primarily but also other potentially more serious infections, is highly prevalent in Taiwan inpatient and outpatient settings. Recognition of this clone can be facilitated by its antimicrobial susceptibility profile. Because the resistance pattern of these isolates differs from that of traditional H-MRSA strains, the antimicrobial susceptibility profile has important implications for treatment. Understanding the roles these strains play in MRSA epidemiology helps physicians choose the most appropriate treatment. Prompt and judicious management and infection control measures should help deter further spread of this virulent pathogen.

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Dr Feng-Jui Chen is a microbiologist at the National Health Research Institutes, Taiwan. His primary research interest is molecular microbiology, particularly the mechanisms of quorum sensing in *S. aureus*.

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Salmonella Paratyphi A Rates, Asia

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Little is known about the causes of enteric fever in Asia. Most cases are believed to be caused by *Salmonella enterica* serovar Typhi and the remainder by *S.* Paratyphi A. We compared their incidences by using standardized methods from population-based studies in China, Indonesia, India, and Pakistan.

Enteric fever still causes substantial illness and death in many parts of the world, especially in poorer nations. *Salmonella enterica* serovar Typhi is believed to cause most enteric fever episodes, and a smaller portion are caused by *S.* Paratyphi (1–3). This assumption, however, may no longer be true. Since 1999, more *S.* Paratyphi A than *S.* Typhi strains have been isolated in the province of Guangxi, southeastern China (4). Increasing isolation rates of *S.* Paratyphi A has also been reported from India (5). This finding has 2 major implications for the prevention of enteric fever. First, licensed typhoid fever vaccines (Vi polysaccharide and live oral Ty21a) do not protect against infections caused by *S.* Paratyphi A, and they may become less useful in controlling enteric fever in regions of Asia. Second, transmission and risk factors for *S.* Typhi and *S.* Paratyphi are different in Indonesia (6), so reduction strategies effective against *S.* Typhi may not protect against *S.* Paratyphi. Since little is known about the current cause of enteric fever in Asia, we compared *S.* Typhi and *S.* Paratyphi A incidence from study sites in China, Indonesia, India, and Pakistan by using standardized epidemiologic and laboratory methods (7).

The Study

After a baseline census, surveillance was conducted in study sites in Karachi, Pakistan; Calcutta, India; North Jakarta, Indonesia; and Hechi City, China, for 12 months to identify typhoid and paratyphoid cases from specific populations at high risk (Table). None of the sites had specific enteric fever control programs in the past. Hechi City, China, is located in Guangxi Zhuang Autonomous Region where Vi vaccines had been used in the past (8,9); however, no such intervention had taken place in Hechi City. The closest county with a vaccination program was ≈80 km away and vaccinated only students (29,000 doses in 2001).

During the surveillance period, persons with fever who lived in each study area were requested to visit participating healthcare providers. We collected 5–10 mL blood from adults with fever ≥3 days' duration into Bactec bottles (Becton Dickinson, Franklin Lakes, NJ, USA). We collected 3–8 mL from children with fever ≥3 days' duration into Pediatric Bactec bottles. The bottles were incubated at 37°C for 7–10 days and visually checked for growth every day. Bottles were subcultured on MacConkey agar on days 1, 2, 4, and 7 or when turbidity was detected. Suspected colonies were screened by using Kligler iron agar, sulfide-indole-motility medium, urea agar, and citrate. Colonies that showed biochemical reactions suggestive of salmonellae were confirmed serologically by Felix-Widal tube agglutination test with specific O and H antisera (Becton Dickinson). All *Salmonella* isolates were confirmed at a reference laboratory (University of Oxford, Wellcome Trust Clinical Research Unit, Ho Chi Minh City, Vietnam).

Incidence rates were calculated by using age-specific denominators of the population living in the catchment area based on the study census. We assumed that each person living in the study area contributed 12 months of person-time to the denominator. The number of disease episodes in eligible individuals was used as the numerator.

Each study received individual approval from the local ethical committees, the institutional review board of the International Vaccine Institute (Seoul, Korea), and the Secretariat Committee for Research Involving Human Subjects, World Health Organization (Geneva, Switzerland).

During the surveillance period, 285 *S.* Typhi episodes and 84 *S.* Paratyphi A episodes were detected at the 4 sites (Table). In Indonesia, 14% of enteric fever episodes were caused by *S.* Paratyphi A, in Pakistan 15%, in India 24%, and in China 64% (Figure). The highest *S.* Typhi incidence was observed in Pakistan (394/100,000/year), and the lowest *S.* Typhi incidence was found in China (15.2/100,000/year). The highest *S.* Paratyphi A incidence was also seen in Pakistan (72/100,000/year), and the lowest *S.* Paratyphi A incidence was seen in Indonesia (13.7/100,000/year).

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Table. Population and enteric fever episodes in 4 Asian countries

Country	Pakistan	India	Indonesia	China
Site	Karachi	Calcutta	North Jakarta	Hechi City
Surveillance period	Aug 2002–Jul 2003	Sep 2003–Aug 2004	Aug 2002–Jul 2003	Aug 2001–Jul 2002
Age group surveyed (y)	2–16	All ages	All ages	5–60.9
Population under surveillance (no.)	15,219	57,075	160,257	98,376
Total enteric fever cases	71	102	154	42
No. *Salmonella enterica* serovar Typhi cases (%)	60 (85)	78 (76)	132 (86)	15 (36)
No. *S.* Paratyphi A cases (%)	11 (15)	24 (24)	22 (14)	27 (64)

Conclusions

The perception that a small proportion of enteric fever cases are caused by *S.* Paratyphi A is probably no longer true in many regions of Asia, especially in southeast China, where *S.* Paratyphi A is already more frequently isolated than is *S.* Typhi. This finding could be signaling the emergence of *S.* Paratyphi A as a pathogen in Asia. Comparison of *S.* Paratyphi A incidence during the last decade is needed to prove this hypothesis. However, none of the sites have comparable surveillance data on *S.* Paratyphi A over time. An alternative explanation is that the incidence of *S.* Typhi is decreasing. Previous reports from vaccine trials have shown successful control of *S.* Typhi but no changes in the incidence of *S.* Paratyphi A (8,10,11). Nonetheless, the reversal of the proportion of *S.* Typhi and *S.* Paratyphi A infections in Hechi City, China, is unlikely to be the result of typhoid fever control in other counties, considering the distance and oral-fecal transmission route of *S.* Typhi and *S.* Paratyphi A.

Economic growth in Asia has also resulted in improved water supply, sanitation, and hygiene; however, we have no reason to assume selective reduction only for transmission of *S.* Typhi. The incidence of typhoid fever per 100,000 in the different countries follows the same pattern as mean gross national income (2003) and the mortality ranking for children <5 years (12).

The sensitivity of blood culture for *S.* Typhi is well described, but little is known about *S.* Paratyphi A. Bacterial loads during infection are probably similar for both *S.* Typhi and *S.* Paratyphi A (J. Wain, unpub. data). Furthermore, *S.* Paratyphi A had been rarely isolated in these regions, which suggests that the increase in isolation rate of *S.* Paratyphi A is possibly caused by an increase in the number of cases of enteric fever caused by *S.* Paratyphi A rather than any bias toward blood culture–positive disease.

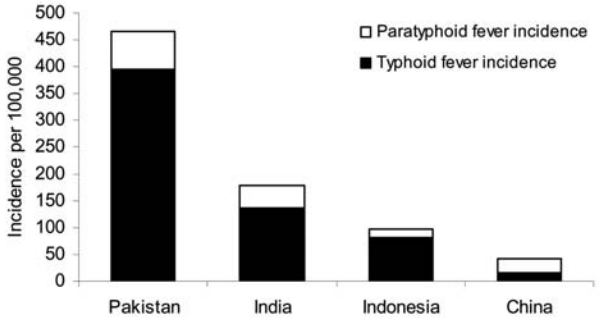

Figure. Incidence of *Salmonella enterica* serovar Typhi and *S.* Paratyphi A in 4 Asian countries.

Besides the limitation that our studies only describe a 12-month period, the population varied between sites. In Pakistan, only children 2–16 years of age were included. As *S.* Paratyphi A infections are more frequently observed in adults, including older patients in surveillance may increase *S.* Paratyphi A incidence rates reported from Pakistan.

In China and India, countries with the largest populations in the world, *S.* Paratyphi A is the causal agent for a substantial proportion of enteric fever episodes that cannot be distinguished clinically from typhoid fever episodes. While similar treatment strategies may work for both organisms, future enteric fever prevention strategies in Asia must focus on *S.* Paratyphi A as well as on *S.* Typhi, especially when considering the emergence of drug-resistant strains (13–15). Future vaccination strategies should include bivalent vaccines that protect against *S.* Typhi as well as *S.* Paratyphi A. Otherwise, the protective effectiveness of typhoid fever vaccines (Vi, Ty21a) against enteric fever may diminish, which could result in a loss of public confidence and decrease public willingness to be vaccinated.

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Social Factors Associated with AIDS and SARS

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We conducted a survey of 928 New York City area residents to assess knowledge and worry about AIDS and SARS. Specific sociodemographic groups of persons were more likely to be less informed and more worried about contracting the diseases.

Public reaction to emerging infectious diseases is a critical factor in controlling the diseases. Informed behavior change may be needed to control disease transmission. Negative public reactions, such as stigmatizing persons at risk for the disease, may greatly hamper prevention and treatment efforts (1,2). The current public health strategy to control emerging infectious diseases includes timely and complete public reporting (3). Providing timely and complete information, however, cannot determine public reaction to the information.

In this study, we examined contrasting relationships between sociodemographic characteristics and knowledge and worry about AIDS and severe acute respiratory syndrome (SARS). AIDS may be considered the prototype of an emerging infectious disease. While AIDS has received considerable public attention since the early 1980s, the strong emotions associated with it create the possibility of nonrational information processing. The stigmatization of persons with or at risk for AIDS has persisted despite public information about the disease (4). In contrast, SARS emerged quite abruptly in 2002–2003 and received intense public media attention, but the disease was declared contained by the World Health Organization in 2003 (5); little public media attention has been paid to SARS since then.

The Study

Data for this study came from a cohort of adults (≥18 years of age) who lived in metropolitan New York City (NYC). The cohort was recruited through a random digit dial telephone survey conducted from March 25 to June 25, 2002. Additional details on the sampling are provided elsewhere (6,7). The response rate was 56%. This rate is typical for well-conducted telephone surveys (7).

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A total of 1,832 respondents was interviewed from September 24, 2003, to February 29, 2004, for this study. We first asked if respondents had heard about SARS and AIDS; persons who had heard about the diseases were asked if they had heard "a great deal," "some," or "not much" about the diseases. We also asked respondents if they were "not at all worried," "somewhat worried," or "very worried" about contracting the diseases.

The analyses were weighted to correct potential selection bias related to the number of household telephones, persons in the household, and oversampling. The analyses were also weighted to make the sample demographically similar to the NYC metropolitan area population according to US Census 2000. The institutional review board of the New York Academy of Medicine approved the study.

Table 1 presents the sociodemographic characteristics of respondents and their relationships to self-reported knowledge of AIDS and SARS. In this analysis, we compared characteristics of respondents who reported knowing "nothing" or "not much" and "some" or "a lot" about the diseases. We considered respondents who reported knowing "nothing" or "not much" to be poorly informed. Five percent of the respondents reported being poorly informed about AIDS, and 21% reported being poorly informed about SARS. Table 2 presents the sociodemographic characteristics of the respondents and shows their relationship to worry about contracting AIDS or SARS. In this analysis, we examined characteristics of respondents who reported that they were "very worried" about contracting AIDS or SARS. There were no meaningful difference in the percentage of subjects who reported being "very informed" about each disease or "very worried" about contracting each disease.

The factors associated with being poorly informed and worried about contracting AIDS and SARS varied; respondents in the lower socioeconomic group were likely less informed and more worried about both of the diseases. Particularly, racial/ethnic minority status, lower formal education, and lower income were associated with being poorly informed and worried.

Being poorly informed about AIDS and being poorly informed about SARS were strongly related. Of respondents who reported being poorly informed about AIDS, 78% reported also being poorly informed about SARS; 18% of the respondents who reported not being poorly informed about AIDS reported being poorly informed about SARS ($p<0.001$). A strong relationship existed between being very worried about both diseases. Of the respondents who reported being very worried about AIDS, 16% reported also being very worried about SARS; 5% of the respondents who were not very worried about AIDS were very worried about SARS ($p = 0.016$).

Finally, we examined the relationships between being informed and worried about contracting AIDS/SARS.

Table 1. Survey findings of respondents' knowledge about AIDS and severe acute respiratory syndrome (SARS) (N = 928)*

Characteristic	Total, n (%)	Poorly informed AIDS, n (%)	p value	Poorly informed SARS, n (%)	p value
Sex					
Male	402 (45.3)	20 (8.0)	0.016	67 (21.7)	0.716
Female	526 (54.7)	13 (2.5)		102 (20.2)	
Race/ethnicity					
White	579 (54.1)	19 (2.9)	0.054	79 (14.6)	<0.0001
Asian	50 (5.0)	5 (16.3)		5 (20.8)	
Black	133 (18.9)	3 (5.7)		30 (17.5)	
Hispanic	131 (19.5)	3 (6.4)		45 (40.6)	
Other	21 (2.6)	2 (11.9)		8 (36.1)	
Age, y					
>65	147 (11.9)	12 (6.6)		52 (37.8)	
55–64	125 (12.4)	5 (9.6)		24 (24.9)	
45–54	185 (18.2)	0 (0.0)		18 (15.7)	
35–44	215 (20.7)	4 (1.8)		30 (14.7)	
25–34	185 (25.8)	7 (3.5)		29 (19.2)	
18–24	61 (11.0)	4 (16.3)		12 (19.6)	0.006
Educational status					
Graduate work	173 (13.8)	2 (3.3)	0.460	10 (12.8)	<0.0001
College degree	306 (30.0)	7 (4.7)		32 (12.8)	
Some college	172 (21.4)	2 (1.8)		23 (17.4)	
High school/general education diploma	186 (25.2)	14 (8.3)		62 (27.2)	
Less than high school	89 (9.6)	8 (7.0)		41 (48.9)	
Marital status					
Married	409 (52.9)	14 (5.6)	0.907	69 (22.4)	0.028
Divorced/separated/widowed	214 (15.8)	14 (5.2)		56 (28.5)	
Never married/unmarried couple	298 (31.3)	5 (4.1)		43 (14.7)	
Household income at baseline					
≥$75,000	262 (33.8)	4 (1.3)	0.062	22 (12.3)	0.002
$40,000–$74,999	217 (27.9)	3 (4.1)		24 (12.7)	
$20,000–$39,999	158 (23.2)	9 (7.0)		42 (28.5)	
<$20,000	130 (15.2)	8 (9.6)		47 (36.8)	
Total	928 (100.0)	33 (5.0)		169 (20.9)	

*Poorly informed respondents reported knowing "nothing" or "little" about the disease.

These analyses were confined to respondents who reported having some information about AIDS/SARS; respondents who reported that they had not heard about the diseases were not asked the follow-up questions. In these respondents, no relationship between having heard and being worried about getting the diseases was shown.

Conclusions

Given the widespread disparities in health among racial/ethnic and socioeconomic groups in the United States (8), that these factors were associated with being less informed and more worried about contracting AIDS or SARS was not surprising. The data presented here, however, are likely not related to access to healthcare services (particularly for SARS) and suggest more fundamental issues in obtaining information and developing realistic concerns about diseases. The high percentage of Spanish-speaking respondents who were poorly informed about AIDS and SARS and very worried about getting SARS suggests possible language and cultural issues in acquiring and processing information.

The data from this study were collected in a major city of an industrialized country and should not be generalized to developing and transitional countries. Nevertheless, if obtaining and evaluating information is adversely affected by factors such as low education level, low income, and ethnic minority status, then properly informing the public may be particularly difficult in developing and transitional countries. The epidemiology of AIDS and SARS has been very different in NYC (>58,097 AIDS cases [9], 9 SARS cases). Despite this difference, strong parallels existed in the relationships of socioeconomic factors to knowledge and worry about both diseases.

The limitations of this study included using single items to measure knowledge and worry about AIDS and SARS and the standard limitations of telephone surveys, e.g., inability to contact households without telephones, moderate refusal rates. However, this study strongly suggests that adequate public knowledge and emotional assessment may be critical to control these diseases.

Our data suggest that socioeconomic class and race/ethnicity factors may help shape public understanding of emerging infectious diseases. Targeted communication to different population subgroups may be required to achieve public understanding of an emerging infectious disease.

Table 2. Survey findings about respondents' worry about AIDS and severe acute respiratory syndrome (SARS)*

Characteristic	Total, n = 928 (%)	Very worried AIDS, n = 917 (%)	p value	Very worried SARS, n = 863 (%)	p value
Sex					
Male	402 (45.3)	20 (6.3)	0.553	10 (2.0)	0.006
Female	526 (54.7)	25 (5.0)		35 (8.0)	
Race/ethnicity					
White	579 (54.1)	8 (1.3)		13 (2.5)	0.028
Asian	50 (5.0)	2 (3.5)		4 (20.0)	
Black	133 (18.9)	15 (8.4)		11 (7.2)	
Hispanic	131 (19.5)	19 (15.4)		15 (8.3)	
Other	21 (2.6)	0 (0.0)		2 (3.8)	
Age, y					
>65	147 (11.9)	4 (1.2)		10 (5.4)	
55–64	125 (12.4)	3 (1.7)		6 (5.5)	
45–54	185 (18.2)	8 (7.5)		6 (7.4)	
35–44	215 (20.7)	11 (4.5)		10 (5.5)	
25–34	185 (25.8)	16 (11.0)		7 (2.6)	
18–24	61 (11.0)	3 (1.1)	0.006	5 (5.9)	0.723
Educational attainment					
Graduate work	173 (13.8)	1 (1.0)	<0.0001	4 (1.7)	0.250
College degree	306 (30.0)	5 (2.0)		13 (4.9)	
Some college	172 (21.4)	7 (1.4)		11 (7.2)	
High school/general education diploma	186 (25.2)	16 (10.4)		8 (3.6)	
Less than high school	89 (9.6)	14 (18.9)		9 (13.3)	
Marital status					
Married	409 (52.9)	15 (4.4)	0.627	13 (5.5)	0.778
Divorced/separated/widowed	214 (15.8)	10 (6.0)		14 (5.4)	
Never married/unmarried couple	298 (31.1)	19 (6.8)		15 (4.0)	
Household income at baseline					
≥$75,000	262 (33.8)	4 (1.6)	<0.001	10 (5.9)	0.197
$40,000–$74,999	217 (27.9)	6 (4.2)		6 (1.6)	
$20,000–$39,999	158 (23.2)	11 (4.7)		10 (6.7)	
<$20,000	130 (15.2)	18 (22.8)		11 (8.2)	
Total	928 (100)	45 (5.6)		45 (5.2)	

*Among those who had heard at least something about AIDS (n = 917) and SARS (n = 863), respectively.

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Assays to Detect West Nile Virus in Dead Birds

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Using oral swab samples to detect West Nile virus in dead birds, we compared the Rapid Analyte Measurement Platform (RAMP) assay with VecTest and real-time reverse-transcriptase–polymerase chain reaction. The sensitivities of RAMP and VecTest for testing corvid species were 91.0% and 82.1%, respectively.

Since the discovery of West Nile virus (WNV) in New York in 1999, an integral part of monitoring has been testing dead bird tissue by using real-time and standard reverse-transcriptase–polymerase chain reaction (RT-PCR) (1–3). The detection limit for WNV by both methods is as low as 0.08 PFU (1.9 $\log_{10}$ PFU/mL), which indicates that RT-PCR is more sensitive than cell culture and more accurately indicates infection, since RNA is more stable than infectious virus in tissues (3). Recent studies have assessed potential time- and cost-saving alternatives such as VecTest (Medical Analysis Systems Camarillo, CA, USA) (4–9). Although studies have found that VecTest, with a detection limit in mosquitoes of 5.17 $\log_{10}$ PFU/mL (10), is less sensitive than RT-PCR for detecting WNV, test sensitivity was generally high when testing swab samples from corvid species (4,6,8,9) and certain noncorvid species such as House Sparrows (*Passer domesticus*) (4) and North American Owls (family *Strigidae*) (7). Disadvantages were occasional atypical results, including false-positives (4).

In this study we evaluated another alternative for WNV detection, the Rapid Analyte Measurement Platform (RAMP, Response Biomedical Corp, Burnaby, British Columbia, Canada). Limited studies conducted at the Centers for Disease Control and Prevention and the Canadian National Microbiology Laboratory indicated that the RAMP WNV test, with detection limits in mosquitoes as low as 3.17 $\log_{10}$ PFU/mL, was more sensitive than VecTest (10). Both tests incorporate immunochromatographic test strips by using labeled antibodies to detect antigen in samples. VecTest uses antibodies bound to gold sol particle labels, while the RAMP test uses antibodies bound to fluorescently labeled latex particles. Development of a visible reddish-purple line in both the test and control zones on the VecTest strip indicates a positive result. The RAMP test strip, enclosed within a cartridge, is inserted into a reader that calculates the ratio between the fluorescence emitted at the test and control zones and displays the results as RAMP units. Values above a background threshold are recorded as positive.

This study compared WNV results from the RAMP and VecTest on oral swab samples from dead birds, with RT-PCR on brain tissue as the standard. Brain swab samples were also tested as an alternate antigen source in the RAMP and VecTest.

The Study

Birds included in this study were received from mid-May to late November 2004 and from mid-February through May 2005 from counties in New York State. Oral swab samples for the RAMP and VecTest were collected with 2 sterile, polyester fiber-tipped plastic applicators held together and moved around the oral cavity and proximal esophagus. One swab sample was twirled in 1.0 mL of VecTest buffer solution in a 5-mL plastic tube. The second swab sample was either twirled in 1.0 mL of RAMP buffer solution in a separate 5-mL plastic tube or placed in an empty 5-mL plastic tube, capped, and frozen at –20ºC for later testing. RAMP tests were run the same day on fresh material or later on frozen samples. Before being tested, all frozen samples were thawed at room temperature; swabs not previously mixed in solution and swabs from thawed carcasses were then mixed in RAMP buffer solution. Samples were taken from the brains of a subset of corvid species by swabbing cerebral parenchyma and processing as for oral samples. The RAMP and VecTest were run according to manufacturers' directions in a class II biosafety cabinet at the New York State Department of Environmental Conservation's Wildlife Pathology Unit. RAMP test values $\geq$50, calculated by the RAMP reader, were recorded as positive. Differences in test performance were assessed by chi-square analysis. Data are expressed as a percentage in text and tables only when n is $\geq$10.

Brain samples for RT-PCR were taken at necropsy and frozen at –20ºC. Brain tissue was analyzed at the Arbovirus Laboratory, Wadsworth Center, New York State Department of Health, as described previously (2,3). RT-PCR was repeated on 54 birds for which results from RAMP, VecTest, or both, contrasted with RT-PCR results. Retests of 6 birds yielded different results from the original tests. Three of these were initially positive and retested negative; the original values were low, which indicated infectivity was focal and undetected on a different sample,

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and the level was below RAMP and VecTest limits of detection. Three originally negative samples retested positive; 2 were highly positive, which indicated a technical error, and 1 kidney tissue sample was positive, although results of a retest with brain tissue were negative.

In this study, oral samples from 679 birds were tested; 193 (28.4%) were WNV-positive by RT-PCR. RAMP sensitivity was 80.8%, compared to 71.0% for VecTest (Table 1). For corvid species (n = 156), RAMP sensitivity (91.0%) was significantly greater than that of VecTest (82.1%) ($p \leq 0.025$). With smaller sample sizes at the species level, sensitivity between RAMP and VecTest did not differ significantly ($p > 0.05$) for 128 American Crows (*Corvus brachyrhynchos*) (91.4% and 84.4%, respectively) and 27 Blue Jays (*Cyanocitta cristata*) (88.9% and 70.4%, respectively) tested, nor for interspecies differences within each test. The detection thresholds of these tests, coupled with viral titers of specimens, may explain these different results.

RAMP confirmed more Common Grackles (*Quiscalus quiscula*) (3/3) and House Sparrows (5/6) as positive than did VecTest (2/3 and 3/6, respectively). With the exception of a few species, both tests performed poorly overall on small sample sizes of other noncorvid species.

To determine if RAMP results were affected by freezing the sample, samples from 13 corvids (10 positive, 3 negative) were retested by using swabs taken from frozen carcasses. Six initially were tested with fresh swabs and 7 with frozen swabs; all retests yielded results similar to initial results. The same results for fresh versus frozen samples were obtained with VecTest (4).

VecTest specificity with oral swabs was excellent in correctly identifying all 486 RT-PCR–negative birds, returning no false-positive results (Table 2). RAMP had high specificity for American Crows (98.5%), Blue Jays (90.9%), and noncorvid species (98.9%).

Brain swab samples from 39 corvids were tested; 27 (69.2%) were RT-PCR–positive. Both RAMP and VecTest performed well, with sensitivities of 92.6% and 88.9%, respectively, and no false-positive results.

Conclusions

Although RAMP was more sensitive than VecTest, both appear adequate for WNV surveillance in dead corvids. RAMP also performed well with oral swabs from Common Grackles and House Sparrows, although sample sizes were small. These findings are similar to previous results for VecTest, which also tested well with House Finches (*Carpodacus mexicanus*), Northern Cardinals (*Cardinalis cardinalis*), and American Kestrels (*Falco sparverius*) (4). As in the previous study, both tests did poorly in RT-PCR–positive raptors.

Table 1. Oral swab RAMP and VecTest sensitivity for real-time RT-PCR–positive birds. New York, 2004–2005*

Species (presented in taxonomic order)	N†	No. positive (%)	
		RAMP	VecTest
Pelicaniformes			
Double Crested Cormorant (*Phalacrocorax auritus*)	1	0	0
Falconiformes			
Cooper's Hawk (*Accipiter cooperii*)	4	0	0
Red-tailed Hawk (*Buteo jamaicensis*)	2	0	0
American Kestrel (*Falco sparverius*)	1	1	1
Charadriiformes			
Ring-billed Gull (*Larus delawarensis*)	1	1	0
Strigiformes			
Great Horned Owl (*Bubo virginianus*)	3	1	0
Passeriformes			
Eastern Kingbird (*Tyrannus tyrannus*)	1	0	0
Red-eyed Vireo (*Vireo olivaceus*)	1	0	0
Blue Jay (*Cyanocitta cristata*)	27	24 (88.9)	19 (70.4)
American Crow (*Corvus brachyrhynchos*)	128	117 (91.4)	108 (84.4)
Fish Crow (*Corvus ossifragus*)	1	1	1
American Robin (*Turdus migratorius*)	7	1	1
Gray Catbird (*Dumetella carolinensis*)	1	0	0
Northern Mockingbird (*Mimus polyglottos*)	2	1	1
Cedar Waxwing (*Bombycilla cedrorum*)	1	1	1
Northern Cardinal (*Cardinalis cardinalis*)	1	0	0
Common Grackle (*Quiscalus quiscula*)	3	3	2
House Finch (*Carpodacus mexicanus*)	2	0	0
House Sparrow (*Passer domesticus*)	6	5	3
Total all species	193	156 (80.8)	137 (71.0)

*RAMP, Rapid Analyte Measurement Platform; RT-PCR, reverse transcription–polymerase chain reaction.
†No. of birds real-time RT-PCR–positive.

Table 2. Oral swab RAMP and VecTest specificity for real-time RT-PCR–negative birds, New York, 2004–2005*

Species (presented in taxonomic order)	N†	No. negative (%)	
		RAMP	VecTest
Galliformes			
Ruffed Grouse (*Bonasa umbellus*)	12	12	12
Columbiformes			
Mourning Dove (*Zenaida macroura*)	24	24	24
Rock Dove (*Columba livia*)	12	12	12
Passeriformes			
Blue Jay (*Cyanocitta cristata*)	22	20 (90.9)	22
American Crow (*Corvus brachyrhynchos*)	198	195 (98.5)	198
Fish Crow (*Corvus ossifragus*)	2	2	2
American Robin (*Turdus migratorius*)	22	20 (90.9)	22
Gray Catbird (*Dumetella carolinensis*)	14	13 (92.9)	14
European Starling (*Sturnus vulgaris*)	11	11	11
Brown-headed Cowbird (*Molothrus ater*)	10	10	10
Common Grackle (*Quiscalus quiscula*)	19	19	19
House Sparrow (*Passer domesticus*)	22	22	22
Other species‡	118	118	118
Total all species	486	478 (98.4)	486

*RAMP, Rapid Analyte Measurement Platform; RT-PCR, reverse transcription–polymerase chain reaction.
†No. real-time RT-PCR–negative birds.
‡52 species, representing 14 orders.

In the previous New York study, VecTests successfully tested brain, kidney, blood, feather pulp, and cloacal samples from corvids and House Sparrows (4). In the current study, RAMP and VecTest worked well with brain as the antigen source. Brain swab samples may be the preferred antigen source when the oral cavity is compromised. Further testing of alternative swab samples is warranted and may identify a superior antigen source; however, testing internal organs may pose greater risks and may not be applicable in field work and nonlaboratory-based surveillance. In addition, further testing, including immunohistochemical tests, on noncorvids should be conducted to accurately assess these tests and identify the distribution of WNV in the oral cavity and internal tissues.

In this study, VecTest produced no false-positive results. Although its specificity was high, RAMP produced 8 false-positive results (range 50.9–147.6). Four of these were near the ≥50 positive indicator level and may have been due to other sources of fluorescence. The remaining 4 false-positives (3 American Crows and 1 Blue Jay), with scores from 74.4 to 147.6, came from birds with oral cavities compromised by blood or fly eggs, which may have biased results.

VecTest results are easily distinguished when a true WNV-positive reaction occurs, but the reddish-purple line may appear faint or thin in other cases and may be subject to interpretation (4). RAMP quantitative results eliminate subjective interpretation, which helps assure replication but limits confidence in lower RAMP-positive scores.

The RAMP system requires an initial purchase of an electronic reader (≈US $3,500), and materials cost $13–$15 per test; VecTest costs $8 per test. If large numbers of specimens are tested, the cost of the RAMP reader per test is minimal. The RAMP test requires a minimum of 1.5 h to run because of the required cartridge drying time; VecTest takes 15–30 min to run after the test strip is placed in the sample solution.

In conclusion, both RAMP and VecTest are useful alternatives to RT-PCR for WNV surveillance in dead corvids and some passerine species when immediate turn-around of large numbers of specimens is valuable. Testing with RAMP is advantageous because of its increased sensitivity; however, follow-up testing with RT-PCR is recommended for low RAMP-positive results near the positive indicator level. Using both tests in a system in which initial testing is conducted with VecTest may also be useful; RAMP could be reserved for high-priority cases in which VecTest results are negative. RT-PCR should still be used to confirm initial viral activity in a new period and area and for research requiring more definitive results.

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West Nile Virus Epidemic, Northeast Ohio, 2002

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Serum samples and sociodemographic data were obtained from 1,209 Ohio residents. West Nile virus immunoglobulin M (IgM) and IgG antibodies were detected by enzyme-linked immunosorbent assay and confirmed. Children were 4.5 times more likely to become infected yet 110 times less likely to have neuroinvasive disease develop.

Since its 1999 North American introduction, West Nile virus (WNV) has emerged as an important cause of illness and death. Although several at-risk populations have been identified, older age remains the major risk factor for developing encephalitis after infection (1–4).

WNV rapidly spread across the United States, resulting in intense epidemic activity in Louisiana, Illinois, Michigan, and Ohio in 2002; Colorado in 2003; and Arizona and California in 2004 (5,6). In Ohio, WNV infections were first recognized in animals in 2001. In 2002, Ohio reported 341 human cases of WNV encephalitis or meningitis (West Nile neuroinvasive disease [WNND], incidence: 28 cases/million population) with 31 deaths. In 2002, Cleveland and surrounding Cuyahoga County (2000 population 1,393,978 of whom 1,302,982 were ≥5 years of age) reported 221 laboratory-confirmed cases of WNV illness, including 155 WNND cases (111 cases/million population) with 11 deaths from July 30 to October 3. All reported WNND patients (median age 61 years, range 11–98 years) were hospitalized (CDC ArboNET Surveillance Network, unpub. data).

Since most WNV infections are asymptomatic (7,8), the true rate of WNV infection can best be estimated by measuring the prevalence of WNV-specific antibody in a recently exposed population. In December 2002, the Cuyahoga County public health community conducted a household-based seroprevalence survey to estimate neighborhood and countywide WNV infection rates.

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The Study

The survey was conducted December 5–12, 2002. Stratified multistage cluster sampling was used to estimate countywide and subpopulation prevalence rates. The county was divided into 3 risk strata (Table 1). Census tracts were sampled within strata with probability proportional to population. Within each census tract, clusters of ≈50 households were formed. At random points, residents were approached for recruitment until 10 participating households were enrolled from each cluster.

Residents ≥5 years of age who had lived in the household since July 1, 2002, were asked to participate by providing a blood sample and responding to a questionnaire. One person from each household completed a questionnaire about the home environment. Questionnaires developed by the Centers for Disease Control and Prevention (CDC) were used (10). Informed consent was obtained from all participants or their legal guardian. Assent was obtained from minors ≥8 years of age. Residents were offered a US $10 gift certificate and test results as compensation. Persons who were pregnant, mentally handicapped, or taking anticoagulants were not enrolled. Institutional review board approval was obtained from University Hospitals of Cleveland.

Serum samples were screened with a WNV-specific immunoglobulin M (IgM) antibody-capture (MAC) enzyme-linked immunosorbent assay (ELISA) (11) and indirect IgG ELISA at Focus Laboratories (Cypress, CA, USA). Positive IgM and IgG were defined as an antibody index ≥2.0 and ≥0.9, respectively. All IgM- and IgG-positive samples were sent to the Viral and Rickettsial Laboratory, California Department of Health Services (Richmond, CA, USA) for confirmatory plaque reduction neutralization tests to identify WNV and St. Louis encephalitis virus (SLEV)–specific neutralizing antibody. At the second laboratory, WNV MAC-ELISAs (12) were repeated and IgG ELISAs for WNV, SLEV, and dengue were performed (13). Laboratory-based case definitions were developed (Table 2).

SPSS version 11.5 (SPSS Inc, Chicago, IL, USA) and SUDAAN version 8.0 (Research Triangle Institute, Research Triangle Park, NC, USA) were used for preliminary analyses and to assess differences in demographics, behavior, and clinical characteristics between seropositive and seronegative persons. Since SUDAAN variance estimation did not accommodate our complex sample design, we developed formulas to provide better estimates of variance and confidence intervals (CIs) using an $\alpha = 0.05$. Unless noted, all analyses were weighted. Individual weights were derived by taking the inverse of the probability of selection.

The standard Horvitz-Thompson estimator was used for point estimation (14). For variance estimates, all

Table 1. WNV seroprevalence*

Seroprevalence	No. positive/no. tested	Weighted % (95% CI)
Overall	34/1,209	1.9 (0.8–4.6)
Age-specific		
5–17 y	4/168	6.5 (4.3–9.5)
18–64 y	25/790	1.3 (0.4–4.5)†
≥65 y	5/219	1.4 (0.4–4.5)‡
Strata-specific		
More human illnesses reported; higher MIR (stratum 1)§¶	16/463	2.5 (0.6–9.2)
Fewer human illnesses reported; varying MIR (stratum 2)#	7/453	1.5 (0.2–4.4)
No human illnesses reported; varying MIR (stratum 3)**	11/293	3.3 (0.4–23.9)

*WNV, West Nile virus; CI, confidence interval; MIR, minimum infection rate.
†Significant difference between 5- to 17-year-old and 18- to 64-year-old patients ($p<0.02$).
‡Significant difference between 5- to 17-year-old and ≥65-year-old patients ($p<0.01$).
§Reference 9.
¶Stratum 1 included neighborhoods with at least 9 reported human cases, a WNV case rate >4.5/10,000, and mosquito MIR ≥15/1,000.
#Stratum 2 included neighborhoods with at least 1 reported human case, a WNV case rate <4.5/10,000, and varying levels of MIR (0–54/1,000).
**Stratum 3 included neighborhoods with no known human cases and varying levels of MIR.

sources of variation that resulted from the selection process were included by using standard Taylor series approximations. To calculate the confidence interval for the true prevalence ratio (PR), we approximated the variance of the logarithm of the sample PR by using standard Taylor series method. The end points of this interval were exponentiated to obtain the interval for PR.

Conclusions

Participants were recruited from 13 Cuyahoga County municipalities and 9 Cleveland neighborhoods. Of 4,676 households visited, 2,318 households had an eligible adult present; of these eligible households, 819 households (35.3%) agreed to participate. Of 1,747 eligible residents in 819 households, 1,251 (71.6%) consented to participate; 42 participants in 13 households had insufficient serum samples and were excluded. The study sample consisted of 1,209 participants from 806 households; they had a mean age of 43.2 years (range 5–94 years) and included 168 (12.4%) children 5–17 years of age. Compared to 2000 Cuyahoga County census demographics, our study sample contained a significantly larger proportion of adults 18–64 years of age (75.7% vs. 63.5%), female participants (57.8% vs. 52.8%), and African Americans (31.8% vs. 27.4%).

Initial screening identified WNV IgM and IgG antibody in 4 serum samples, IgG only in 90 serum samples, and IgM only in 2 specimens. Based on criteria listed in the Table 2, confirmatory testing of the 96 samples identified 27 confirmed and 7 probable WNV-infected persons. The countywide seroprevalence rate was 1.9% (95% CI 0.8–4.6) (Table 1), which suggests that 10,400–59,900 residents were infected. Based on 155 WNND cases reported from Cuyahoga County, ≈1 WNND case occurred for every 160 infected persons (95% CI 1:67–1:386).

Seroprevalence varied significantly between age groups ($p<0.05$) (Table 1). Based on reported WNND cases and age-stratified seroprevalence rates, we estimate that 1 case of WNND occurred per 4,167 infected children 5–17 years of age, per 154 infected adults 18–64 years of age, and per 38 infected persons ≥65 years of age (Figure). Strata-specific seroprevalence values ranged from 1.5% to 3.3% but were not statistically different (Table 1).

In 2002, Cuyahoga County experienced its largest epidemic of arboviral encephalitis and meningitis, yet only 1.9% of the county's population became infected during

Table 2. Laboratory-based definitions used for confirmatory testing*†‡

Case	Definition
Confirmed WNV infection	WNV IgM MAC-ELISA positive and WNV PRNT titer ≥1:20 and WNV PRNT titer ≥2-fold than SLEV PRNT titer **or** WNV PRNT titer ≥1:20 and WNV PRNT titer ≥4-fold than SLEV PRNT titer
Probable WNV infection	WNV PRNT titer ≥2-fold than SLEV PRNT titer
Previous SLEV infection	SLEV PRNT titer ≥1:20 and SLEV PRNT titer ≥2-fold than WNV PRNT titer
Probable nonspecific flavivirus infection	Negative WNV and SLEV PRNT results **and** Negative WNV IgM MAC-ELISA results **and** Positive WNV, SLEV, or dengue IgG EIA results **and** No history of YFV or JEV vaccination
Previous infection	History of YFV or JEV vaccination, WNV IgM MAC-ELISA negative, and WNV and SLEV PRNT negative

*WNV, West Nile virus; IgM, immunoglobulin M; MAC-ELISA, IgM antibody capture enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; SLEV, St. Louis encephalitis virus; EIA, enzyme immunoassay; YFV, Yellow fever virus; and JEV, Japanese encephalitis virus.
†All specimens referred for confirmatory testing were positive for WNV IgG during initial screening.
‡Case definitions were developed in consultation with the Centers for Disease Control and Prevention and the Ohio Department of Health.

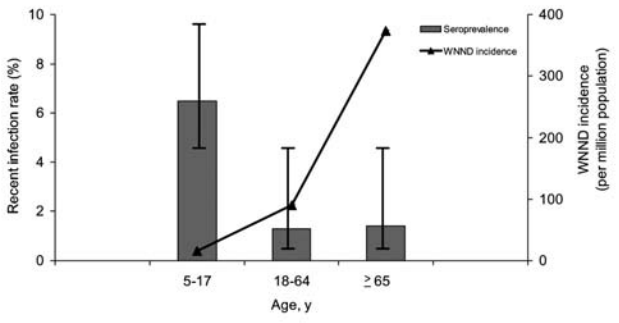

Figure. Comparison of age-stratified seroprevalence rates (gray bars) to the age-stratified incidence of West Nile neuroinvasive disease (WNND) (black line). Seroprevalence rates were measured in the 2002 seroprevalence study. The incidence of WNND was based on cases reported through the local disease reporting system during the 2002 transmission season.

this first WNV transmission season. In the 733-km^2 area of Cuyahoga County, 155 cases of encephalitis and meningitis (WNND incidence: 111 cases/million population) occurred; the seroprevalence was 1.9% countywide and 2.5% in the selected highest risk survey stratum.

Little is known about WNV infection rates in children (15). In contrast to a previous study (8), our study demonstrated an age-dependent risk for WNV infection. The antibody prevalence in the 5- to 17-year age group was significantly greater than in older age groups. These data suggest that children were 4.5 times more likely to be infected than older persons. In this study, children reported spending more time outdoors and using less personal protective measures, which likely contributed to their higher seroprevalence rate. In 2002, only 4 cases of WNND were reported in the 5- to 17-year age group, resulting in a WNND:infection ratio of 1:4,200 compared to a 1:38 ratio among persons ≥65 years of age. Thus, the risk for WNND after infection may be as much as 110× greater in adults ≥65 years of age, as compared to children. Inclusion of a larger number of children in this study compared to previous studies allowed these age-stratified analyses to be completed.

Although WNV seroprevalence was similar to those measured in previous outbreaks (7,8), our study was the first to demonstrate that the risk for WNV infection can be age-dependent. Children had a higher rate of infection than adults, but serious neurologic disease developed in few of them. This finding has implications for public health practice and emphasizes the need for children to use protective measures to prevent mosquito bites to further lower their risk for infection with WNV and other mosquitoborne viruses.

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Quarantine Stressing Voluntary Compliance

**Cleto DiGiovanni,* Nancy Bowen,†
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A 1-day table-top exercise in San Diego, California, in December 2004 emphasized voluntary compliance with home quarantine to control an emerging infectious disease outbreak. The exercise heightened local civilian-military collaboration in public health emergency management. Addressing concerns about lost income by residents in quarantine was particularly challenging.

In a study of quarantine implementation in the greater Toronto area during its 2003 outbreak of severe acute respiratory syndrome (SARS) (1), investigators identified several factors that influenced compliance with quarantine restrictions (with rare exceptions, identified contacts of SARS patients in the area were quarantined in their own homes). Key factors were fear of income lost while quarantined, consistent information about the threat and measures to contain it, and adequate logistic and psychosocial support to those confined. Using these findings, we organized a 1-day table-top exercise in San Diego, California, in late 2004 that revolved around an emerging infectious disease caused by an easily transmitted novel respiratory virus.

The Study

Our twin objectives were to promote coordinated implementation of quarantine measures by the several local military and civilian agencies and jurisdictions in San Diego County and to emphasize initial decisions that encouraged voluntary compliance. In this exercise, quarantine referred to restriction at home of possible carriers of the agent, who were identified through contact tracing. We also challenged local military commanders to preserve operational readiness as scenario events unfolded.

Before this exercise, quarantine plans in San Diego County were in their formative stages. We did not, therefore, organize a conventional table-top exercise that tested an existing plan and identified its gaps. Instead, we used the exercise as a stimulant for further developing that plan; throughout the several months that preceded the exercise, we worked closely with participants to ensure that the exercise achieved their objectives and goals.

Contact tracing, issuing verbal quarantine orders and instructions, and delivering news to the public were generally well coordinated. When concerns about privacy issues arose in framing public announcements, a US Department of Health and Human Services representative assured participants that Health Insurance Portability and Accountability Act Privacy Rule provisions would not restrict release of protected health information, as necessary, to inform the public of the crisis and instruct them about protective measures.

The region's military public affairs officers formed a joint information bureau to prepare press releases and sent a representative to the county's emergency operations center to coordinate military-civilian announcements to the public. Civilian and military mental health providers, however, lacked coordination in the delivery of their services.

County officials established a phone bank with a single 800-number for the use of all county residents (military and civilian), staffed it with operators from several agencies, and provided the operators with Internet-based training to promote consistency in information released to the public. Service organizations provided logistic support to those in home quarantine.

Military commanders pledged cooperation with civilian public health and emergency operations center officials but were unable to commit resources in advance because they needed to protect their operational readiness. The San Diego County superintendent of schools, who coordinates all public schools in the county, including those on military bases, organized Web- and television-based distance-learning programs so that quarantined students could continue to receive instruction and submit assignments.

Civilian law enforcement officials characterized the public health crisis in the exercise scenario as "unprecedented." They also frequently expressed concerns about carrying out enforcement measures that were requested by civilian public health authorities and urged county officials to emphasize public education to minimize the need for enforcement.

When members of the community who were ordered into quarantine raised questions about payment of their wages and salaries while away from their jobs, answers varied. Military personnel were assured of continuing income, but some government employees and the privately employed or self-employed received no assurances. This issue remained unresolved throughout the exercise because no program or funds to compensate for income lost by still-healthy contacts while in quarantine existed at the local, state, or federal level. In the absence of income insurance, officials could only appeal to the public's sense of civic responsibility to comply with quarantine restrictions.

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Border health officials agreed that health warnings could be posted, and perhaps health-related information provided, at the county's border crossings with Mexico, but any general increase in intensity of health screenings there that lengthened processing of border crossers even minimally would impede the flow of traffic and become untenable. (The San Ysidro border crossing, between San Diego County and Mexico, is the busiest US port of entry. Future construction of new traffic lanes may allow more time for health screenings, when necessary.) Making border health screenings even more problematic at the time of this exercise was the shortage of both federal and county personnel to carry them out.

Conclusions

Several key lessons were identified for future exercise planning. First, memoranda of agreement (MOA) or understanding (MOU) should be developed. Although San Diego County has had a variety of real-world disasters, it had relatively little experience with an evolving public health crisis as depicted in the scenario. Thus, forming collaborative and coordinated incident command among local civilian and military officials took some effort. The lack of either formal collaborative agreements or even informal collaborative relationships among local civilian and local military commanders in the overall management of a declared communitywide public health emergency was highlighted by this exercise. In areas with multiple jurisdictions, whether civilian, military, federal, or tribal, planning for quarantine must be based on collaborative command and control, best articulated in MOA or MOU.

Second, early collaboration of nonmedical officials in exercise planning should be obtained. Quarantine measures are initiated by health officers, but they are largely implemented by nonmedical personnel. Success of our exercise required active collaboration by a wide variety of local public officials and nonmedical military officers; some required convincing that they had crucial roles in what they initially thought was a purely medical exercise. Communities planning to exercise quarantine in collaboration with local military commanders should enlist, early in their planning, the cooperation of local military force protection as well as medical corps officers. Private sector participation in this exercise was valuable. Implementing quarantine in a community will have an immediate impact on the business sector, and its cooperation will promote compliance. Administrative, nursing, and medical representatives from the private hospitals in the community also need to participate and develop consistent approaches to human resources issues, such as pay and benefits for quarantined employees, including those in work quarantine, i.e., those who may have to leave their homes and return to work because of critical staff shortages during an epidemic.

A third key lesson is to include likely challenges, even sensitive ones without current solutions. This exercise did not resolve the critical issue of income protection for those in quarantine but did identify the lack of this protection as a potential impediment to voluntary compliance with quarantine, highlight its importance for further discussion, and force participants to craft appeals to the residents' sense of civic responsibility to mitigate concerns of income loss.

A fourth lesson is to use exercises to help develop quarantine plans, not only to test them. Web-based guidance by the federal government to civilian public health authorities (2) and Department of Defense directives to military commanders (3) will encourage quarantine planning. Staging an exercise will develop this capacity and pinpoint deficiencies in interagency collaboration.

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Anaplasma phagocytophilum–infected Ticks, Japan

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We report *Anaplasma phagocytophilum* infection of *Ixodes persulcatus* and *I. ovatus* ticks in Japan. Unique *p44/msp2* paralogs (and/or 16S rRNA genes) were detected in tick tissues, salivary glands, and in spleens of experimentally infected mice. These findings indicate the public health threat of anaplasmosis in Japan.

Anaplasma phagocytophilum (formerly known as the agent of human granulocytic ehrlichiosis), *Ehrlichia phagocytophila*, and *E. equi* (1) are tickborne human pathogens of veterinary importance. They cause an emerging infectious and febrile systemic illness now known as human granulocytic anaplasmosis. The first case of human infection by *A. phagocytophilum* was reported in 1994 (2). Since then, an increasing number of cases have been recognized in the United States. Severities of this disease range from asymptomatic seroconversion to death, and severe illness is frequently documented. In Europe, the first human cases of this disease were described in 1997 (3), and serologic and polymerase chain reaction (PCR) analyses suggest that *A. phagocytophilum* is distributed throughout Europe and in some parts of the Middle East and Asia (4–6).

In nature, *A. phagocytophilum* is believed to be maintained in a tick-rodent cycle. The known vectors for this agent are *Ixodes* ticks, i.e., *Ixodes scapularis* and *I. pacificus* in the United States, *I. ricinus* mostly in Europe, and *I. persulcatus* in Russia (7) and China (5). Exposure to *A. phagocytophilum*–infected tick bites is the most common route of human infection, except for perinatal transmission or contact with infected mammalian blood (8,9).

In Japan, several *Ixodes* species, such as *I. persulcautus*, *I. ovatus*, and *I. monospinosus*, are potential vectors for transmission of *Borrelia* spp., *Rickettsia* spp., or *Ehrlichia* spp. (10–12). However, little information is available regarding the ecologic and epidemiologic features of clinical cases of infection with *A. phagocytophilum* in Japan. We report infection with *A. phagocytophilum* in *Ixodes* ticks in central Japan determined by molecular epidemiologic approaches.

The Study

In 2003 and 2004, a total of 273 unfed and adult *Ixodes* ticks (114 *I. persulcatus* and 159 *I. ovatus*) were collected in central Japan (Figure 1). Of these, 123 live ticks were dissected, and DNA was isolated from whole tissues of 73 ticks and salivary glands of 50 ticks by using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). For detection of *A. phagocytophlilum* DNA, a nested PCR using primers designed based on the highly conserved region of *p44/msp2* paralogs of (p3726 [5′-GCTAAGGAGTTAGCTTATGA-3′], p3761, p4183, and p4257) was conducted (12–14). Four (12.1%) of 33 *I. persulcatus* ticks collected at the Utsukushinomori (UM) site in Yamanashi Prefecture were positive by PCR (Table). Sixteen (7 *I. persulcatus* and 9 *I. ovatus*) (32%) of 50 salivary glands from ticks collected at the Takabachi and Mizugazuka sites in Shizuoka Prefecture were positive by PCR. Data indicated that *I. persulcatus* and *I. ovatus* in Japan are naturally infected with *A. phagocytophilum* and that ticks at certain sites are highly infected.

We further examined the infection of immunocompromised mice with *A. phagocytophilum* in ticks by using the procedure described previously (12). Briefly, whole tissues from 150 live ticks (55 *I. persulcatus* and 95 *I. ovatus*) were pooled and intraperitoneally injected into 15 ddY male mice (6–15 pooled ticks per mouse) treated with the

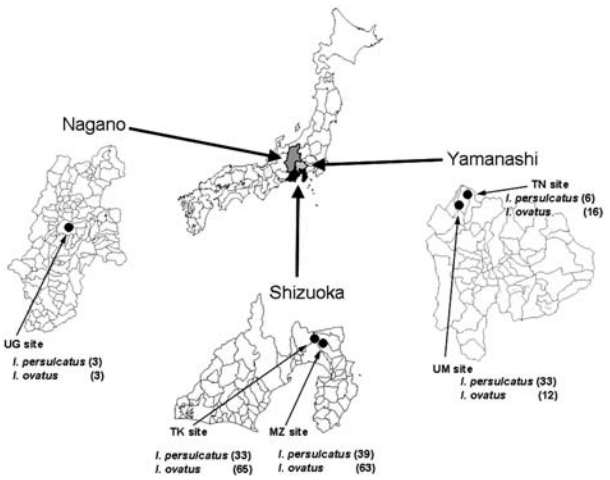

Figure 1. Areas in Shizuoka, Nagano, and Yamanashi Prefectures of Japan where *Ixodes persulcatus* and *I. ovatus* ticks were collected in 2003 and 2004. Closed circles indicate collection sites. Numbers of ticks collected at each site are shown in parentheses. UG, Utsukushigahara; TK, Takabachi; MZ, Mizugazuka; TN, Tennyosan; UM, Utsukushinomori.

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Table. Polymerase chain reaction (PCR) detection of *A. phagocytophilum p44/msp2* paralogs from *Ixodes* ticks or spleens of mice experimentally infected with tick tissues

	Whole tissue†		Salivary gland†	Experimental infection with ticks‡		
Collection site, year*	Female	Male	Female	Female	Male	Total
I. persulcatus						
Yamanashi, 2004						
TN	0/2	0/4				0/6
UM	2/16	2/17				4/33
Nagano, 2004						
UG	0/3	0/0				0/3
Shizuoka, 2004						
TK			6/9			6/9
MZ			1/8			1/8
Shizuoka, 2003						
TK				0/14 (2)	0/10 (1)	0/24 (3)
MZ				0/22 (2)	0/9 (1)	0/31 (3)
Total	2/21	2/21	7/17	0/36 (4)	0/19 (2)	11/114 (6)
I. ovatus						
Yamanashi, 2004						
TN	0/8	0/8				0/16
UM	0/9	0/3				0/12
Nagano, 2004						
UG	0/1	0/2				0/3
Shizuoka, 2004						
TK			9/17			9/17
MZ			0/16			0/16
Shizuoka, 2003						
TK				0/32 (3)	1/16 (2)	1/48 (5)
MZ				0/26 (2)	0/21 (2)	0/47 (4)
Total	0/18	0/13	9/33	0/58 (5)	1/37 (4)	10/159 (9)

*TN, Tennyosan; UM, Utsukushinomori; UG, Utsukushigahara; TK, Takabachi; MZ, Mizugazuka.
†No. positive/no. examined. One hundred twenty-three ticks were dissected (whole tissues from 73 ticks and salivary glands from 50) were individually examined by PCR.
‡No. of positive mouse spleens/no. of ticks examined (no. of mice used). Six to 15 ticks were pooled and homogenized (55 *I. persulcatus* and 95 *I. ovatus*), and intraperitoneally injected into ddY male mice.

immunosuppressant cyclophosphamide. PCR was conducted with DNA isolated from blood and spleens of these mice. Only 1 of 9 spleens from *I. ovatus*-injected mice was positive by PCR (Table). We previously detected *Ehrlichia* spp. DNA in *I. ovatus*–injected mice, but did not detect *A. phagocytophilum* DNA in *I. ovatus*– or *I. persulcatus*–injected mice (12) because we used only a few immunocompromised mice, i.e., most had normal immune systems. Thus, we treated all 15 mice used in the present study with cyclophosphamide. Results indicate that *A. phagocytophilum* in *I. ovatus* can be infective for immunocompromised mice, although the efficiency of infection was low (1/95 [1.1%]).

The *p44/msp2* amplicons from 8 PCR-positive ticks and 1 PCR-positive mouse were cloned into a pCR2.1 vector with the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Recombinant clones were randomly selected and 28 recombinant *p44/msp2* clones were sequenced with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). A phylogenetic tree was constructed based on the alignment of Japanese *p44/msp2* sequences and the most closely related paralogs (220–400 bp) by using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/), followed by the neighbor-joining method with 1,000 bootstrap resamplings (Figure 2). In this tree, the *p44/msp2* sequences obtained from *I. ovatus* were located mostly in clusters different from those where sequences from *I. persulcatus* were located, except for Tick41-1. This finding suggests that *A. phagocytophilum* in *I. ovatus* may encode *p44/msp2* paralogs distinct from those of *A. phagocytophilum* in *I. persulcatus*. A previous study suggested that the *p44/msp2* sequences from the United States and the United Kingdom can be divided into 27 similarity groups based on >90% similarities of DNA sequences, and most sequences from the United Kingdom are distinguishable from those from the United State because of the similarities <79% (15). Of 28 Japanese *p44/msp2* sequences in this study, 11 sequences with similarities >85.6% to the previously identified paralogs were probably divided into 8 similarity groups (Figure 2). Of the remaining 17 sequences with similarities <73.1%, 11 members that were grouped into 2 distinctive clusters (Figure 2) and 6 members that were individually located (Figure 2, arrows) were distinguishable from the 8 similarity groups. Thus, some *p44/msp2* paralogs of Japanese *A. phagocytphilum* are unique and distinct from those of

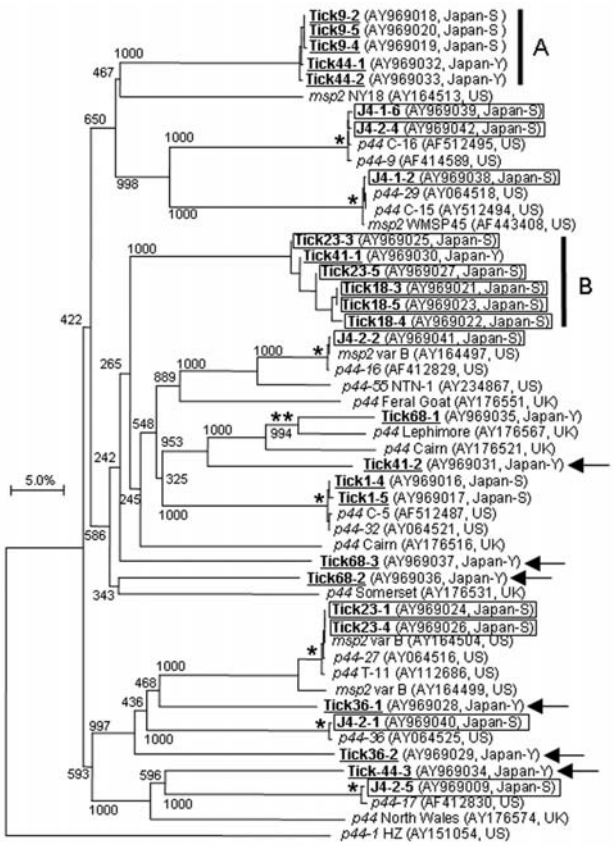

Figure 2. Phylogram of *Anaplasma phagocytophilum p44/msp2* including Japanese paralogs. A) Cluster from *Ixodes persulcatus*. B) Cluster from *I. ovatus*, except for Tick41-1. The tree was constructed using the neighbor-joining method. Numbers on the tree indicate bootstrap values for branch points. Japanese *p44/msp2* paralogs from *Ixodes persulcatus* and *I. ovatus* are underlined and boxed, respectively, in bold. A single star shows *p44/msp2* clusters with 99.2%–100% similarities and double stars show a cluster with 85.6% similarity. Two vertical bars and 6 arrows indicate Japanese *p44/msp2* clusters and paralogs, respectively, which are distinct from the previously identified *p44/msp2* (<73.1% similarity). A horizontal bar indicates percentage of sequence divergence. Accession numbers and location (Japan-Y [Yamanashi], Japan-S [Shizuoka], US [United States], and UK [United Kingdom]) are in parentheses.

A. phgocytophlium in other countries, although multiple copies of *p44* in the genome of an organism should be considered (13).

A partial sequence of the 16S rRNA gene of *A. phagocytophilum* (1.4 kb) from a *p44/msp2* PCR-positive mouse was amplified from spleen DNA with primers ER5-3, ER-R1, AP-F1, and AP-R1 (12), cloned, and sequenced. Similarities among 6 Japanese recombinant 16S rRNA sequences (GenBank accession nos. AY969010–AY969015) were 99.3%–99.6%. When compared with *A. phgocytophilum* human agent U02521, the similarities were 99.6%–99.8% between individual 16S rRNA cloned sequences and human agent U02521. Because we used pooled ticks to examine infection in mice, these sequence diversities may depend on genetic variants (or a heterogeneous population) of *A. phagocytophilum* from individual ticks. When the amplicon was directly sequenced, its sequence was identical with that of human agent U02521.

Conclusions

We demonstrated that *A. phagocytophilum* infects *Ixodes* ticks in Japan, that both *I. persulcatus* and *I. ovatus* ticks are naturally infected with *A. phgocytophilum*, that *A. phagocytophilum* may be transmitted by *Ixodes* ticks because of organisms in the salivary glands of unfed and female adult ticks, and that immunocompromised mice can be infected with *A. phagocytophilum*. This study provides new information on the ecologic, biologic, and public health significance of *A. phagocytophilum* and emphasizes the threat of anaplasmosis in Japan.

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Tickborne Relapsing Fever in Israel

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We evaluated the epidemiology of relapsing fever from 1971 to 2003 in Israel. In civilians, incidence declined from 0.35 to 0.11 cases per 100,000 persons annually; in military personnel it averaged 6.4 cases per 100,000 persons annually. These data imply that the pathogen and vector continue to exist in Israel.

Relapsing fever is caused by infection with *Borrelia* species that can genetically vary their surface antigens, which causes repeated stimulation of the immune system and recurring episodes of fever. Two distinct types of relapsing fever exist, louseborne relapsing fever and tickborne relapsing fever (TBRF) (1,2). The TBRF *Borrelia* sp.is transmitted to humans by an infected *Ornithodoros* tick bite (2).

Human TBRF is generally contracted only in the geographic range of the tick vectors. *Ornithodoros* species of ticks belong to the *Argasidae* (soft tick) family that feeds nocturnally. The primary reservoirs of *Borrelia* spp. are rodents and lagomorphs (2). Many *Ornithodoros* species have been reported worldwide, each with its characteristic *Borrelia* species (2–5).

After an incubation period of ≈7 days, TBRF typically begins abruptly with fever, chills, headache, myalgia, arthralgia, and abdominal pain. Death is rare. Diagnosis is usually made by observing spirochetes on peripheral blood smears. Antimicrobial drug treatment is occasionally associated with the Jarisch-Herxheimer reaction (6).

Since 1946, all cases of relapsing fever described in Israel have been tickborne. *B. persica*, transmitted by *Ornithodoros tholozani* is thought to be the cause of TBRF in Israel. The *O. tholozani* tick was first identified in 1937 and is prevalent in Middle Eastern and central Asian countries. It is primarily found in dark, moist areas such as caves and abandoned buildings (7). In Israel, TBRF has traditionally been called cave fever because of the known correlation to cave exposure (8). The only survey conducted in Israel described the incidence of TBRF from 1954 to 1967 and concluded that the incidence of TBRF was declining (9).

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The Study

In Israel, all cases of TBRF in civilians are reported to the Ministry of Health and investigated by an epidemiologist nurse. Cases in soldiers are reported to the military health branch of the Surgeon General Headquarters of the Israel Defense Force. An epidemiologic investigation of cases in soldiers is conducted by the reporting physician. We evaluated the demographic, clinical, and geographic data from reports of civilians from 1971 to 2003 and of soldiers from 1975 to 2003.

We included confirmed cases, in which a person had both febrile illness and a positive peripheral blood smear, and associated cases, in which a person had a relapsing febrile illness and was associated with a confirmed case. Tests for trends were conducted with PEPI software, version 4.0 (Sagebrush Press, Salt Lake City, UT, USA). A total of 606 cases were reported during the study years, 283 in civilians, and 323 in soldiers.

The yearly civilian incidence is shown in Figure 1A. The yearly incidence average in civilians declined from 0.35 cases per 100,000 population from 1975 to 1985 to 0.11 cases per 100,000 population from 1986 to 2003 ($p<0.001$). The peak in civilian cases in 1984 and 1985 was unexplained; 2 cases occurred in immigrants from Ethiopia 3 months after they arrived in Israel. The

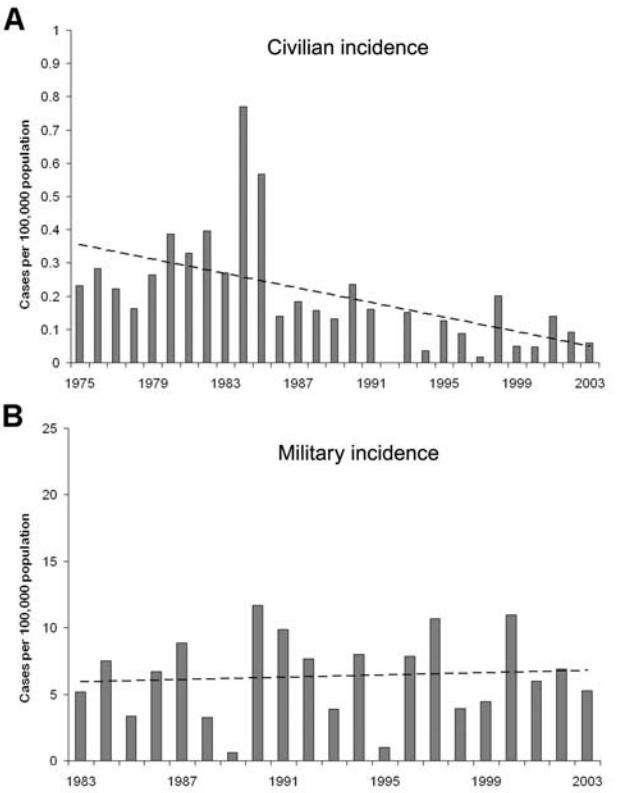

Figure 1. Incidence of tickborne relapsing fever in Israel. A) among civilians, 1975–2003; B) among soldiers, 1983–2002. Dotted line indicates prevalence.

incidence of TBRF in the military from 1983 to 2003 has been relatively constant, with an average of 6.4 cases per 100,000 population annually (Figure 1B).

The patients included 529 men (87%) and 77 women (13%). Of 359 patients for whom this information was available, 304 (85%) were of Jewish origin and 55 (15%) were of non-Jewish origin, similar to the proportions in the Israeli population. Information on exposure circumstances was available for 256 cases. The most commonly reported exposures were visiting caves (64%), visiting abandoned buildings (5%), and engaging in other outdoor activity (19%). In 12% of the cases, exposure to caves was excluded, but the exposure circumstances were not identified. In soldiers, 43% of cases were associated with outdoor exposure, usually related to prolonged periods of lying on the ground. In civilians, 49% were students and 16% were outdoor workers. One hundred cases occurred in clusters with an average of 3.3 (range 2–10) people in each cluster.

Figure 2. Civilian yearly incidence of tickborne relapsing fever, Israel, 1951–2003.

Conclusions

Soldiers comprised more than half of the total cases in this study, with an average incidence 36 times greater than in civilians. The higher incidence in soldiers may be due to increased risk of exposure because of their specific activities or better case reporting in the Israel Defense Force.

During the last century in industrialized countries, a significant decline in many infectious diseases has been observed (10). This decline can be attributed to elimination or significant reduction of pathogens or decreased exposure to the pathogens.

This study has shown a decrease in the incidence of TBRF in civilians, with no similar decrease in soldiers. This decrease becomes even more evident when compared to the incidence in Israel during the 1950s and 1960s (Figure 2). That the incidence has remained similar over time among military personnel indicates that the pathogen and the vector continue to exist in Israel and that soldiers are at a high risk of contracting it because of their activities. Infection has occurred although Israel Defense Force commanders are aware of TBRF and all soldiers and commanders receive annual guidelines on preventing TBRF. Furthermore, entering caves is prohibited, and most TBRF cases in the military (67%) were not due to cave exposure. The declining incidence among civilians is likely due to a decrease in the rate of exposure with increased urbanization. A civilian subpopulation that continues to be at risk is schoolchildren; most cases occurred after field trips.

Although TBRF was reported throughout the year, most cases occurred during summer and fall. Possible explanations include higher *Ornithodoros* tick activity, higher human outdoor activity, and lighter clothes worn during these seasons. TBRF can be contracted throughout Israel, despite the varying climatic conditions (Table). In soldiers, 63% of cases were contracted in the hot, arid, southern regions of Israel. The high number of cases in the southern regions is probably because most military training activity is conducted there. In civilians, more cases were contracted in the northern and central regions of Israel where more persons travel. That 64% of the cases are associated with caves might be explained by the relatively stable conditions in caves that are favorable to ticks.

Most cases that occurred in the military were in men. Although women also served in the Israeli military, only a fraction of them served in positions that required outdoor exposure. The illness was also more preponderant in civilian men. This tendency has been observed in previous reports, and likely results from men's high-risk behavior.

In conclusion, TBRF continues to be endemic in Israel and still poses a considerable hazard for soldiers and civilians. Although TBRF has been substantially reduced in civilians, the incidence in soldiers has not declined. Sufficient information on the habits of the *O. tholozani* tick and its reservoirs is not available. To reduce the incidence of TBRF, further studies are warranted to accurately map *Ornithodoros* tick infestations. Further studies are also needed to identify *Borrelia* reservoirs in Israel and to study the habits of its vector.

Table. Geographic distribution (%) of tickborne relapsing fever cases

Geographic region	Civilians	Military	Total
Israel			
North	36	18	29
Center	50	12	35
South	10	63	32
Other			
Africa	4	0	2
Lebanon	0	7	3
Total			100

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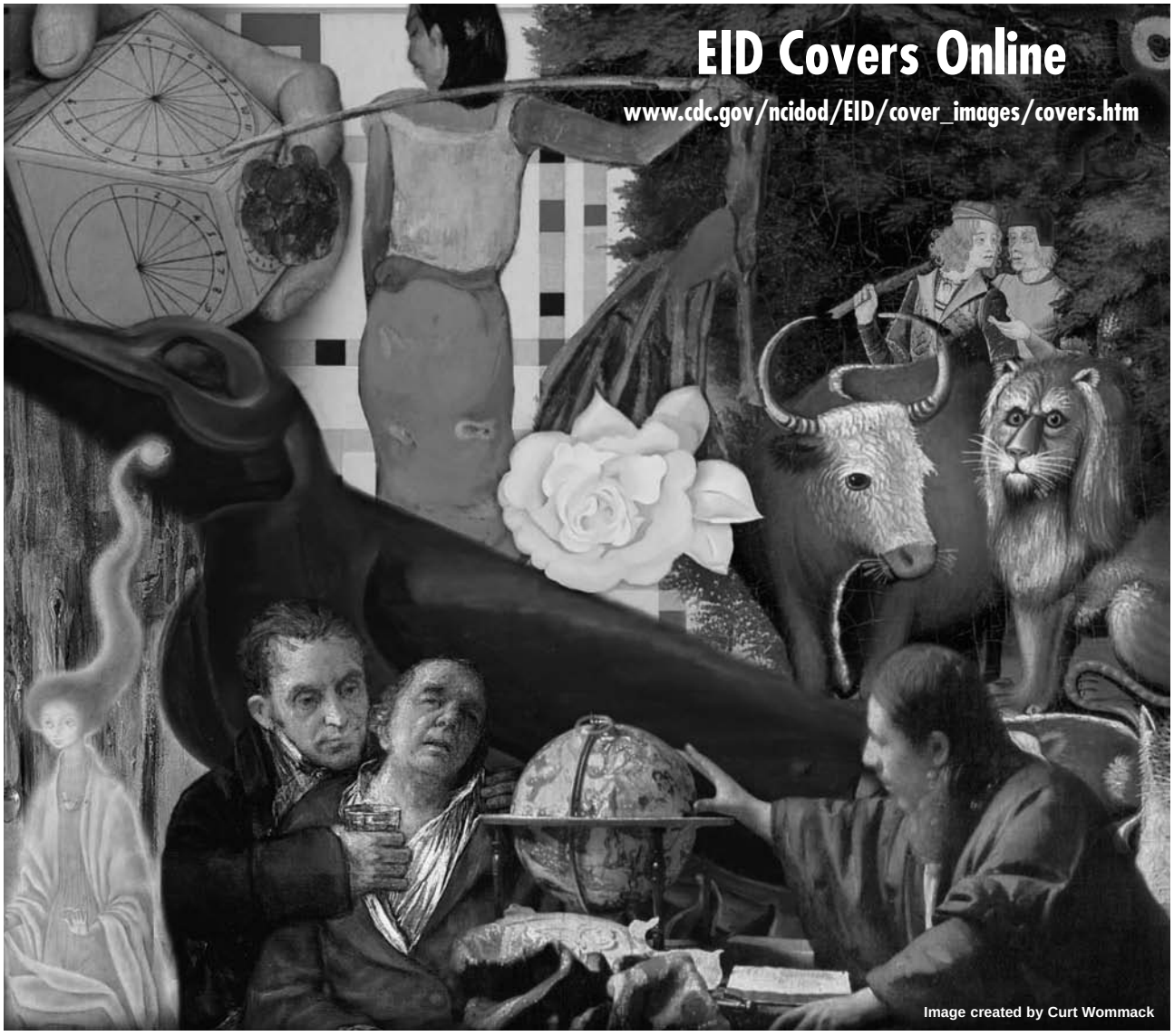

Slow Epidemic of Lymphogranuloma Venereum L2b Strain

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We traced the *Chlamydia trachomatis* L2b variant in Amsterdam and San Francisco. All recent lymphogranuloma venereum cases in Amsterdam were caused by the L2b variant. This variant was also present in the 1980s in San Francisco. Thus, the current "outbreak" is most likely a slowly evolving epidemic.

Since the end of 2003, an ongoing lymphogranuloma venereum (LGV) proctitis outbreak has been reported in industrialized countries, first in the Netherlands, followed by neighboring European countries and the United Kingdom, and now in the United States and Canada (1–4). We recently identified a new LGV variant designated L2b (GenBank accession no. AY586530) in all our cases in 2002 and 2003 that suggests this LGV outbreak was new (5). Until now, only men who have sex with men (MSM) are affected, and most are HIV co-infected. Although these infections, which can be caused by LGV serovars L1, L2, L2a, and L3, are often characterized by severe inflammatory symptoms, delayed or incorrect diagnosis has increased both the risk for transmission and the development of severe sequelae. Successful treatment of LGV proctitis requires a 3-week course of doxycycline followed by a test of cure, whereas in the case of *Chlamydia trachomatis* proctitis caused by serovars D–K, a 1-week course will suffice.

In a recent article on this LGV outbreak (3), 2 issues were stressed: 1) the lack of an easy diagnostic tool and 2) whether lymphogranuloma venereum is a new problem or whether it has been present but undiagnosed. Indeed, among the obstacles to the correct diagnosis of LGV is the lack of a commercially available assay to specifically distinguish between *C. trachomatis* infections caused by LGV serovars and infections caused by less invasive *C. trachomatis* serovars. A definitive diagnosis of LGV is currently made with nucleic acid sequence-based tests, like polymerase chain reaction (PCR)–based restriction fragment length polymorphism (RFLP) analysis, which are only available in a few specialized laboratories. We recently developed a real-time PCR (TaqMan and RotorGene) that can specifically distinguish LGV infections from infections with other *C. trachomatis* serovars, which facilitates diagnosis (6).

We used this new diagnostic tool to determine whether the LGV outbreak and its cause are a new phenomenon or whether LGV *C. trachomatis* serovars have been present much longer but have gone undiagnosed. First, we determined if the newly identified Amsterdam L2b variant was already present in the MSM population before 2002 by using stored samples collected from MSM with and without proctitis who sought treatment at the sexually transmitted infections (STI) outpatient clinic in Amsterdam. Second, we performed the same analysis on archived specimens from MSM in San Francisco, California, collected 20–25 years ago.

The Study

From MSM who attended the Amsterdam Municipal Health Service STI Outpatient Clinic in 2000 and 2001, randomly selected stored specimens of *C. trachomatis* DNA–positive (as assessed by ligase chain reaction, Abbott Laboratories, Chicago, IL, USA) rectal samples were tested for the *C. trachomatis* variant by real-time PCR (6). From 2002 to 2005, MSM with symptomatic proctitis (i.e., purulent discharge, rectal ulceration, bleeding, or edematous mucosa) and MSM without symptoms were included.

From the San Francisco region, 51 LGV–positive isolates from symptomatic MSM were analyzed (7). The isolates were collected in medical clinics (e.g., ambulatory care, emergency room, screening, acute care) from 1979 to 1985 (Table). LGV was assessed at the time of collection, according to phenotypic properties observed during cell culture. Although the growth characteristics of LGV serovars can be distinguished from serovars D–K, cell culture for *C. trachomatis* is no longer available in most clinical settings.

C. trachomatis serovar typing was performed as described previously (5). Briefly, amplification of the *ompA* gene (1.1 kb) was performed in a nested PCR format. Serovars and variants were initially identified by their RFLP patterns after polyacrylamide gel electrophoresis. The *ompA* nucleotide sequences were subsequently analyzed by automated DNA sequencing on an ABI 310 sequencer (PE Biosystems, Foster City, CA, USA). The sequences obtained from *C. trachomatis*–infected MSM in 2000 and 2001 in Amsterdam and from MSM in San

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Table. Number of lymphogranuloma venereum (LGV) L2b variants identified in *Chlamydia trachomatis* DNA–positive rectal swabs in Amsterdam (2000–2005) and San Francisco (1979–1985)

Location	Year*	No. L2b /no. samples
Amsterdam	2000	2/67
	2001	4/28
	2002	40/127
	2003	69/276
	2004	52/297
	2005	26/161
San Francisco	1979	0/1
	1980	0/0
	1981	2/8
	1982	10/29
	1983	5/9
	1984	0/3
	1985	1/1

*In 2002 and 2003, 45 LGV L2b variants of 109 isolates have been described in detail (5).

Francisco were compared to the recently identified L2b variant to determine if the strain was present earlier. The Table presents the results of this analysis.

In the Amsterdam *C. trachomatis* DNA–positive rectal samples, LGV strains were detected by real-time PCR in 2 of 67 samples in 2000 and in 4 of 28 samples in 2001. Sequencing showed that in all 6 LGV strain–positive samples, the L2b variant was present. Also in 2002 and 2003, 109 L2b-positive samples of 403 *C. trachomatis* DNA–positive rectal samples were identified, of which 45 were strain L2b, and these have been described in a previous publication (5). All 51 San Francisco specimens (from 51 patients) were positive for LGV variants by real-time PCR. By sequencing variable segment 2 of the *ompA* gene (VS-2), we identified 15 as serovar L1, 18 as serovar L2 prototype, and 18 as the L2b variant. We sequenced the complete *ompA* gene of 5 of these 18 L2b variants that originated in San Francisco; all were identical to the recently described L2b variant circulating in Amsterdam. Four nucleotide changes were found when compared to reference serovars L2, L2a, and the variant L2′, including 1 change that encoded the previously undescribed change at amino acid 162, AAT→AGT (5).

Conclusions

The L2b LGV variant identified as the cause of all the LGV proctitis in the recent outbreak among MSM in Amsterdam appears to have been circulating in Amsterdam in 2000. Moreover, we showed that this L2b variant was present in the 1980s in San Francisco with exactly the same mutations in the complete *ompA* gene. However, since we only sequenced the *ompA* gene, and although the sequence was identical in old and new L2b strains, we cannot exclude the possibility that it could involve different strains of *C. trachomatis* that differ in other parts of the genome, although this is unlikely.

Since LGV causes potentially severe infections with possibly irreversible sequelae if adequate treatment is not begun promptly, early and accurate diagnosis is essential. Sequence-based nucleic-acid tests that can discriminate between LGV serovars and less invasive *C. trachomatis* species can help detect cases and prevent further transmission of LGV.

In conclusion, our results suggest that we are dealing with the same LGV variant >25 years later, and the current LGV outbreak in industrialized countries has most likely been a slowly evolving epidemic with an organism that has gone unnoticed in the community for many years and is now being detected by new technologies. The numbers detected in 2005 in Amsterdam suggest that a considerable reservoir exists, which emphasizes the need for ongoing public health awareness.

Ms Spaargaren is a medical microbiologist in charge of the public health laboratory of the Municipal Health Service of Amsterdam. One of her research interests is the epidemiology, pathogenesis, and immunogenesis of *C. trachomatis* infections in the human urogenital tract.

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Adventitious Viruses and Smallpox Vaccine

To the Editor: Recently, Murphy and Osburn (1) strongly argued for testing old smallpox vaccine stocks made in animal skin for adventitious infectious agents such as viruses, mycoplasmas, and eventually, prions. Their argument appears clearly justified after unexpected cases of myopericarditis occurred during recent campaigns of smallpox vaccinations in the United States (2).

To the long list of bovine viruses cited in this paper, it seems necessary to add another, the pseudocowpox virus, a widespread parapoxvirus that may infect humans. During the 1960s, this virus was identified in vaccine lymph from a heifer at the Institut Pasteur, Paris (3).

In humans, this virus is responsible for limited skin lesions, more frequently in immunocompromised patients. Mainly farmers and butchers are affected. Pseudocowpox virus is easily differentiated from orthopoxviruses such as vaccinia virus by the virus's peculiar form on transmission electron microscopy scan, but polymerase chain reaction is probably the best detection method (4). In fact, many other more hazardous viruses may be found in the oldest stocks of smallpox vaccine and deserve more attention than previously considered.

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Fluoroquinolone Use in Food Animals

To the Editor: Two recent articles (1,2) show that fluoroquinolone use in food animals is associated with infections by antimicrobial drug–resistant strains of *Campylobacter* in humans. These infections cause problems in treating illnesses as well as increased rates of illness and death (3). Despite a large body of scientific evidence and a judicial review (1–3) that show harmful results in many persons, some members of the poultry and pharmaceutical industries argue that fluoroquinolone use in food animals has no adverse effects in humans (4) and continue to supply these drugs for use in poultry (2,5). The use of these drugs has caused rapidly increasing resistance rates in most countries. In the United States, 19% of *Campylobacter* isolates from humans are now ciprofloxacin resistant (2), and resistance rates >80% are seen in Spain (5). By contrast, in Australia, where fluoroquinolones were never approved for use in food animals, domestically acquired infections with fluoroquinolone-resistant *Campylobacter* spp. are rarely found in humans (6). Drug-resistant *Escherichia coli* is also of concern. In Spain, humans frequently acquire fluoroquinolone-resistant *E. coli* associated with fluoroquinolone use in poultry (7).

In the United States, better controls in meat and poultry slaughter and processing, as well as improved food-safety education campaigns, have resulted in 28% fewer *Campylobacter* infections annually since 1996 (8). However, ≈1.8 million persons (600 per 100,000) are likely to contract symptomatic *Campylobacter* infections per year (3,8), and fluoroquinolone resistance is now 19% (2). Thus, the risk of a person's contracting fluoroquinolone-resistant *Campylobacter* infection is 114 per 100,000 per year. If 80% of *Campylobacter* infections are foodborne (3), and 90% of these infections are acquired from poultry (9), then ≈82 of 100,000 persons per will contract ciprofloxacin-resistant *Campylobacter* infections from poultry each year. Most persons with *Campylobacter* infections would not benefit from antimicrobial drug therapy. However, if only 10% of infected persons would benefit from antimicrobial drug therapy, fluoroquinolone use in poultry could cause ≈82 persons per million to have a compromised response to therapy. In the United States (population 300 million), this number translates to >24,000 persons annually.

Data on the number of animals that receive fluoroquinolones are difficult to find. Bayer (manufacturer of the only fluoroquinolone used in poultry in the United States) states that Baytril (enrofloxacin) is used in <1% of US broiler flocks (4). This statistic allows us to estimate how many persons will potentially have an adverse outcome compared to the number of animals receiving fluoroquinolones. If 24,000 persons in the United States have an adverse outcome annually after <84 million chickens (1% of 8.4 billion) are treated with enrofloxacin, then ≈285 persons are at risk of having an adverse outcome for every 1 million chickens treated.

This risk seems needless. In Australia, consequences from not using these agents in food animals (i.e., neither therapeutic nor prophylactic use is approved) have not been seen. Thus, I do not agree with Iovine and Blaser (1), who would allow fluoroquinolones to be used to treat sick food production animals. Bayer claims that "Baytril is used for therapeutic purposes only..." (4). Thus, continuation of fluoroquinolone use for these therapeutic purposes will allow the consequent development of resistant bacteria in humans, which will include resistant strains of *Campylobacter*, *E. coli*, and *Salmonella*. Discontinuing fluoroquinolone use by mass dosing (the current practice for poultry [10]) would decrease the amount of the drug used. However, why use fluoroquinolones at all? Narrower spectrum antimicrobial drugs (e.g., sulfonamides, amoxicillin) could be used to adequately treat sick animals. Surely *E. coli* drug resistance in food animals in the United States cannot be at a level that makes fluoroquinolones indispensable. If resistance levels to narrower spectrum antimicrobial drugs are at high levels, does this finding not imply that major changes concerning antimicrobial drug use in food animals are needed?

Better methods are needed to accurately estimate how many persons are negatively affected annually because of the misuse of antimicrobial drugs in food animals. Compromised therapeutic outcomes occur in many persons throughout the world because of fluoroquinolone-resistant *Campylobacter* infections (10). Fluoroquinolone use is not essential for food animal production or the welfare of animals. Many ways to keep animals healthy and productive exist other than treating or trying to prevent infections with the mass use of antimicrobial drugs such as fluoroquinolones.

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In response: We agree with Dr Collignon's view that using fluoroquinolones in the poultry industry imposes a "needless risk" of harm to humans by promoting the emergence of fluoroquinolone-resistant *Campylobacter* infections and consequent increased illness in humans (1). We support the therapeutic use of fluoroquinolones in poultry if only animals that are ill are treated (2). The widespread practice of adding fluoroquinolones to the drinking water of a hen house with thousands of birds, or of an entire flock, promotes the emergence of resistant *Campylobacter* strains. Unless veterinary practices limit fluoroquinolone use exclusively to sick birds, the only responsible recourse is to ban the use of fluoroquinolones in the poultry industry altogether, in agreement with the Food and Drug Administration's decision (3).

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In response: We thank Dr Collignon for his comments regarding the human health impact after fluoroquinolone use in food animals (1) Similar conclusions concerning the human health consequence of using fluoroquinolones in poultry in the United States were reached by the US Food and Drug Administration (FDA) in a quantitative risk assessment in 2000 (1). FDA concluded that fluoroquinolone use in poultry has resulted in the emergence and dissemination of fluoroquinolone-resistant *Campylobacter* that infects thousands of persons each year in the United States. Therefore, since 2000, FDA has sought to discontinue the use of fluoroquinolones in poultry. On July 25, 2005, FDA announced the withdrawal of fluoroquinolones for use in poultry effective as of September 12, 2005.

The debate regarding the use of antimicrobial agents in food animals and their impact on human health has been longstanding. For many years, public health officials have raised concern regarding the use of antimicrobial drugs in food animals that are of importance to human health. Industry representatives have stated that these concerns are unfounded. In our study, we found no fluoroquinolone resistance among a sample of *Campylobacter jejuni* strains collected from persons in 1990 (2). In 1995, fluoroquinolone use was approved in the United States for food animal use, specifically for poultry. Between 1997 and 2001, we noted a significant increase in fluoroquinolone resistance among human *Campylobacter* strains in the United States, monitored through Centers for Disease Control and Prevention surveillance (13%–19%, logistic regression odds ratio 2.4, 95% confidence interval 1.4–4.1). This finding means that despite a 31% decline in the overall incidence of *Campylo-bacter* infections from 1997 to 2001, the incidence of fluoroquinolone-resistant *Campylobacter* infections increased (3,4). More recently published data show that persons with fluoroquinolone-resistant infection have a longer duration of diarrhea and are more likely to have invasive disease or die than persons with fluoroquinolone-susceptible infections (5,6). These data demonstrate, as Dr Collignon indicates, the human health consequences of increasing fluoroquinolone resistance among *Campylobacter*.

Campylobacter is a zoonotic pathogen and most often associated with consumption of poultry. Our study found that in 1999, 10% of grocery store–purchased chickens yielded fluoroquinolone-resistant *Campylobacter* (2). More recently, retail food testing in 2002 performed by FDA found that 14% of retail chicken samples were contaminated by fluoroquinolone-resistant *Campylo- bacter* (7). Studies of commercial poultry flocks before, during, and after fluoroquinolone treatment found that only a small proportion of flocks had fluoroquinolone-resistant *Campy-lobacter* infections before fluoroquinolone treatment, but that fluoroquinolone-resistant strains quickly emerged during treatment and often persisted after treatment (8,9). As Dr Collignon describes, in Australia, fluoroquinolone-resistant *Campylo-bacter* strains have not been detected in domestically acquired human infections; this finding has been attributed to the fact that fluoroquinolones are not licensed for use in food animals (10). We agree with Dr Collignon that convincing data indicate that use of antimicrobial agents that are of human importance among food animals has an adverse human health impact and that the time has come to find alternatives that promote food-animal health while minimizing the induction of antimicrobial resistance.

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Ehrlichia ruminantium, Sudan

To the Editor: *Ehrlichia ruminantium*, the causative agent of heartwater, is transmitted by *Amblyomma* spp. ticks. *Amblyomma variegatum* ticks, which are found in the Caribbean and sub-Saharan Africa, except in certain areas of southern Africa, are major vectors of *E. ruminantium* (1–3). *A. lepidum* is also an important vector of heartwater, especially in eastern Sudan (4). However, few epidemiologic data exist on infection rates of *Amblyomma* spp. ticks and distribution of *E. ruminantium* in Sudan. A polymerase chain reaction (PCR) assay that uses DNA probe pCS20 has been developed for detecting *E. ruminantium* (5). Another PCR assay for the major antigen protein 1 gene (*map1*) has been used to differentiate strains of *E. ruminantium* (6,7). These PCR assays have high sensitivity and specificity for the amplification of *E. ruminantium* DNA (6,8). For epidemiologic study of *E. ruminantium* in Sudan, we used PCR to detect *E. ruminantium* DNA in ticks. We also sequenced PCR products to identify the genotype of *E. ruminantium.*

The pCS20 DNA fragment of *E. ruminantium* was detected in 8 (8.2%) of 97 *A. variegatum* ticks and 2 (1.9%) of 106 *A. lepidum* ticks (χ^2 = 3.123, by Yates correction). The nucleotide sequences (279 bp) obtained from 5 *A. variegatum* ticks and 1 *A. lepidum* tick were identical (GenBank accession no. AB218277). The sequences were similar to those of Welgevonden, Vosloo, and Ball3 strains from southern Africa and Gardel strain from the Caribbean islands (similarity = 99.64%). The pCS20 sequences obtained in this study were different from those of strains from western Africa.

An 855-bp *map1* nucleotide sequence obtained from 1 *A. lepidum* tick was provisionally named Gedaref (GenBank accession no. AB218278). The nucleotide sequence of Gedaref was found to be closely related to those of Senegal and Pokoase strains from western Africa and to South Africa Canine and Kümm1 strains from southern Africa (similarity = 90.53%– 97.43%). Gedaref clustered with these 4 strains and with 6 other strains, including Kiswani from eastern Africa and Antigua from the Caribbean islands (Figure). In contrast, the nucleotide sequence of Gedaref showed 84.8% similarity with that of Um Banein, which has been known as the only strain of *E. ruminantium* in Sudan. Um Banein formed another cluster with Gardel, Lutale, and Umpala strains from southern Africa (Figure). The *map1*

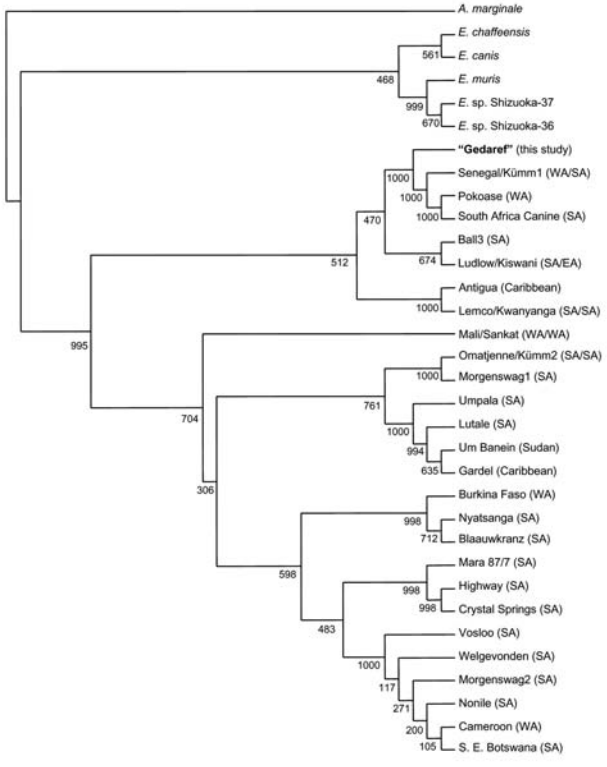

Figure. Neighbor-joining phylogram based on *map1* nucleotide sequences of *Ehrlichia ruminantium* strains. Ninety-seven *Amblyomma variegatum* ticks were obtained from cattle in the suburbs of Juba in southern Sudan, and 106 *A. lepidum* ticks were obtained from camels in the suburbs of Gedaref in eastern Sudan in 2000. The amplicon used included all 3 variable regions in the *map1* sequence (nucleotide positions 472–1377) (7). The nucleotide position refers to GenBank accession no. X74250. The amplicon without primer sequences (855 bp) was subjected to sequencing analysis. Sequence homogeneity was determined and multiple alignment analyses were conducted as previously described (9). *A. marginale* strain Pawhuska major surface protein 4 (GenBank accession no. AY127078) was used as an outgroup. WA, western Africa; SA, southern Africa; EA; eastern Africa. Kiswani is identical to Ludlow, Kümm1 is identical to Senegal, Kümm2 is identical to Omatjenne, Kwanyanga is identical to Lemco, and Sankat is identical to Mali (6).

coding sequence of Gedaref was closely related to those of strains Senegal, Ball3, South Africa Canine, and Pokoase (similarity = 92.61%–97.97%). Gedaref and these 4 strains formed a cluster and branch with Um Banein (similarity = 87.6%).

The novel *E. ruminantium* genotype Gedaref was detected in *A. lepidum* by PCR assays. This work has shown that another strain of *E. ruminantium*, in addition to the Um Banein strain, is present in Sudan. Since the Um Banein strain of *E. ruminantium* was isolated from sheep in 1984 (4), no other strain had been reported in Sudan. The *map1* sequence of Gedaref has diverged from that of Um Banein. Gedaref formed a cluster with several strains that originated in southern and western Africa. As previously reported, the variation of *map1* sequences of *E. ruminantium* strains does not reflect the geographic distribution of the strains (6). However, Gedaref was distinctively differentiated from the Um Banein strain. Gedaref was closely related to 3 southern African strains and a Caribbean strain in the pCS20 sequence but different from western African strains. The pCS20 sequence has been highly conserved among strains from western Africa (10), and the distribution of *A. lepidum* is limited to eastern Africa (3). If one considers the distribution of *A. lepidum*, results of genetic analyses in the pCS20 gene regions of Gedaref are important for epidemiologic research on *E. ruminantium*.

We detected pCS20 DNA specific for *E. ruminantium* in *A. variegatum*. This tick is widely distributed in Africa and is the most efficient vector of heartwater (3). Nevertheless, detection of *E. ruminantium* in *A. variegatum* in Sudan had not previously been reported. Our results show that *A. variegatum* is also an important vector of *E. ruminantium* in Sudan. *A. variegatum* ticks are also found North and South America, Southeast Asia, and Australia (3). Thus, the potential spread of *E. ruminantium* to livestock is a continuous threat in these regions from the importation of tick-infested and subclinically infected wild animals from Africa and the importation of livestock from Caribbean islands (1,2). We believe that *A. variegatum* plays an important role in the spread of heartwater because of its ability to adapt to various environments.

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Borrelia spielmanii Erythema Migrans, Hungary

To the Editor: Lyme disease is the most frequent tickborne human infection in the northern hemisphere. At least 5 species of the *Borrelia burgdorferi* sensu lato complex, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. bissettii*, and *B. lusitaniae*, have a pathogenic role in human Lyme disease in central Europe (1–3). A sixth pathogenic strain, A14S, has been isolated from 1 Dutch (4) and 2 German patients with erythema migrans (5). This strain was also detected in 4 questing *Ixodes ricinus* ticks in Germany (6,7) and 1 in the Czech Republic (8). A14S has recently been described as a new species, *B. spielmanii* (9); its main reservoir host is probably the garden dormouse (*Eliomys quercinus*), but *B. spielmanii* could not be detected in mice or voles. Richter et al. (9) could not find ticks harboring *B. spielmanii* in 3 of 5 examined areas in Germany. They were present almost exclusively in a single area where the prevalence of infection with this genotype was 15 (6%) of 251. We describe the isolation of this novel Lyme disease spirochete from a human patient with erythema migrans in Hungary.

Since 1999, we have regularly isolated *Borrelia burgdorferi* sensu lato from skin biopsy specimens of erythema migrans and acrodermatitis chronica atrophicans taken from patients at the Center for Tick-borne Diseases, Budapest, Hungary. To identify the *Borrelia* species occurring in Hungarian Lyme disease patients, we have started to molecularly analyze cultured isolates that originate from erythema migrans of different patients. DNA was isolated from 8 bacterial pellets by using QIAamp DNA mini kit (Qiagen, Hilden, Germany). Primers BSL-F and BSL-R were used; these amplify an ≈250-bp region of the outer surface protein (osp) A gene from all Lyme disease spirochetes (10). We added 2 μL extracted DNA to a 20-μL reaction mixture composed of 1.0 U HotStart-Taq DNA polymerase, 200 μmol/L of each dNTP, 25 pmol of each primer, and 1.5 mmol/L $MgCl_2$ (HotStartTaq Master Mix, Qiagen). An initial denaturation step at 94°C for 15 min was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Final extension was done at 72°C for 5 min. Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel that was prestained with ethidium bromide and viewed under UV light. After purification, the dideoxy chain termination (Applied Biosystems Division, Foster City, CA, USA) was used for sequencing. Obtained sequences were checked with Chromas v.1.45 and compared to sequence data available from GenBank by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). New sequences were submitted to GenBank.

Six sequences (DQ007298, DQ007299, DQ007300, DQ007301, DQ007302, DQ007303) showed 100% homology to *B. afzelii* Khab 625 strain (AY502599). One (DQ007297) of the remaining 2 samples showed 99.6% similarity with *B. burgdorferi* B31 (AE000790), and the other (AY995900) showed 99.21% similarity with *B. spielmanii* (AF102057).

The patient whose culture showed *B. spielmanii* was a 42-year-old woman with a homogenous erythema migrans, diagnosed on September 24, 1999. The erythema was 10 cm in diameter on the front surface of the knee at the first visit (see online Figure, available at http://www.cdc.gov/ncidod/EID/vol11no11/05-0542-G.htm). The immunoglobulin M (IgM) and IgG *Borrelia* immunoblot that applied *B. afzelii* (ACA1) antigen was negative in serum drawn on the seventh day after the appearance of erythema migrans. The patient did not remember a tick bite and had not traveled abroad during the previous 6 months. She complained of an "extremely unusual," intense, serous nasal discharge that started 3 weeks before the appearance of erythema migrans and of a moderate headache; both disappeared spontaneously 2 weeks before treatment.

Our results show at least 3 distinct species of *B. burgdorferi* sensu lato in Hungary. In addition to *B. burgdorferi* sensu stricto and *B. afzelii*, known throughout Europe, we detected the recently described species *B. spielmanii* among randomly selected samples. Together with 2 previous publications (4,5), our observation also suggest that *B. spielmanii* has a pathogenic role in human Lyme disease. Although *B. spielmanii* is distributed more focally than other species of the *B. burgdorferi* sensu lato complex (9), it occurs from the Netherlands through Germany and Czech Republic to Hungary (4,5,7,8).

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Profiling *Mycobacterium ulcerans* with *hsp65*

To the Editor: *Mycobacterium ulcerans* is an emerging human pathogen responsible for Buruli ulcer, a necrotizing skin disease most commonly found in West Africa, but outbreaks have also been reported in the Americas, Australia, and Asia (1). Environmental sources of infection and mode of transmission are not completely known. *M. ulcerans* grows slowly at 32°C, requiring 6–8 weeks for colonies to be visible in primary culture. Differentiation from *M. marinum*, which also causes skin infections, is important, since *M. marinum* can usually be treated with antimicrobial agents, whereas *M. ulcerans* most often does not respond favorably to drug therapy, and treatment is usually by surgical excision (2). *M. shinshuense*, initially isolated from a child in Japan, is phenotypically and genetically related but biochemically distinct from *M. ulcerans* (3).

In the last decade, several DNA-based techniques for mycobacterial identification have been developed. Rapid molecular detection and differentiation of organisms that cause skin infections directly from tissue or exudates could be of great value for early treatment. Some techniques, especially those that include nucleic acid amplification, could be used directly on clinical samples. The accepted standard for molecular identification of mycobacteria is sequencing analysis of 2 hypervariable regions identified in 16S rRNA gene. *M. marinum* and *M. ulcerans* share identical 5′-16S rDNA and 16S-23S rRNA gene spacer sequences (4). Polymerase chain reaction (PCR)-dependent methods are based on the 16S rRNA gene (5), the *hsp65* gene (6) or the insertion sequence IS*2404* (7). Recently, a novel category of variable number tandem repeats that could distinguish *M. marinum* and *M. ulcerans* genotypes has been described (8).

Polymorphisms in the 3′-16S rDNA region discriminate *M. ulcerans* from *M. marinum* and *M. shinshuense* (5). These polymorphisms also allow the separation of *M. ulcerans* into 3 subgroups according to geographic origin and variable phenotypic differences. IS*2404* discriminates *M. ulcerans* from *M. marinum* (7). It has been used in restriction fragment length polymorphism analysis applied to a comparable number of *M. ulcerans* and *M. marinum* strains, confirming that this sequence is present in high copy numbers in *M. ulcerans* but absent in *M. marinum*. Nevertheless, an unusual mycobacterium was recently isolated that is closely related to *M. marinum* by phenotypic tests, lipid pattern, and partial 16S rDNA sequencing but presents low copy numbers of this element (9).

PCR-restriction enzyme analysis (PRA) of a 441-bp fragment of the *hsp65* gene is a rapid, easy, and inexpensive method for identifying mycobacteria (10). Devallois et al. (6) described the PRA-*hsp65* pattern of 1 *M. ulcerans* strain ATCC 33728 that originated in Japan. This isolate was considered a new species that resembled *M. ulcerans* and was named *M. shinshuense* (3).

We report here the usefulness of PRA-*hsp65* to differentiate *M. ulcerans* strains from different geographic areas. Since Buruli ulcer cases have been reported on 5 continents, we studied 33 *M. ulcerans* strains that originated from Africa (Benin, Zaire, Ghana, Congo, Angola, Côte d'Ivoire, Togo), Asia (China, Malaysia), Australia (Papua New Guinea, Australia), the Caribbean (Mexico, Surinam, French Guiana), 1 *M. shinshuense* from Japan, 1 *M. marinum* isolate and 1 IS*2404*-positive *M. marinum* isolate from France (9). All strains were identified at the Institute

of Tropical Medicine, the World Health Organization Collaborating Centre for the Diagnosis and Surveillance of *Mycobacterium ulcerans* Infection by IS*2404* PCR and biochemical tests (online Table, available from http:www.cdc.gov/ncidod/EID/vol11no11/05-0234.htm#table).

DNA extracted from cultures by 3 freeze-boiling cycles was used for amplification, according to the protocol described by Leao et al. (10). Gel images were analyzed by using GelCompar II v. 2.5 (AppliedMaths, Sint-Martens-Latem, Belgium). Two distinct *M. ulcerans* PRA-*hsp65* patterns were identified. Of 36 strains, 34 had a PRA-*hsp65* pattern indistinguishable from that of *M. marinum* [*Bst*EII and *Hae*III (bp) of 235/210/0 and 145/105/80] at the Swiss PRAsite (http://app.chuv.ch/prasite/index.html). Two strains, 1 each from Japan and China, showed a different pattern [*Bst*EII and *Hae*III (bp) of 235/210/0 and 190/105/80], that described by Devallois et al. (6).

We have shown that PRA-*hsp65* analysis performed on several *M. ulcerans* strains from different geographic areas produced different patterns. In fact, the unique PRA-*hsp65* profile of the *M. ulcerans* strain previously published (6) was the most rarely found pattern among the profiles found in this study. This work helps to clarify the PRA-*hsp65* patterns of *M. ulcerans* found in different countries. Because the epidemiology of Buruli ulcer is poorly understood, new molecular tools are still needed to differentiate *M. ulcerans* from different geographic settings, mainly in Africa, where the disease is more prevalent. The PRA-*hsp65* method represents a rapid, easy, and inexpensive technique to differentiate *M. shinshuense* from *M. ulcerans* and *M. marinum*.

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Spelling of Emerging Pathogens

To the Editor: Language is about comprehension; provided the parties in a discussion can understand each other, variations in pronunciation of individual words may be tolerated or disregarded. In modern English, numerous examples of variant pronunciations exist that cause no problems of comprehension (e.g., either, tomato, laboratory, fertile). These arise from several causes; regional practice is likely the most important factor, but the speaker's education and social background, personal preferences, and even etymologic theories also play a part. It would be futile and, some would feel, undesirable to attempt to impose uniformity by prescribing approved pronunciations if communication is not endangered. Moreover, both language and pronunciation are subject to constant change.

The same is not true regarding the spelling of organisms' names.

Although we accept variation in pronunciation, we should not accept variation in the spelling of binomial names. Common spelling variants and the citation frequency (PubMed) of 4 organisms, *Acinetobacter baumannii*, *Coccidioides immitis* (the fungal causal agent of coccidioidomycosis), *Coxiella burnetii* (the causal agent of Q fever), and *Tropheryma whipplei* (the causal agent of Whipple disease), are detailed in the Table. Common spelling mistakes occur with double letters (e.g., nn, ii), as well as complicated strings of consecutive vowels (e.g., *Coccidioides*). However, a defense to such criticism is that various authors have adopted the spelling of a previous taxonomic description that has become outdated, e.g., *C. burneti* (previous) and *C. burnetii* (current). Historic change in the spelling of these names is the primary reason they are published and cited in PubMed with different spellings. However, even disregarding historic taxonomic variants, ≈14.8% of *Tropheryma whipplei*, 14.3% of *Acinetobacter baumannii*, 12.3% of *Coxiella burnetii*, and 1.9% of *Coccidioides* citations are spelled incorrectly in PubMed. These relatively large percentages may mean that relevant literature is overlooked in searches.

Authors should be aware that previous taxonomic spelling of binomial names exist and check their historic evolution in the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.cict.fr). Authors should cite previous spelling when such a change has been recent and they may wish to include previous spellings in literature searches. Additionally, the most current and formally accepted spelling must be used when preparing a manuscript for publication.

The origins of incorrect and variant spellings of binomial names may lie in an array of sources, including original mispronunciation with subsequent incorrect phonetic transcription. Written language is rarely a phonetic transcript of vocal acoustics, however, it interfaces with several factors that prevent us from spelling words the way they sound. Orthography, which promotes the practice of writing words with the proper letters according to standard usage and conventionally correct spelling, is further complicated by the use of Greek or Latin words, each with their own linguistic peculiarities.

Table. Common spellings of binomial names of organisms*

Organism name [no. citations in PubMed]*	Spelling variants [no. citations in PubMed]	Date official spelling first described
Acinetobacter baumannii [844]	*A. baumanii* [117] *A. baumanni* [18] *A. baumani* [6]	1986†
Coccidioides‡ [1,209]	*Coccidiodes* [17] *Coccidoides* [4] *Cocidioides* [2] *Coccidioidis* [1]	1896§
Coxiella burnetii [1,531]	*C. burneti* [374] *C. burnetti* [199] *C. burnettii* [16]	1980¶
Tropheryma whipplei [52]	*T. whippelii* [118] *T. whippleii* [5] *T. whippeli* [4]	2001#

*Organism name in List of Bacterial Names with Standing in Nomenclature; search conducted June 2005.
†Approved name described by Bouvet and Grimont (ref 1).
‡*Coccidioides* is not a bacterium but a fungus; however, this name is described in the Index Fungorum.
§First described by Stiles (ref 2).
¶Approved name described by Skerman et al. (ref 3); first described by Derrick (ref 4) as *Rickettsia burneti*, the cause of Q fever.
#Approved names described by La Scola et al. (ref 5); 1992, Relman et al. (ref 6) tentatively proposed the name "*T. whippelii*."

Although we may not be able to standardize phonetic pronunciation of binomial names locally, nationally, or internationally, we should be constantly conscious of their spelling. As authors and peer reviewers, we should strive to achieve uniformity in written media to promote enhanced communication with our peers in infectious diseases.

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Staphylococcus aureus Bacteremia, Europe

To the Editor: In their article, Collignon et al. (1) present a table comparing absolute numbers and incidence rates of *Staphylococcus aureus* bacteremia (SAB) in Australia to those of 5 other countries, and state that "some data are available from other countries for comparison" and "only 2 countries, Denmark and England, appeared to have comprehensive collection systems."

We would like to add data from the European Antimicrobial Resistance Surveillance System (EARSS) to their table. EARSS is a multinational surveillance system that links national networks by collecting comparable and validated data on the prevalence of antimicrobial resistance of 5 microorganisms, including *S. aureus* (2). A total of 30 countries participate in EARSS. To ensure representativeness of the data it publishes, EARSS has set criteria, which can be found elsewhere (3).

In 2003, through a questionnaire, we collected background information on all hospitals served by laboratories participating in EARSS, including the estimated hospitals' catchment populations. Proportion of the country population covered by EARSS was then calculated by dividing the sum of catchment populations by the total population of the country (4). Catchment populations of hospitals providing single specialty or supraregional type of care were not counted to avoid overlap with other hospitals within the same country. Only the countries that provided denominator data for at least 60% of the isolates were included to ensure that the sample of hospitals was still representative of the country as a whole.

The number of SAB and the incidence of SAB per 100,000 inhabitants in 2003 were calculated from EARSS data, adjusted for population coverage, and are presented in the Table. These are crude estimates of the true number of SAB and should, thus, be interpreted with caution. For example, we assumed that the isolates for which hospital background information was not available did not differ from isolates for which we had this information, and that hospitals that participated in EARSS in 2003 were a representative sample of the countries' hospitals. Additionally, the incidence of SAB was positively correlated with the blood culturing rate (Spearman $r = 0.74$, $p = 0.002$), which means that the incidence of SAB is likely to have been underestimated in countries that reported few blood cultures. Although some countries did not report their blood culturing rate, the incidences of SAB in these countries were among the highest and are unlikely to be underestimated. For example, the EARSS data for Denmark and Ireland nicely fitted those presented in Table 4 of the article by Collignon et al. (1). Finally, reporting to the EARSS system greatly improved over the years, which is why this study was performed on the last available year, 2003.

Table. Absolute numbers, rates of *Staphylococcus aureus* bacteremia (SAB), and percentage of methicillin-resistant *Staphylococcus aureus* (MRSA), European Antimicrobial Resistance Surveillance System (EARSS), 2003*

Country	Population† (4)	% population covered by EARSS‡	Blood culture sets/1,000 inhabitants	No. SAB reported to EARSS	No. SAB for country§	SAB/100,000 inhabitants§	% MRSA
Austria	8,188,207	42.7	NA	871	2,038	25	15
Bulgaria	7,537,929	100	2	157	149	2¶	31
Croatia	4,422,248	81.3	7	360	443	10	37
Czech Republic	10,249,216	92.3	11	1,387	1,503	15	7
Denmark	5,384,384	46.2	NA	671	1,451	27	<1
Estonia	1,408,556	100	<1	98	98	7¶	5
Finland	5,190,785	94.3	27	727	771	15	1
Hungary	10,045,407	100	1	859	859	9¶	14
Iceland	280,798	100	28	64	64	23	0
Ireland	3,924,140	89.2	NA	1,108	1,243	32	42
Israel	6,116,533	39.7	42	368	926	15	43
Malta	400,420	100	4	122	122	31	43
Poland	38,622,660	24.3	3	166	684	2¶	19
Romania	22,271,839	59	<1	85	144	<1¶	46
Slovenia	1,935,677	100	17	299	296	15	13
Spain	40,217,413	24.3	21	1,391	5,731	14	25
Sweden	8,878,085	100	28	1,855	1,760	20	<1

*Only countries that provided hospital background information for at least 60% of the isolates were included; NA, not available.
†Source: (4).
‡Population coverage rate as calculated from EARSS hospitals that provided background information was adjusted for nonresponding hospitals as follows: population coverage as calculated divided by proportion of isolates with hospital background information.
§The total number of SAB per country was calculated as follows: number of *S. aureus* isolates in EARSS divided by adjusted proportion of population covered.
¶These rates are grossly underestimated because of the very low blood culturing rate.

Nevertheless, one cannot exclude underreporting of SAB by EARSS participating hospitals since EARSS is a voluntary reporting system. For example, England reported 18,403 SAB cases or an incidence of 37 SAB per 100,000 inhabitants from April 2002 to March 2003 through its mandatory surveillance scheme (5), whereas an estimate for the United Kingdom from the EARSS database would only give 7,800 SAB cases for 2003. However, it is impossible to determine whether this discrepancy was due to poor voluntary reporting of SAB cases, a lower blood culturing rate in EARSS participating hospitals, or a poorly representative sample of the country's hospitals. Data from the United Kingdom were excluded from the present study on the basis of the latter possibility; denominator information for <60% of the isolates was available.

In conclusion, EARSS is the first comprehensive surveillance system on antimicrobial resistance in Europe. Within certain limitations, EARSS can also provide valuable information on blood-culturing practices and the incidence of SAB in Europe. The system is continuously being improved, and additional information on the representativeness of EARSS data is being collected. This will allow us to improve the quality and accuracy of the reported incidence rates. In the future, the system should allow reporting of similar data for an even larger number of European countries and for additional microorganisms, such as *Escherichia coli*.

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Family Clustering of Avian Influenza A (H5N1)

To the Editor: The unprecedented epizootic of avian influenza A (H5N1) in Asia poses a serious threat of causing the next global influenza pandemic. H5N1 viruses, to which humans have little or no immunity, have demonstrated the capacity to infect humans and cause severe illness and death (1–4). Fortunately, these viruses have not yet demonstrated the capacity for efficient and sustained person-to-person transmission, although limited person-to-person transmission was the cause of at least 1 family cluster of cases (5). Since family clusters of H5N1 illness may be the first suggestion of a viral or epidemiologic change, we have been monitoring them with great interest.

Through our regional contacts and public sources, we have monitored family clusters and other aspects of H5N1 in Southeast Asia. A cluster was defined as ≥2 family members with laboratory-confirmed H5N1 or ≥2 family members with severe pneumonia or respiratory death, at least one of which had confirmed H5N1. To determine if family cluster events had increased over time, we divided the number of cluster events by the total number of days in 2 discrete periods and calculated rate ratios (RR) and 95% confidence intervals (CI). To determine whether the increase in family clustering was attributable to an increase in the number of cases, we divided the number of family units with ≥2 laboratory-confirmed cases by the total number of family units in the period. Percentage of deaths was also compared.

From January 2004 to July 2005, 109 cases of avian influenza A (H5N1) were officially reported to the World Health Organization (WHO) (6). During this time, 15 family clusters were identified (Table). Of the 11 (73%) clusters that occurred in Vietnam, 7 were in northern Vietnam. Cluster size ranged from 2 to 5 persons, and 9 (60%) had ≥2 persons with laboratory-confirmed H5N1. Cluster 6 in Thailand was well documented and was likely the result of limited person-to-person transmission (5). For the other clusters, epidemiologic information was insufficient to determine whether person-to-person transmission occurred. In at least 3 clusters in Vietnam (Table; clusters 5, 7, and 11), >7 days occurred between the onset of the first and the next case, suggesting that simultaneous acquisition from a common

Table. Family clusters of influenza A (H5N1) in Southeast Asia, January 2004–July 2005*

Cluster	Onset of index case	Country	Age (y)/Sex	Relation to index case	H5N1	Onset	Outcome
1	Dec 03	Vietnam (N)	12/F	Self	+	Dec 25	D
			30/F	Mother	+	Jan 1	D
2	Dec 03	Vietnam (N)	5/M	Self	+	Dec 29†	D
			7/F	Sister	NT	NN	D
3	Jan 04	Vietnam (N)	31/M	Self	NT	Jan 7†	D
			30/F	Sister	+	Jan 10	D
			28/F	Wife	+	Jan 10	R
			23/F	Sister	+	Jan 11	D
4	Jan 04	Thailand	6/M	Self	+	Jan 8	D
			33/F	Mother	NT	Jan 8	D
5	Jul 04	Vietnam (S)	19/M	Self	NT	Jul 23	D
			22/F	Cousin	NT	NN	D
			25/F	Sister	+	Jul 31	D
6	Sep 04	Thailand	11/F	Self	NT	Sep 2	D
			26/F	Mother	+	Sep 11	D
			32/F	Aunt	+	Sep 16	R
7	Dec 04	Vietnam (N)	46/M	Self	+	Dec 26	D
			42/M	Brother	+	Jan 10†	R
			36/M	Brother	+	Not ill	Not ill
8	Jan 05	Vietnam (S)	17/M	Self	+	Jan 10†	D
			22/F	Sister	NN	NN	Unknown‡
9	Jan 05	Vietnam (S)	35/F	Self	+	Jan 14	D
			13/F	Daughter	+	Jan 20	D
10	Jan 05	Cambodia	14/M	Self	NT	NN	D
			25/F	Sister	+	Jan 21	D
11	Feb 05	Vietnam (N)	21/M	Self	+	Feb 14	Unknown‡
			14/F	Sister	+	Feb 23	Unknown‡
			80/M	Grandfather	+	Not ill	Not ill
12	Feb 05	Vietnam (N)	69/M	Self	+	Feb 19	D
			61/F	Wife	+	Not ill	Not ill
13	Mar 05	Vietnam	13/F	Self	NT	Mar 9§	D
			5/M	Brother	+	Mar 12†	R
			Adult/F	Aunt	P	NN	Unknown‡
14	Mar 05	Vietnam (N)	39/M	Self	+	Mar 22†	Unknown‡
			Adult/F	Wife	+	Mar 22†	Unknown‡
			4 mo/NN	Child	+	Mar 22†	Unknown‡
			3/NN	Child	+	Mar 22†	Unknown‡
			10/NN	Child	+	Mar 22†	Unknown‡
15	Jul 05	Indonesia	8/F	Self	+¶	Jun 24	D
			1/F	Sister	NT	Jun 29	D
			38/M	Father	+	Jul 2	D

*D, respiratory death; N, north; NT, not tested; NN, not noted; P, pending; R, recovered; S, south.
†Date of hospitalization.
‡Had respiratory symptoms, was hospitalized (unknown for #13), and outcome was unknown.
§Date of death.
¶Serologically confirmed; classified as a probable case by the World Health Organization.

source was unlikely. In cluster 11, 2 nurses assisted in the care of the index case-patient and subsequently were hospitalized with severe pneumonia; 1 had laboratory-confirmed H5N1.

Family clusters were slightly more likely to have occurred between December 2004 and July 2005 than in the first year of the outbreak (9 clusters in 243 days or 3.7 per 100 days vs. 6 clusters in 365 days or 1.6 per 100 days, respectively; RR 2.3, 95% CI 0.8–6.3). The difference was similar when the periods were limited to the same 8 months, 1 year apart (RR 1.8, 95% CI 0.6–5.4). Twenty-five (61%) of the 41 patients in the 15 family clusters died; the 7 persons who recovered or were not ill experienced secondary cases.

Family clusters are still occurring; however, they do not appear to be increasing as a proportion of total cases. The proportion of families that were part of a cluster was similar from December 2004 to July 2005 to the proportion in the first year (6/55, 11% vs. 3/41, 7%, respectively, p = 0.7). However, the proportion of deaths dropped significantly, from 32 of 44

(73%) during December 2003 to November 2004, to 23 of 65 (35%) during December 2004 to July 2005 ($p<0.0001$).

Although reports of H5N1 family clusters slightly increased, the increase was not statistically significant. Nevertheless, we believe any cluster of cases is of great concern and should be promptly and thoroughly investigated because it might be the first indication of viral mutations resulting in more efficient person-to-person spread. Family clustering does not necessarily indicate person-to-person transmission, as it may also result from common household exposures to the same H5N1-infected poultry or from other exposures, such as to uncooked poultry products.

The decrease in proportion of deaths during 2005 is another epidemiologic change that should be monitored closely because it may reflect viral adaptation to the human host. Surveillance for human cases of avian influenza has been intensified in recent months, perhaps resulting in the identification of less severe cases. Alternatively, more widespread laboratory testing may be associated with false-positive results. No evidence to date shows genetic reassortment between H5N1 and human influenza A viruses (7). Viruses isolated from case-patients need to be immediately sequenced and characterized in relation to previously circulating viruses to see whether they are evolving.

Recent modeling studies suggest that containing a pandemic at its source may be possible because emergent pandemic viruses may be less transmissible than commonly assumed (8), and antiviral treatment and chemoprophylaxis may slow the spread (9). Although the logistics of an attempt to contain the beginning of a potential influenza pandemic are formidable, we believe it is not beyond the capability of the modern global public health system. As WHO (10) has called for, countries should intensify their pandemic preparedness plans and strengthen international collaborations.

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Imported Tickborne Relapsing Fever, France

To the Editor: Tickborne relapsing fevers caused by *Borrelia* species are characterized by ≥1 recurrent episodes of fever accompanied by headache, myalgia, arthralgia, abdominal pain, and eventually by hepatic or neurologic manifestations. In the Old World, *Borrelia duttonii* is endemic in sub-Saharan East Africa (1) and *B. crocidurae* and *B. hispanica* are distributed in West Africa and Mediterranean countries (2). In North America, *B. hermsii*, *B. turicatae*, and *B. parkeri* cause mild and sporadic fever cases, although several outbreaks have been reported (3). Relapsing fevers in disease-nonendemic countries are infrequently diagnosed and probably underdiagnosed (4). We report 3 patients with relapsing fever diagnosed in France in travelers from disease-endemic countries.

Patient 1, a 29-year-old French man, was admitted to Hôtel-Dieu in Paris for a fourth recurrence of a flulike syndrome. Three weeks earlier, he had traveled through Spain and Morocco, when high-grade fever, chills, myalgia, and arthralgia suddenly developed. Symptoms quickly resolved after treatment with salicylate and acetaminophen, but 3 relapses

occurred within 20 days. He had a low-grade fever and persisting myalgia. Results of a clinical examination were normal. Analyses showed elevated levels of C-reactive protein (CRP) (368 mg/L), creatine kinase (269 IU/L), and lactate dehydrogenase (1,149 IU/L). Because of previous travel in Africa, Giemsa-stained blood smears were examined for malaria parasites. They showed helical bacteria suggestive of *Borrelia*. Polymerase chain reaction (PCR) of a blood sample and sequencing of the 16S rRNA gene identified this bacterium as *B. hispanica*. Cefotaxime was administered for 72 hours and replaced with doxycycline, 100 mg twice a day for 10 days. One month later, the patient was free of symptoms.

Patient 2, a 21-year-old Malian woman, was admitted to Hôpital de Mantes in Mantes-la-Jolie and delivered a normal baby with the gestational age of 36.2 weeks. One month earlier, she experienced a spontaneously resolving fever with myalgia. Biologic analyses of the mother showed anemia (hemoglobin 9.8 g/dL) with an inflammatory syndrome (CRP 164 mg/L). Giemsa-stained blood smears showed spirochetes. Molecular analyses identified these as *B. crocidurae*. Results of this analysis in the newborn were negative. The patient was treated with doxycycline, 100 mg twice a day for 10 days, and quickly recovered.

Patient 3, a 21-year-old Mauritanian woman, was admitted to Avicenne Hospital in Bobigny with a febrile illness that lasted 4 days. She had been traveling for 2 months through Senegal and Mauritania. The day of her return to France, high-grade fever (temperature 41°C), chills, headache, diarrhea, and arthralgia developed. Results of a clinical examination were normal. Laboratory investigations showed anemia (hemoglobin 9.8 g/dL) and thrombocytopenia (64,000 platelets/μL). Giemsa-stained blood smears showed spirochetes. Molecular analyses identified this bacterium as *B. crocidurae*. The patient was treated with doxycycline, 100 mg twice a day for 10 days, and the patient quickly recovered.

Relapsing fevers caused by *Borrelia* spp. are rarely reported in travelers from disease-endemic countries. Because most infections are benign, cases are probably neglected. Since cultivation of the causative agents can be difficult, diagnosis relies on microscopic detection of helical bacteria in stained blood smears. Blood samples should be obtained during febrile episodes, but as shown in patient 2, spirochetes may be visualized on blood smears when the patient is no longer febrile. Quantitative buffy-coat analysis that increases detection sensitivity has been reported (5). Serologic tests are being developed to diagnose infection with *B. recurrentis* (6).

Detection of *Borrelia* DNA by PCR amplification from the blood is highly sensitive and specific. Identification can be achieved by sequencing the 16S rRNA gene (7) (Figure). Given the high level of sequence conservation (7), mutations can be informative. The identifying nucleotides for *B. crocidurae*, *B. hispanica*, and *B. duttoni* were at positions 65, 181, 381, and 596. Therefore, sequence analysis of the first 600 nucleotides (nt) in the 16S rRNA gene is sufficient to differentiate *B. crocidurae*, *B. hispanica*, and *B. duttonii*.

The complete *Borrelia* sequence obtained from the patient 1 showed 99.93% identity with *B. hispanica* (1 nt difference), 99.79% with *B. crocidurae* (3 nt differences), and 99.72% identity with *B. duttonii* (4 nt differences). This patient was infected with *B. hispanica* during his travel through Spain or Morocco, which is consistent with the distribution of this species. Comparison of 1,430 nt from sequences from patients 2 and 3 showed 99.93% identity with the sequence of *B. crocidurae* (1 nt difference). *B. crocidurae* occurs mostly in sub-Saharan countries of West Africa (Senegal, Mali, and Mauritania), where patients 2 and 3 were likely to have been infected.

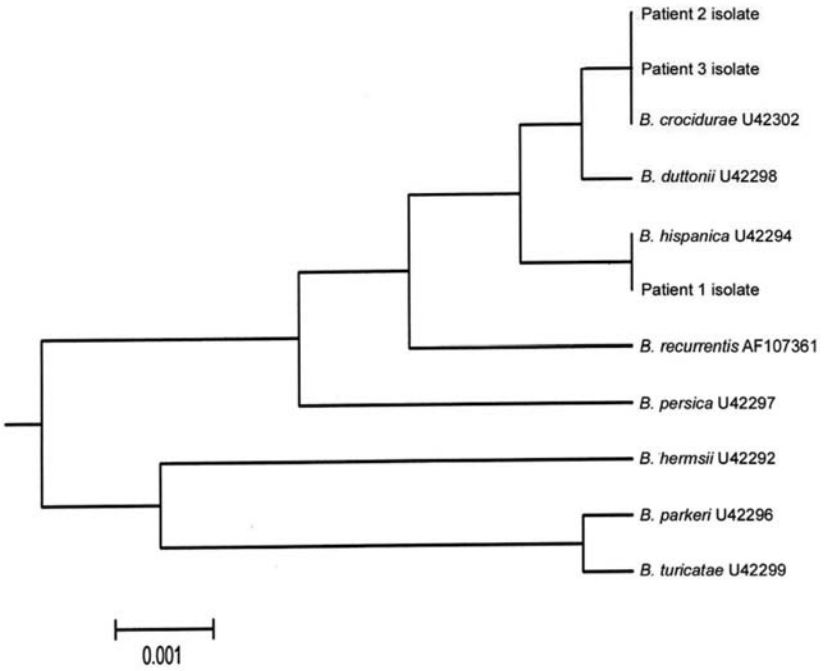

Figure. Unweighted pair group with mathematical average rooted tree of complete sequences of the *Borrelia* 16S rRNA gene. Sequences from databanks are indicated by their accession numbers.

Physicians should be alert for relapsing fever in travelers, and this diagnosis should be considered in febrile patients from disease-endemic regions. Diagnosis relies upon examination of stained blood smears. Where available, molecular methods are highly efficient to detect and identify bacterial species. Other tickborne infections (e.g., those with *Rickettsia* spp.) should also be considered in patients returning from disease-endemic countries (8). The recommended treatment is doxycycline, although it can cause a Jarish-Herxheimer reaction in some patients.

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Neonatal *Moraxella osloensis* Ophthalmia

To the Editor: *Moraxella osloensis* is an aerobic, gram-negative, lactose-nonfermenting coccobacillus. It is a commensal of the human upper respiratory tract and occasionally of the skin and urogenital tract (1). Unlike *M. catarrhalis*, *M. osloensis* is rarely pathogenic in humans. However, several cases of serious infections caused by this organism have been documented (2–6). While cases of nongonococcal, nonchlamydial, neonatal ophthalmia have been reported in which the causative agent was *M. catarrhalis* (7,8), to our knowledge, this case is the first report of neonatal ophthalmia due to *M. osloensis*.

A 3-week-old, previously healthy boy was seen at the emergency department with a 48-hour history of yellow drainage from and swelling in both eyes. One day before admission, the drainage increased; the child could not open his eyes spontaneously. He had been eating well and was normally active. Aside from mild fussiness, no other symptoms were noted.

The child was born full-term without complications to a gravida 6, para 5–6 mother. He received normal newborn care, including topical erythromycin ointment to the eyes. Aside from some mild jaundice at 6 days of age, he had been healthy. The mother denied any history of sexually transmitted disease.

On examination, the infant's temperature was 38°C rectally, heart rate 144 beats/min, respirations 26/min, and blood pressure 94/60 mm Hg. The child appeared well developed and was fussy but showed no symptoms of toxicity. Both eyelids were markedly swollen and erythematous, and a yellow, purulent discharge was noted bilaterally. The sclera and conjunctivae were injected bilaterally. An ophthalmologist recorded that the red reflex was intact bilaterally and the corneas were clear. Intravenous cefotaxime, oral erythromycin, and topical erythromycin ointment to the eyes were recommended. The leukocyte count was 11,400 cells/mm^3 with a normal differential. Hemoglobin level, hematocrit, platelet count, and bilirubin level were all within normal range. Urinalysis results as well as urine, blood, and cerebrospinal fluid cultures were negative. Secretions from the eyes were collected and sent for Gram stain and bacterial culture as well as chlamydial culture. Gram stain showed few gram-variable cocci.

The child's eyes were flushed with copious amounts of normal saline, and a dose of intravenous cefotaxime and ampicillin was administered in the emergency department. He was admitted to the hospital for presumed ophthalmia neonatorum. The following day, decreased lid swelling, erythema, and eye discharge were observed, with trace conjunctival injection and minimal chemosis.

The child's condition improved markedly during the next 24–48 hours. Cultures of the secretions obtained from the eye grew presumed *Neisseria* species as a pure culture. The isolate was sent to the Allegheny

County Health Department for further testing and speciation. It was first tested with a fluorescein-conjugated antibody for *Neisseria gonorrhoeae*; results were negative. A RapID NH panel (Remel, Lenexa, KS, USA) was performed that identified the isolate as *M. osloensis* with a 99.7% probability. Ideally, the isolate would have undergone more comprehensive genotypic and phenotypic characterization. However, as a presumed *Neisseria* species, it was subjected to the usual testing protocol at the health department. Chlamydial culture was performed by using buffalo green monkey kidney cells (Viromed, Minnetonka, MN, USA) grown under standard conditions. No viral inclusions were seen, and the culture did not react with chlamydial antibodies (Trinity Biotech, Bray, Ireland). Because the child responded rapidly to antimicrobial drug treatment, no further workup of the bacterial isolate was considered. The child was healthy 3 days later and was discharged to his home with topical erythromycin and instructions to his parents to follow up with his primary care physician.

Neonatal ophthalmia is a potentially serious, sight-threatening infection that may be caused by sexually transmitted pathogens. Accordingly, this clinical presentation warrants prompt diagnosis and appropriate therapy. At the same time, suspicion of a sexually transmitted disease causes immense social turmoil. Specific bacterial cultures are essential for precise microbiologic diagnosis and treatment.

Cultures of conjunctival specimens from our patient grew *M. osloensis*. Clinically, this patient's infection was indistinguishable from other causes of neonatal ophthalmia. The differential diagnosis includes other agents such as *N. gonorrhoeae*, *Chlamydia trachomatis*, *M. catarrhalis*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*. Rarely, gram-negative enteric organisms may be implicated (9). Viruses, such as adenovirus or herpesvirus, are also a potential cause but were unlikely in this case.

Finally, social issues must be considered. When an infant is seen with neonatal ophthalmia, a physician will often presume it to be gonococcal or chlamydial and assume the mother is positive for these infections. Recognizing that *Moraxella* species, including *M. osloensis*, may produce an identical clinical picture should limit presumptions regarding sexually transmitted diseases until a precise microbiologic diagnosis is made.

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African Tick-bite Fever in French Travelers

To the Editor: African tick-bite fever (ATBF) is caused by *Rickettsia africae* and remains the most common tickborne rickettsiosis in sub-Saharan Africa (1,2). We describe an outbreak of ATBF in 10 of 34 French tourists on their return from South Africa in March 2005. Fever, skin rash, and multiple eschars on the legs developed in the index case-patient (patient 9, Table). After informed consent was obtained, the tourists completed a questionnaire for epidemiologic and clinical data. Acute- and convalescent-phase serum samples were collected when possible for serologic analysis performed at the Unité des Rickettsies. The samples were tested against a panel of antigens including *R. typhi*, *Francisella tularensis*, *Coxiella burnetii*, *Borrelia burgdorferi*, *Anaplasma phagocytophylum*, *R. felis*, *R. helvetica*, *R. conorii* subsp. *conorii* strain Malish, *R. africae*, *R. sibirica mongolotimonae*, *R. massiliae*, and *R. slovaca*, as previously described (3). A case of symptomatic confirmed ATBF was defined as clinical illness and positive serologic results against *R. africae*, whereas a case of probable ATBF was defined as typical clinical symptoms without

definite serologic evidence of *R. africae* infection.

Of the 34 travelers, 30 completed the questionnaire and 20 consented to give at least 1 serum sample. After their return to France, symptoms compatible clinically with ATBF developed in 10 of the travelers (Table) and 9 had positive serologic results and/or a seroconversion for spotted fever group–rickettsia, including *R. africae* (Table). The median time from illness onset to serum testing was 19 days. Thus, 9 of the travelers had probable and 1 had possible (no serum was available) ATBF. Including both probable and possible cases, the illness rate for the whole group was 33.3% (10/30). None of the travelers reported a history of tick bite. The delay between probable exposure and onset of symptoms was 3–10 days (mean ± standard deviation 6.1 ± 1.9 days). Multiple eschars on the legs or arms were seen in 7 (70%) of 10 patients. Eight patients received doxycycline (200 mg per day) for a mean of 10.8 ± 5.9 days (range 5–20), 1 patient received pristinamycin for 8 days, and 1 patient received no treatment. All patients recovered fully without sequela; however, 6 patients reported convalescent-phase asthenia and 1 reported chronic insomnia, which had not occurred previously, for 2 months after the illness. Among the 10 remaining travelers, for whom a serum sample was available, with no clinical evidence of ATBF, 5 were positive for *R. africae* with only immunoglobulin M (IgM) at a titer of 1:32 in 4 cases and IgG at 1:128 with IgM at 1:32 in 1 case (an acute-phase serum from this patient showed IgG at 1:32 and IgM at 1:32). The 5 other travelers had negative serologic results. Results of serologic testing for other bacteria were negative for all travelers. Twenty-four travelers (80%), including the 10 symptomatic patients, reported using topical insect repellent daily.

Most cases of ATBF are reported in clusters of travelers exposed to ticks during game hunting or safaris, as described here (1,3–5). The estimated incidence of African tick-bite fever in safari travelers is 4%–5.3% (4) but higher incidence may be reported as emphasized in our study. In our study, epidemiologic and clinical data for the 10 symptomatic patients were obtained in accordance with current knowledge of ATBF (2).

Skin biopsy samples remain the best tool to isolate or detect *R. africae* (2,6). However, specific serologic tests, especially immunofluorescence assays, remain the most widely used microbiologic test worldwide (7). No commercially available test for ATBF exists but due to extensive cross-reactions between spotted fever group rickettsiosis, commercial kits based on the detection of *R. conorii* antibodies can be used for the diagnosis of ATBF. Most tourists reported using topical insect repellents without any efficacy. Applying repellents to exposed skin provides little protection against ticks because they can crawl underneath clothing and bite untreated portions of the body (8). Thus, treating clothing with synthetic pyrethroid insecticide is recommended to complement the topical repellant (8).

In conclusion, our study emphasizes the importance of ATBF as a common cause of flulike illness in travelers returning from South Africa, but with a higher rate than malaria, typhoid fever, or other tropical fevers. The most important clinical clues are the presence of clustered cases with multiple inoculation eschars. Healthcare professionals who are providing advice should inform persons traveling to endemic areas of Africa of the risk of contracting ATBF and the importance of protecting themselves against tick bites. Chemoprophylaxis with doxycycline is not recommended,

Table. Epidemiologic, clinical, and serologic information for 10 patients with African tick-bite fever*

Patient	Sex/age (y)	Tick bite	Delay before onset (d)	Fever	Headache	Myalgia	Eschar (site)	Skin rash	1st serum† IgG/IgM	2nd serum† IgG/IgM	Diagnosis
1	M/62	No	7	Yes	No	No	Multiple (legs)	No	NA	NA	Probable
2	F/58	No	6	Yes	No	Yes	Multiple (legs, arms)	No	64/32	64/128	Confirmed
3	M/58	No	6	No	Yes	No	Single (trunk)	No	64/32	128/16	Confirmed
4	F/51	No	6	No	Yes	Yes	Multiple (legs, trunk)	No	0/64	128/16	Confirmed
5	M/58	No	5	Yes	No	Yes	Multiple (legs)	No	512/0	512/0	Confirmed
6	F/57	No	5	No	No	Yes	Yes (unknown)	Yes	NA	32/16	Confirmed
7	M/65	No	5	Yes	Yes	Yes	Multiple (hands)	No	128/64	512/128	Confirmed
8	F/59	No	10	No	No	No	Multiple (legs, arms, trunk)	No	64/8	128/32	Confirmed
9	M/53	No	3	Yes	Yes	Yes	Multiple (legs)	Yes	0/0	1,024/512	Confirmed
10	M/51	No	8	Yes	No	Yes	No	Yes	32/32	64/64	Confirmed
Total (%)		0		60	40	70	90	30			

*NA, not available; Ig, immunoglobulin; male-to-female ratio, 60%; mean age = 57.2 ± 4.5 years.
†Identical results obtained with both *Rickettsia africae* and *R. conorii* antigens.

however, this recommendation may be evaluated in future clinical trials.

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Microbe: Are We Ready for the Next Plague?

Alan Zelicoff and Michael Bellomo

Amacom, New York, NY, 2005
ISBN: 0-8144-0865-6
Pages: 256; Price: US $23.00

Microbe: Are We Ready for the Next Plague? by Alan Zelicoff and Michael Bellomo is a comprehensive, yet succinct, account of the threat to public health posed by microbial pathogens. What distinguishes this book from the surfeit of recent books hyping the threat of bioterrorism are its balanced perspective and elucidation of naturally emerging disease threats, such as severe acute respiratory syndrome (SARS) or West Nile virus, as exotic entities requiring a rapid and effective response; Mother Nature is quite the bioterrorist herself. Early recognition that an event has occurred is key to containment of the nascent epidemic.

The authors provide sufficient basic science background to bring the uninitiated up to speed on a variety of exotic and recently introduced microbes in engagingly titled chapters such as "The Birds that Fell from the Sky" (West Nile), "Corona of Death" (SARS), and "Something in the Water" (*Cryptosporidium*). In addition, they describe hantavirus pulmonary syndrome, mad cow disease, and Legionnaires' disease in the context of recent public health emergencies. The authors also explain why both smallpox and anthrax are more than abstract concerns as agents of bioterrorism, on the basis of weaponization history, intrinsic attributes, and realistic scenarios. An account of the 1970 smallpox outbreak, which occurred in Aralsk, Kazakhstan, as a consequence of open air testing of a smallpox weapon by the Soviets is an eye-opener; there should be no doubts about capability and intent after reading this story.

The scenarios are well chosen and informative; they highlight the importance of early recognition that "something has happened" and breaking the disease cycle close to the index case. The unifying theme of the book is the importance of syndrome-based surveillance in achieving this goal. The authors dismiss BIOWATCH (air-monitoring devices to detect and identify microbes in aerosol clouds) as a well-intended but expensive "work in progress," to put it charitably. BIOSENSE is a national surveillance system that they say has not been implemented in any substantive way. I reluctantly find myself in agreement with these assessments and receptive to their suggestions to implement an emerging diseases reporting system based on syndromic reporting.

Healthcare providers recognize syndromes, not microbial diseases. How long did it take to recognize monkeypox in 2003? The hantavirus associated with lethal pulmonary syndrome in New Mexico in 1993 was recognized only when the pattern emerged among previously healthy young adults living in rustic conditions on a Navajo reservation. The authors describe a product they dub Syndromic Reporting Information System (SYRIS) as a "beta test" product that has been deployed on a limited, regional basis and promises to provide a near instantaneous map of syndromic reports and to comply with all Centers for Disease Control and Prevention requirements for electronic reporting systems. Like most good ideas, simplicity is central to the SYRIS concept; it is likely to succeed because participating doctors, nurses, and veterinarians (most of the exotic pathogens are zoonoses) can report syndromic occurrences in 15 seconds or less and will be rewarded with instantaneous feedback and tailored reports and alarms. While this section does read a bit like an infomercial, the concept is sound and worthy of serious consideration by public health officials and policy makers. This book is the best of its genre and is recommended for anyone interested in understanding and managing the risks associated with emerging microbial threats.

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The AIDS Pandemic: Impact on Science and Society

Kenneth H. Mayer and H.F. Pizer, editors

Elsevier Academic Press, Amsterdam, the Netherlands, and Boston, Massachusetts, 2005
ISBN: 0-12-465271-9
Pages: 537; Price: US $84.95

As we enter the third decade of the AIDS pandemic, numerous texts explore the many aspects of AIDS and its consequences. Mayer and Pizer's premise is that AIDS has transformed many of the disciplines that it has touched. For the most part, this well-written volume supports their thesis. The authors, all established researchers, tackle many of the major issues, including virology, immunology, vaccines, microbicides, and sexually transmitted diseases, as well as

the global impact of HIV/AIDS. Each chapter provides a well-referenced overview of its topic with many references as recent as 2003.

One of the real strengths of this book is a chapter on quantitative science that explores not only the history of HIV clinical trials, but also the design and importance of clinical trials in general. This chapter should be required reading for those considering clinical research in HIV. The chapters on Africa and Asia ably contrast the differences in these areas of highest prevalence. Another strength is the discussion of HIV in correctional facilities and the challenge of caring for this population, including their coexisting conditions and illicit drug use. Lastly, the discussion of the economics of AIDS is especially welcome in this era of efforts to increase access to drugs worldwide.

Overall, this book fills a valuable niche. A relatively concise text, it reviews many aspects of HIV with a focus on how each topic has evolved over the years. A few tables are small, but overall the diagrams and charts are clear and legible. This book would be of interest to infectious disease fellows, HIV caregivers, and those involved in public health and health policy. I heartily recommend this book and plan to keep it handy for future reference.

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Tick-Borne Diseases of Humans

Jesse L. Goodman, David T. Dennis, and Daniel E. Sonenshine, editors

ASM Press, Washington, DC, 2005
ISBN: 1-55581-23-4
Pages: 440, Price: US $119.95

During the past 2 decades, the scientific landscape of tickborne diseases has changed remarkably. In part because of advances in molecular biology, more than 10 new rickettsial diseases, several ehrlichial diseases, and novel agents of *Borrelia* and *Babesia* genera have been recognized. This renaissance of interest in tickborne infections benefits from advances in molecular phylogenetics and diagnostics, immunology, and informatics that provide tantalizing insights into the complexities of vectorborne infection. Tick-Borne Diseases of Humans is a well-referenced textbook that encompasses these new insights in vector biology and reviews the emerging epidemiology and clinical science of these diseases as they occur across the globe. The editors' goal of providing a "comprehensive" resource is admirably fulfilled.

The book, consisting of 20 chapters by 40 contributors, is divided into 3 sections. The first section includes excellent reviews of tick biology and systematics, tick-pathogen interactions, host responses, and vector management. This section provides a superb overview. While thorough and up-to-date, occasional redundancy occurs between chapters by different authors that could have been streamlined with additional editing. A concise and well-written chapter on the clinical approach to diagnosis and management of these diseases also seems misplaced; it would fit in better at the start of the next section.

Section 2 of the book includes summaries of major and lesser known tickborne infections. These chapters each provide detailed information on specific vectors and pathogens and on the epidemiology and clinical characteristics of the diseases they cause. While the description of the molecular biology and vector ecology of these infections is generally excellent, the clinical discussions often lack the nuance and detail of current infectious diseases texts. Nevertheless, each chapter provides current and well-referenced information on disease manifestations, diagnosis, and treatment. Several chapters, i.e., those on

Correction: Vol 11, No. 8

In "Laboratory Exposures to Brucellae and Implications for Bioterrorism" by Pablo Yagupsky and Ellen Jo Baron, an error occurred in the dosage for rifampin prophylaxis.

On page 1184, first column, first paragraph, the correct dosage of rifampin is 600 mg once daily.

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol11no08/04-1197.htm

We regret any confusion this error may have caused.

Correction: Vol. 11, No. 10

In "Methicillin-resistant *Staphylococcus aureus* Necrotizing Pneumonia," by Monica Monaco et al., an error occurred on page 1647, in the first full sentence of the third column. The sentence should read "On day 3 of admission, antimicrobial drug therapy was changed to linezolid (600 mg 2 times a day)."

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol11no10/05-0776.htm

We regret any confusion this error may have caused.

anaplasmosis, relapsing fever, and Lyme borreliosis, are superb in all aspects.

Section 3 includes a series of global maps that depict the distribution of different tick vectors or the diseases they cause. While useful overall, maps on this scale do not convey the focality of tick distribution, and their organization in the text (i.e., mixing of vector maps with disease maps) could be improved. The maps are followed by a color atlas of tickborne diseases with plates depicting typical skin lesions and other clinical findings along with examples of microscopic pathology. This part of the book is visually compelling, although the reproductions of microscopic pathology are often small and therefore difficult to view in detail. Section 3 concludes with an almanac of the geographic distribution of ticks and the diseases they cause. This information is often difficult to find, and its inclusion in a chapter of the text is useful.

In summary, Tick-Borne Diseases of Humans is an excellent resource for a diverse audience. Vector biologists (whether molecular or ecologic in focus), infectious disease physicians, and those involved in the public health surveillance and control of these diseases will find this book to a valuable addition to current texts.

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Phoenix and Birds (detail)
China (c. 16th century)
Ink and colors on silk
(213.4 cm x 113 cm)
Honolulu Academy of Arts, Hawaii, USA
Gift of Charles M. and Anna C. Cooke Trust Fund, 1928 (141.1)

Phoenix and Fowl: Birds of a Feather

Polyxeni Potter*

A treasured aviary, the vast collection of bird paintings in Chinese art, reflects longstanding global fascination with our feathered friends. Balanced on two legs, like humans, and able to fly and swim, birds have been viewed as an engineering miracle in the East and West and have been studied by artists and scientists alike.

Traditional Chinese painting goes back 6,000 years to the Neolithic period and is found on early pottery decorated with brush images of humans and animals (1). Painting of flowers and birds originated on this primitive pottery, as well as on bronze and silk adorned with simple but brightly colored designs (2). The genre, which is seen throughout Chinese art history, flourished during the Song dynasty (1101–1125 AD).

During the Yuan dynasty (1279–1368), the Mongol wars and general turmoil under Genghis Khan overshadowed a strong artistic legacy enriched by diverse foreign influences. The period saw suspension of artists' and intellectuals' rights and retreat to traditional styles of painting (3). Need for greater artistic expression coincided with the return of native rule during the Ming dynasty.

The Ming dynasty (1368–1644) was a time of cultural restoration and expansion for the Chinese, a "scholar's culture" of thriving literary and artistic communities populated by writers, poets, and artists, many of them outstanding masters with extraordinary skills and breadth (4). Revived interest in local culture was often expressed in landscape images of mountains or other nature scenes painted on scrolls. Monochrome and color woodblock printing developed and advanced at this time, as did porcelain production and diversification. Yet, artists worked primarily in a revival of Song academic styles, prescribed by a conservative court for its glorification and prestige (5).

Genius is the most important quality in a painter, knowledge comes next, and "the single brush stroke is the source of all things," wrote painter and member of the Ming royal house, Shitao (1642–1707) (6). Unlike canvas, silk, which was used in painting even before paper was invented, was unforgiving of errors and required exceptional skill and confidence. Many renowned Chinese painters were also expert calligraphers and poets, who often made literary references in landscape painting, emphasizing the connection between disciplines and adding complexity to the work.

Phoenix and Birds, on this month's cover, exhibits many of the qualities of Ming dynasty silk scroll painting. The narrative content (The Five Human Relationships Represented by Five Different Birds) is expressed with surely executed lines and subtle colors in vertical format. Shadow, light, and proportion are used to create a third dimension. A central figure, the phoenix, dominates the scene. This legendary bird, part of global mythology, is described here in the Chinese tradition. Like the dragon, with which it is often associated, the phoenix, or *fenghuang*, exemplifies the union of yin and yang (polar opposites complementing each other in nature and underlying order within the universe). In some legends, the *fenghuang* is created from desirable parts of other creatures: cock's beak, swallow's face, fowl's forehead, snake's neck, goose's breast, tortoise's back, stag's hind, fish's tail. Its song reflects the notes of the

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musical scale, its feathers five fundamental colors, its figure the celestial bodies: head symbolizes the sky; eyes, the sun; back, the moon; feet, the earth; tail, the planets (7).

This emperor of birds is anchored on a rock, its royal plumes and fearless stare signaling preeminence. Below are two cranes, symbols of wisdom and longevity. They seem aware of their surroundings and of two other waterfowl fraternizing in the foreground.

In this harmonious bird scene, the unknown artist injects a measure of Confucian values, the need for each creature to act not singly but in connection with others, through five relationships: parent-child, husband-wife, sibling-sibling, friend-friend, ruler-subject, in networks of individual persons, the family, the state, the universe (8). This conglomeration of myth and Confucian wisdom within the Asian tradition has timeless implications. And in today's context, troubled by the specter of pandemic avian flu, Phoenix and Birds seems prophetic.

The bird ensemble captures issues at the heart of our current predicament: unknown pathogen origins, exotic composites of unlikely elements, increasing complexity, vast public health implications. The imperial phoenix with its patchwork beauty, perched high on the mount is not much different from the frolicking cranes or the humble fowl crouching anonymously in the foreground. All participate in nature's play. More than the sum of its unlikely parts, the phoenix recalls the flu virus and its wild recombination. Less conspicuously, the migrating waterfowl signal this species' importance as reservoir hosts and dissemination agents, bringing the virus to creatures absent from this painting (domestic poultry, swine). The circle is complete as new opportunities arise for recombination with local mammalian strains to form a new virus with pandemic potential. Confucian relationships meet nature's whim.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the December issue for the following topics:

Role of Multisector Partnerships in Controlling Emerging Zoonotic Diseases

Community Epidemiology Framework in Theory and Practice

Bushmeat Hunting and Deforestation in Predicting Zoonosis Emergence

Human Granulocytic Anaplasmosis and *Anaplasma Phagocytophilum*

Francisella tularensis in the United States

Host Range and Emerging and Reemerging Pathogens

Person-to-Person Transmission of Andes Hantavirus

European Bat Lyssaviruses in the Netherlands

Infection with SARS-CoV from Palm Civet

Echinococcosis in Tibetan Populations

Porcine and Human Noroviruses

Intergenogroup Recombination in Sapoviruses

Distribution of Viral Load in SARS Outbreak

Rabies Postexposure Prophylaxis, New York, 1995–2000

PanAsia Strain of Foot-and-Mouth Disease Virus Serotype O

Complete list of articles in the December issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 12–14, 2005
6th International Conference on Typhoid Fever and Other Salmonelloses
Guilin, China
Contact: tandongmei112@yahoo.com.cn or yyyjin@126.com

November 13–18, 2005
Fourth MIM Pan-African Malaria Conference
Yaoundé, Cameroon
http://www.mim.su.se/conference2005

December 5–9, 2005
National Viral Hepatitis Prevention Conference
Hyatt Regency Hotel on Capitol Hill
Washington, DC, USA
http://www.nvhpc.com

December 10, 2005
2005 Pre-Meeting Course: Immune Regulation: Parasites and Chronic Infectious Diseases
Hilton Washington Hotel and Towers
Washington, DC, USA
Contact: 847-480-9592 or astmh@astmh.org
http://www.astmh.org

December 10, 2005
2005 Clinical Pre-Meeting Course: Anti-Malaria Chemoprophylaxis
Hilton Washington Hotel and Towers
Washington, DC, USA
Contact: 847-480-9592 or astmh@astmh.org
http://www.astmh.org

December 10–14, 2005
American Public Health Association 133rd Annual Meeting and Exposition
Philadelphia, PA, USA
http://www.apha.org

December 11–15, 2005
ASTMH 54th Annual Meeting
Hilton Washington Hotel and Towers
Washington, DC, USA
Contact: 847-480-9592 or astmh@astmh.org
http://www.astmh.org

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

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

EMERGING INFECTIOUS DISEASES

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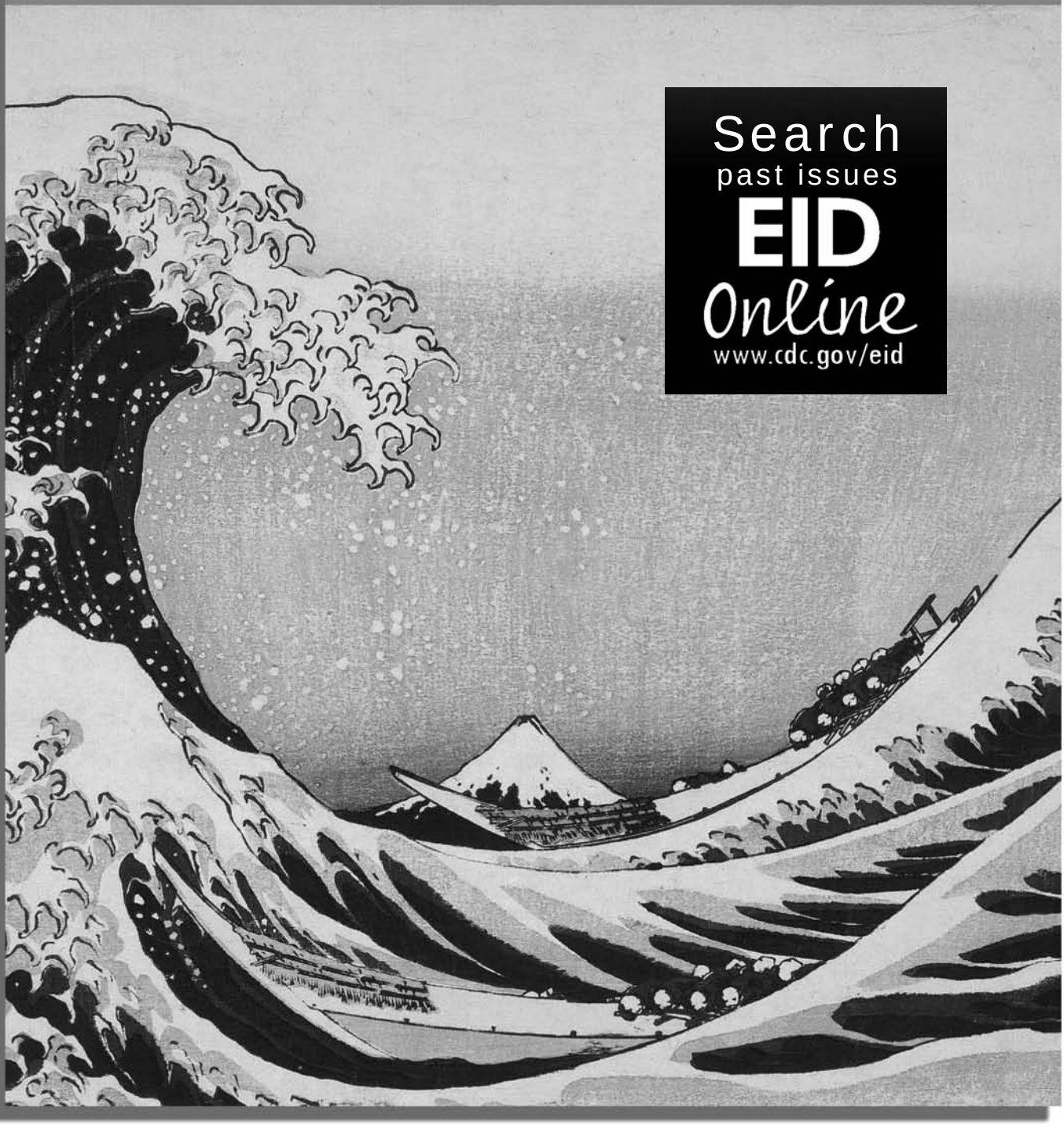
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.11, No.10, October 2005



EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

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

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

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

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

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

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

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.